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Short Communication

Effects of Urea Removal on the Stable Isotopes $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in Rays from the Coastal Waters of Peninsular Malaysia

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Abstract

This is the first reported record of stable isotope values for elasmobranch rays within Malaysian waters, and serves as an important baseline methodological information for future studies investigating stable isotope values in both shark and ray species in the region. This study examined the effects of urea removal on the stable isotopes values of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in muscle tissues sampled from several elasmobranch rays species, namely *Pastinachus atrus*, *Rhinoptera javanica*, *Himantura gerrardi*, *Himantura uarnak* and *Aetobatus ocellatus*. Individual ray species were collected in July and August of 2018, from the coastal waters of Terengganu State, Malaysia. Urea removal was performed by soaking muscle tissue samples in deionised water for at least 24 hours before being dehydrated. The resulting stable isotope values of these samples were compared to samples that did not undergo the urea removal process. Stable isotope values were individual and species-specific, however, the effect of urea removal was significant for $\delta^{15}\text{N}$ values but did not significantly affect the $\delta^{13}\text{C}$ values. We conclude that removing urea from elasmobranch samples before stable isotope analysis is advisable to draw correct conclusions about the animal's diets.

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1. Introduction

Understanding a species ecological role within its environment requires a good knowledge about that species diet and trophic level (Couturier *et al.*, 2013; Bornatowski *et al.*, 2014). This information can help conservation planning, especially in areas where their prey's seasonal abundance and distribution are known (Bachok *et al.*, 2004; Couturier *et al.*, 2013). Stable isotope analysis (SIA) is a more recent approach to examining the diets of mobile animals such as elasmobranchs, which include shark and ray species (Burgess *et al.*, 2016). However, one of the main characteristics that differentiate sharks and rays from other fish is the high urea content in their body tissues (Hussey *et al.*, 2012). The retention of nitrogen-rich urea ((NH₂)₂CO) in the muscle tissue of elasmobranchs aids in the maintenance of osmotic balance and is one of the significant distinctions between elasmobranchs and teleosts (Fisk *et al.*, 2002). Urea has the potential of affecting the stable isotope values of muscle tissue due to its high nitrogen content. Hence, significant experimental work is necessary to test the key assumptions that underpin the implementation of SIA, especially for sharks and rays, which have distinctive stable-isotope dynamics due to their physiology (Hussey *et al.*, 2012).

In the past, gut content analysis was commonly used to study the trophic linkages between species. However, it can only provide information on the most recently consumed foods and is biased towards foods that are hard to digest and therefore remains longer in the gut (Christensen and Moore, 2009). This causes stomach content studies to only relay information on the temporary, locally available food. SIA can reflect an elasmobranch's diet, which has been integrated over time (Carlisle *et al.*, 2012), because the heavier stable isotopes (e.g., $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) are generally conserved at each trophic level (DeNiro and Epstein, 1978), and can therefore reflect information on dietary carbon sources (Figure 1) and trophic position (MacNeil *et al.*, 2005). Different types of animal tissues, such as muscle tissue and red blood cells, have lower or higher isotope incorporation rates respectively and therefore can reveal information about an animal's long and short-term diets (Fisk *et al.*, 2002; MacNeil *et al.*, 2005; Hussey *et al.*, 2012).

Sampson *et al.* (2010) utilized SIA to describe the diet and trophic position of bent fin devil rays, *Mobula thurstoni*, and spintail devil rays, *Mobula japonica*, in the Gulf of California, and Peel *et al.* (2019), used

the method to characterize the feeding ecology of reef manta rays, *Mobula alfredi*, at a remote coral reef in the Western Indian Ocean. In addition, Borrell *et al.* (2011) used SIA to analyze 13 species of elasmobranch caught off Gujarat, India. SIA has been used to describe the isotopic niche breadth and overlap among 128 individuals representing 11 sympatric elasmobranch species present in Winyah Bay, South Carolina (Pankow *et al.*, 2021). Although limited, several studies have been conducted using SIA to study the food web structure, and trophic levels of marine organisms around Malaysia's coastal waters (Newell *et al.*, 1995; Chong *et al.*, 2001; Zulkifli *et al.*, 2014, 2016; Mukhtar *et al.*, 2016). However, no studies have investigated the diet of rays using SIA in Malaysian waters. This study aimed to determine the effects of urea extraction on the stable isotope values $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in elasmobranch muscle tissues collected in the coastal waters of Terengganu, Malaysia.

2. Materials and Methods

2.1 Sample Collection

The present study was conducted using tissue samples collected from several different ray species caught around Bidong Island in the coastal waters of Terengganu in Peninsular Malaysia. Three stations were set up at the south-eastern, south-western, and northern parts of Bidong Island hereafter referred to as stations A, B, and C, respectively. Samples were collected using stingray gillnets as part of a study to examine the influence of habitat and seasonal divisions on the abundance and distribution of stingray species around Bidong Island (Mohidin *et al.*, 2021). Samplings were conducted in July and August 2018. A sampling permit was obtained from the Department of Fisheries Malaysia (Prk.ML. S.04/32/2 Jld.9 (22)).

A total number of 13 individual rays consisting of five different species were caught; including *Pastinachus atrus*, *Rhinoptera javanica*, *Himantura gerrardi*, *Himantura uarnak*, and *Aetobatus ocellatu*, and used for this study. All of the species collected are considered Vulnerable (VU) by the IUCN checklist, and *Pastinachus atrus* is Least Concerned (LS). The individual weight of the rays caught was relatively smaller in July as compared to August (Table 1).

Each caught specimen was weighed, measured, and identified following the protocols from Yano *et al.* (2005), Ahmad *et al.* (2014), and Last *et al.* (2016). Muscle tissue samples were obtained from each specimen and frozen at -20°C until further analysis.

Table 1. Detailed description of the rays collected and the results of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ from non-treated and treated (urea extracted) muscle samples. VU = Vulnerable, LC = Least Concern

Date	St.	Species	IUCN Red List status	Common name/ Malay name	Weight (kg)	DW (cm)	Non-treated		Treated	
							$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰)	$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰)
July	C	<i>Pastinachus atrus</i>	LC	Banana-tail ray/	7.1	65.2	-12.86	7.27	-13.19	7.93
	A	<i>Pastinachus atrus</i>	LC	Pari daun	8.0	66.8	-13.09	6.49	-14.41	8.56
	A	<i>Pastinachus atrus</i>	LC		13.4	81.8	-14.44	8.18	-17.42	7.40
	B	<i>Rhinoptera javanica</i>	VU	Javaneses cownose ray/	10.8	97.6	-10.11	5.04	-10.41	6.09
	B	<i>Rhinoptera javanica</i>	VU	Pari kelawar jawa	6.9	80.8	-15.44	5.81	-14.26	6.61
	C	<i>Himantura gerrardi</i>	VU	Whitespotted whipray/ Pari bintik putih	10.6	75	-13.52	7.09	-13.44	7.79
Au- gust	A	<i>Pastinachus atrus</i>	LC	Banana-tail ray/ Pari daun	23.0	93.2	-19.40	3.53	-14.47	6.87
	C	<i>Himantura gerrardi</i>	VU	Whitespotted whipray/ Pari bintik putih	12.3	75.4	-16.50	5.74	-17.63	5.54
	C	<i>Himantura gerrardi</i>	VU		14.6	79.8	-20.38	5.21	-19.21	6.21
	A	<i>Himantura gerrardi</i>	VU		8.3	67.2	-21.04	5.13	-22.31	5.41
	C	<i>Himantura uarnak</i>	VU	Reticulate whipray/ Pari rimau	17.6	91.2	-18.64	5.78	-18.86	5.80
	B	<i>Aetobatus ocellatus</i>	VU	Whitespotted eagle ray/ Pari lang bintik putih	32.0	143.2	-24.53	3.52	-23.23	4.58
B	<i>Rhinoptera javanica</i>	VU	Javaneses cownose ray/ Pari kelawar jawa	51.0	167.6	-26.28	4.05	-20.63	6.50	

2.2 Sample Preparation

Samples that had been collected and frozen in the field were removed from the freezer and thawed. From these original samples, two sets of pure muscle tissue, measuring approximately 1 cm³ each, were extracted and rinsed with deionised water. Care was taken to clean the dissecting instruments thoroughly between each sample extraction. One of the two muscle tissue samples was oven-dried from each specimen without any further treatment (hereafter referred to as 'non-treated' muscle). These non-treated samples were placed in a petri dish and oven-dried at 60 °C for 36 h to 48 h (Couturier *et al.*, 2013). The dried samples were then finely ground using a pestle and mortar. Urea removal was performed following the methods of Burgess *et al.* (2016), by soaking each sample in deionised water for 24 h. Subsequently, samples were rinsed again, placed in the petri dish, and oven-dried at 60°C for 48 h to 36 h, then finely ground using a pestle and mortar. According to Kim and Koch (2012) and Li *et al.* (2016), the immersion of elasmobranch muscle

tissue in deionised water for a prolonged period is efficient in extracting urea from the tissue. All ground samples from each treatment were transferred into 2 ml microcentrifuge tubes, labelled, and sent to the Malaysian Nuclear Agency (MNA) for SIA.

2.3 Stable Isotope Analysis (SIA)

SIA was performed by the MNA by combusting approximately 1.5 mg of the powdered sample at 1000°C using a SerCon ANCA GSL elemental analyser interfaced via continuous flow to a SerCon GEO20–20 isotope–ratio mass spectrometer. In each sample, the ratio of ¹³C/¹²C for carbon and ¹⁵N/¹⁴N for nitrogen was compared to an internationally recognised standard. For carbon, this standard (R_{standard}) is the ¹³C/¹²C ratio of Vienna Pee Dee Belemnite, whereas, for nitrogen, it is the ¹⁵N/¹⁴N ratio in atmospheric nitrogen (air). The stable isotope values were obtained using the equation $\delta X = [(R_{\text{sample}} / R_{\text{standard}}) - 1] \times 1000 \text{ ‰}$, where X is ¹³C or ¹⁵N, and R is the ratio of ¹³C/¹²C or ¹⁵N/¹⁴N (Peterson and Fry, 1987). Results were expressed in parts per thousand (‰) deviation from these standards. Stable

isotope values which were measured in triplicates were averaged to obtain a single value per sample.

2.4 Data Analysis

Wilcoxon signed-rank tests were used to compare the difference between the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of urea extracted and non-extracted samples. Mann-Whitney U test was used to compare the weights and stable isotope values of the urea extracted samples between months. Spearman rank correlation was used to determine significant correlation between the stable isotope values and the weights of rays. All analysis were performed using IBM SPSS V28.0.1.0 (142).

3. Result and Discussion

3.1 Effects of Urea Extraction in Muscle Samples on the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ Values

In this study, urea extraction did not significantly affect the $\delta^{13}\text{C}$ values ($Mdn = -16.81$ and $Mdn = -17.07$ for the non-treated and urea extracted samples respectively), as indicated by the Wilcoxon signed-rank test, $Z = -0.175$, $P = 0.861$). However, the $\delta^{15}\text{N}$ values were significantly enriched in the urea-extracted samples ($Mdn = 6.56$) as compared to the non-treated samples ($Mdn = 5.51$), $Z = -2.621$, $P = 0.009$ (Figure 1). Previous studies have reported that the presence of with decreased $\delta^{15}\text{N}$ values but may not directly affect the $\delta^{13}\text{C}$ (Carlisle et al., 2012; Nielsen et al., 2019). Our study's results are consistent with other reports that muscle from ray species, including the bluespotted maskray *Neotrygon kuhlii* (Müller and Henle, 1841)

(Burgess and Bennett, 2016), cowtail stingray *Pastinachus ater* (Macleay, 1883), and Australian whipray *Himantura australis* (Last et al., 2016; Crook et al., 2019), were not significantly affected by the extraction of urea. Meanwhile, it has been well accepted that the extraction of urea increases the $\delta^{15}\text{N}$ value of the tissues, although the extent of this effect varies among ray species (Burgess and Bennett, 2016; Carlisle et al., 2012). Thus, the urea-extraction method used in this study has efficiently removed the urea content in

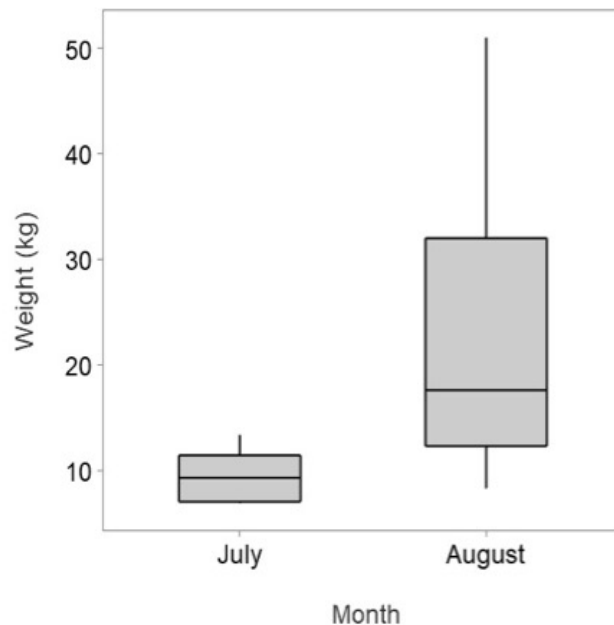


Figure 2. Boxplot showing comparison of weights of rays caught in July and August. The values were significantly different between July and August ($P = 0.015$, $n = 13$).

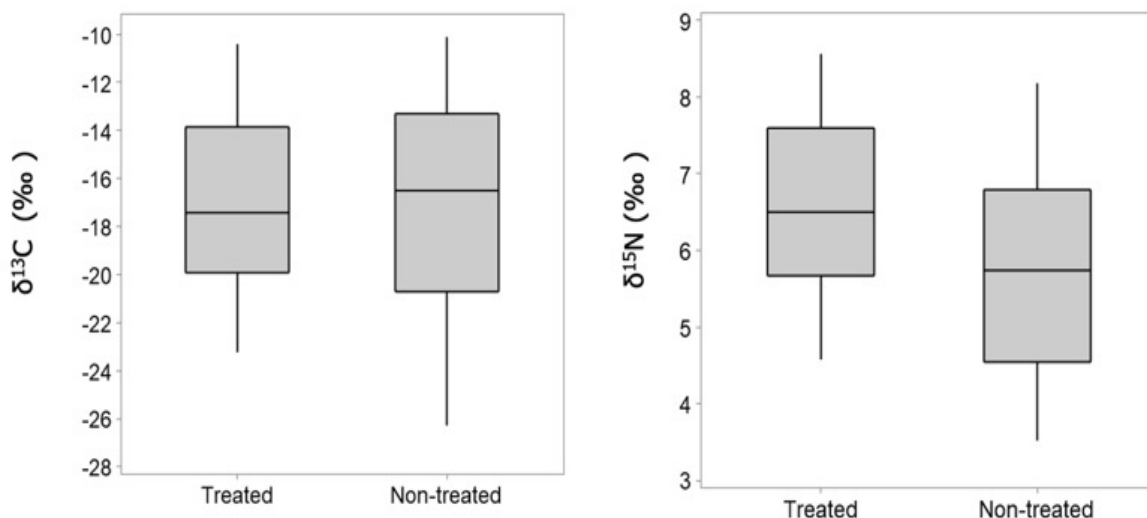


Figure 1. Comparison between the stable isotope values (‰) for treated (urea-extracted) and non-treated muscle tissues of rays ($n = 13$). The $\delta^{13}\text{C}$ values were not significantly different between treatment ($P = 0.861$) but the $\delta^{15}\text{N}$ values were significantly different between treatments ($P = 0.009$).

the ray muscle tissue samples. Overall, it is important that researchers correctly estimate $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values for their study species, as this will affect their perceived trophic dynamics in the examined ecosystem. Lower $\delta^{15}\text{N}$ values caused by excessive urea in shark and ray tissue samples may result in these individuals being assumed to occupy lower positions within their food web. This can lead to misleading conclusions about the mesopredatory roles of these vulnerable species in the marine environment, among others. urea in elasmobranch tissues is generally associated

3.2 Stable Isotopes Ecology of Rays from Kuala Terengganu Coastal Waters

The $\delta^{13}\text{C}$ values from the urea extracted samples ranged from -23.23 ‰ to -10.41 ‰ for the *Aetobatus ocellatus* and *Rhinoptera javanica* respectively (Table 1). Meanwhile, the $\delta^{15}\text{N}$ values ranged from 4.58 ‰ to 8.56 ‰ for the *Aetobatus ocellatus* and *Pastinachus atrus*. Rays caught in July ($Mdn = 9.30$) were significantly smaller than those caught in August ($Mdn = 17.60$), with ($U = 4.00, P = 0.015$) (Figure 2).

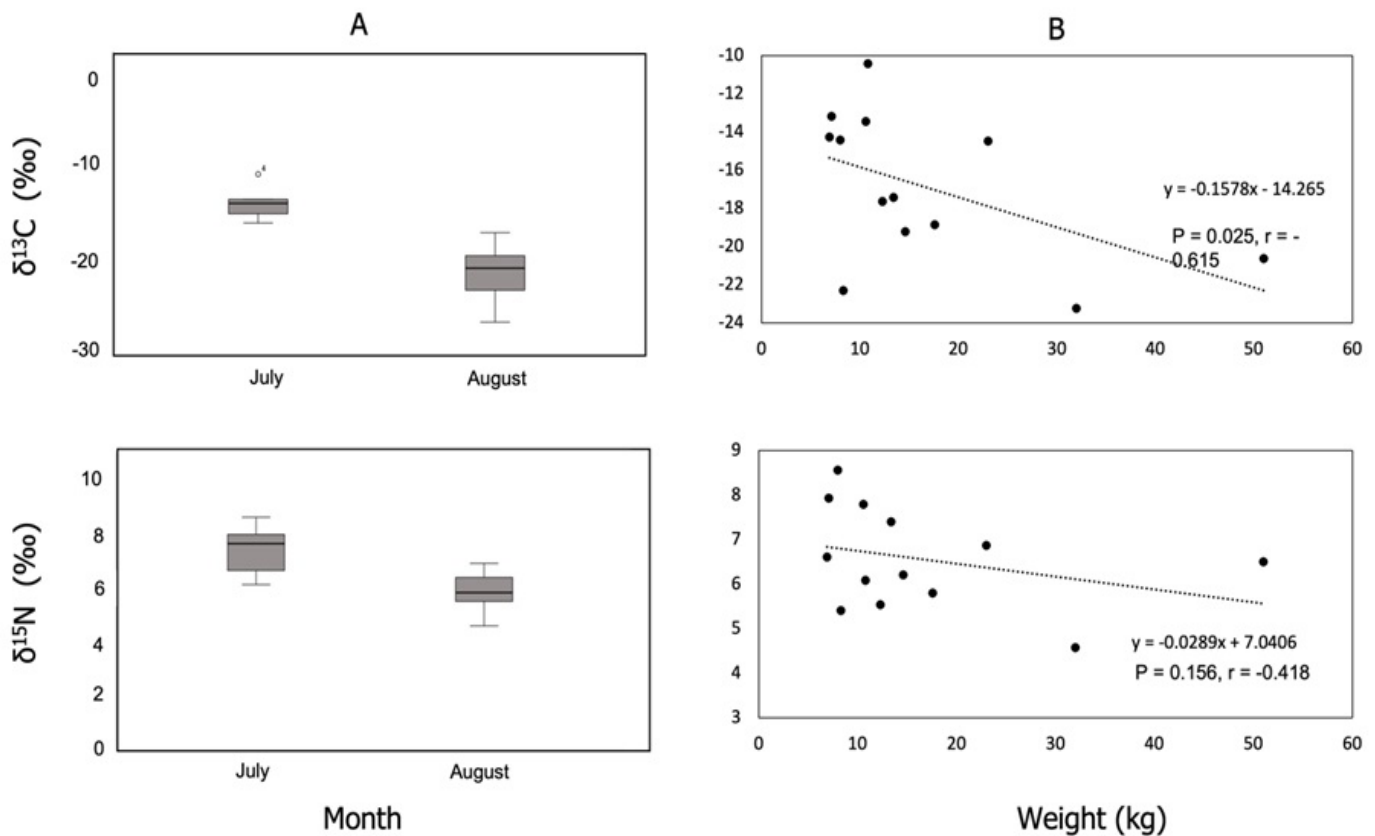


Figure 3. The $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of rays (from urea extracted samples) in July and August. The $\delta^{13}\text{C}$ values were significantly depleted in August (A) but the $\delta^{15}\text{N}$ values were not significantly different. Meanwhile, Figure 3B shows the significant Spearman rank correlation between $\delta^{13}\text{C}$ values with ray's sizes ($P = 0.025$), but the $\delta^{15}\text{N}$ values were not significantly correlated with ray's sizes ($P = 0.156$).

Previous research has shown that urea concentrations can differ between species and animals of different sizes due to the urea-trimethylamine oxide concentrations in their habitat, the time since the last feeding (Goldstein *et al.*, 1968; Ballantyne, 1997; Pillans *et al.*, 2005; Wood *et al.*, 2007). Variable urea content among species and tissues of elasmobranch may significantly complicate stable isotope analysis. Thus, more experimental research is needed to clarify the effect of urea on stable isotope values across elasmobranch species and study locations (Hussey *et al.*, 2012).

Overall, the $\delta^{13}\text{C}$ values were significantly different between July ($Mdn = -13.85$) and August ($Mdn = -19.04$), ($U = 1.00, P = 0.004$). Besides, the $\delta^{15}\text{N}$ values were also significantly different ($U = 4.00, P = 0.015$) between July ($Mdn = 7.60$) and August ($Mdn = 5.8$) (Figure 3A). The sizes of the rays were significantly correlated between $\delta^{13}\text{C}$ values but not with the $\delta^{15}\text{N}$ values (Figure 3B). The largest and most common ray species found in this study, namely *Rhinoptera javanica* (Müller & Henle, 1841), has been reported to feed on various prey, including bivalves, such as clams and

oysters, and crustaceans (Michael, 1993). Therefore, the difference in $\delta^{13}\text{C}$ values from July to August in *R. japonica*, may be attributed to a substantial dietary shift at an individual level (English et al., 2015). Such shifts may be driven by fluctuations in prey abundance (O'Shea et al., 2013), although assumptions such as these can only be confirmed through further investigations into invertebrate benthos populations in the studied habitat.

Dietary shifts among rays are common, especially for opportunistic generalists (Collins et al., 2007). In the present study, *Himantura gerrardi* (Gray, 1851) showed a wide variation of diet range as indicated by the $\delta^{13}\text{C}$ values, which were visibly more enriched in July (-13.44 ‰) than in August (-22.31 ‰). This observation agreed with previous records by O'Shea et al. (2013), who reported that *Himantura uarnak* (Gmelin, 1789), which is of the same genus as *H. gerrardi*, consumed a generalist diet of penaeids, annelids, and brachyurans. Meanwhile, *Pastinachus atrus* (Macleay, 1883) has higher $\delta^{15}\text{N}$ values, probably as a resemblance of its main diet, which, according to O'Shea et al. (2013), consists of up to 70% of annelids such as polychaetes.

The lowest $\delta^{15}\text{N}$ value was found in *Aetobatus ocellatus* (Kuhl, 1823), also known as the ocellated eagle ray. It is a large-sized ray occurring in the tropical waters of the Indo-West Pacific (Berthe et al., 2018). Low $\delta^{15}\text{N}$ values are expected due to the benthopelagic nature of *Aetobatus ocellatus*. Diets of related species, such as *Aetobatus narinari* (Euphrasen, 1790), indicate that individuals from this genus are specialists of hard-shelled prey which feed predominantly on animals such as molluscs, which can make up 78 - 98% of its diet, as well as crustaceans, polychaetes, and echinoderms (Schluessel et al., 2010).

4. Conclusion

We conclude that removing urea from elasmobranch samples before stable isotope analysis is advisable to draw correct conclusions about the animal's diets. This study supports the relevance of the popular utilization of SIA to increase our understanding of the feeding ecology of elasmobranchs and also helps to guide researchers in choosing appropriate sample preparation procedures for SIA for the elasmobranch species. This will ultimately improve the accuracy of SIA results and subsequent inferences about the trophic interactions, feeding ecology, and roles of elasmobranchs in the marine ecosystem in Malaysia.

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Authors' Contributions

All authors have contributed to the final manuscript. The contribution of each author as follow, MMA, LWY and ZB; collected the data, drafted the manuscript, and designed the figures. RM conducted the stable isotope analysis; MB, SH; devised the critical revision of the article. All authors discussed the results and contributed to the final manuscript; FH; conducted the sample collection.

Conflict of Interest

The authors declare that they have no competing interests.

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