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Article

Compression of trophic discrimination in $^{15}N/^{14}N$ within amino acids for herbivorous gastropods

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Abstract

Nitrogen isotope ratios of amino acids have been widely used to estimate the trophic position of organisms in ecological food webs. However, un-explainable compression/expansion of the trophic discrimination factor (TDF) has been reported in diverse consumer species. In particular, significant compression has been reported in herbivorous gastropods and bivalves, resulting in the estimated trophic position of them is lower than 2.0. In the present study, we report three diverse TDF for the gastropods *Turbo sazae* (7.5±0.5‰), *Haliotis discus* (5.3±1.0‰), and *Lyncina vitellus* (3.0±0.7‰), as TDF found in *H. discus* and *L. vitellus* is considerably smaller than that generally found in many species (7.6±1.3‰). The compression of TDF, however, well corresponds to the amount of proteinous mucus released from these species, implying that a part of the metabolic flux of amino acids is used for the production of proteinous mucus. Therefore, we suggest that understanding of TDF for consumer species with respect to 'balance of metabolic flux' between deamination of amino acids (i.e., energy production) and construction of own proteins including additional proteinous materials is required, to enhance accuracy in the application of nitrogen isotope ratios of amino acids to the food web studies.

1. Introduction

The illustration of food web structure and its complex network among species in ecosystems is one of the major challenges in natural sciences including biology, ecology, and biogeochemistry, which is highly useful for better understanding the role of energy transfer through diverse organisms in the biosphere. Stable isotope analysis of bulk nitrogen ($^{15}N/^{14}N$) and carbon ($^{13}C/^{12}C$) in organisms and their tissues has been widely used in diverse food web studies, owing to the general trend of trophic enrichment in ^{15}N (by 3-4‰) and ^{13}C (0-1‰) between diet and consumer species (e.g., DeNiro and Epstain, 1978; Minagawa and Wada, 1984). More recently, applying gas chromatography/isotope ratio mass spectrometry (GC/IRMS), we dramatically updated the food web studies by compound-specific isotope analysis of nitrogen within amino acids (e.g., McCelland and Montoya, 2002; Chikaraishi et al., 2007; McCarthy et al., 2007). For instance, the trophic position (i.e., $TP_{x/y}$: position of organisms in the trophic hierarchy of food webs) for diverse species (e.g., Chikaraishi et al., 2014) has been 'simply' calculated by comparison between nitrogen isotope ratios of two types of amino acids ('trophic' represented by glutamic acid; and 'source' represented by phenylalanine), with the following equation (1), suggested by Chikaraishi et al. (2009):

$$TP_{x/y}=[(\delta^{15}N_x-\delta^{15}N_y+\beta_{x/y})/TDF_{x/y}]+1 \quad (1)$$

where $\beta_{x/y}$ represents the isotopic difference between amino acids x and y in primary producers (e.g., phytoplankton or higher plants, in general) at the bases of food web, and $TDF_{x/y}$ represents difference in the trophic discrimination factor (TDF; i.e., change in the $\delta^{15}N$ value during each shift of trophic level in the food web pyramid) between amino acids x and y ($TDF_{x/y}=TDF_x-TDF_y$). The error of trophic positions estimated with nitrogen isotope ratios of glutamic acid and phenylalanine ($TP_{glu/phe}$) is known as better (within 0.2 unit) than the that estimated with nitrogen isotope ratios of bulk organisms (larger than 0.5 unit, e.g., Bowes and Thorp, 2015). This new methodology hence has widely applied for many ecological studies during the last decade (e.g., Steffan et al., 2013; Chikaraishi et al., 2014; Lorrain et al., 2015; Choi et al., 2017).

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In the application of this new methodology, universality on the scale of $TDF_{glu/phe}$ (i.e., the stepwise enrichment in ^{15}N between glutamic acid and phenylalanine) is required, to calculate the accurate and precise TP of organisms in diverse ecosystems. Chikaraishi et al. (2009) first established that $TDF_{glu/phe}$ is 7.6±1.3‰ (based on TDF_{glu}=8.0±1.2‰ and TDF_{phe}=0.4±0.5‰) for marine zooplankton and fish, and this value is further confirmed in diverse organisms including fungi, bacteria, insects, fish, and mammals (e.g., Steffan et al., 2015). However, the compression of $TDF_{glu/phe}$ is frequently found in high-TP ordered consumers such as gentoo penguin (McMahon et al., 2015) and harbor seals (Germain et al., 2013), whereas the expansion of that is certainly found in flowers and sprouts in some plant species (Takizawa and Chikaraishi 2014; Takizawa et al., 2017). These results imply the presence of a large variation in TDF among natural organisms (McMahon and McCarthy, 2016). Several processes have been hypothesized to explain the variability of TDF: compression caused by the nitrogen excretion system for ammonia and urea/uric acid (Germain et al., 2013); and expansion caused by phenological increase in the deamination of amino acids (Takizawa and Chikaraishi, 2017; Takizawa et al., 2017). These metabolic isotope consequences can thus induce the apparently un-explainable $TP_{glu/phe}$ in organisms (e.g., TP>1, 'heterotrophy' even for primary producers). It is noted that unexpected low $TP_{glu/phe}$ (i.e., compression of TDF) was also reported for the herbivorous gastropods and bivalves in the previous studies (e.g., TP=1.7 for *Haliotis discus*, Chikaraishi et al., 2014; TP=1.0-1.8 for *Mytilus californianus*, Vokhshoori and McCarthy, 2014; TP=1.6-1.8 in *Limecola balthica*, Ek et al., 2018), although any specific mechanisms responsible for the compression of TDF (resulting in low TP) have not yet been proposed.

Inherently, the scale of TDF should be correlated with the metabolic flux of amino acids in organisms, as between expansion into life energy production and deposit into biomass construction. Indeed, Goto et al. (2018) demonstrated that nitrogen isotopic fractionation associated with the deamination of glutamic acid well follows the Rayleigh fractionation model with the fractionation factor (α) being 0.9938±0.0005:

$$\delta^{15}N_{glu,t}=(1000+\delta^{15}N_{glu,0})\times F^{(0.9938-1)}-1000 \qquad (2)$$

where $\delta^{15}N_{glu,0}$ and $\delta^{15}N_{glu,t}$ represent the isotopic ratios of glutamic acid presenting before and remaining against deamination, respectively; and F ($0<F<1$) represents the proportion of the remaining pool of glutamic acid against deamination. According to this equation (2), 8.0±1.2‰ for TDF_{glu} corresponds to that 72±3% of the diet-derived glutamic acid is deaminated in organisms at each trophic shift in food webs. Also, the compression and expansion of TDF can be explained by less and much metabolic flux to the deamination for glutamic acid in organisms, respectively.

In the present study, we first attempted to explain the compression of TDF (i.e., TP<2) in herbivorous gastropods and bivalves, based on the metabolic flux specific to these species. Some species of gastropods such as abalone generate proteinous mucus to enhance settlement ability (Tamaki et al., 1997). Also mussels produce proteinous byssus to settle at the subfloor (Waite et al., 1998). According to this knowledge, we hypothesize that the production of proteinous materials will change the metabolic balance regarding to amino acids in specific herbivorous gastropods and bivalves. We therefore determined nitrogen isotope ratios of amino acids ($\delta^{15}N_{AAs}$) of three species of herbivorous gastropods that have significant difference in the production of proteinous mucus, to evaluate the relationship between the scale of TDF and the production of proteinous mucus. Furthermore, we discuss the how the metabolic balance of amino acids controls the $\delta^{15}N_{AAs}$ values in organisms.

2. Materials and Methods

Sampling of gastropods and proteinous mucus

Samples were collected in 2015 from a stony shore in Yugawara (35.15°N, 139.13°E), Japan. We caught three specimens for each of the three gastropods *Turbo sazae* (reported as *Batillus cornutus*, in Chikaraishi et al., 2014), *Haliotis discus*, and *Lyncina vitellus* with similar size within species (Table 1) by hand. Samples were immediately cleaned with streamed seawater in sampling site, to remove other materials on the shell surface. The cleaned samples were put individually into a plastic bag with 50 ml of seawater, and thoroughly shook for 5 minute by hand to release the proteinous mucus. The proteinous mucus released (in sea water) and gastropod muscle remained were separated, dried with a freeze drier, and homogenized with a Tube-Mill (IKA).

Analysis of $\delta^{15}N_{AAs}$

The nitrogen isotope ratios of glutamic acid and phenylalanine in respective muscle and proteinous mucus from three specimens of each species of the gastropods were determined by GC/IRMS, according to the procedure described in Chikaraishi et al. (2009). Briefly, the sample was hydrolyzed by 12M of HCl at 110ºC for approximately 12 hours. After removal of hydrophobic materials with *n*-hexane/dichloromethane (3/2, v/v), the hydrolysate was derivatized with thionyl chloride/2-propanol (1/4, v/v) for 2 h at 110ºC and subsequently pivaloyl chloride/dichloromethane (1/4, v/v) for 2 h at 110ºC. The derivatives were then extracted with *n*-hexane/dichloromethane (3/2, v/v). The $\delta^{15}N_{AAs}$ values were determined with a GC/IRMS instrument

Table 1. Mean (1σ standard deviation, $n=3$) of proteinous mucus and shell size for three gastropods analyzed in the present study.

	Turbo sazae		*Haliotis discus*		*Lyncina vitellus*	
	Mean	1σ	Mean	1σ	Mean	1σ
Shell length (mm)	40.0	0.0	35.0	1.0	19.7	0.6
Shell hight (mm)	30.7	1.2	49.0	1.7	30.3	0.6
Shell width (mm)	29.3	1.2	12.0	0.0	14.7	0.6
Dry Wt. of mucus with 50ml of seawater (mg)	1253.4	1.4	1256.1	0.9	1267.3	2.0
Corrected dry wt. of mucus (mg/L)[1]	0.0	28.4	53.3	17.8	276.7	40.8

[1]Normalized with the dry weight of seawater for *T. sazae*.

using an isotope ratio mass spectrometer (Thermo Finnigan Delta Plus XP) coupled to a gas chromatograph (Agilent Technologies 6890N) linked via a GC-C/TC III interface. Combustion and reduction furnaces were used at 950ºC and 550ºC respectively, and a programmable temperature vaporizing (PTV) injector (Gerstel) and an HP ultra-2 capillary column (50 m length, 0.32 mm i.d., 0.52 μm film thickness, Agilent Technologies) were used in the GC. Helium was used as the carrier gas in a constant flow mode at a rate of 1.4 mL/min, and the GC oven temperature program was set according to the procedure described in Chikaraishi et al. (2009).

Calculation of the $TP_{glu/phe}$ and TDF

$TP_{glu/phe}$ was calculated using equation (3), which is given by equation (1):

$$TP_{glu/phe} = [(\delta^{15}N_{glu} - \delta^{15}N_{phe} - \beta)/TDF] + 1 \quad (3)$$

where 7.6‰ and 3.4‰ were used for TDF and β, respectively, according to Chikaraishi et al. (2009). Species-specific TDF was also calculated as equation (3), with the assumption that TP of gastropods is exactly 2.0, as the functional trophic position of herbivores.

3. Result and discussion

The weight of dried seawater including proteinous mucus is slightly different among the three species. Since amino acids are not detected in the mucus fraction from *T. sazae*, it can be employed as blank to calculate amount of proteinous mucus extracted from *H. discus* and *L. vitellus*. The proteinous mucus from *L. vitellus* is abundant compared to that from *H. discus*, although the size of shell of the former is much smaller than that of latter (Table 1).

Nitrogen isotope ratios of phenylalanine ($\delta^{15}N_{phe}$) of the muscle tissue are similar and have little variation among the three species (3.6±1.1‰ for *T. sazae*; 3.4±0.9‰ for *H. discus*; 3.9±1.7‰ for *L. vitellus*), which are also fallen in the $\delta^{15}N_{phe}$ range for diverse species (e.g., seaweeds, gastropod, crab, and fish) collected from the same stony shore in the previous study (Chikaraishi et al., 2014). It is known that the $\delta^{15}N_{phe}$ value can be useful as an indicator of nitrogen isotopic baseline for the habitat of organisms of interests, because of the negligible trophic elevation in the $\delta^{15}N_{phe}$ value from diets to consumers (e.g., reviewed in Ohkouchi et al., 2017). Hence, no substantial difference in the $\delta^{15}N_{phe}$ value among these three species in this study and diverse species in the previous study clearly indicates that the three species are grown within the same stony shore in Yugawara.

On the other hand, nitrogen isotope ratios of glutamic acid ($\delta^{15}N_{glu}$) have a great diversity among the three species (14.5±1.6‰ for *T. sazae*; 12.2±1.7‰ for *H. discus*; 10.3±1.5‰ for *L. vitellus*). It is known that the subtraction of the $\delta^{15}N_{phe}$ value from the $\delta^{15}N_{glu}$ value within a single organism can be useful as an indicator of the trophic position of organisms in food webs, because of the negligible (for phenylalanine) and considerable (for glutamic acid) elevations in the $\delta^{15}N$ value from diets to consumers (e.g., reviewed in Ohkouchi et al., 2017). Appling the equation (3) with TDF=7.6‰, we estimate that the $TP_{glu/phe}$ is 2.0 for *T. sazae* but 1.7 for *H. discus* and 1.4 for *L. vitellus*. The $TP_{glu/phe}$ of the former two species is consistent with that of the different individual, belonging to the same species in the previous studies (Chikaraishi et al., 2014), although no study has reported so far the $TP_{glu/phe}$ of *L. vitellus*. The intermediate $TP_{glu/phe}$ between 1.0 and 2.0 (i.e., mixotrophic strategy) is, ecologically irrational for the trophic function (i.e., herbivores) of these gastropods. Thus, as previous studies (e.g., Vokhshoori and McCarthy, 2014) have suggested, these un-explainable $TP_{glu/phe}$ are potentially derived from the compression of the TDF specific to the gastropods. Moreover, because the presence of different metabolic reactions/processes/pathways within similar taxonomic group is very unlikely, it is predicted that the

Table 2. Trophic discrimination factor ($TDF_{glu/phe}$) for the muscle and proteinous mucus.

Species	Muscle			Proteinous mucus			Δ^1		
	Individual	Mean	1σ	Individual	Mean	1σ	Individual	Mean	1σ
	7.0			-			-		
Turbo sazae	7.5	7.5	0.5	-			-		
	8.0			-			-		
	6.5			6.8			-0.3		
Haliotis discus	4.9	5.3	1.0	5.1	5.4	1.3	-0.2	-0.1	0.3
	4.6			4.3			0.3		
	2.8			2.5			0.3		
Lyncina vitellus	3.7	3.0	0.7	3.2	2.5	0.6	0.5	0.4	0.1
	2.4			1.9			0.4		

[1] Δ indicates offset of TDF between muscle and proteinous mucus.

compression is caused by the variation in the metabolism flux of amino acids among the gastropod species.

The species-specific TDF varies among the three species of gastropods: 7.5±0.5‰ for *T. sazae*; 5.3±1.0‰ for *H. discus*; and 3.0±0.7‰ for *L. vitellus.* The TDF for *T. sazae* is very similar or almost identical to the standard value (i.e., 7.6‰) reported in the previous study (Chikaraishi et al., 2009), whereas that for the other two species is lower than that for *T. sazae* as well as the standard value (Table 2, $p<0.05$). These results imply that TDF is variable but not substantially constant within gastropods. Interestingly, the compressed TDF is apparently, inversely correlated with amount of the proteinous mucus in these species (Tables 1 and 2). According to the general rule of isotopic mass balance, the compression of TDF for the muscle tissue will be resulting in the expansion of TDF for other materials (i.e., proteinous mucus is a candidate in this study). However, no substantial difference in the $\delta^{15}N$ value (and also TDF) of glutamic acid and phenylalanine is found between muscle and proteinous mucus in both gastropods (*Haliotis discus* and *Lyncina vitellus*) (Fig. 1b, Table 2), indicating no further isotopic fractionation associated with the production of proteinous mucus in the species. Thus, it seems that both muscle tissue and proteinous mucus are isotopically homogeneous or are composed of almost the same-ordered metabolized amino acids within a species, but the order is different among the three species of gastropods.

Goto et al. (2018) demonstrated that TDF of glutamic acid is highly dependent on the flux of deamination based on the Rayleigh fractionation model. Applying to the species-specific TDF of the gastropods in this study, we expect that the flux of deamination is approximately 0.72 for *T. sazae* and small proportion for both *H. discus* and *L. vitellus*. To estimate the metabolic flux, we used the following equation (4), which is given by the equation (2), according to Goto et al. (2018):

$$\delta^{15}N_{glu,\ gastropod} = (1000 + \delta^{15}N_{glu,\ diet}) \times F^{(0.9938-1)} - 1000 \quad (4)$$

In the equation (4), F indicates remaining pool of glutamic acid after deamination. The $\delta^{15}N_{glu}$ value in the diet resources ($\delta^{15}N_{glu,\ diet}$) is estimated using an average $\delta^{15}N_{phe}$ value in the three species of gastropods ($\delta^{15}N_{phe,\ gastropod}$) with the following equation (5).

$$\delta^{15}N_{glu,\ diet} = \delta^{15}N_{phe,\ gastropod} - 0.4 + 3.4 \quad (5)$$

where 0.4 and 3.4 are derived from the TDF_{phe} and β value, respectively (Chikaraishi et al. 2009). By solving the equation (4), $[1-F]$ for *T. sazae* is 0.71, as large as the value (0.72) for the standard TDF (8.0‰) species. In contrast, $[1-F]$ for *H. discus* and *L. vitellus* is 0.58 and 0.43, respectively, being significantly lower than the standard value, by 0.14 and 0.29, respectively (Fig. 2). These results suggest that the compression of TDF found in *H. discus* and *L. vitellus* reflects a reduced isotopic fractionation responsible for low flux on the deamination of amino acids. The results also imply that the abundance of amino acids dedicated to the energy production is small in the gastropods than in other herbivores. This is probably consistent with negative correlation between TDF and abundant proteinous mucus in the gastropods.

In this study, there is abundant proteinous mucus from the gastropods that have compression of TDF (Table 1 and 2). Also, no substantial difference in the TDF is found between proteinous mucus and muscle for these gastropods (Fig. 1b), indicating that both mucus and muscle are composed of almost the same order metabolized amino acids within a species. Moreover, according to the Rayleigh model, we found that the size of TDF for the gastropods is reflected by variation in the deamination flux of amino acids (Fig. 2). Based on these results, we hypothesize that the diverse of TDF within gastropods is explained by the metabolic fate and its flux of dietary proteins as follows:

1. TDF of amino acids in consumers is highly dependent on the balance of metabolic flux between deamination and assimilation of amino acids (Fig. 3a). However, we expect that this balance is generally similar among diverse species, as $[1-F]$ is approximately 0.72, because

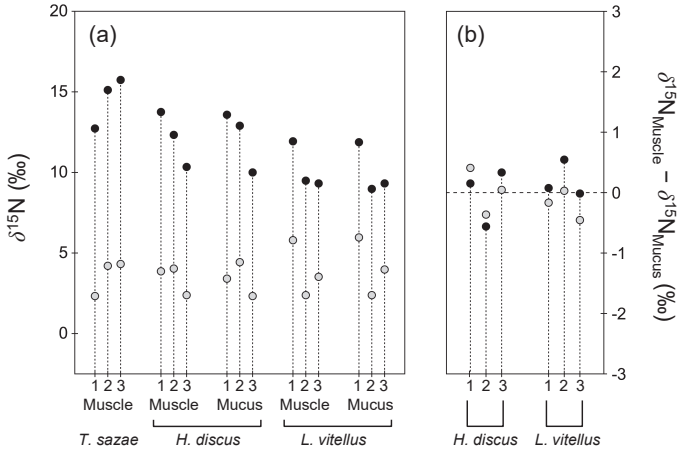

Fig. 1. (a) Nitrogen isotope ratios of glutamic acid ($\delta^{15}N_{glu}$, black filled cycle) and phenylalanine ($\delta^{15}N_{phe}$, gray filled cycle) of muscle tissue and proteinous mucus for three specimens of each gastropods species, and (b) difference in the δ^{15}N value. Nitrogen isotopic difference between muscle tissue and proteinous mucus in the *Haliotis discus* and *Lyncina vitellus*.

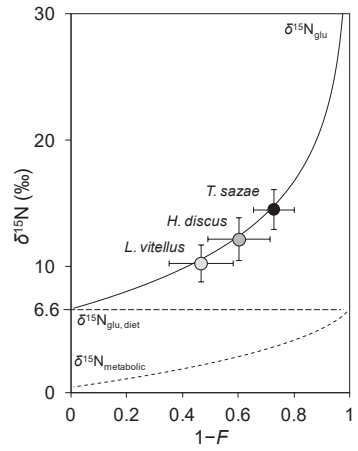

Fig. 2. Relationship between deamination flux [1−*F*] and $\delta^{15}N_{glu}$ value on the Rayleigh fractionation model: nitrogen isotopic fractionation of glutamic acid with $\alpha = 0.9938$ (Goto et al. 2018). The $\delta^{15}N_{metabolic}$ value is determined by two source mass balance, $\delta^{15}N_{metabolic} = [\delta^{15}N_{glu, diet} - (\delta^{15}N_{glu} \times F)]/(1-F)$.

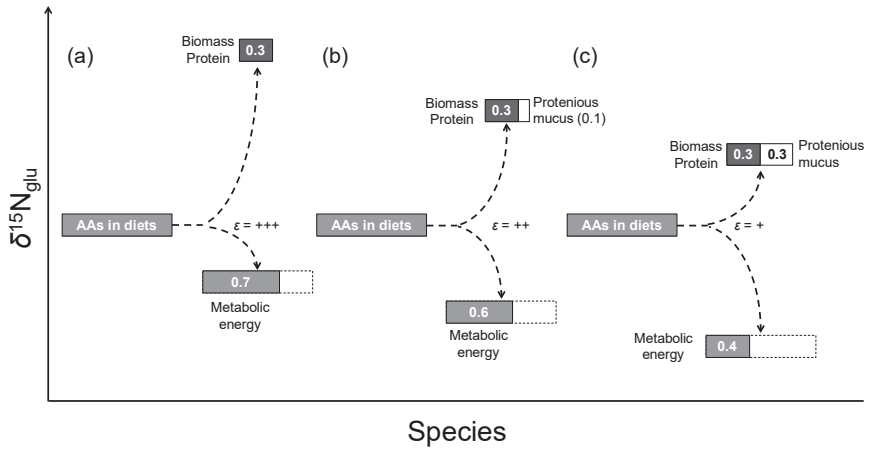

Fig. 3. Schematic view of the isotopic fractionation (ε) in amino acid metabolism associated with the balance among the production of metabolic energy, biomass, and additional proteinous materials, for the three gastropods species (a) *Turbo sazae,* (b) *Haliotis discus*, and (c) *Lyncina vitellus*.

7.6 ± 1.3‰ for $TDF_{glu/phe}$ is generally applicable to diverse species.

2. Some consumer species have distinct scale of TDF for amino acids, because specific production of additional proteinous materials can change the balance of the metabolic fate and its flux of amino acids. For instance, several gastropods such as *H. discus* and *L. vitellus* have a small [1−*F*], which are probably associated with the specific production of proteinous mucus. TDF is thus variable regarding to the production rate of proteinous mucus (Fig. 3 b,c).

4. Implications

It is well known that some species can frequently produce a large amount of specific functional proteinous materials for their life strategies, such as proteinous mucus for gastropod and byssus for mussels as well as trap net for spiders and ink for squids. Based on the results of this study, we can speculate that TDF of amino acids potentially reduced for these species responsible for the production of proteinous materials. Accordingly,

more studies are required to evaluate the compassion/expansion of TDF for these species and its contribution to natural ecosystems. On the other hand, the illustration of the relationship between TDF and *F* described in this study can be useful for better understanding of the metabolic balance of amino acids within organisms of interests in biological, ecological, and biogeochemical studies.

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