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Comparisons between tissues, preservation, and desiccation methods on stable isotopes $\delta^{13}C$ and $\delta^{15}N$ of spot-tail sharks (*Carcharhinus sorrah*) from the South China Sea

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Abstract

This study presents the $\delta^{13}C$ and $\delta^{15}N$ values of spot-tail sharks (*Carcharhinus sorrah*), focuses on the inter-tissue comparisons between fin and muscle tissues; the effects of ethanol preservation as compared with freezing and evaluations of oven- and freeze-drying desiccation methods. The average $\delta^{13}C$ and $\delta^{15}N$ values significantly differed between fin and muscle tissues and were correlated for selected treatments. Ethanol preservation did not affect $\delta^{13}C$ but significantly enriched $\delta^{15}N$ in the muscles, whereas both desiccation methods produced similar results. Freezing samples for preservation is recommended for stable isotope analysis, whereas desiccation methods can be chosen at the researchers' discretion.

Introduction

Stable isotope analysis (SIA) is useful in investigating the foraging dynamics and trophic roles of sharks in their ecosystems across time and space (Li, Hussey & Zhang, 2016). The analysis of carbon and nitrogen isotopes in particular has been extensively applied in ecological studies, including shark research, as it allows scientists to investigate the migratory histories, diet shifts and trophic changes of sharks (Minagawa & Wada, 1984; Hobson & Welch, 1992; Kline Jr. *et al.*, 1993; Vander Zanden & Rasmussen, 1999; Phillips & Eldridge, 2006). These interpretations are made possible because, unlike in the traditional examination of gut contents that can only provide a snapshot of an animal's most recent foraging inputs, SIA can relay information on the individual's assimilated diet as it is incorporated into the body tissues over time (Hobson, Gibbs & Gloutney, 1997; Halley, Minagawa, Nieminen & Gaare, 2010).

SIA requires only a very small amount of tissue samples, as little as 0.5–1.0 mg in dry weight (Centre for Stable Isotopes, University of New Mexico), to produce reliable results. This feature enables biologists to sample large numbers of specimens and specimens that are endangered or found within marine protected areas or no-take zones, without causing long-term harm to the population. White muscles are one of the most commonly used tissues in the stable isotope investigations of sharks (Fisk, Tittlemier, Pranschke & Norstrom, 2002; Estrada, Rice, Lutcavage & Skomal, 2003; Domi, Bouquegneau & Das, 2005; MacNeil, Skomal & Fisk, 2005; Logan & Lutcavage, 2010). Although only a small amount of muscles is needed for the analysis, muscle extraction using a biopsy punch may still be harmful to small individuals, such as neonate

sharks. To provide a safer alternative, this study aims to examine if muscle tissues can be substituted with more easily extracted fin tissues in the isotope studies of young sharks.

Another limitation faced by scientists seeking to sample shark tissues for SIA in remote field locations is the limited access to freezing facilities that can properly preserve biological samples (Hobson, Gibbs & Gloutney, 1997). This limitation has led to the substitution of various solutions, including alcohol, formalin, formalin–ethanol, dimethyl sulfoxide, and salt solutions, to preserve tissue samples until further processing is possible. Some of these solutions have indeed been shown to alter the stable isotope ratios of the preserved tissues (Hobson *et al.*, 1997). However, the degree of ^{13}C or ^{15}N enrichments or depletions induced by these preservatives varies between species, tissue types, preservation duration and studies, often with contradicting results (Hobson *et al*.,1997; Kaehler & Pakhomov 2001; Halley, Minagawa, Nieminen & Gaare, 2008), which causes problems in the interpretation of SIA results. To determine a viable method to preserve shark tissues without freezing them, we tested the effects of ethanol preservation on the $\delta^{13}C$ and $\delta^{15}N$ values of shark fin and muscle tissues. Ethanol was chosen as it has repeatedly been reported to have an insignificant effect on the isotopic values of several animal tissues (Hobson *et al.*, 1997; Arrington & Winemiller, 2002; Barrow, Bjorndal & Reich, 2008; Halley *et al.*, 2008). It is also easy to obtain and is relatively inexpensive.

The aims of this study are 1) to compare the $\delta^{13}C$ and $\delta^{15}N$ values of the muscle and fin tissues of spot-tail sharks (*Carcharhinus sorrah*) (Müller & Henle, 1839), to assess if the sampling of shark fin tissues can provide a viable alternative to that of muscle tissues, 2) to compare the effects of preservation in 70% ethanol and preservation by freezing on the isotopic values of shark fins and muscles and 3) to compare the effects of oven- and freeze-drying on the isotope values of shark fins and muscles to determine if both methods can be used interchangeably for SIA.

Methodology

For the examination of the $\delta^{13}C$ and $\delta^{15}N$ isotopic values of *C. sorrah* fin and muscle tissues, samples were obtained from six neonate specimens estimated to be 2–7 months old, with a mean (± SD) total length of 71.1 cm (± 11.7) and mean (± SD) weight of 1876.4 g (± 903.4). All sharks were caught off the coast of Terengganu, Malaysia in the South China Sea by a fisherman in July 2018 and were kept frozen at −20 °C for four days prior to the analysis (Kim &Koch, 2012). Fin samples were cut from the trailing end of each shark's dorsal fin and divided into three pieces of approximately the same size (N = 18; 6 sharks x 3 samples) (Hussey, Chapman, Donnelly, Abercrombie & Fisk, 2011). White muscle tissues were extracted along the shark's lateral line, approximately at the height of its dorsal fin, and subsequently cut into three pieces of approximately 1 cm^3 each (N = 18; 6 sharks x 3 samples).

In the laboratory, all samples were defrosted and rinsed with distilled water before further treatment. One set of fin and muscle tissue samples (N = 6) were oven-dried at 60 °C for 48 hours or until completely dry (Kaehler & Pakhomov, 2001; Barrow, Bjorndal & Reich, 2008; Kim &Koch, 2012). These samples served as a control sample and are henceforth referred to as 'FO' (for frozen and oven-dried). Meanwhile, the second set of samples (N = 6) were deep-frozen at −80 °C for 24 hours prior to being freeze-dried for 24 h. These samples are referred to as 'FF' (for frozen and freeze-dried). The last set of samples (N = 6) were immersed i*n* a solution of 70% ethanol for 14 days, subsequently rinsed with distilled water and oven-dried at 60 °C for 48 hours or until completely dry. These samples are referred to as 'EO' (for ethanol preserved and oven-dried). All the samples were ground into homogenous powder using a pestle and mortar and transferred into 2 ml sterile plastic vials. Vials were stored in air-tight bags filled with silica desiccants and sent to the Malaysian Nuclear Agency for SIA.

Approximately 1.5 mg of powdered tissue from each sample was combusted at 1000 °C using a SerCon ANCA-GSL elemental analyser interfaced via continuous flow to a SerCon GEO20-20 isotope-ratio mass spectrometer. Stable isotope abundances were measured in triplicates for each sample by comparing the ratio of the two most abundant isotopes (e.g. $^{13}C/^{12}C$ and $^{15}N/^{14}N$) in the sample to the international standards. The results were expressed in terms of parts per thousand (‰) deviation from the standard, using the following equation:

$$\delta X = [(R_{sample} / R_{standard}) - 1] \times 1000 \text{ ‰}, (1)$$

where *X* is ^{13}C or ^{15}N and *R* is the isotopic ratio $^{13}C/^{12}C$ or $^{15}N/^{14}N$ (Peterson & Fry, 1987). Standards used for carbon and nitrogen were secondary standards referenced to a relative known international standard, i.e. Vienna Pee Dee Belemnite (VPDB), and atmospheric nitrogen (air), respectively.

All data analyses were performed using IBM SPSS Statistics 20. For $\delta^{13}C$ and $\delta^{15}N$ (‰), the residuals of pairs of compared tissues or treatments were tested for normality using the Shapiro–Wilk test. The residuals of the isotopic values were distributed normally, and pairwise comparisons were performed using two-tailed paired t-tests. Pearson's correlation analyses were used to test for possible correlations between the isotopic values of fin and muscle tissues. Data were $\log_{10}$ transformed when necessary.

Results

The $\delta^{13}C$ values in *C. sorrah* muscles were significantly lower than those in the fins for all

treatments: FO ($P < 0.001$), EO ($P = 0.021$) and FF ($P = 0.003$) (Table 1 and Figure 1). Conversely, the $\delta^{15}N$ values in the muscles were significantly higher than those in the fins: FO ($P = 0.005$), EO ($P = 0.001$) and FF ($P = 0.026$). Despite the difference, there were significant correlations between the stable isotope values of fins and muscles in the EO ($r = 0.832$; $P = 0.040$) and FF samples ($r = 0.962$; $P = 0.002$) for $\delta^{13}C$ and $\delta^{15}N$ respectively (Figure 2).

Different preservation methods in EO and FO did not affect the $\delta^{13}C$ values for both type of tissues namely fins ($P = 0.081$) and muscles ($P = 0.530$). Similarly, the different preservation methods in EO and FO did not affect ($P = 0.952$) the $\delta^{15}N$ values of fins (Table 2). However, the $\delta^{15}N$ of muscles in EO was significantly enriched than FO ($P < 0.001$). Meanwhile, the $\delta^{13}C$ and $\delta^{15}N$ were not affected by the different desiccation methods namely FO and FF for fins ($P = 0.110$ and 0.097) and muscles ($P = 0.638$ and 0.431), respectively.

Discussion

The differences in the $\delta^{13}C$ and $\delta^{15}N$ values in the fin and muscle tissues of *C. sorrah* observed in this study are common and have been previously reported in other types of sharks, such as shortfin mako *Isurus oxyrhynchus*, thresher *Alopias vulpinus* and blue shark *Prionace glauca* (Logan & Lutcavage, 2010; Matich *et al.*, 2010; Matich, Haithaus & Layman, 2010; Hussey *et al.*, 2011). This is due to the differences in the turnover rate between the fins and muscles (MacNeil, Drouillard, & Fisk, 2006). However, shark size is an important factor in the differences because the younger sharks may retain their maternal signatures for an extended period (Matich *et al.*, 2010). Meanwhile, significant correlations between the fin and muscle tissues observed for $\delta^{13}C$ and $\delta^{15}N$ values suggest that a shark's fin can be used as an alternative for muscle tissues in SIA studies. However, the stable isotope values of shark fins should

Table 1. Mean (±SD) and range values (‰) of $\delta^{13}C$ and $\delta^{15}N$ from the different tissue types of *C. sorrah* namely fin and muscle, and in different treatments namely frozen and oven dried (FO), preservation in ethanol and oven-dried (EO) and frozen and freeze-dried (FF)

Tissue	N	Treatment	$\delta^{13}C$ (‰)		$\delta^{15}N$ (‰)	
			Mean (±SD)	Range	Mean (±SD)	Range
Fins	6	FO	−15.2 (0.5)	−15.8 to −14.6	13.8 (0.7)	13.2 to 15.1
	6	EO	−16.2 (1.0)	−17.2 to −14.8	13.78 (1.0)	12.9 to 15.4
	6	FF	−15.8 (0.8)	−16.8 to −14.8	12.25 (2.0)	10.0 to 14.4
Muscles	6	FO	−16.8 (0.4)	−17.5 to −16.2	14.7 (0.7)	13.6 to 15.7
	6	EO	−17.0 (1.0)	−18.1 to −15.6	16.0 (0.7)	15.3 to 16.8
	6	FF	−16.9 (0.7)	−17.9 to −15.9	13.8 (3.0)	10.4 to 17.1

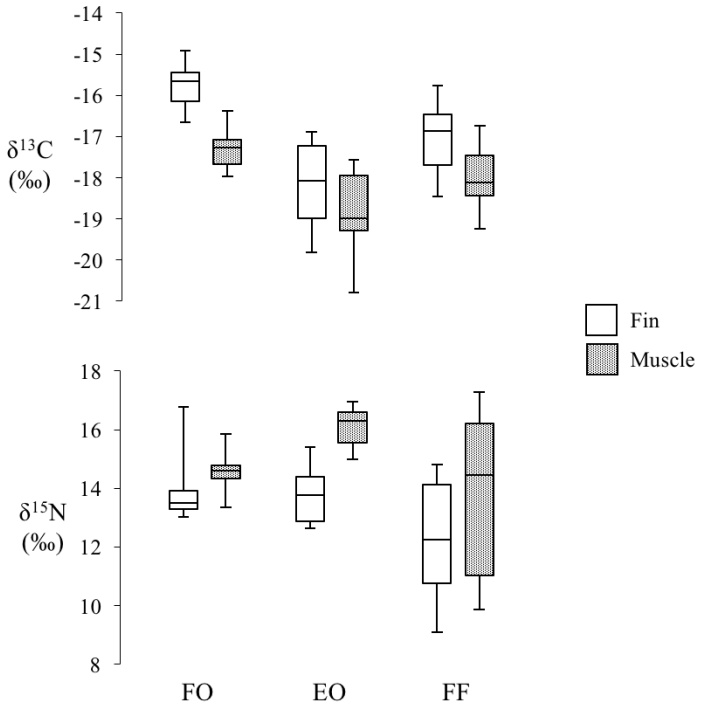

Figure 1. Stable isotope values of $\delta^{13}C$ and $\delta^{15}N$ (‰) from the fin and muscle tissues of the spot-tail shark *C. sorrah* (N = 6) in different preservation methods (FO-EO) and desiccation methods (FO-FF). FO to represent frozen and oven-dried; EO to represent ethanol preserved and oven-dried; and FF to represent frozen and freeze-dried sample treatments.

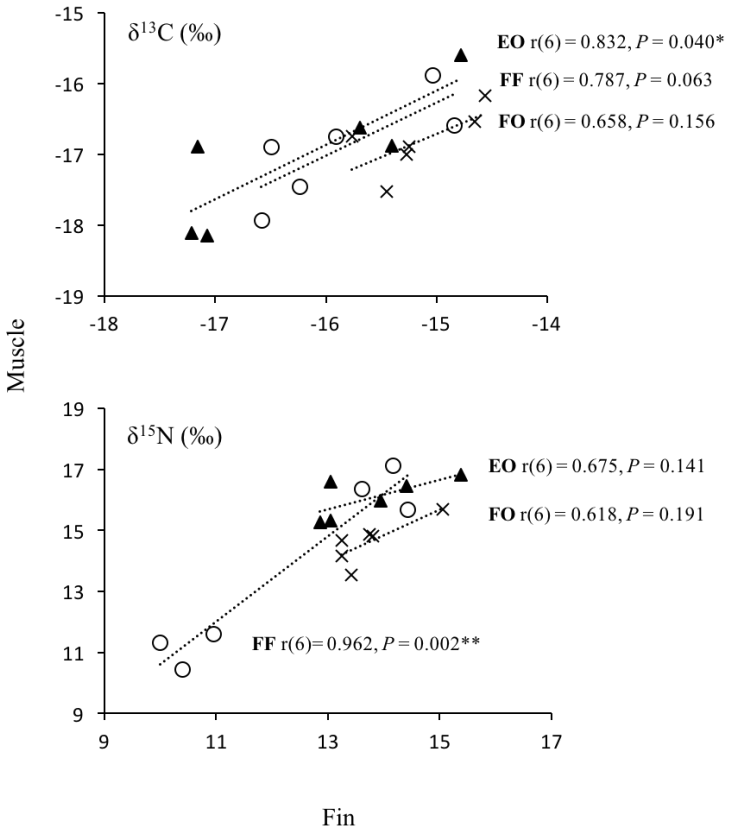

Figure 2. Pearson correlation coefficient between the fin and muscle tissues of the $\delta^{13}C$ (‰) and $\delta^{15}N$ (‰) isotopic values, from the spot-tail shark *C. sorrah* in different sample treatments namely FO (cross), EO (triangle) and FF (circle). Asteriks * and ** denote significant difference at P < 0.05 and P < 0.01 respectively.

Table 2. Pairwise comparisons of the $\delta^{13}C$ and $\delta^{15}N$ values (‰) within and between treatments for tissue types, preservation methods, and desiccation methods (N = 6). Data show the mean difference for the $\delta^{13}C$ and $\delta^{15}N$ values (‰) and the paired-test results. Asterisks *, ** and *** denote the significant difference at P < 0.05, P < 0.01 and P < 0.001 respectively

Pairwise comparison		$\delta^{13}C$ (‰)		$\delta^{15}N$ (‰)	
		Mean diff	Paired-test results	Mean diff.	Paired-test results
Tissue types (Fins–Muscles)	FO	−1.61	t(5) = −10.52, P< 0.001***	+0.90	t(5) = 4.83, P= 0.005**
	EO	−0.80	t(5) = −3.30, P= 0.021*	+2.30	t(5) = 7.69, P= 0.001**
	FF	−1.07	t(5) = −5.44, P= 0.003**	+1.48	t(5) = 3.11, P= 0.026*
Preservation methods (FO–EO)	Fins	−1.05	t(5) = −2.18, P= 0.081	+0.03	t(5) = 0.64, P= 0.952
	Muscles	−0.23	t(5) = −0.67, P= 0.530	+1.42	t(5) = 8.63, P< 0.001***
Desiccation methods (FO–FF)	Fins	−0.65	t(5) = −1.94, P= 0.110	−1.48	t(5) = −2.04, P= 0.097
	Muscles	−0.10	t(5) = −050, P= 0.638	−0.90	t(5) = −0.86, P= 0.431

not be compared directly to those of shark muscle tissues. Thus, applying tissue- and element-specific diet-tissue discrimination factors is crucial to standardise the isotopic values of different shark tissues and allow for more meaningful comparisons to be made in future studies.

Past research has shown that the effects of ethanol preservation on the isotopic values of animal tissues greatly varies across species. For example, ethanol preservation has been reported to cause a significant increase in the $\delta^{13}C$ values of longnose skate *Raja rhina* muscles (Kim & Koch, 2012) but a significant decrease in the $\delta^{13}C$ of Arctic charr *Salvelinus alpinus* muscles (Kelly, Dempson & Power, 2006), while not affecting the $\delta^{15}N$ of either. Meanwhile, others have recommended 70% ethanol as a suitable preservative, as it did not affect the isotopic values of various teleosts and aquatic invertebrates (Arrington & Winemiller, 2002; Le Bourg, Lepoint & Michel, 2019), green turtles (Barrow, Bjorndal & Reich, 2008), quail blood and muscles and sheep blood (Kaehler & Pakhomov, 2001).

This present study shows that ethanol preservation does not significantly affect $\delta^{13}C$ in either shark fin or muscle tissues, but the effects on $\delta^{15}N$ values were varied. These variations may, in part, be attributed to the different structures and compositions of shark fins

and muscles (Every *et al.*, 2016), which, in turn, may have led to the differential incorporation of the ethanol solution into these tissues (Gearing, 1991; Ponsard & Amlou, 1999; Hobson *et al.*, 1997). Therefore, the $\delta^{13}C$ values of ethanol-preserved shark tissues can be safely compared with those of frozen samples, but further investigations are needed to ascertain its effects on $\delta^{15}N$ across the general shark population.

The study also suggests that oven- or freeze-drying methods to desiccate *C. sorrah* fin and muscle tissue samples prior to SIA can be fairly used and compared. This is supported by the fact that, although oven-drying is widely used to desiccate the tissues of marine organisms, such as octopus, sea stars, sea turtles, teleosts and sharks, for SIA (Barrow, MacNeil, Skomal & Fisk, 2005; Kaehler & Pakhomov, 2001; Arrington & Winemiller, 2002; Bjorndal & Reich, 2008; Logan & Lutcavage, 2010; Kim & Koch, 2011), freeze-drying has also been used in stable isotope studies of sharks (Barria, Navarro & Coll, 2018). Our results confirm that both desiccation methodss produce similar SIA results for *C. sorrah* and can therefore be freely chosen by researchers in stable isotope studies of sharks. However, it is also important to note that the variation in the $\delta^{15}N$ values for the FF samples are quite high. Therefore, future application of the $\delta^{15}N$ values from this study should be treated with care especially when it involves the calculation for discrimination factors or mixing models.

As a conclusion, although the stable isotopes $\delta^{13}C$ and $\delta^{15}N$ differ between shark fins and muscles, correlations between the two tissues suggest that by applying tissue-specific discrimination factors, reliable comparisons can be made between them. The use of 70% ethanol to preserve shark tissues for SIA has no effect on the $\delta^{13}C$ values of samples, but its effect on $\delta^{15}N$ requires further investigation. Lastly, oven- and freeze-drying are suitable methods to desiccate shark tissues for SIA.

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