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Diet-tissue Stable Isotopic Fractionation of Tropical Sea Cucumber, *Holothuria scabra*

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Abstract

To provide a basis for a stable carbon and nitrogen isotope ratio ($\delta^{13}C$ / $\delta^{15}N$) analysis to determine the assimilated organic matter in sea cucumber, *Holothuria scabra*, diet-tissue fractionations were experimentally determined by mono-feeding rearing with diatom. While $\delta^{15}N$ fractionation of the whole body wall (2.4‰) was similar to the commonly accepted value (2.6 – 4‰), $\delta^{13}C$ fractionation of the body wall (4.2‰) showed considerable discrepancy with the commonly accepted value (0 – 1‰) due to the high content (35% dry wt/wt) of calcareous spicules ($CaCO_3$) in the body wall, which had significantly higher $\delta^{13}C$ (-8.6‰) than the organic fractions. Computational elimination of spicules based upon spicule content and spicule $\delta^{13}C$ reduced the $\delta^{13}C$ fractionation of the body wall to 1.5‰, close to the common value. $\delta^{13}C$ fractionation after spicule removal by acid decarbonation and subsequent rinsing (3.2‰) did not agree with the common value, and $\delta^{15}N$ fractionation was significantly elevated by decarbonation. $\delta^{15}N$ and $\delta^{13}C$ fractionations of the intestine (1.5 and 2.2‰, respectively) did not agree with the common values. Since $\delta^{13}C$ and $\delta^{15}N$ of the feces did not differ significantly from those of the diet, feces may be used to determine ingested organic matter in the wild.

Discipline: Aquaculture
Additional key words: calcium carbonate, C/N ratio, spicule

Introduction

Many species of tropical sea cucumbers in many countries in the Pacific and Indian Oceans are being over-exploited for processing into bêche-de-mer (dried form)[5, 8, 18]. As the demand for sea cucumbers increases and fishery stocks dwindle in many areas of these countries, there is a growing demand to produce sea cucumbers by aquaculture and stock enhancement using juveniles produced in the hatchery. There is also growing interest in co-culturing sea cucumbers alongside other organisms to utilize organic debris and thus mitigate eutrophication within and around the aquaculture facilities[4, 22, 33, 37, 46]. To this end, an increasing number of studies have recently been performed to develop techniques for hatchery, aquaculture and stock enhancement of sea cucumbers, especially sandfish, *Holothuria scabra*, the most valued species of tropical sea cucumbers[3, 4, 28, 37].

The hatchery production of *H. scabra* seeds has got underway in countries, such as Vietnam, India, New Caledonia and the Philippines[1, 11, 36], but the high mortality and slow growth rate of cultured juveniles have been problematic. Despite the various food items used in broodstock maintenance and juvenile production and grow-out of *H. scabra*, including *Spirulina* spp, *Schizochytrium* spp, *Navicula* spp, homogenized *Sargassum* spp, ground shrimp feed, shrimp head meal, soya bean powder, rice bran, chicken manure and seagrass powder[1, 3, 11, 36], juveniles often grow much faster in sea pens or earthen ponds where no artificial feeding is conducted. Information about *H. scabra* diets is indispensable in improving hatchery, aquaculture and reseeding techniques, as well as monitoring environmental conditions for wild stocks.

H. scabra are benthic detritus feeders, which ingest muddy sandy sediment and assimilate the organic matter

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it contains. Previous studies suggested that *H. scabra* have the ability to identify sediments with higher organic content but have no food preference[18, 28]. The gut contents of *H. scabra* reportedly include bacteria, copepods, diatoms and fragments of macroalgae, molluscan shells, foraminiferans and sediments[18]; however, the relative contribution of each type of organic matter as a food source is poorly understood. Unlike, for instance, piscivorous fish whose gut contents can be easily recognized, it is very difficult to identify origins of detrital organic particles by visual observation, even with the aid of a microscope. It is also more difficult to determine which organic particles in the gut are assimilated by *H. scabra*. To ascertain assimilated food items, tracer analysis, such as stable isotope ratio method is useful.

Carbon and nitrogen stable isotope ratios ($\delta^{13}C$ and $\delta^{15}N$) are widely used for food web studies, in which assimilated food items are determined based upon stable isotopic fractionation (enrichment) between diet and animal tissues. It is generally accepted that isotopic fractionation has similar values (i.e. 0 – 1‰ for $\delta^{13}C$ and 2.6 – 4‰ for $\delta^{15}N$) regardless of food items or animal species[30, 31, 32, 34], and this criterion has been employed in numerous food web studies. However, some studies showed that isotopic fractionation is species- and tissue-specific and pointed out the need for laboratory determination of the fractionation of each species[10, 35, 47]. Diet-tissue fractionations of sea cucumbers are unknown, and in the few studies on the stable isotope ratio of sea cucumbers reported to date, commonly accepted fractionation values were assumed[15, 20, 42].

The body wall of echinoderms contains microscopic calcareous spicules (ossicles), which mainly comprise $CaCO_3$[26, 41, 45], and thereby possibly affect the measurement of $\delta^{13}C$ fractionation. Unlike the axial skeleton and exoskeleton of other marine organisms however, the mechanical removal of microscopic spicules is difficult. In this study, the stable isotopic fractionation between diet and tissues was determined in *H. scabra* juveniles with special reference to the influence of calcareous spicules on $\delta^{13}C$ analysis.

Materials and methods

1. Production of microalgal feed

Two species of diatoms (*Chaetoceros calcitrans* and *Navicula ramossisima*) were cultured as feed at the Aquaculture Department of the Southeast Asian Fisheries Development Center, (SEAFDEC/AQD) in Iloilo, the Philippines. To culture *C. calcitrans* (planktonic diatom), F-medium fertilizer[17], containing ferric chloride, sodium nitrate and sodium biphosphate, trace metals, vitamin stock, sodium silicate and sodium EDTA was used. For *N. ramossisima* (benthic diatom) culture, TMRL fertilizer (Tungkang Marine Laboratory)[24, 39], containing ferric chloride, sodium nitrate, sodium silicate and sodium biphosphate) was used. The diatoms were cultured under constant indoor conditions to avoid possible fluctuation in $\delta^{13}C$ and $\delta^{15}N$ values, and collected daily from the culture tanks before being used for the feeding trial. For stable isotope analysis, the diatoms were taken on the final day of the experiment and kept frozen at -80℃.

2. Mono-feeding culture of *H. scabra*

Newly hatched *H. scabra* larvae were obtained by the artificial spawning induction of broodstock at the sea cucumber hatchery of SEAFDEC/AQD on May 18, 2009. The broodstock used was collected in Lawi (10.328 N, 122.311 E) and Panobolon (10.248 N, 122.340 E), Guimaras, Philippines.

H. scabra larvae were fed with *C. calcitrans* until the initiation of settlement. Settlement commenced 14 days after hatching on corrugated plates propagated with *N. ramossisima*. During the transition stage from the pelagic to the benthic phase, the larvae and newly settled juveniles were fed with a mixture of *C. calcitrans* and *N. ramossisima* for 20 days. Mono-feeding rearing using *N. ramossisima* was subsequently conducted for 50 days. In a 50 L glass fiber tank, *H. scabra* juveniles (approximately 5 mm in body length, 0.01 g in body weight) were placed, and sufficient *N. ramossisima* was fed *ad libitum*. The tank was aerated, and the water was partially (75 - 80%) changed every 2 days after siphoning the excess food and feces at the bottom. No substrates were added to the tank. The rearing sea water was pumped off the coast of SEAFDEC/AQD, sand-filtered, UV-sterilized, and filtered once at 5 μm and 3 times with 1 μm filters before use respectively to minimize contamination of other food materials.

After 50 days of mono-feeding rearing, juveniles were randomly sampled (n = 10, 20.6 ± 7.07 mm in body length and 0.66 ± 0.46 g in body weight, ± SD) and kept frozen at -80℃ until stable isotope analysis. Feces of *H. scabra* juveniles collected from the rearing tanks were also kept frozen at -80℃ pending analysis.

3. Isolation of calcareous spicules from the *H. scabra* body wall

Spicules were isolated from *H. scabra* cultured at SEAFDEC/AQD (n = 10, 25 - 98 mm in body length). *H. scabra* were degutted, oven-dried at 50℃ for one week, and the dry weight was measured to the nearest 0.01 mg with a micro balance. The dried body was then cut into small pieces, placed in a 50 mL centrifuge tube, complete-

ly dissolved in 10% commercial bleach[36], rinsed with distilled water (DW) 3 times and oven-dried. The dry weight of the obtained spicules was measured to the nearest 0.01 mg to obtain the % content in the body wall and stored at room temperature until being analyzed.

4. Stable isotope analysis

H. scabra samples from mono-feeding culture were thawed in a refrigerator, the intestine and body wall were separated, the intestinal contents rinsed with DW, lyophilized, and ground into a fine powder. A fraction of the powdered samples was defatted by the conventional Folch method[12] using methanol and chloroform. *H. scabra* and feed samples were also defatted to eliminate the effect of lipids known to have a lower $\delta^{13}C$ than other tissue fractions[9, 43]. The defatted samples were then oven-dried at 50˚C overnight and sealed in a tin container to measure $\delta^{15}N$ and $\delta^{13}C$. To remove calcareous spicules (decarbonation), a fraction of the defatted body wall was also treated with ample 1N HCl overnight, rinsed with DW 3 times and oven-dried for the measurements. *H. scabra* feces and diatom samples were rinsed 3 times with DW, lyophilized, ground into a fine powder and measured after defatting (n = 3 for pooled and well-homogenized *N. ramossisima* and feces, and n = 1 for *C. calcitrans*). Spicule samples were measured without additional treatments.

The $\delta^{15}N$ and $\delta^{13}C$ of the prepared samples were analyzed using an EA-1108 elemental analyzer (Carlo Erba) coupled with an isotope ratio mass spectrometer (Finnigan Mat ConFlo II, Mat 252). The isotope ratios were expressed in the form of per mil (‰) deviation from international standards (i.e. fossil calcium carbonate for C and air for N): $\delta^{13}C$, $\delta^{15}N = (R_{sample} / R_{standard} -1) \times 1000$, where R is $^{13}C/^{12}C$ and $^{15}N/^{14}N$. The instrumental precision was 0.2‰[44]. Atomic carbon: nitrogen (C/N) ratios of the samples were analyzed at the same time.

5. Mathematical and statistical analyses

Diet tissue isotopic fractionation was calculated as the difference in $\delta^{15}N$ and $\delta^{13}C$ between the mean values in *N. ramossisima* and *H. scabra* tissues. The effect of acid treatment on the body wall $\delta^{13}C$ was examined by comparing $\delta^{13}C$ of the acid decarbonated body wall ($\delta^{13}C_{acid}$) and the spicule-free body wall ($\delta^{13}C_{spcl\ free}$), which was estimated based upon $\delta^{13}C$ of spicules ($\delta^{13}C_{spcl}$), $\delta^{13}C$ of the whole body wall containing spicules ($\delta^{13}C_{body\ wall}$) and spicule content in the body wall (S): $\delta^{13}C_{spcl\ free} = (\delta^{13}C_{body\ wall} - \delta^{13}C_{spcl} \times S)/(1 - S)$. The C/N ratio of the spicule-free body wall ($C/N_{spcl\ free}$) was also estimated based upon the carbon content in the whole body wall ($C_{body\ wall}$), spicule content in the body wall (S), carbon content in the spicule (C_{spcl}) and sample dry weight (W), and were compared with the C/N ratio of the acid decarbonated body wall (C/N_{acid}). The carbon content of the spicule-free body wall ($C_{spcl\ free}$) was estimated as follows: $C_{spcl\ free} = C_{body\ wall} - (W \times S \times C_{spcl})$, and $C/N_{spcl\ free} = C_{spcl\ free} / N_{body\ wall}$, where $N_{body\ wall}$ is the nitrogen content of the whole body wall.

The difference in the $\delta^{15}N$, $\delta^{13}C$ and C/N ratios was tested by 1-way analysis of variance (ANOVA) and a Tukey test for a posteriori comparison among samples. To compare the mean values of a pair of data, a two-tailed t-test was used. A P value of less than 0.05 was considered statistically significant.

Results

1. $\delta^{15}N$ and $\delta^{13}C$ of *H. scabra* after mono-feeding rearing

Although *C. calcitrans* and *N. ramossisima* are both diatoms, they had largely different stable isotope signatures: $\delta^{15}N$ and $\delta^{13}C$ of *C. calcitrans* were -1.0 and -22.2‰ (n = 1), respectively; those of *N. ramossisima* were -7.5 ± 0.1 (± SD, n = 3) and -17.6 ± 0.1‰, respectively (Fig.1).

The mean $\delta^{13}C$ of the whole body wall and intestine of *H. scabra* juveniles after mono-feeding rearing with *N. ramossisima* was -13.4 ± 0.5 and -15.3 ± 0.6‰ (n = 10), respectively, and the mean $\delta^{15}N$ of the body wall and intestine was -5.1 ± 1.1 and -6.0 ± 0.9‰, respectively (Fig.1). The mean $\delta^{13}C$ and $\delta^{15}N$ of feces (n = 3) was -17.7 ± 0.02 and -7.8 ± 0.2‰, respectively (Fig. 1). Isotopic differences between *N. ramossisima* and the whole body wall and

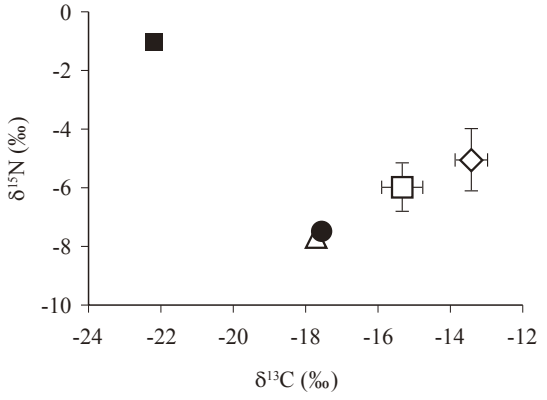

Fig. 1. $\delta^{13}C$ and $\delta^{15}N$ of *H. scabra* juvenile after 50 day mono-feeding culture

◇: whole body wall (n = 10), □: intestine (n = 10), △: feces (n = 3), ●: *N. ramossisima* (n = 3), ■: *C. calcitrans* (n = 1). *C. calcitrans* was fed to the larvae prior to the mono-feeding culture of juveniles with *N. ramossisima*. Error bars show standard deviation.

the intestine were significant ($P < 0.01$) except for $\delta^{15}N$ between *N. ramossisima* and the intestine ($P = 0.07$).

2. Effects of acid treatment on $\delta^{13}C$, C/N ratio and $\delta^{15}N$

The dry weight content of spicules in the body wall varied greatly, ranging from 18.3 to 56.2%, with a mean of 35.1 ± 13.0% ($n = 10$). The carbon weight content of spicules ranged from 10.7 to 12.7%, with a mean of 12.1 ± 0.8% (Table 1). The mean $\delta^{13}C$ of spicules was -8.6 ± 2.2‰ (Table 1), while the amount of nitrogen in the spicules was below the detection level of the elemental analyzer in all analyzed samples.

The mean C/N ratio of the whole body wall (4.6 ± 0.2, $n = 10$) was reduced by acid decarbonation (3.4 ± 0.1) to a value significantly lower than that of the spicule-free body wall (3.7 ± 0.1) (Fig.2, $P < 0.0001$). The mean $\delta^{13}C$ significantly decreased in the order of the whole body wall (-13.4 ± 0.5‰), acid decarbonated body wall (-14.4 ± 0.4‰) and spicule-free body wall (-16.0 ± 0.7‰) (Fig.2, $P < 0.0001$). Acid decarbonation significantly elevated the mean $\delta^{15}N$ in the body wall from -5.1 ± 1.1 to -4.4 ± 0.8‰ (Fig. 2, $P < 0.001$).

Table 1. Spicule content in the body wall, and $\delta^{13}C$ and carbon content of spicule in *H. scabra* juvenile

Spicule content (% dry weight ± SD)	Spicule $\delta^{13}C$ (‰ ± SD)	Spicule C content (% dry weight ± SD)
35.1 ± 13.0	-8.6 ± 2.2	12.1 ± 0.8

3. Diet-tissue fractionations

Diet-tissue fractionations were calculated based upon the above values and are summarized in Table 2. $\delta^{13}C$ fractionation in the whole body wall (4.2 ± 0.5‰) exceeded that in the acid decarbonated body wall (3.2 ± 0.4‰), intestine (2.2 ± 0.6‰) and spicule-free body wall (1.5 ± 0.7‰), while the $\delta^{15}N$ fractionation in the acid decarbonated body wall (3.1 ± 0.8‰) exceeded that in the whole body wall (2.4 ± 1.1‰) and intestine (1.5 ± 0.9‰). The differences in fractionation amongst these values were all significant ($P < 0.001$). Isotopic fractionations between *N. ramossisima* and feces were very small ($\delta^{13}C$: -0.10 ± 0.02‰, $\delta^{15}N$: -0.27 ± 0.2‰).

4. Food item and feces

The mean C/N ratio of feces of *H. scabra* juveniles fed with *N. ramossisima* (6.7 ± 0.06) significantly exceeded that of *N. ramossisima* (4.7 ± 0.006) (Fig. 3, $P < 0.01$, $n = 3$). There was no significant difference in $\delta^{15}N$ and $\delta^{13}C$ between *N. ramossisima* and the feces ($P > 0.1$, $n = 3$).

Discussion

Although information on food requirements is essential for improving aquaculture and stock enhancement techniques, little is known about the food assimilation of *H. scabra* due to its detrital feeding habit[18]. $\delta^{13}C$ and $\delta^{15}N$ fractionation between diet and animal tissue is a consistent

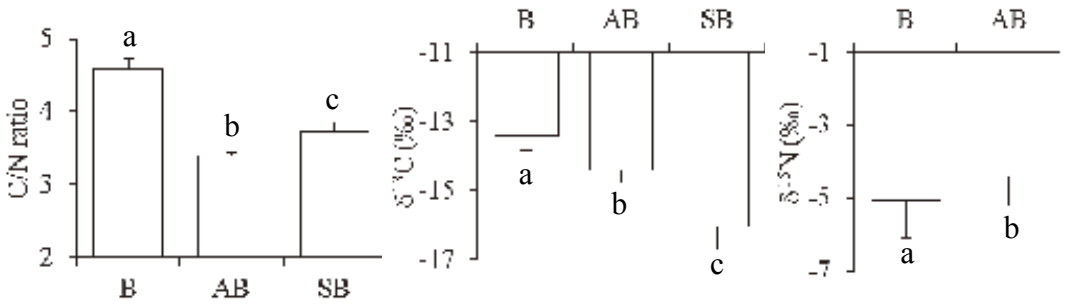

Fig. 2. C/N ratio, $\delta^{13}C$ and $\delta^{15}N$ of the body wall, acid decarbonated body wall and spicule-free body wall of *H. scabra* juvenile

B: body wall ($n = 10$), AB: acid decarbonated body wall ($n = 10$), SB: spicule-free body wall ($n = 10$). Error bars show standard deviation. Different letters (a, b and c) indicate statistically significant differences ($P < 0.05$).

feature of stable isotope studies to determine assimilated food. In this study, diet-tissue fractionations (enrichments) in $\delta^{13}C$ and $\delta^{15}N$ of *H. scabra* were determined by mono-feeding rearing as a basis for stable isotope analysis.

The negative $\delta^{15}N$ values of *N. ramossisima* and *H. scabra* fed with *N. ramossisima* are highly unusual in natural environments, which may be attributed to the artificial sodium nitrate contained in the TMRL fertilizer for the algal culture. The unusual $\delta^{15}N$ values are useful in confirming the absence of contamination of naturally available food sources. Large difference in $\delta^{15}N$ between *C. calcitrans* and *N. ramossisima* is also considered attributable to the difference in dissolved inorganic nitrogen in the artificial fertilizers. The difference in $\delta^{13}C$ between *C. calcitrans* and *N. ramossisima* may be attributable to that between planktonic and benthic lifestyle associated with boundary layer diffusion resistance[13].

This study did not examine the time-course change in the $\delta^{13}C$ and $\delta^{15}N$ of *H. scabra* juveniles during mono-feeding rearing with *N. ramossisima*. However, based upon the approximate 66-fold increase in body weight of the juveniles during the mono-feeding period, the effects of residual tissues from the larval and settlement periods, during which *C. calcitrans* was used as the diet, on stable isotope ratios at the time of sampling are considered negligible (i.e. only a 1.5% contribution in the complete absence of tissue turnover). Thus, it is safe to ascertain that the $\delta^{13}C$ and $\delta^{15}N$ values of the *H. scabra* juveniles had already peaked at sampling.

The mean $\delta^{15}N$ fractionation values obtained for the whole body wall of *H. scabra* juveniles after 50 days' mono-feeding with *N. ramossisima* (2.4‰) resembled the commonly accepted range of 2.6 – 4‰, while the mean $\delta^{13}C$ fractionation for the whole body wall (4.2‰) was about four times higher than the common range of 0 – 1‰[30, 31, 32, 34]. The mean $\delta^{15}N$ fractionation for the intestine (1.5‰) was about half the common range, while the mean $\delta^{13}C$ fractionation for the intestine (2.2‰) was about double the common range. Therefore, the use of the generally accepted $\delta^{13}C$ fractionation values for the whole body wall and intestine of *H. scabra* can result in erroneous interpretation of the data, resulting in up to a fourfold overestimation of trophic shift and a mismatch with the $\delta^{15}N$ interpretation.

Some studies indicate that isotopic fractionation is species- and tissue-specific[35, 47]. DeNiro & Epstein (1978)[10] indicated that different fractionation values seen in different tissues depend upon the relative distribution of component fractions, such as lipids, proteins and carbohydrates. Since the fat contents of organisms often change seasonally, and lipid is known to have a lower $\delta^{13}C$ than other tissue fractions[9, 43], samples for stable isotope analyses are generally defatted to reduce this variation. Although the samples in this study were defatted, there was still a large discrepancy in $\delta^{13}C$ fractionation between the body wall and the commonly accepted value.

In this study, it was hypothesized that the presence of spicules may affect the $\delta^{13}C$ of the body wall. Echinoderms have calcareous spicules composed mostly of magnesian calcite ($CaCO_3$ containing about 5% $MgCO_3$) and a small amount of proteinaceous matrix (about 0.1%) occluded within the spicules[41, 45]. Compared to asteroids and echinoids, less is known about the molecular contents and formation process of the spicules in holothurians. This study indicated that *H. scabra* spicules are also composed mostly of $CaCO_3$; namely the obtained carbon weight content (12.1 ± 0.8%) of the spicules agreed well with the carbon content of $CaCO_3$ (i.e. 12%). Inorganic carbon composing the spicules may affect the $\delta^{13}C$ of the whole body wall if it has a $\delta^{13}C$ value differing significantly from the organic fractions of the body tissues. In this study, spicules were found to have significantly higher $\delta^{13}C$ (-8.6‰) than the

Table 2. Diet-tissue isotopic fractionation of *H. scabra* juvenile fed with *N. ramossisima*

H. scabra	$\delta^{15}N$ (‰ ± SD)	$\delta^{13}C$ (‰ ± SD)
Whole body wall	2.4 ± 1.1	4.2 ± 0.5
Acid-decarbonated body wall	3.1 ± 0.8	3.2 ± 0.4
Spicule-free body wall	---	1.5 ± 0.7
Intestine	1.5 ± 0.9	2.2 ± 0.6
Feces	-0.3 ± 0.2	-0.1 ± 0.02

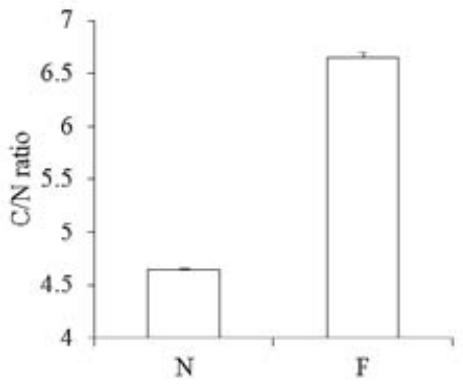

Fig. 3. C/N ratio of feces of *H. scabra* juvenile fed with *N. ramossisima*

F: feces (n = 3), N: *N. ramossisima* (n = 3) Error bars show standard deviation. Different letters (a and b) indicate statistically significant differences ($P < 0.01$).

whole body wall (-13.4‰, $P < 0.001$), and they occupied a considerable fraction (35.1%) of the body wall by dry weight. By computationally removing the effect of the spicules, it was shown that the $\delta^{13}C$ fractionation in the organic fractions of the body wall (1.5‰) resembled the commonly accepted range of 0 – 1‰. Thus, it is possible to say that the high $\delta^{13}C$ fractionation value exhibited by the whole body wall may be substantially explained by the spicule content. The reasons for unusual $\delta^{13}C$ fractionation value seen in the intestine, which does not contain spicules, however, are unknown. Since *H. scabra* juveniles were fed with *N. ramossisima* cultured under constant conditions, the difference in tissue turnover rate between the intestine and body wall (i.e. the difference in time lag due to the diet shift)[19, 25] did not significantly affect stable isotope signatures. Further studies on the $\delta^{13}C$ value of fraction components in the intestine, especially protein and carbohydrate[8], are necessary.

Although the $\delta^{13}C$ fractionation of the acid decarbonated body wall (3.2‰) was smaller than that of the whole body wall (4.2‰), it was still about three times higher than the common range. Acid decarbonation seemed to remove spicules as indicated by the decrease in the C/N ratio (i.e. spicules contain C but not N, meaning their removal decreases the C/N ratio). However, the C/N ratio (3.4) was significantly smaller than that calculated for the spicule-free body wall (3.8). These indicate that compounds other than spicules may also be removed by the acid decarbonation process. This is also evinced by the fact that acid decarbonation significantly affected the $\delta^{15}N$ fractionation value of the body wall, as is also reported in fish[35]. Jecob et al. (2005)[21] reported that acid treatment showed a significant effect on $\delta^{13}C$ and $\delta^{15}N$ in various invertebrates and fish samples containing no $CaCO_3$, and suggested that rinsing acid-treated samples with DW may remove water and acid soluble contents from them, causing a bias in $\delta^{13}C$ and $\delta^{15}N$. Thus, acid decarbonation of spicules is not recommended for the sample preparation of stable isotope analysis in *H. scabra*.

It is recommended to calculate spicule-free $\delta^{13}C$ for each *H. scabra* samples by the formula employed in this study: $\delta^{13}C_{spcl\ free} = (\delta^{13}C_{body\ wall} - \delta^{13}C_{spcl} \times S)/(1 - S)$, since it is unknown whether the $\delta^{13}C$ fractionation obtained for the whole body (4.2‰) is constant in other developmental stages and geological locations. The spicule content in the body wall also showed considerable individual variation. Echinoderms are considered to indirectly deposit $CaCO_3$ to the spicules after extracting dissolved calcium and bicarbonate ions (HCO_3^-) from sea water. The source of the carbon of sea urchin spicules is considered to pass through primary mesenchyme cells, which are responsible for spicule formation, and carbon from sea water does not directly precipitate in spicules[45]. A study using radioactive tracers (^{45}Ca and ^{14}C) showed that respiratory CO_2 contributes to the carbon supply for spicule formation in sea urchins[41]. Similar results are also reported in other animal groups. In molluscan bivalves, 10 – 30% of carbon incorporation in the shells is derived from metabolic CO_2[2, 16, 27], while species-dependent, non-equilibrium $\delta^{13}C$ fractionation effects are known to occur in the formation of calcareous tests in foraminiferans[40]. Similar studies should be performed on holothurians to elucidate the relative carbon supply of HCO_3^- in sea water and metabolic CO_2 in spicule formation.

Since differences in $\delta^{13}C$ and $\delta^{15}N$ between *N. ramossisima* and feces of *H. scabra* fed with *N. ramossisima* were insignificant, fecal stable isotope ratios may be used as proxies for ingested (as opposed to assimilated) organic matter of *H. scabra* in the wild. Fecal stable isotopes are widely used in diet studies in mammals and birds[6, 23], since fractionation is not considered to occur during food assimilation[14]. However, a recent study[7] indicated that while fecal $\delta^{13}C$ is a reliable proxy for diet in mammals, $\delta^{15}N$ is altered by digestion for unidentified reasons contingent on diet composition. Further study on the diet-fecal $\delta^{15}N$ relationship in *H. scabra* should be conducted using different food items to confirm the validity of fecal isotope proxies. A possible drawback of using feces for stable isotope analysis, however, is that the data may not represent organic matter integrated over time, but only that of data immediately before sampling.

Further studies using the stable isotopic fractionation shown in this study may help elucidate suitable food items for the seed production and aquaculture of *H. scabra*, as well as suitable conditions for co-culture with other organisms.

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