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Carbon isotopic changes of 16 proteinogenic amino acids during trophic transfer in a cultured marine consumer

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Abstract

Stable carbon isotopic compositions of amino acids ($\delta^{13}C_{AA}$) in organisms potentially record information on carbon sources and flow in food webs. In this study, we report $\delta^{13}C$ values of 16 proteinogenic amino acids in marine consumers and evaluate their changes associated with the trophic transfer by using a newly developed analytical method based on multidimensional preparative liquid chromatography and elemental analysis with isotope-ratio mass spectrometry. Our targets in this study are cultured black rockfish (*Sebastes melanops*) and its isotopically-known diet, as well as wild blue mackerel (*Scomber australasicus*) as a representative marine consumer. $\delta^{13}C$ values of AAs in marine consumers have a variation as large as 30‰, with glycine, serine, and threonine being most ^{13}C-enriched and leucine and phenylalanine being most ^{13}C-depleted. On the other hand, the $\delta^{13}C_{AA}$ variation among species is relatively small, showing that $\delta^{13}C$ values of amino acids in marine organisms are mainly determined by their central biochemical pathways rather than species-specific processes. The $\delta^{13}C_{AA}$ differences ($\Delta^{13}C = \delta^{13}C_{consumer} - \delta^{13}C_{diet}$) between the cultured fish and its diet are small for most amino acids (− 0.6‰ on average), with two amino acids synthesized from glycolytic intermediates, serine (+ 4.4‰) and alanine (− 2.6‰), having the largest positive and negative deviation, respectively. It indicates a larger extent of de novo synthesis of glycolytic amino acids in fish. Some essential amino acids also show small but significant $\Delta^{13}C$, like threonine (+ 1.7‰) and methionine (− 2.4‰). In summary, this study provides a new assessment of $\delta^{13}C$ compositions of amino acids in marine consumers and their changes during the trophic transfer, covering a nearly complete set of proteinogenic amino acids. It should offer significant refinements for future studies.

Keywords Carbon isotope, Amino acids, Fish, Compound-specific isotope analysis, Trophic transfer

1 Introduction

Tracing the carbon sources and flow in the marine food web is an important and challenging issue in marine ecological science. While the high complexity and dynamic nature make it difficult to directly observe the trophic connections in the marine food web, stable isotope analysis (SIA) has been utilized as a powerful approach over the decades (Minagawa and Wada 1984; Peterson and Fry 1987; Newsome et al. 2007; Ohkouchi et al. 2015). Because of their abundance and ubiquity in the marine food web, amino acids (AAs) in marine organisms are used as representative materials for the studies of marine food web structures. Particularly,

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stable nitrogen isotopic compositions of individual AAs ($\delta^{15}N_{AA}$) in marine organisms have been extensively applied for monitoring trophic relationships among species (e.g., McCarthy et al. 2007; Chikaraishi et al. 2009. See McMahon and McCarthy 2016; Ohkouchi et al. 2017; Ohkouchi 2023 for more detailed reviews). Likewise, stable carbon isotopic compositions of AAs ($\delta^{13}C_{AA}$) have the potential of recording the sources and flow of carbon in the food web. Indeed, this possibility has been explored with some success (see Ohkouchi et al. 2015; Whiteman et al. 2019; Yun et al. 2022 for general reviews, and references therein). However, while nitrogen makes up the amino groups that are easily added to and removed from AAs, carbon forms the backbone of the AA molecules, which means that carbon skeletons of AAs are derived from a large variety of precursors and more biochemical reactions are involved in their formation. Thus, AA carbon isotopic compositions can be affected by more factors and become more difficult to understand and predict compared to those of nitrogen.

In theory, $\delta^{13}C$ values of AAs are determined by the isotope effects associated with enzymatic reactions in both their biosynthetic pathways and metabolism (Hayes 2001). According to their biosynthetic origins, proteinogenic AAs except His can be classified into 5 major groups, including 3-phosphoglycerate (3PG) family (Gly, Ser), phosphoenolpyruvate (PEP) family (Phe, Tyr), pyruvate family (Ala, Val, Leu), Asp family (Asp, Asn, Thr, Ile, Met), and α-ketoglutarate family (Glu, Gln, Pro, Arg) (Fig. 1). Lys is synthesized from Asp in most organisms, while in some protists and fungi it is synthesized from α-ketoglutarate (Xu et al. 2006; Wu 2009). $\delta^{13}C$ values of AAs in primary producers are determined by those of their precursor molecules (e.g., 3PG, PEP, pyruvate, oxaloacetate, and α-ketoglutarate), and altered by the isotope effects associated with enzymatic reactions in their formation. In terms of metabolism, AAs are classified into two groups: essential amino acids (EAAs) and nonessential amino acids (NEAAs), based on their ability of de novo synthesis in heterotrophs.

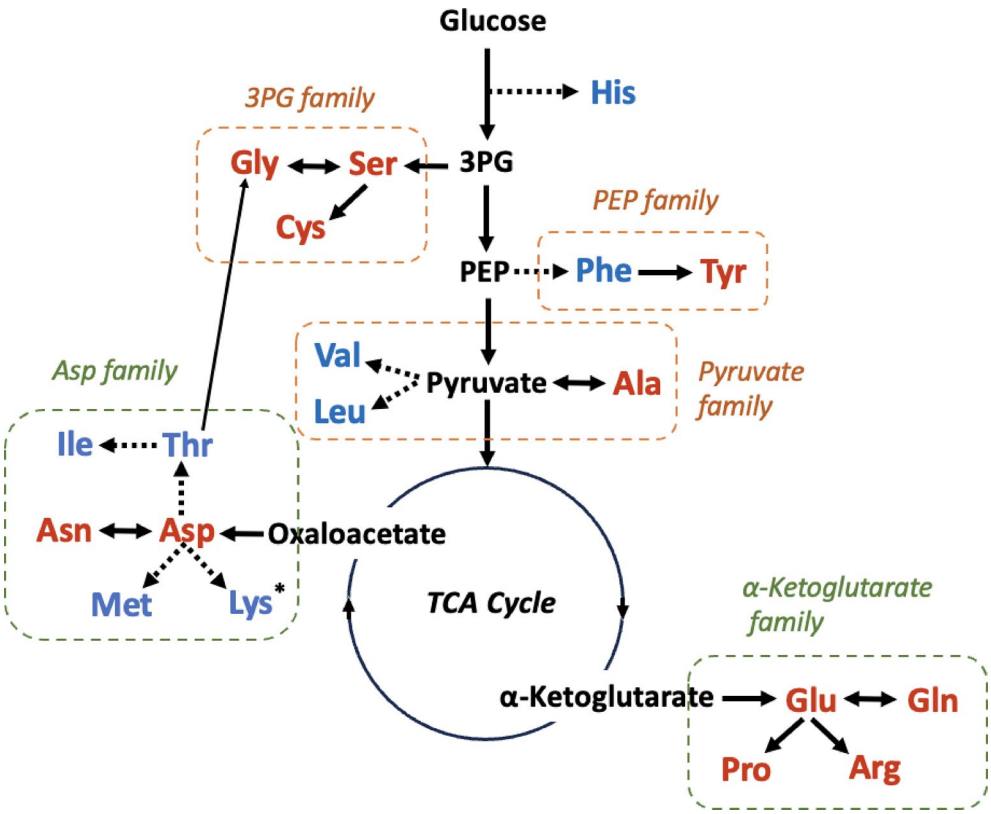

Fig. 1 A general overview of metabolic relationships among amino acids and their precursors in the central biochemical pathway. Essential amino acids are marked in blue, while nonessential amino acids are marked in red. Arrows represent the possible transformations between amino acids and their precursors, with solid arrows representing those existing in animals and dashed arrows indicating those only existing in primary producers. The directions of arrows indicate the dominant direction of transformation during the biosynthesis of amino acids, although most of the reactions are reversible. For the abbreviations, see the appendix. *Lys might be synthesized from α-ketoglutarate in some organisms

$\delta^{13}C$ values of EAAs ($\delta^{13}C_{EAA}$) in heterotrophs strongly reflect those in their diet, as all EAAs originate from dietary protein (Wu 2009). In the absence of other factors, the change on $\delta^{13}C$ values of EAAs during trophic transfer is limited. In other words, the difference in $\delta^{13}C$ values of AAs in the consumer and diet (i.e., $\Delta^{13}C = \delta^{13}C_{consumer} - \delta^{13}C_{diet}$) is expected to be negligible. Larsen et al. (2009, 2013) demonstrated that in different types of aquatic and terrestrial primary producers, EAAs show distinct $\delta^{13}C$ patterns, which can be used as "fingerprints" of their primary production sources. This "fingerprinting" approach can be applied to trace the carbon sources of both marine (e.g., Arthur et al. 2014) and freshwater (e.g., Besser et al. 2022) organisms. Especially, $\delta^{13}C_{EAA}$ "fingerprints" play a vital role in distinguishing the contributions of carbon input from different producers in the ecosystems with multiple carbon sources, such as intertidal (Elliott Smith et al. 2021), coral (McMahon et al. 2016; Fox et al. 2019; Skinner et al. 2021), and mangrove food webs (Larsen et al. 2012; Harada et al. 2022; Thibodeau et al. 2023). In contrast, $\delta^{13}C_{NEAA}$ are determined by both isotopic routing from the diet and de novo biosynthesis of AAs. Because the materials for the syntheses of NEAAs also come from other nutrients such as carbohydrates and lipids in the diet, $\delta^{13}C_{NEAA}$ values are considered different from the $\delta^{13}C$ baseline of the food web, thus can not be used as "fingerprints" of primary production sources. Instead, they can be potentially used for studying the dietary information and nutrient utilization of organisms (Choy et al. 2013; Wang et al. 2019).

Although the basic theoretical background of using $\delta^{13}C_{AA}$ to trace the sources and flow of carbon in the food web has been established for almost two decades, to date, our knowledge on the detailed controlling mechanisms of $\delta^{13}C_{AA}$ patterns in marine organisms, especially the effect of trophic processes on $\delta^{13}C_{AA}$ values, is still limited. Relatively large $\Delta^{13}C_{EAA}$ values in marine consumers were reported in some previous studies, which could potentially compromise the $\delta^{13}C$ "fingerprint" of EAAs. For instance, Lerner et al. (2021) reported up to 4.3‰ enrichment in ^{13}C in His during the trophic transfer of Chinook salmon (*Oncorhynchus tshawytscha*) based on a culture experiment. Such a large $\Delta^{13}C$ factor might be explained in many ways, due to the incomplete turnover of AAs (Whiteman et al. 2018), incorporation of EAAs synthesized by gut microbes (Newsome et al. 2011), or the isotopic fractionation associated with decarboxylation and other biochemical reactions in the metabolism (Melzer and Schmidt 1987; Takizawa et al. 2020). Furthermore, the controlling factors of $\delta^{13}C_{NEAA}$ are still less well understood compared to those of EAAs due to a higher complexity of the sources of NEAAs in marine consumers (Vane et al. 2025). All these problems limit the further applications of the $\delta^{13}C_{AA}$ approach in marine ecological studies.

In this study, we applied a newly developed method, using multidimensional high-performance liquid chromatography (HPLC) and elemental analysis with isotope-ratio mass spectrometry (Sun et al. 2020, 2023, 2024) to determine $\delta^{13}C$ compositions of underivatized AAs in marine consumers. Although this method is more time-consuming than the conventional method based on gas chromatography/combustion/isotope-ratio mass spectrometry (GC/C/IRMS), it does not require the chemical derivatization and thus does not require any correction for the carbon added by derivatization (Docherty et al. 2001; Chikaraishi et al. 2010), ensuring high accuracy of analysis. Moreover, we were able to measure most proteinogenic AAs including His, Met, and Arg, which often lack in previous studies due to the difficulties in the derivatization. Using this method, we aim to compare a nearly complete set of $\delta^{13}C$ values of AAs in black rockfish (*Sebastes melanops*) and its isotopically-known and homogeneous diet for a better understanding of carbon isotopic changes of AAs during trophic transfers, and study the variations of AA $\delta^{13}C$ compositions in marine consumers.

2 Experimental

2.1 Sampling location and controlled feeding experiment

Black rockfish (*S. melanops*) were reared at Japan Fisheries Research and Education Agency at Miyako, NE Japan, in 2007. After hatching, they were fed on rotifer until 31 days old, and their diet was shifted to a commercial diet pellet designed for carnivorous fish, Yellowtail Soft-dry, which consists of 42.8% protein, 26.1% lipid, 10.1% ash, and 10.6% moisture (Watanabe et al. 1991). *S. melanops* were then fed 3 times per week for 10 weeks. They grew from 2 to 6 cm in length in this period, which is long enough to allow most of the biomass to turn over to the new diet (Le Cren 1951). After the 10 weeks, *S. melanops* were collected (Fig. S1), euthanized, and stored at − 18 °C. Wild blue mackerel (*S. australasicus*) was collected from NW Pacific in 2016. The dorsal muscle of *S. melanops* and *S. australasicus* was collected and freeze-dried. Dry muscle samples were rinsed with methanol, extracted with *n*-hexane/dichloromethane mixture (3:2, v/v) three times in order to remove lipids, dried again, and stored at − 18 °C before the following HPLC separation and isotope analysis.

2.2 Sample pre-treatment and cation-exchange chromatography

For each analysis, ~ 10 mg of dry fish muscle or diet pellet was weighed. For a comparison, muscle collagen of *S.*

melanops in ~ 100 mg of dry muscle was also extracted and analyzed (see the Supplementary material for the detailed method). Samples were hydrolyzed with 500 µL of 12 M HCl at 110 °C for 12 h to release free AAs. After cooling down, hydrolysates were extracted with 1 mL of *n*-hexane/dichloromethane mixture (3:2, v/v) three times to further remove lipids and fatty acids in the samples. Then, hydrolysates were dried under a gentle N_2 flow and redissolved in 500 µL of 0.1 M HCl. Samples were then filtered through a membrane filter (GHP Nanosep MF, pore size: 0.45 µm, Pall Life Sciences, USA), which was pre-rinsed with 0.5 mL of 0.1 M HCl three times.

For a better HPLC separation performance, we conducted cation-exchange chromatography purification to remove metal ions and inorganic salts in the samples, following the protocol of Takano et al. (2010). It was previously reported that the resin treatment has no isotopic fractionation effect for underivatized amino acids and the analytical accuracy regarding carbon and nitrogen isotopic compositions has been verified (Takano et al. 2021). In short, a column was packed with 5 mL of Bio-Rad AG50 WX8 cation-exchange resin (200–400 mesh) suspended in two bed volumes of distilled water. The resin was conditioned three times with (a) 15 mL of 1 M HCl, (b) 15 mL of 1 M NaOH, and (c) 15 mL of 1 M HCl, respectively, and flushed with 15 mL of distilled water after each conditioning. Then, the hydrolysates were loaded onto the resin. The resin-packed column was then washed with 25 mL of distilled water to remove any metal ions and inorganic salts existing in the samples. Finally, AAs in the samples were recovered by flushing the column with 15 mL of 10% ammonia solution ($NH_3 \cdot H_2O$). The solutions were collected and dried, and AAs were redissolved in 0.5 mL of 0.1 M HCl.

2.3 Isolation of underivatized amino acids by HPLC

The detailed procedure of the isolation of individual underivatized AAs from biological samples is described in Sun et al. (2020). In short, the HPLC system (1100 series, Agilent Technologies, USA) was equipped with a fraction collector and a reversed-phase CAPCELL PAK C18 MG semi-preparative scale column (20 mm × 250 mm, particle size 5 µm, Osaka Soda Co. LTD, Japan) for the first chromatographic separation. Distilled water with 0.1% (v/v) trifluoroacetic acid (solvent A) and acetonitrile with 0.1% (v/v) trifluoroacetic acid (solvent B) were used as mobile phases. The flow rate and column temperature of the first column, CAPCELL PAK C18 MG column, were set at 2 mL min^{-1} and 30 °C, respectively. The column was flushed with 100% solvent A at 2 mL min^{-1} for 3 h before the injection of samples for conditioning. The HPLC system was connected to a charged aerosol detector (Corona CAD, Thermo Fisher Scientific) to confirm the retention time of the AAs, or to a fraction collector to collect isolated AAs. In the first chromatographic separation, hydrolysates of fish muscle and diet pellet samples recovered from the cation-exchange chromatography were injected to HPLC, and AAs from hydrolysates of fish muscle and diet pellet samples were separated by the HPLC column. Some AAs shown as single peaks with baseline separation were collected individually, and co-eluted AAs were collected together in several fractions for carrying over as follows.

Fractions containing multiple AAs were dried, resuspended in 0.1 M HCl, and injected to the second column, Primesep A column (4.6 mm × 250 mm, particle size 5 µm, SIELC Technologies, USA). The flow rate of the Primesep A column was 1 mL min^{-1}, and the column temperature was set at 30 °C. For conditioning, we flushed the column with 100% solvent A for 1 h before injection. We collected and dried the eluates following the same procedures described above, dissolved the dried AAs into 500 µL of 0.1 M HCl, and filtered the solution through GHP Nanosep membrane filters. Individual AA solutions were transferred to glass vials and stored at 4 °C.

Before isotopic analyses, collected individual AAs were dried and rinsed with 100 µL of diethyl ether twice to remove impurities (Ishikawa et al. 2018). The precipitates were then redissolved into 0.1 M HCl solution. The majority of each AA solution was used for EA/IRMS measurement, with a small portion used for quantification and purity check, following the protocol in Furota et al. (2018).

2.4 Elemental and carbon isotopic analysis by EA/IRMS

$\delta^{13}C$ values of isolated AAs were determined using a nano-EA/IRMS system (Ogawa et al. 2010) consisting of a modified elemental analyzer (Flash EA1112, Thermo Finnigan, USA), continuous flow interface (ConFloIII, Thermo Finnigan), and an isotope-ratio mass spectrometer (Delta plus XP IRMS, Thermo Finnigan). Solutions containing approximately 7–12 µg of isolated individual AAs were transferred to pre-cleaned tin capsules and dried at 95 °C before analysis. Approximately 50 µg of diet pellet and dry muscle of *S. melanops* were also weighed and transferred to a pre-cleaned tin capsule for analysis. Total carbon and nitrogen contents in the samples were determined by calibrated ion currents with *m/z* 44 and 28, respectively, in the IRMS (Isaji et al. 2020). Carbon isotopic composition is expressed as conventional δ notation relative to Vienna Pee Dee Belemnite (VPDB):

$$\delta^{13}C = \left(\frac{R_{Sample}}{R_{VPDB}} - 1 \right) \times 1000(‰),$$

where R represents the $^{13}C/^{12}C$ ratio.

The $\delta^{13}C$ values were calibrated using three to five inter-laboratory determined standards and commercial reference materials ranging from −26.86‰ to +0.18‰: *L*-tyrosine (BG-T), *L*-proline (BG-P), *L*-alanine (BG-A), DL-alanine (KERKU-01) (Tayasu et al. 2011), *L*-glutamic acid, and *L*-valine (Shoko Science, Japan). Isotope and quantity analyses of standards and samples were performed with quantities of 0.8–22 μgC. The analytical error of nano-EA/IRMS measurement, estimated through repeated analysis of BG-T during sample analysis, is within ± 0.39‰ (s.d. 1σ, n = 13; Sun et al. 2020).

The whole procedure of the analysis is summarized in Fig. S2.

3 Results

3.1 Isolation of individual amino acids

Figure 2 shows representative chromatograms of the first and second HPLC column separations of marine consumer samples. Among 20 underivatized proteinogenic AAs, Cys and Trp could not be confirmed from HPLC chromatograms because they were decomposed during the acid hydrolysis. Furthermore, Gln and Asn were converted into Glu and Asp during the acid hydrolysis, respectively. Other 16 AAs were successfully isolated from the samples. In the separation of the first HPLC column, baseline separation of 9 AAs, including Glu, Arg, Pro, Val, Met, Tyr, Ile, Leu, and Phe, was achieved. Other co-eluted AAs were collected in three fractions. Fraction 1 contained Gly and Ser, fraction 2 contained Asp and Lys, and fraction 3 contained Ala, His, and Thr. These three fractions were injected to HPLC equipped with the Primesep A column, as described in Sect. 2.3. On the second HPLC column, separation of all AAs in the three fractions was achieved. Three AAs co-eluted with inorganic ions, including Gly and Thr co-eluting with sodium, and His co-eluting with calcium. These AAs were collected together with the co-eluting inorganic ions, since sodium and calcium should not affect the isotope analysis by EA/IRMS. Overall, all 16 proteinogenic AAs recovered from the acid hydrolysis were collected as individual AAs for $\delta^{13}C$ analyses. The muscle collagen of *S. melanops* was extracted in order to further purify the sample for the chromatographic separation of AAs. However, because the successful isolation of 16 proteinogenic AA in muscle samples was achieved, AAs in the muscle collagen were not further processed after the first HPLC column separation (see the Supplementary material for details).

3.2 $\delta^{13}C_{AA}$ compositions in marine consumers and $\Delta^{13}C_{AA}$ values between fish and diet

$\delta^{13}C$ values of AAs in cultured *S. melanops* and wild *S. australasicus* are shown in Table S1 and plotted in Fig. 3, with the data of a wild spear squid (*Heterololigo bleekeri*) reported in Sun et al. (2020). Note that $\delta^{13}C$ values of Glu and Asp also contain the contributions from Gln and Asn, respectively; thus, they are marked as Glx and Asx in Fig. 3. $\delta^{13}C_{AA}$ shows a large variation from +5‰

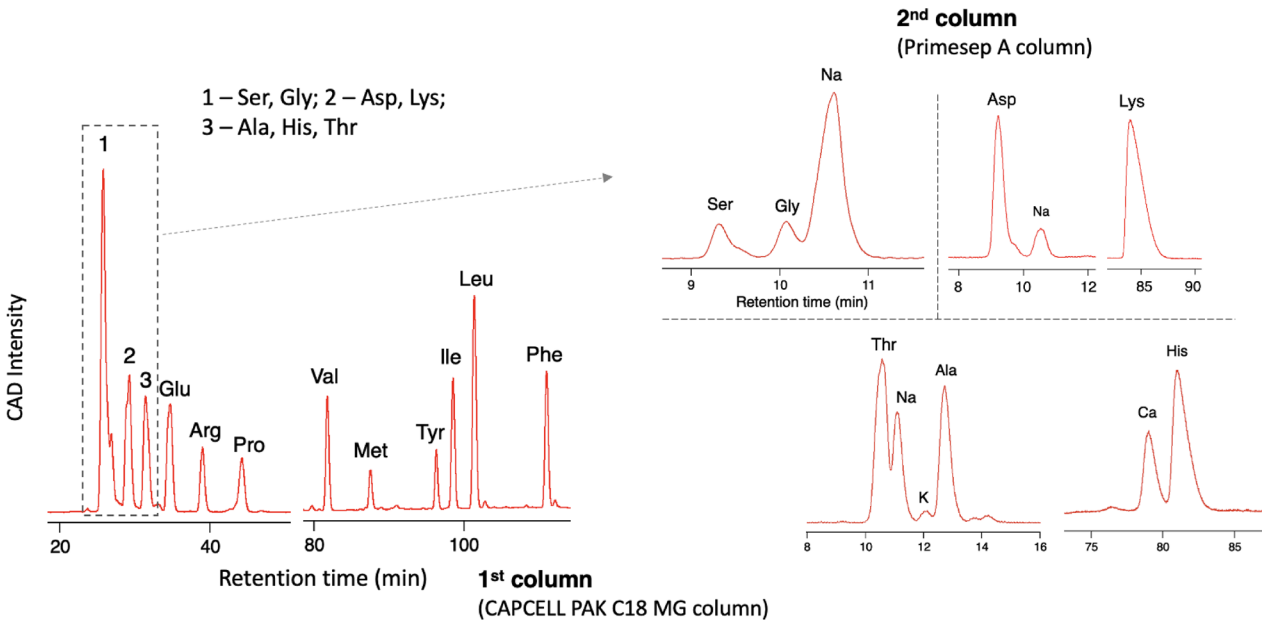

Fig. 2 Representative chromatograms of the hydrolysate of marine consumer samples (*S. melanops* muscle) on the multidimensional HPLC. Peaks 1, 2, 3 on the first column containing multiple amino acids were collected and injected to the second column for further separation, while other peaks were collected as individual amino acids in separate fractions

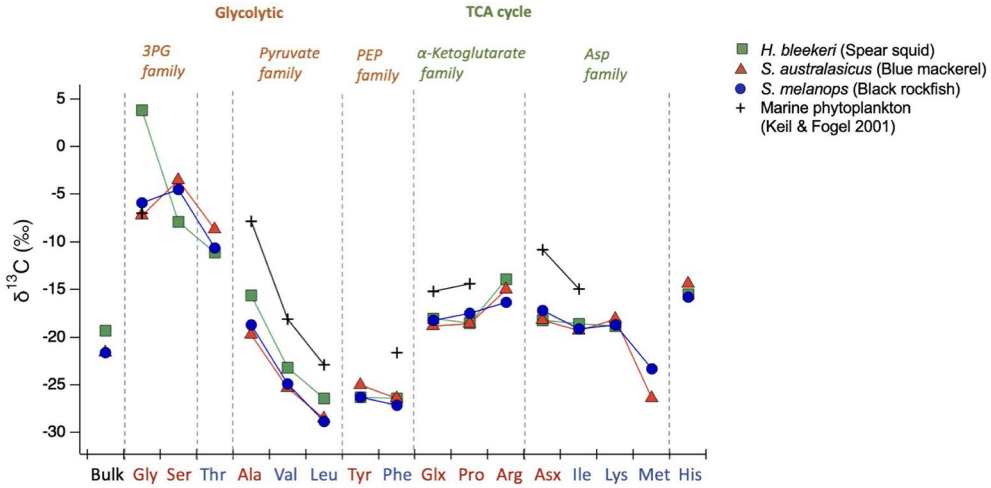

Fig. 3 $\delta^{13}C$ values of amino acids in three marine consumer (*S. melanops*, *S. australasicus, H. bleekeri*) and primary producer samples ($n = 1$, 2 or 3. For $n = 2$ and 3, the average value of measurements are shown). Essential amino acids are marked in blue, while nonessential amino acids are marked in red. Glx: Glu + Gln; Asx: Asp + Asn. Green square: *H. bleekeri* (Spear squid, Sun et al. 2020); red triangle: *S. australasicus* (Blue mackerel); blue circle: *S. melanops* (Black rockfish); black cross: marine phytoplankton (Keil and Fogel 2001). Note that Thr is normally considered as a member in the Asp family; however, due to its close metabolic relationship and similar $\delta^{13}C$ value with amino acids in the 3PG family, it is listed next to amino acids in the 3PG family

to − 30‰. In general, Gly, Ser, and Thr are ^{13}C enriched, while Leu, Tyr, and Phe are ^{13}C depleted. Although these three samples, including wild fish, cultured fish, and wild mollusk, belong to different taxonomic groups and have significantly different life histories, their $\delta^{13}C_{AA}$ showed a surprisingly similar pattern. Two AAs in the PEP family, Phe and Tyr, show similar values for all three species, likely due to their close relationship in the biosynthetic pathway (Kanehisa et al. 2014). Also, three AAs in the pyruvate family, Ala, Val, and Leu, show almost identical $\delta^{13}C$ patterns, which are also similar with the pattern in marine phytoplankton (Keil and Fogel 2001), despite the fact that their values are lower in marine consumers. All AAs which are synthesized from intermediates in the tricarboxylic acid (TCA) cycle also have similar $\delta^{13}C$ values among species, with Thr being enriched and Met being depleted. Two AAs in the 3PG family are an exception. Gly in *H. bleekeri* is 10‰ enriched in ^{13}C compared to those in other two fish species. In contrast, Ser in *H. bleekeri* is 3‰ depleted.

Figure 4 illustrates $\delta^{13}C$ values of each AA as well as bulk tissue of cultured *S. melanops* and its diet, and the corresponding $\Delta^{13}C$ values are shown in Fig. 5. For all 16 AAs, the $\Delta^{13}C$ values were generally small (− 0.6‰ on average), as was the small $\Delta^{13}C$ value for the bulk tissue (0.2‰). EAAs generally had $\Delta^{13}C$ values close to zero, with an average value of − 0.7‰ and standard deviation of 1.3‰. For NEAAs, the mean $\Delta^{13}C$ value was also negligibly small (0.1‰). However, there was a relatively large variation (2.2‰, 1σ) compared to EAAs. Among all AAs we analyzed, two NEAAs synthesized from glycolytic precursors, Ser (+4.4‰) and Ala (− 2.6‰) had the largest positive and negative $\Delta^{13}C$ values, respectively.

4 Discussion

$\Delta^{13}C_{AA}$ values observed in the cultured *S. melanops* in this study were generally small (+4.4‰ ~ − 2.6‰) compared with many previous studies on fish and other consumers (e.g., McMahon et al. 2010; Liu et al. 2018; Takizawa et al. 2020; Xu et al. 2022). For instance, Takizawa et al. (2020) reported large ^{13}C enrichment factors up to around 18‰, while McMahon et al. (2010) reported that many AAs showed a depletion during the trophic transfer, down to − 8‰. Near-zero $\Delta^{13}C$ values of EAAs further empirically support the approach of using $\delta^{13}C_{EAA}$ "fingerprints" to trace the carbon sources of AAs in the food web (Larsen et al. 2009, 2013), although $\Delta^{13}C$ values of Thr (+1.7‰) and Met (− 2.4‰) are larger than those of the others, which may cause some uncertainties in the identification of carbon sources using the $\delta^{13}C_{EAA}$ "fingerprint."

The small $\Delta^{13}C_{AA}$ values observed in this study may be due to the similar AA compositions of *S. melanops* and its diet (Fig. 6). The absolute values of the difference between the AA relative abundance in the *S. melanops* and its diet are relatively small, varying between 0 and 2% with an average of 0.7%. The similar AA compositions in the diet and *S. melanops* potentially reduces the imbalance of AA incorporation during the trophic transfer and causes a small extent of de novo synthesis of NEAAs by

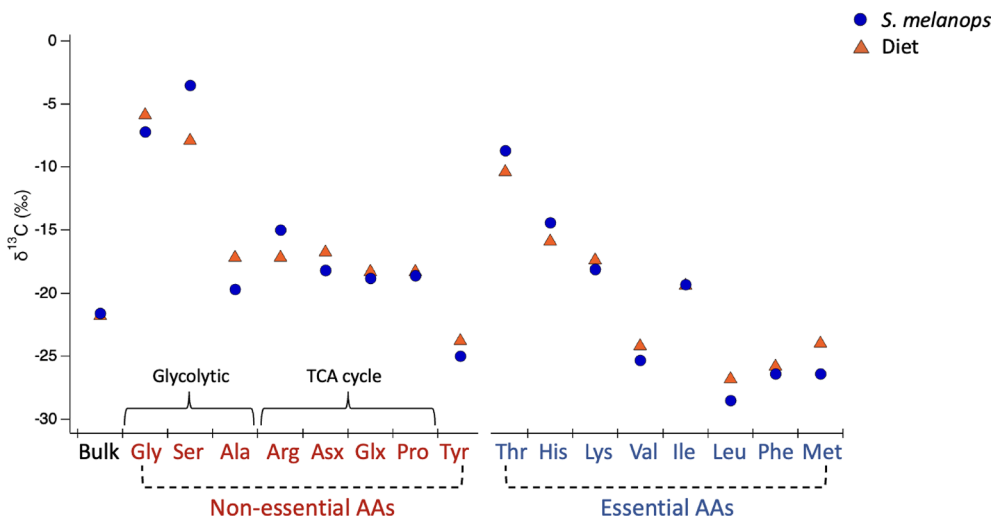

Fig. 4 $\delta^{13}C$ values of amino acids in black rockfish (*S. melanops*) and the diet pellet ($n = 1$, 2 or 3. For $n = 2$ and 3, the average value of measurements are shown). Blue circle: *S. melanops;* orange triangle: diet

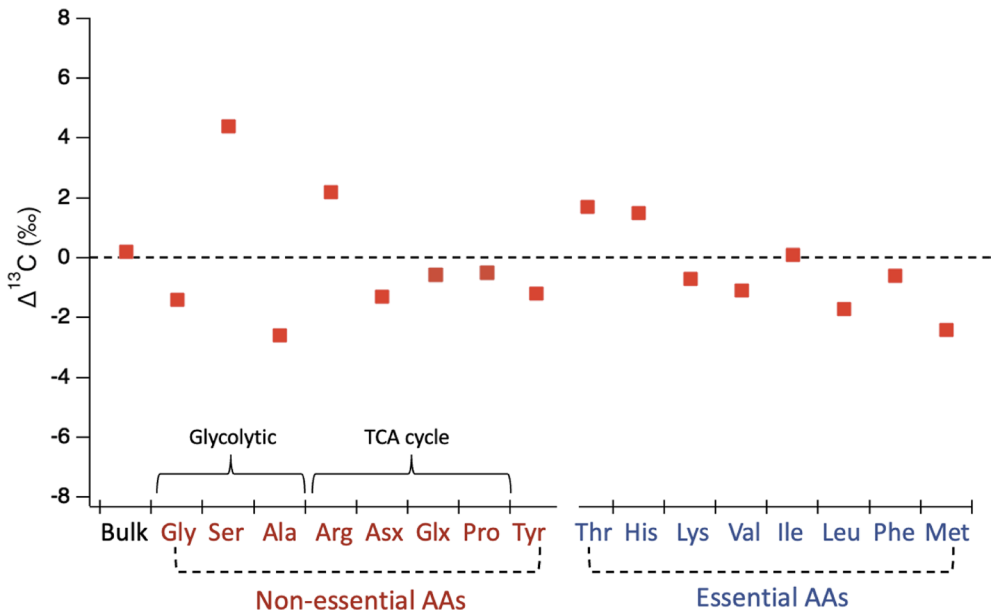

Fig. 5 $\Delta^{13}C$ factors between amino acids in black rockfish (*S. melanops*) and the diet pellet. $\Delta^{13}C = \delta^{13}C_{fish} - \delta^{13}C_{diet}$

S. melanops. However, despite of the small $\Delta^{13}C_{AA}$ values in general, our results clearly suggested that the $\delta^{13}C_{EAA}$ values in consumers are not necessarily identical to those in the diet. It shows that other factors may also control $\delta^{13}C_{EAA}$ values, which is consistent with some previous results (Newsome et al. 2011; Lerner et al. 2021). One possibility is that fish may utilize carbon skeletons of EAAs to synthesize some other necessary biomolecules in the metabolism, and those biochemical reactions may be associated with carbon isotopic fractionations. Also, microbes living in the gut of fish can synthesize EAAs, which may be incorporated into fish's biomass (e.g., Newsome et al. 2011; Whiteman et al. 2018). In this case, $\delta^{13}C_{EAA}$ compositions could also reflect $\delta^{13}C$ values of AAs synthesized by gut microbes. While it is difficult to precisely estimate the contribution of AAs synthesized by gut microbes with our current data, it is reported that the microbial contribution of AAs to fish can largely vary depending on the quality of food (Newsome et al. 2011). In some other consumers like mammals or insects, up to 60% of some EAAs are reported to be derived from gut microbes (Ayayee et al. 2016; Newsome et al. 2020). The

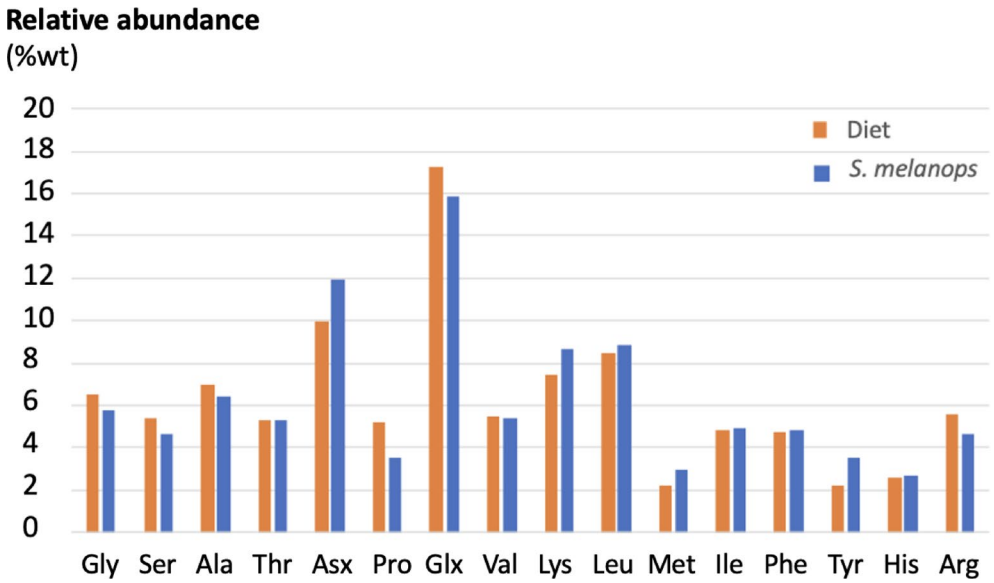

Fig. 6 Amino acid relative abundance (weight percentage) in the hydrolysates of black rockfish (*S. melanops*) muscle (right column, blue) and the diet pellet (left column, orange)

detailed controlling mechanisms of $\delta^{13}C_{EAA}$ values in marine consumers need to be further systematically studied in the future.

Compared to EAAs and NEAAs synthesized from intermediates in the TCA cycle, NEAAs having glycolytic precursors have a larger variation in $\delta^{13}C$ values among organisms and show larger differences with those in marine planktons (Keil and Fogel 2001; McCarthy et al. 2004). It is likely due to the relatively large extent of de novo synthesis of AAs from intermediates derived from carbohydrate (Wang et al. 2019), although with the current data, we cannot exclude the possibility of carbon isotopic fractionation in the metabolism. Gly has four major synthetic pathways (Kanehisa et al. 2014). While in animals, a large portion of Gly is synthesized from Ser through the enzymatic reaction catalyzed by serine hydroxymethyltransferase (SHMT), Gly can also be synthesized from Thr, glyoxylate, and choline (Kanehisa et al. 2014). The complexity of the biosynthetic sources of Gly makes it difficult to interpret the change in its $\delta^{13}C$ values. Ser is closely related to Gly in the synthetic and metabolic pathways through the one-carbon (C1) pathway. As a result, $\delta^{13}C$ values of Ser and Gly are found to have a linear correlation in fish (Wang et al. 2018). Notably, the $\Delta^{13}C_{Gly}$ value between *S. melanops* and its diet is − 1.4‰, showing a slight ^{13}C depletion rather than enrichment during the trophic transfer. Previous studies reported variable $\Delta^{13}C_{Gly}$, up to around + 20‰ (McCarthy et al. 2004; Takizawa et al. 2020) and down to − 8‰ (McMahon et al. 2010). This is probably due to the fact that Gly has diverse synthetic pathways. Moreover, the metabolism of Gly can also affect its $\delta^{13}C$ values in consumers. Gly is mainly metabolized through Gly cleavage system (Wu 2009), in which Gly is converted to carbon dioxide and the aminomethyl group on glycine-cleavage complex H protein-lipoyllysine. Since this reaction involves the cleavage of C–C bond and is catalyzed by glycine dehydrogenase, a pyridoxal 5'-phosphate (PLP)-dependent enzyme (Hasse et al. 2013), it is expected that a carbon isotopic fractionation will be associated with the metabolism of Gly (Ohkouchi et al. 2015). Although our data support previous studies which found that Gly in marine samples in general has higher $\delta^{13}C$ values compared to their terrestrial counterparts, the actual cause of ^{13}C enrichment in marine organisms remains largely unknown, which needs careful evaluation in the future. An implication of this research is that ^{13}C enrichment in Gly might be contributed from the imbalanced AA compositions between the diet and consumer. While other AAs show more similar $\delta^{13}C$ values among three species, $\delta^{13}C$ value of Gly in *H. bleekeri* is ~ 10‰ higher than that in other two fish species. Since Gly has a larger requirement for the soft tissue formation in squid than in fish muscle (Rajapakse et al. 2005), it is likely that Gly uptake is insufficient, which requires a large extent of de novo biosynthesis during squid's growth, resulting in ^{13}C enrichment in Gly. In this study, we found that the isotopic changes of Gly and Ser during the trophic transfer have different directions. Unlike Gly, Ser shows a + 4.4‰ ^{13}C enrichment. It indicates that while Gly and Ser are both in general enriched in ^{13}C in marine consumers (Wang et al. 2018) because they have common precursors, the C1 pathway which

converts between Gly and Ser may have an isotope effect, which enriches ^{13}C on one side and ^{12}C on the other. It can potentially become an important factor that determines the $\delta^{13}C$ values of Gly and Ser in organisms.

Another glycolytic NEAA, Ala, shows the largest depletion of − 2.6‰ between *S. melanops* and its diet among all 16 proteinogenic AAs, which is consistent with previous studies reporting a general ^{13}C depletion of Ala between two trophic levels (McMahon et al. 2010; Liu et al. 2018; Rogers et al. 2019; Wang et al. 2019; Lerner et al. 2021). Ala is synthesized from and closely related to pyruvate by a single enzymatic reaction catalyzed by alanine transaminase (ALT) in the central biochemical pathway (Fig. 1). The ^{13}C depletion of Ala during the trophic transfer may indicate that there is a high metabolic demand for Ala in *S. melanops*. Pyruvate is an important precursor for the synthesis of many biomolecules in organisms. McNeil et al. (2020) reported a ^{13}C depletion up to 9‰ on the C-2 position of Ala during the transamination reaction which converts Ala to pyruvate in the first step of Ala metabolism. This kind of depletion likely results in the ^{13}C depletion of Ala in three marine consumers compared to that in marine primary producers.

In this study, we successfully analyzed AAs like Arg and His which often lack in previous studies because of analytical difficulties. This study provides us some first insights into the $\delta^{13}C$ values of these AAs in marine organisms. Arg is found to be relatively enriched in ^{13}C among three AAs in the α-ketoglutarate family (Fig. 3), and it shows an enrichment of 2.2‰ from diet to *S. melanops* (Fig. 5). Considering the AA balance between *S. melanops* and its diet (Fig. 6), it is likely that the ^{13}C enrichment is due to the steps in the metabolism of Arg in *S. melanops*. However, we cannot exclude the possibility that de novo synthesis of Arg in *S. melanops* also contributed to the ^{13}C enrichment, because although adult fish are known to have low activities of Arg biosynthetic enzymes such as pyrroline-5-carboxylate synthase, ornithine transcarboxylase, and carbamoyl phosphate synthase III, Arg synthesis in fish keeps active during the juvenile stage (Wang et al. 2021). Since $\delta^{13}C$ values of Arg were rarely reported in previous researches, more studies are necessary in order to elucidate their controlling mechanisms. His is another AA that is technically difficult to measure by the conventional GC/C/IRMS method; thus, only a few records of its $\delta^{13}C$ values in marine organisms exist. In this study, we found $\Delta^{13}C$ value of 1.5‰ for His, which shows a slight ^{13}C enrichment, smaller than reported values in Lerner et al. (2021) and Hesse et al. (2022). Since fish strictly lack the ability of synthesizing His de novo, the causes are likely to be the steps in the metabolism of His, for instance, the decarboxylation of His (Hesse et al. 2022), and the synthesis of His by gut microbes. The detailed mechanism also requires further investigations.

16 proteinogenic AAs analyzed in this study represent more than 96% (wt%) of carbon in proteins in fish (Ryu et al. 2021). Thus, the weighted-average $\delta^{13}C$ value of AAs obtained in this study (− 0.6‰) can represent that of the whole protein to the largest extent. It is well known in isotope ecology that there is a slight enrichment in ^{13}C of bulk organic matter between two adjacent trophic levels in the food web, ranging from 0 to 1.5‰ (0.8‰ on average, Fry and Sherr 1989; Wada et al. 2013; Ohkouchi et al. 2015). Its actual reason and mechanisms are still under debate. Our study can shed light on this long-standing question. Compared with the ^{13}C enrichment of 0.2‰ in bulk tissues, the weighted-average value of AAs shows a slight depletion of − 0.6‰. Because our study covers the majority of AAs in protein in terms of carbon amount, we conclude that the isotopic change of protein is not likely to be the source of ^{13}C enrichment in bulk tissue during the trophic transfer. The discrepancy between bulk tissue and AAs indicates that the ^{13}C enrichment in bulk tissue is likely contributed from the enrichment in other pools of organic matter, for instance, carbohydrates, lipids, or nucleotides.

5 Conclusions and future work

In this study, we provided a new assessment of $\delta^{13}C$ values of AAs in marine consumers and their changes during trophic processes, covering 16 proteinogenic AAs. $\delta^{13}C_{AA}$ values of marine consumers have a large variation up to 30‰, with Gly, Ser, and Thr being most ^{13}C-enriched and Leu and Phe being most ^{13}C-depleted. The results from the culture experiment of *S. melanops* support the idea that $\delta^{13}C$ values in marine organisms are mainly determined by their central biochemical pathways and validate the approach of using $\delta^{13}C_{EAA}$ "fingerprints" to trace the carbon source of AAs in the food web. Comparison of $\delta^{13}C_{AA}$ values between the cultured *S. melanops* and its diet indicates a larger extent of de novo synthesis of AAs synthesized from glycolytic precursors in the fish. As previously reported, EAAs also show some nonzero $\Delta^{13}C$ values, suggesting that mechanisms other than the direct isotopic routing may also control $\delta^{13}C_{EAA}$ patterns. This study provides us some first insights into the $\delta^{13}C$ compositions of some rarely measured AAs, and should offer significant refinements for future studies.

The number of organisms analyzed in this study is limited. To further investigate the distributions of $\delta^{13}C_{AA}$ values in marine organisms, analyzing more marine organisms covering a more completed hierarchy of the marine food web is necessary. It would also be useful to study the change in $\delta^{13}C_{AA}$ values between different trophic levels, for example, from marine phytoplankton

to zooplankton, and under different dietary protein contents. It will allow us to explore the possibility that varying degrees of de novo synthesis of AAs caused by differences in the dietary protein content can produce a large $\delta^{13}C_{AA}$ variation. These studies will be necessary for better understanding the variation of $\delta^{13}C_{AA}$ values in marine organisms and evaluating the uncertainties in the determination of carbon sources in the marine food web through the $\delta^{13}C_{EAA}$ "fingerprinting" approach. It is also promising that we can use $\delta^{13}C$ values of glycolytic NEAAs like Gly, Ser, and Ala to study the nutrient intake and consumption in marine consumers, in order to achieve a more comprehensive understanding of the dynamics of trophic relationships in the aquatic food web.

Abbreviations

AA	Amino acid
EAA	Essential amino acid
NEAA	Nonessential amino acids
3PG	3-Phosphoglycerate
PEP	Phosphoenolpyruvate
TCA	Tricarboxylic acid
Gly	Glycine
Ala	Alanine
Glu	Glutamate/glutamic acid
Ser	Serine
Cys	Cysteine
Thr	Threonine
Asp	Aspartate/aspartic acid
Val	Valine
Pro	Proline
Arg	Arginine
Met	Methionine
His	Histidine
Lys	Lysine
Leu	Leucine
Ile	Isoleucine
Tyr	Tyrosine
Phe	Phenylalanine
Gln	Glutamine
Asn	Asparagine
Trp	Tryptophan
HPLC	High-performance liquid chromatography
CAD	Corona-charged aerosol detector
EA/IRMS	Elemental analysis/isotope-ratio mass spectrometry
VPDB	Vienna Pee Dee Belemnite
SHMT	Serine hydroxymethyltransferase
PLP	Pyridoxal-5′-phosphate
ALT	Alanine transaminase

Supplementary Information

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Additional file 1.

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Author contributions
All authors together designed the experiment project. NOO, NFI, and YS conducted the pre-treatment of fish samples. YS performed the isolation and collection of individual AAs, and NOO and YS conducted isotope analyses. YT, NOO, and NO outlined the analytical scheme of preparative ion-paring chromatography and assessed the wet chemical procedures for underivatized amino acids. YS interpreted the data and wrote the first draft. All authors participated in the discussion and contributed to the final manuscript.

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Data availability
The dataset supporting the conclusions of this article is included within the article and its additional files.

Declarations

Competing interests
The authors declare that they have no competing interest.

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