# A new method to reconstruct fish diet and movement patterns from $\delta^{13}C$ values in otolith amino acids

**Kelton W. McMahon, Marilyn L. Fogel, Beverly J. Johnson, Leah A. Houghton, and Simon R. Thorrold**



**Abstract:** Fish ecologists have used geochemical values in otoliths to examine habitat use, migration, and population connectivity for decades. However, it remains difficult to determine an unambiguous dietary $\delta^{13}C$ signature from bulk analysis of otolith. Studies to date have focused on the aragonite component of otoliths with less attention paid to the organic fraction. We describe the application of compound-specific stable isotope analysis (SIA) to analyze amino acid (AA) $\delta^{13}C$ values from small amounts (<1 mg) of otolith powder. We examined $\delta^{13}C$ values of otolith and muscle AAs from a reef-associated snapper (*Lutjanus ehrenbergii* (Peters, 1869)) collected along a carbon isotope gradient (isoscape) from seagrass beds to coral reefs. Carbon isotope values in otolith and muscle samples were highly correlated within and among coastal habitats. Moreover, $\delta^{13}C$ values of otolith AAs provided a purely dietary record that avoided dilution from dissolved inorganic carbon. Otolith AAs served as a robust tracer of $\delta^{13}C$ values at the base of the food web, making compound-specific SIA a powerful tool for dietary reconstructions and tracking the movement of fishes across isoscapes.

**Résumé :** Depuis des décennies, les écologistes des poissons emploient les valeurs géochimiques des otolithes pour étudier l'utilisation de l'habitat, la migration et la connectivité entre les populations. Il reste, cependant, difficile de déterminer une signature $\delta^{13}C$ alimentaire non ambiguë par analyse globale de l'otolithe. Jusqu'à maintenant, les études se sont intéressées à la composante aragonite des otolithes et ont porté moins d'attention à la fraction organique. Nous décrivons l'utilisation de l'analyse des isotopes stables (SIA) de composés spécifiques pour l'analyse des valeurs de $\delta^{13}C$ des acides aminés (AA) sur de petites quantités (<1 mg) de poudre d'otolithe. Nous avons déterminé les valeurs de $\delta^{13}C$ des AA du muscle et des otolithes chez des vivaneaux (*Lutjanus ehrenbergii* (Peters, 1869)) associés aux récifs et prélevés le long d'un gradient d'isotopes de carbone (isoscape) allant d'herbiers marins à des récifs coralliens. Les valeurs des isotopes de carbone dans les otolithes et les échantillons de muscle sont fortement corrélés au sein des habitats côtiers et entre ceux-ci. De plus, les valeurs de $\delta^{13}C$ des AA des otolithes fournissent un rapport entièrement alimentaire qui évite toute dilution par le carbone inorganique dissous. Les AA des otolithes servent de traceurs robustes des valeurs de $\delta^{13}C$ à la base du réseau alimentaire, ce qui fait de l'analyse SIA de composés spécifiques un outil puissant pour reconstituer les régimes alimentaires et pour suivre les déplacements des poissons le long d'isoscapes.

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## Introduction

The use of geochemical values in animal tissues as tags to track movement patterns of animals across isotope gradients in the environment (isoscapes) has become increasingly popular in terrestrial and aquatic systems (West et al. 2010). These studies have conducted bulk tissue stable isotope analyses (SIA) on a variety of tissues, including bird feathers, whale baleen, and fish scales (Hobson 1999; Rubenstein and Hobson 2004). Some of the most comprehensive examples of this approach have been conducted using fish otoliths to address questions of habitat residency, migration, and population connectivity (reviewed by Campana and Thorrold (2001) and Elsdon et al. (2008)). To date, studies have focused almost exclusively on the inorganic aragonite fraction of otoliths to provide information on the environment inhabited by individuals at different life history stages (Secor et al. 1995; Thorrold et al. 2001; Kennedy et al. 2002). Recent work has suggested that the bulk carbon isotope composition of otoliths may also record a significant dietary component

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**K.W. McMahon.** Massachusetts Institute of Technology and Woods Hole Oceanographic Institution, Joint Program in Oceanography and Ocean Engineering, Woods Hole Oceanographic Institution, Woods Hole, MA 02543, USA.
**M.L. Fogel.** Carnegie Institution of Washington, 5251 Broad Branch Road NW, Washington, DC 20015, USA.
**B.J. Johnson.** Department of Geology, Bates College, Lewiston, ME 04240, USA.
**L.A. Houghton and S.R. Thorrold.** Biology Department, Woods Hole Oceanographic Institution, Woods Hole, MA 02543, USA.

**Corresponding author:** Kelton W. McMahon (e-mail: kmcmahon@whoi.edu).

(Elsdon et al. 2010). These results raise the intriguing possibility of using otolith geochemistry to retrospectively identify both lifetime movement patterns and diets of fishes.

Despite considerable promise, interpreting carbon isotope values in otoliths remains a difficult proposition. The carbon deposited in otoliths comes from both metabolic sources and dissolved inorganic carbon (DIC). These two potential sources have $\delta^{13}C$ values that may differ by as much as 20‰. Most studies have found that DIC contributes the majority of otolith carbon (Thorrold et al. 1997; Tohse and Mugiya 2004; Solomon et al. 2006), and therefore, dietary values in otoliths are inevitably diluted by DIC. More importantly, the relative contribution of these two end members is highly variable within and among species, making it difficult to mathematically correct for the DIC dilution effect. Indeed, variations in bulk otolith $\delta^{13}C$ appear to reflect a number of factors, including metabolism (Kalish 1991; Weidman and Millner 2000; Stephenson et al. 2001), diet $\delta^{13}C$ and trophic position (Gauldie 1996; Begg and Weidman 2001), DIC $\delta^{13}C$ (Schwarcz et al. 1998), and environmental conditions (Mulcahy et al. 1979; Kalish 1991). Therefore, it remains difficult to determine an unambiguous dietary $\delta^{13}C$ value from bulk analysis of otoliths.

One potential method for avoiding the confounding effect of DIC-derived carbon on otolith $\delta^{13}C$ values is to focus on otolith protein that may constitute up to 10% (by weight) of an otolith (Degens et al. 1969; Sasagawa and Mugiya 1996; Murayama et al. 2002). Analyzing otolith proteins may provide a purely dietary record that avoids the effect of both DIC dilution and variable metabolic carbon contribution. This protein value represents a mixture of amino acids (AAs) directly routed from dietary protein and AAs biosynthesized from a bulk carbon pool consisting of dietary proteins, lipids, and carbohydrates (Schwarcz 1991; Ambrose and Norr 1993; McMahon et al. 2010). Bulk protein SIA is not, however, without challenges. For instance, it can be difficult to distinguish between changes in $\delta^{13}C$ associated with diet or trophic shifts versus changes due to movement among habitats with different $\delta^{13}C$ values at the base of the food web ($\delta^{13}C_{Base}$ (Post 2002)). This is particularly true when tracking the ontogenetic shifts of highly migratory fishes, where juveniles and adults often occupy different habitats and different trophic levels (Cocheret de la Morinière et al. 2003; Graham et al. 2007).

Compound-specific SIA is a more powerful tool for examining diet and habitat use than conventional bulk SIA alone (Fantle et al. 1999; Popp et al. 2007; McMahon et al. 2010). Several studies have shown that bulk $^{13}C$ fractionation factors of 0‰ to 1‰ are underlain by little or no fractionation in essential AAs and fractionation factors of more than 7‰ in many non-essential AAs (Hare et al. 1991; Jim et al. 2006; McMahon et al. 2010). Although plants and bacteria can synthesize essential AAs de novo, most animals have lost the necessary enzymatic pathways to synthesize these AAs at a rate sufficient for normal growth (Borman et al. 1946; Reeds 2000). Therefore, essential AAs must be incorporated directly from the diet. As a result, $\delta^{13}C$ values of consumer essential AAs represent the isotopic signature of primary producers at the base of a food web ($\delta^{13}C_{Base}$) without the confounding influence of trophic fractionation. Conversely, non-essential AAs can either be synthesized de novo from a bulk carbon pool or directly incorporated from dietary protein into consumer tissue through isotopic routing (Schwarcz 1991). Non-essential AA $\delta^{13}C$ values may, therefore, provide valuable information about metabolic processing and have been shown to correlate with diet quality and composition (McMahon et al. 2010). Carbon isotope analysis of AAs has been applied to several other biominerals, including egg shells (Johnson et al. 1993, 1998), mollusk shells (Engel et al. 1994; Silfer et al. 1994; O'Donnell et al. 2007), bones (Hare et al. 1991; Howland et al. 2003), and teeth (Bada et al. 1990) to reconstruct past climates, examine diagenesis and mineral authenticity, and assess seasonal or ontogenetic shifts in consumer diet. Compound-specific SIA has also recently been applied to fish muscle to assess diet and habitat use (Popp et al. 2007; McMahon et al. 2010). However, researchers have yet to apply compound-specific SIA to accretionary tissues in fishes, including otoliths, that may allow for retrospective analyses of diet and movement.

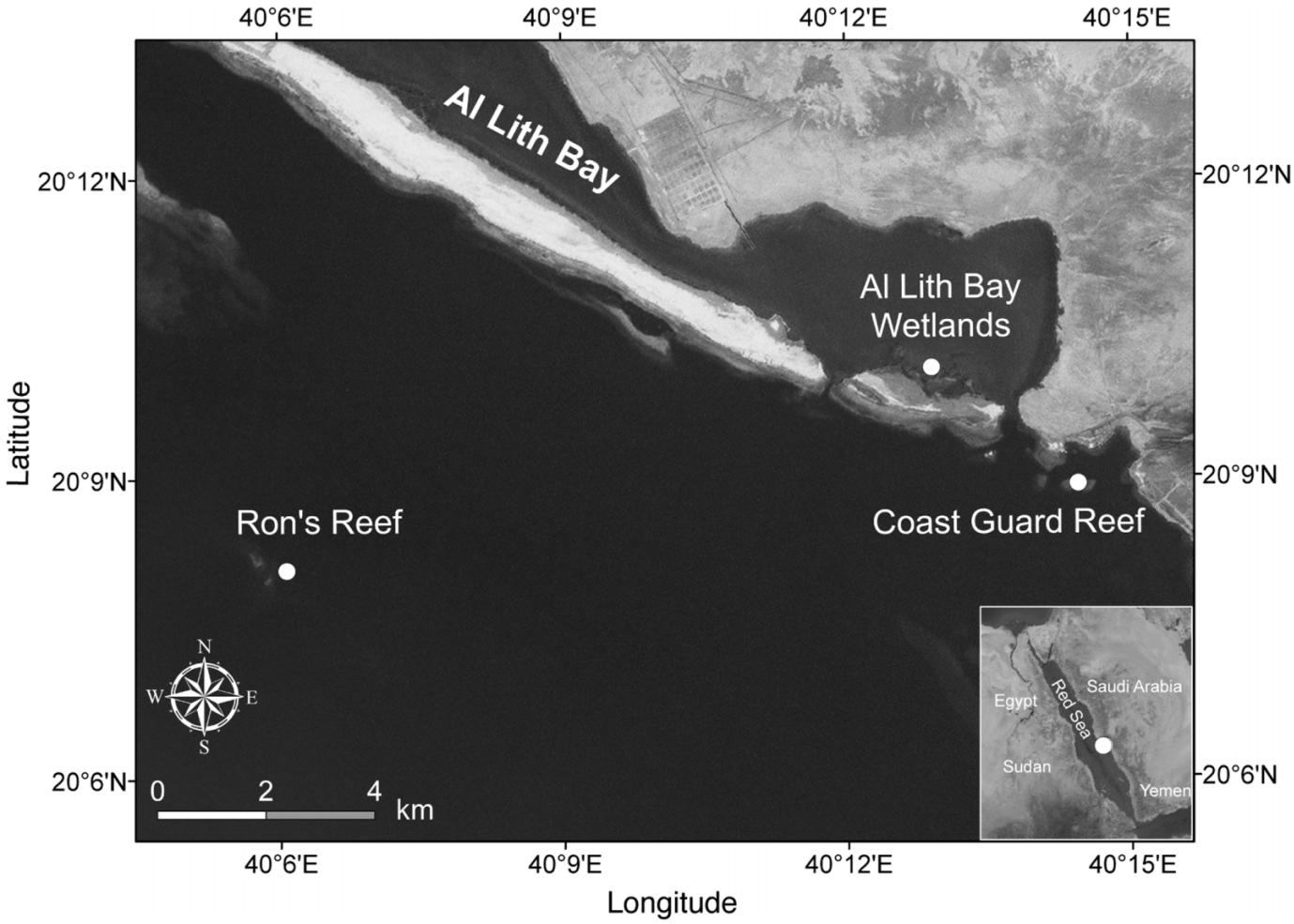
Here, we present a method for stable carbon isotope analysis of AAs in otoliths. To test the method, we compared $\delta^{13}C$ values of individual AAs in muscle tissue and otoliths from wild caught Ehrenberg's snapper, *Lutjanus ehrenbergii* (Peters, 1869), collected from three isotopically distinct habitats near Al Lith, Saudi Arabia, in the Red Sea. We hypothesized that the $\delta^{13}C$ values of otolith AAs would be strongly correlated with those of muscle AAs, providing access to dietary isotope values in otoliths that avoid the DIC dilution effect observed in bulk otolith SIA. We also hypothesized that otolith AA $\delta^{13}C$ values would provide a reliable tracer of residence in isotopically distinct habitats. Our study provides ecologists with a new tool for reconstructing dietary histories and establishing $\delta^{13}C_{Base}$ values to track fish movement through isoscapes.

## Materials and methods

### Field collections

*Lutjanus ehrenbergii* were collected at three locations along a cross-shelf transect from Al Lith, Saudi Arabia, in the Red Sea in March 2009 (Fig. 1). Associated with coral reefs as adults, juvenile *L. ehrenbergii* are abundant in coastal wetland habitats, making them a model species for examining residence along an isotopic gradient. Juvenile *L. ehrenbergii* (total length (TL) = 77 ± 6 mm) were collected from seagrass beds in Al Lith Bay (Al Lith Bay wetlands) using cast nets. Adult *L. ehrenbergii* were speared from a reef 2 km from the entrance of Al Lith Bay (Coast Guard Reef; TL = 209 ± 48 mm) and from a shelf reef approximately 14 km off the coast of Al Lith (Ron's Reef; TL = 232 ± 5 mm)). Sagittal otoliths and white muscle tissue were dissected from each fish in the field. Otoliths were cleaned of residual surface tissue with water and stored dry in 1.5 mL vials. White muscle samples from the dorsal surface of each fish were frozen on the boat prior to transport to an onshore laboratory. In the lab, white muscle samples were frozen at –20 °C and then lyophilized (freeze-dried) for 48 h. Paired otoliths and freeze-dried muscle samples were transferred back to Woods Hole Oceanographic Institution, Woods Hole, Massachusetts, USA, for further preparation and analysis. Seventy-three fish, including 26 fish from Al Lith Bay wetlands, 23 fish from Coast Guard Reef, and 24

**Fig. 1.** Three locations for Ehrenberg's snapper, *Lutjanus ehrenbergii*, collected along a cross-shelf transect from Al Lith, Saudi Arabia, in the Red Sea in March 2009. Juvenile *L. ehrenbergii* were collected from seagrass beds in Al Lith Bay (Al Lith Bay wetlands), and adult *L. ehrenbergii* were collected from a reef adjacent to Al Lith Bay (Coast Guard Reef) and 14 km offshore (Ron's Reef).




fish from Ron's Reef, were analyzed for paired bulk otolith and muscle $\delta^{13}C$ values. Of those fish, five were randomly selected per location for paired compound-specific SIA of otoliths and muscle. Percentage protein for *L. ehrenbergii* muscle and otoliths was determined at the New Jersey Feed Laboratory, Trenton, New Jersey, USA (AOAC Method 994.12; AOAC International 2005).

### Sample preparation

A single sagittal otolith was randomly selected from each fish. All otolith samples were scrubbed and rinsed in ultrapure water, cleaned ultrasonically for 5 min in ultrapure water, and then air-dried under a class-100 positive-flow fume hood for 24 h. Whole otoliths from juvenile *L. ehrenbergii* were used for SIA. For adult *L. ehrenbergii*, we extracted otolith powder after the last annulus of the whole otolith, corresponding to the most recent several months of growth, using a Merchantek MicroMill (Electro Scientific Industries, Portland, Oregon, USA) to provide the closest temporal match possible between muscle and otolith material. Otolith powder was milled onto weigh paper and weighed to the nearest microgram.

Otolith material was homogenized with a mortar and pestle and then subdivided into two portions for bulk inorganic and compound-specific SIA. Approximately 50 µg of otolith material was transferred to a glass reaction vessel and analyzed for bulk $\delta^{13}C$ on a Thermo Finnigan Mat 253 isotope ratio monitoring mass spectrometer (irm-MS) with a Kiell III carbonate device at Woods Hole Oceanographic Institution, Woods Hole, Massachusetts, USA, following the methods of Ostermann and Curry (2000). External precision of the mass spectrometer for $\delta^{13}C$ measurements in carbonate standards was ±0.03‰ (Ostermann and Curry 2000). Approximately 10 mg of otolith material from each fish was processed to isolate individual AAs. Samples of homogenized otolith powder were acid-hydrolyzed in 4 mL Teflon-lined screw cap vials along with 0.1 mL of 6 mol·L$^{-1}$ HCl per milligram of otolith under a $N_2$ atmosphere at 110 °C for 20 h. Samples were neutralized with ultrapure water and evaporated to dryness under a gentle stream of $N_2$ to remove HCl. Samples were stored frozen until they were derivatized just prior to compound-specific SIA.

Freeze-dried, homogenized white muscle samples from each fish were also subdivided into two portions. Approximately 1 mg of muscle was weighed into a tin cup and analyzed for bulk $\delta^{13}C$ values using a Europa Hydra 20/20 irm-

MS at the UC Davis Stable Isotope Facility, Davis, California, USA. A second portion of each sample (~500 μg) was acid-hydrolyzed with 1 mL of 6 mol·L$^{-1}$ HCl per milligram of freeze-dried muscle tissue as described previously for the otolith samples. Dried, neutralized samples were also stored frozen until derivatization.

**Compound-specific stable isotope analysis**

Amino acids contain highly polar functional groups (e.g., carboxylic acid groups), making them difficult to analyze by gas chromatography – combustion – isotope ratio monitoring mass spectrometry (GC-C-irm-MS) (reviewed by Klee 1985). As a result, amino acids must first be derivatized through the addition of less polar functional groups. Acid-hydrolyzed otolith and muscle samples were therefore derivatized prior to analysis using an approach modified from Silfer et al. (1991) and Johnson et al. (1998). All reactions were performed with analytical grade reagents in muffled glassware to prevent contamination. First, each sample underwent an acid-catalyzed esterification. Each sample vial received 0.8 mL of a 2-propanol and acetyl chloride solution (4:1 by volume). Vials were put under an atmosphere of $N_2$ and placed on a heating block at 110 °C for 1 h. The reactions were then quenched in an ice bath, and the otolith samples were quantitatively transferred to new 4 mL vials using dichloromethane (DCM), leaving behind salts associated with the acid hydrolysis of carbonate. All samples were dried under a gentle stream of $N_2$. To remove any remaining acidified isopropanol, samples were brought up in 0.5 mL of DCM and dried under $N_2$ three times. Samples were then acylated with 0.5 mL of trifluoroacetic anhydride (TFAA) and 0.5 mL of DCM under an atmosphere of $N_2$ at 110 °C for 10 min. Again, reactions were quenched in an ice bath, and excess TFAA was removed as described above using three rinses of DCM.

The derivatization process alters the $\delta^{13}C$ values of sample AAs through addition of exogenous carbon associated with the added functional groups and kinetic fractionation associated with the derivatization reactions (Rieley 1994; Docherty et al. 2001). An AA standard, consisting of all AAs of interest, was created from individual AAs (Sigma-Aldrich Co., St. Louis, Missouri, USA). The $\delta^{13}C$ value of each AA was determined via elemental analyzer irm-MS at Woods Hole Oceanographic Institution prior to creating the composite AA standard. The AA standard was concurrently derivatized with each batch of samples. Derivatization correction factors were determined for each AA of interest based on the known $\delta^{13}C$ value of the AAs in the standard prior to derivatization and the postderivatization $\delta^{13}C$ values determined with each sample batch. The correction factors were applied to each sample to adjust for the introduction of exogenous carbon and kinetic fractionation during derivatization.

Derivatized samples were brought up in DCM and injected on column in splitless mode at 260 °C and separated on an HP Ultra-1 column (50 m length, 0.32 mm inner diameter, and 0.5 μm film thickness; Hewlett Packard, Wilmington, Delaware, USA) in an Agilent 6890N Gas Chromatograph (GC) at Woods Hole Oceanographic Institution. Sample concentrations were adjusted to achieve a minimum 2 V output for all AAs. Gas chromatography conditions were set to optimize peak separation and shape as follows: initial temperature 75 °C held for 2 min; ramped to 90 °C at 4 °C·min$^{-1}$, held for 4 min; ramped to 185 °C at 4 °C·min$^{-1}$, held for 5 min; ramped to 250 °C at 10 °C·min$^{-1}$, held 2 min; ramped to 300 °C at 20 °C·min$^{-1}$, held for 8 min. The separated AA peaks were combusted online in a Finnigan GC-C continuous flow interface at 930 °C and then measured as $CO_2$ on a Thermo Finnigan Mat 253 irm-MS. Standardization of runs was achieved using intermittent pulses of a $CO_2$ reference gas of known isotopic composition. All compound-specific SIA samples were analyzed in duplicate along with AA standards of known isotopic composition. The glutamic acid and aspartic acid peaks contained unknown contributions from glutamine and asparagine, respectively, due to conversion to their dicarboxylic acids during acid hydrolysis. The relative abundance (%) of individual AAs in otoliths and muscle were calculated from mass 44 peak area based on standards of known concentration.

**Data analysis**

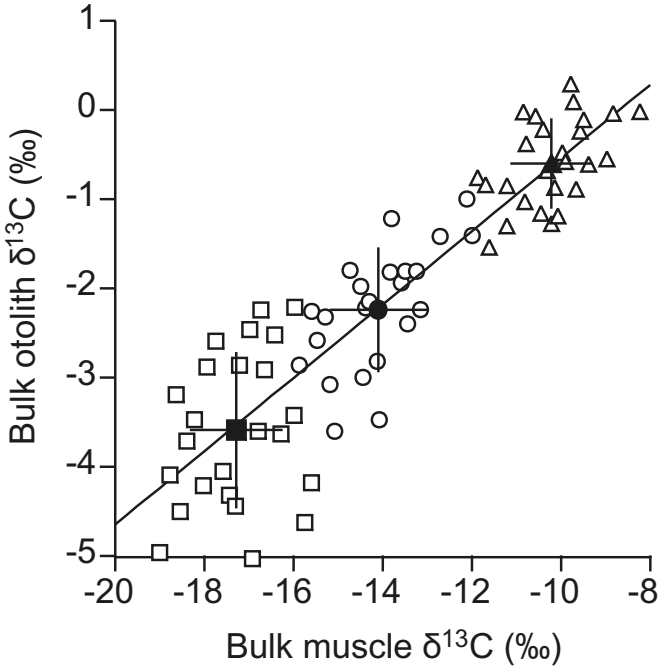
Stable isotope ratios were expressed in standard delta (δ) notation:

$$\delta^{13}C_{sample} = \left( \frac{{}^{13}C/{}^{12}C_{sample}}{{}^{13}C/{}^{12}C_{std}} \right) \times 1000$$

where the standard for carbon was Vienna PeeDee Belemnite (VPDB). We determined the relationship between paired bulk muscle and otolith $\delta^{13}C$ values from 73 fish collected at the three sites using linear regression. We tested for differences in $\delta^{13}C$ of bulk muscle and otoliths among sites using separate one-way analyses of variance (ANOVAs) and Tukey's honestly significant difference (HSD) post-hoc tests ($\alpha$ = 0.05). Bulk muscle and otolith data met the assumptions of normality and equality of variances. The relationships between $\delta^{13}C$ values of individual AAs from paired otolith and muscle samples were determined by linear regression analyses for each AA ($n$ = 15 fish per AA). Differences in $\delta^{13}C$ values of non-essential and essential AAs in *L. ehrenbergii* otoliths among sites were determined using separate multivariate ANOVAs (MANOVA; $\alpha$ = 0.05). We assigned AAs as non-essential or essential according to Karasov and Martínez del Rio (2007).

Minimum sample sizes necessary for compound-specific SIA of otolith and muscle were determined by extrapolation of sample sizes used in this study down to the GC-C-irm-MS lower limit of detection for the least abundant AAs. Three aliquots of the same AA standard were derivatized at the same time under the same reaction conditions (for within-batch variability) on three separate days (for among-batch variability). To examine the variability in AA $\delta^{13}C$ values within and among derivatization batches, the mean relative standard deviation (RSD) within batch and among batch was calculated across all 11 AAs. The desktop stability of derivatives was assessed by analyzing three aliquots of the same AA standard a total of 20 times each over the course of nine days. Overall external precision of the $\delta^{13}C$ measurements after correcting for the fractionation associated with derivatization was 0.80‰ ± 0.96‰ (standard deviation (SD)), averaged across all AAs.

**Fig. 2.** Linear relationship between bulk otolith and bulk muscle $\delta^{13}C$ values from *Lutjanus ehrenbergii* collected from three isotopically distinct habitats near Al Lith, Saudi Arabia, in the Red Sea ($n$ = 73 fish; open symbols), including mean (± standard deviation (SD); solid symbols) muscle and otolith $\delta^{13}C$ values for Al Lith Bay wetlands (triangles; $n$ = 26 fish), Coast Guard Reef (circles, $n$ = 23 fish), and Ron's Reef (squares; $n$ = 24 fish).




## Results

We found a significant linear relationship between paired bulk muscle and otolith $\delta^{13}C$ values of *L. ehrenbergii* (linear regression, $y = 0.38x + 3.31$, $R^2 = 0.83$; Fig. 2). *Lutjanus ehrenbergii* collected from three locations near Al Lith, Saudi Arabia, had distinct $\delta^{13}C$ values for bulk otoliths (one-way ANOVA, df = 2, 14, $F = 13.3$, $p < 0.05$) and bulk muscle (one-way ANOVA, df = 2, 14, $F = 58.9$, $p < 0.05$). However, *L. ehrenbergii* from Coast Guard Reef and Ron's Reef had statistically similar otolith $\delta^{13}C$ values, with $p < 0.05$ for all other pairwise comparisons. The overall range in $\delta^{13}C$ values among locations was much larger for bulk muscle (7.2‰) than it was for bulk otolith (2.6‰). Individuals from seagrass habitats in the Al Lith Bay wetlands had the most positive $\delta^{13}C$ values for muscle (mean ± SD = –10.3‰ ± 1.0‰) and otoliths (–0.7‰ ± 0.6‰), whereas fish from the offshore reef had the most negative $\delta^{13}C$ values for muscle (–17.5‰ ± 0.8‰) and otoliths (–3.3‰ ± 1.1‰). Fish from the reef adjacent to Al Lith Bay had intermediate $\delta^{13}C$ values for muscle (–14.6‰ ± 1.2‰) and otoliths (–2.3‰ ± 0.6‰).

Protein comprised 0.6% ± 0.1% of otoliths and 92.3% ± 1.3% of muscle for *L. ehrenbergii* ($n$ = 3). Eleven individual AAs from muscle and otolith protein with sufficient peak size and GC baseline resolution were identified and analyzed for $\delta^{13}C$ via GC-C-irm-MS (Fig. 3). Glutamic acid and aspartic acid were the most abundant AAs in *L. ehrenbergii* muscle and otolith, whereas leucine and threonine were the most abundant essential AAs in muscle and otolith (Table 1). Based on the least abundant AA in our analyses, isoleucine for otoliths and proline for muscle, and the lower limit of detection for the MAT 253 irm-MS, the minimum sample size needed to conduct compound-specific SIA on *L. ehrenbergii* was 500–1000 µg for otoliths and 10–15 µg for muscle. The derivatization process made the $\delta^{13}C$ values of the AAs in the standard more negative, although the shifts were not uniform among AAs. Variability in the $\delta^{13}C$ values of derivatized AA standards was much smaller within derivatization batches (mean RSD = 0.8% ± 0.2% SD) than among batches (2.2% ± 1.6% SD). Repeated injections of the same derivatized standard were very consistent, showing low variability in $\delta^{13}C$ values (mean SD for all AAs = 0.35‰ ± 0.14‰ SD) for the first 160 h after derivatization (Fig. 4). After approximately 160 h, the $\delta^{13}C$ values of AAs in the standard became significantly more variable (1.25‰ ± 0.57‰) and tended to become more positive with time. The shift was not consistent across all AAs, as serine and threonine typically became unstable first. Similar patterns were also observed in the fish muscle and otolith samples.

We found a strong linear relationship between individual otolith and muscle AA $\delta^{13}C$ values (linear regression, $y = (0.84 \pm 0.25)x - (1.72 \pm 3.66)$, $R^2 = 0.70 \pm 0.22$) of *L. ehrenbergii* (Fig. 5). Otolith $\delta^{13}C$ values in AAs generally tracked the patterns observed in the bulk muscle and otoliths, although the otolith AA $\delta^{13}C$ range was closer to the bulk muscle range, particularly for several of the essential AAs (Fig. 6). Individual AAs from otoliths of fish collected in the Al Lith Bay wetlands typically had the most positive $\delta^{13}C$ values, and those from the offshore reef often had the most negative $\delta^{13}C$ values, with otolith AAs of fish collected in Coast Guard Reef intermediate (Fig. 6). We found significant differences in otolith $\delta^{13}C$ values for non-essential AAs (MANOVA, Pillai's trace = 0.97, df = 6, 8, $F = 48.04$, $p < 0.05$) and essential AAs (MANOVA, pillai trace = 0.92, df = 5, 9, $F = 21.37$, $p < 0.05$).

## Discussion

Stable isotope analysis (SIA) of AAs in otolith protein provides a new way to retrospectively address questions of diet, habitat use, and migration in fishes. The method avoids many of the complications associated with conventional bulk SIA of fish otoliths, including DIC dilution of dietary signatures and variable metabolic carbon contribution to otolith $\delta^{13}C$ values. We tested this new approach by sampling muscle and otoliths from Ehrenberg's snapper, *L. ehrenbergii*, collected along a carbon isotope gradient from coastal seagrass habitats to offshore coral reefs in the Red Sea. Fish from Al Lith Bay wetlands had the most positive muscle $\delta^{13}C$ values (–10.4‰), which likely reflected the carbon contribution of seagrasses within the bay. Seagrasses are C4 primary producers at the base of the food web, with $\delta^{13}C$ values between –8‰ and –12‰ (Hemminga and Mateo 1996). In contrast, *L. ehrenbergii* muscle tissue from the reef 14 km offshore had the most negative $\delta^{13}C$ values (–17.5‰), reflecting a marine phytoplankton $\delta^{13}C_{Base}$ signature that typically ranges from –17‰ to –21‰ (Descolas-Gros and Fontungne 1990). Fish from the reef adjacent to Al Lith Bay had intermediate $\delta^{13}C$ values for muscle (–14.6‰) that presumably indicated carbon inputs from both seagrasses and phytoplankton sources. The observed $\delta^{13}C$ isoscape proved an ideal system to test

**Fig. 3.** A representative gas chromatogram of derivatized individual amino acids from an otolith of *Lutjanus ehrenbergii*. $CO_2$ ref, intermittent pulses of a $CO_2$ gas reference of known isotopic composition. Amino acids: Gly, glycine; Ser, serine; Asp, aspartic acid; Glu, glutamic acid; Pro, proline; Ala, alanine; Thr, threonine; Ile, isoleucine; Val, valine; Phe, phenylalanine; Leu, leucine.

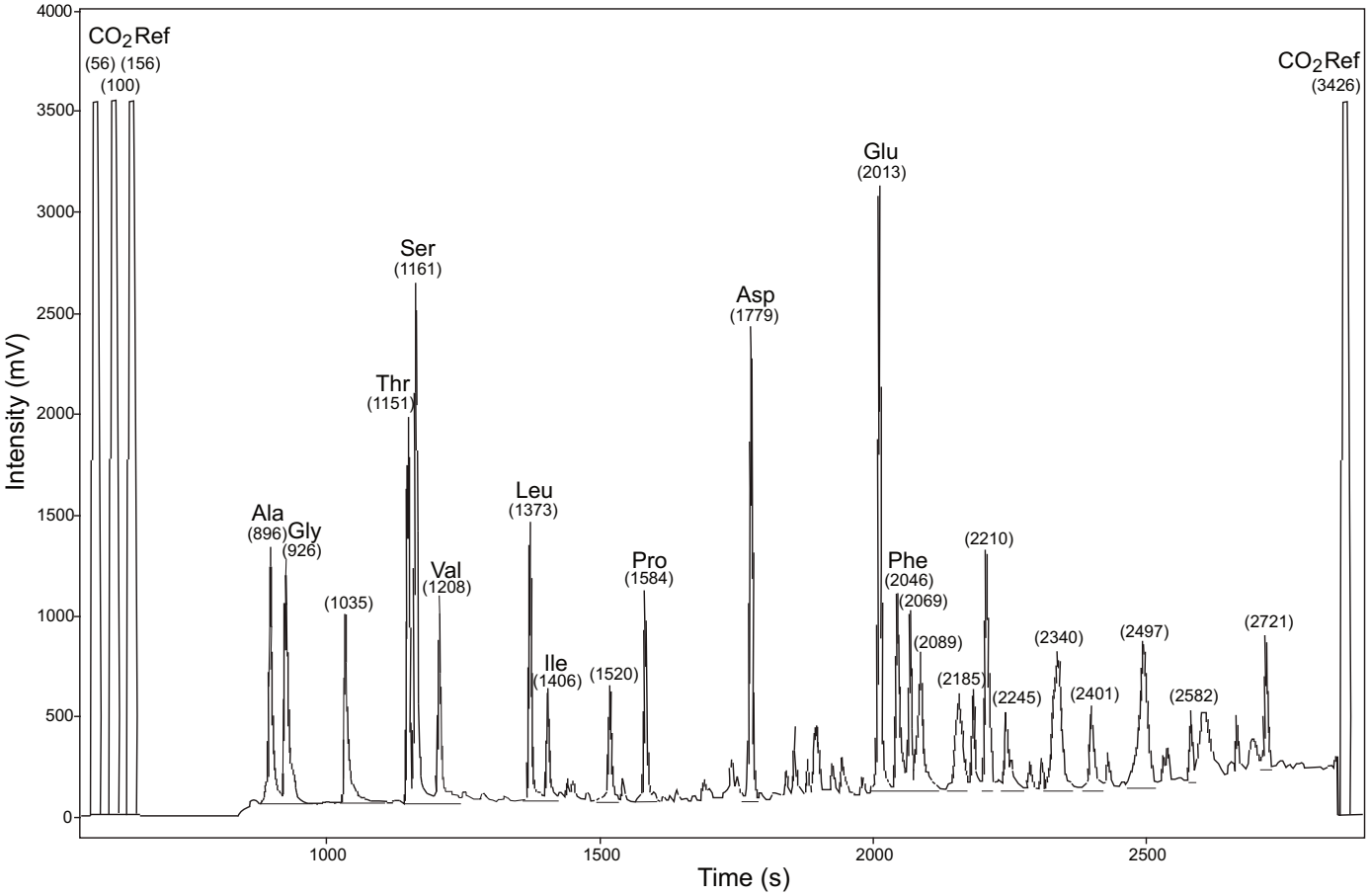
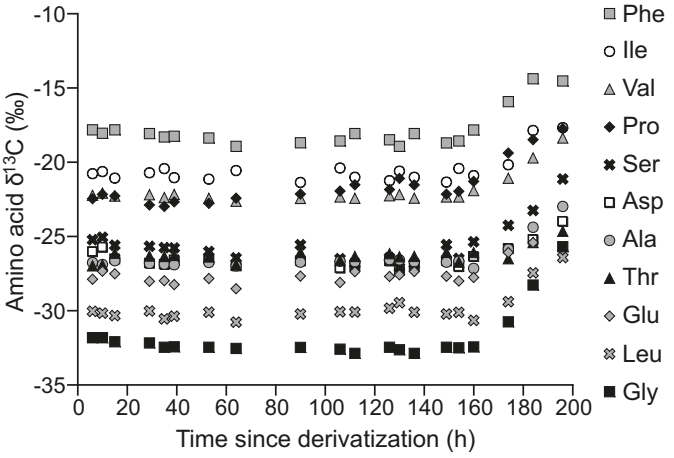



**Table 1.** The relative abundance (mean percentage ± standard deviation (SD)) of 11 individual amino acids in otolith and muscle of *Lutjanus ehrenbergii* calculated from mass 44 peak area and standards of known concentration via gas chromatography.

| Amino acids | Otolith | Muscle |
|---|---|---|
| Non-essential | | |
| Gylcine | 7.2±0.6 | 5.8±0.2 |
| Serine | 9.2±0.5 | 5.1±0.6 |
| Aspartic acid | 16.5±1.0 | 16.6±0.3 |
| Glutamic acid | 27.0±1.2 | 26.1±1.2 |
| Proline | 9.8±1.5 | 4.6±2.5 |
| Alanine | 6.6±0.8 | 7.5±0.3 |
| Essential | | |
| Threonine | 6.6±0.8 | 5.4±0.1 |
| Isoleucine | 3.2±0.9 | 5.1±0.1 |
| Valine | 4.0±0.3 | 5.6±0.2 |
| Phenylalanine | 4.0±0.3 | 5.3±0.1 |
| Leucine | 5.9±0.5 | 13.0±0.4 |
| Percentage protein | 0.6±0.1 | 92.2±1.3 |

**Note:** For relative abundance, $n$ = three sites with five fish per site; for percentage protein of otolith and muscle, $n$ = three fish.

**Fig. 4.** $\delta^{13}C$ values of derivatized amino acid standards analyzed 20 times over the course of nine days via gas chromatography – combustion – isotope ratio monitoring – mass spectrometry. Amino acids: Gly, glycine; Ser, serine; Asp, aspartic acid; Glu, glutamic acid; Pro, proline; Ala, alanine; Thr, threonine; Ile, isoleucine; Val, valine; Phe, phenylalanine; Leu, leucine.




the ability of AAs in otoliths to accurately record $\delta^{13}C_{Base}$ signatures compared with conventional bulk otolith SIA.

Bulk otolith $\delta^{13}C$ values were significantly different among the three habitats and significantly correlated with bulk muscle $\delta^{13}C$ values. Several recent studies have suggested that bulk otolith records some dietary information even though most of the carbon comes from DIC in the water column (Dufour et al. 2007; Mateo 2009; Elsdon et al. 2010).

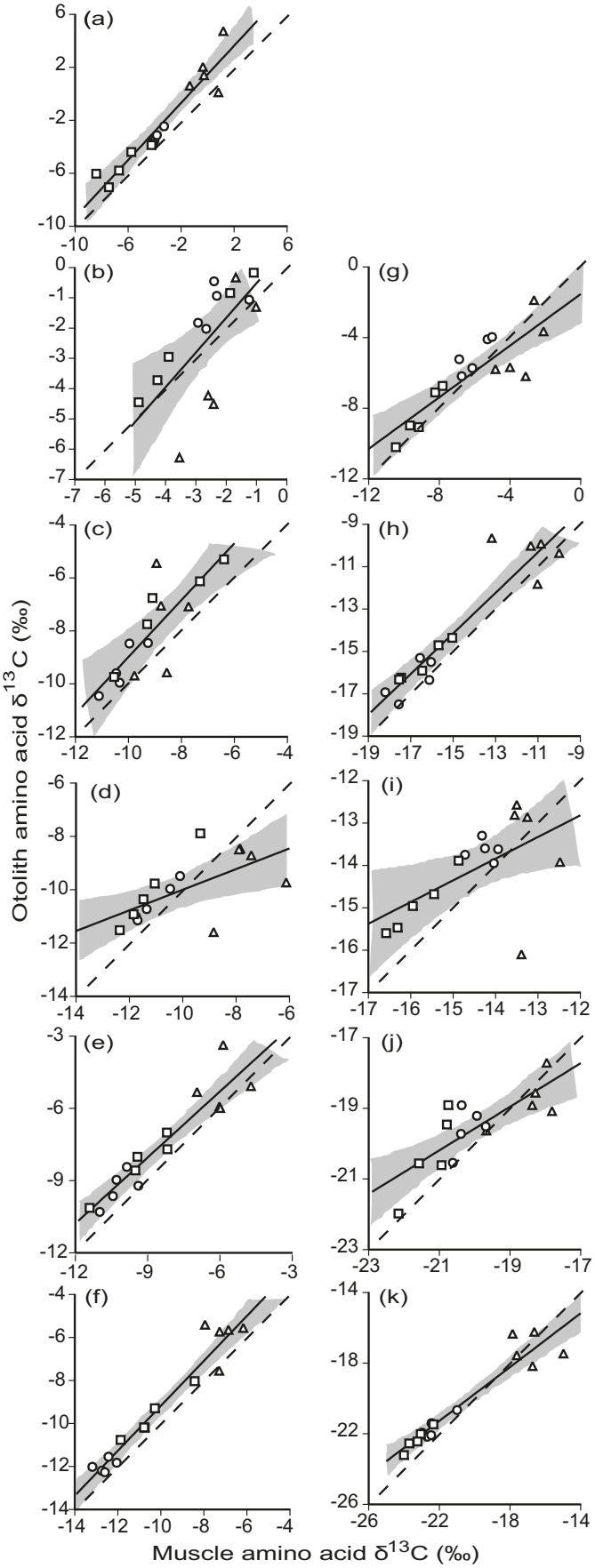
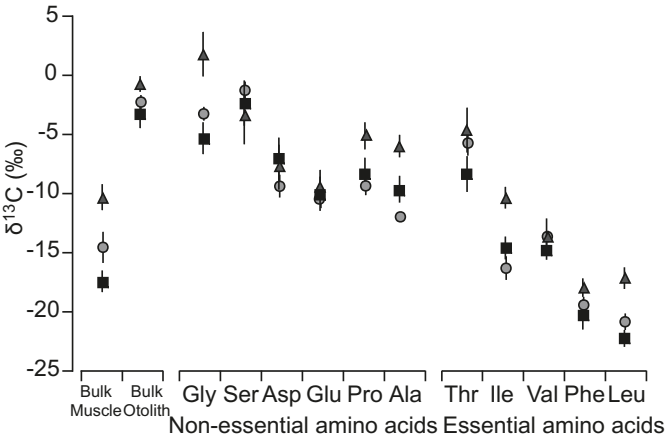



**Fig. 5.** Linear relationships (solid line with shaded 95% confidence intervals) of paired otolith and muscle amino acid $\delta^{13}C$ values for 11 individual amino acids from *Lutjanus ehrenbergii* collected from Al Lith Bay wetlands (triangles), Coast Guard Reef (circles), and Ron's Reef (squares) near Al Lith, Saudi Arabia, in the Red Sea ($n$ = 15 fish). Panels *a* to *f* represent non-essential amino acids: (*a*) glycine, (*b*) serine, (*c*) aspartic acid, (*d*) glutamic acid, (*e*) proline, and (*f*) alanine. Panels *g* to *k* represent essential amino acids: (*g*) threonine, (*h*) isoleucine, (*i*) valine, (*j*) phenylalanine, (*k*) leucine. Broken line represents the 1:1 line. Scales are not the same among panels.

**Fig. 6.** Bulk muscle and otolith $\delta^{13}C$ values (mean ± SD) and otolith non-essential and essential amino acid $\delta^{13}C$ values (mean ± SD) from *Lutjanus ehrenbergii* collected from three isotopically distinct habitats: Al Lith Bay wetlands (triangles), Coast Guard Reef (circles), and Ron's Reef (squares) near Al Lith, Saudi Arabia, in the Red Sea ($n$ = five fish per site). Amino acids: Gly, glycine; Ser, serine; Asp, aspartic acid; Glu, glutamic acid; Pro, proline; Ala, alanine; Thr, threonine; Ile, isoleucine; Val, valine; Phe, phenylalanine; Leu, leucine.




However, this variability in bulk otolith $\delta^{13}C$ among sites may have been due to a number of factors, including changes in metabolic rate with ontogeny (Kalish 1991; Weidman and Millner 2000; Stephenson et al. 2001), changes in diet $\delta^{13}C$ and trophic position shifts between juvenile and adult fish (Gauldie 1996; Begg and Weidman 2001), changes in seawater DIC $\delta^{13}C$ with distance offshore (Schwarcz et al. 1998), or other environmental conditions (Mulcahy et al. 1979; Kalish 1991). Although there were significant differences in bulk otolith $\delta^{13}C$ values across habitats, it remains difficult to determine an unambiguous dietary $\delta^{13}C$ value from bulk $\delta^{13}C$ analysis of otoliths. Furthermore, otolith values recorded less than half of the $\delta^{13}C$ range seen in muscle tissue. As a result, the ability to discriminate among habitats using the bulk otolith data was reduced compared with muscle $\delta^{13}C$ values. This was particularly true when comparing fish from Coast Guard Reef and Ron's Reef, where muscle $\delta^{13}C$ values showed significantly larger differences between habitats (mean difference = 3‰) than otolith $\delta^{13}C$ values (mean difference = 1‰).

Muscle AA $\delta^{13}C$ values accounted for most of the observed variation in otolith AA $\delta^{13}C$ values with a slope of the relationship near to 1. Otolith AAs, therefore, recorded nearly identical dietary information to that of muscle AAs.

This is perhaps not surprising considering both muscle and otoliths likely receive AAs for protein synthesis from a common AA pool in the blood. Our results also suggest that any fractionation during transport of AAs from blood to the site of protein synthesis and subsequent release from the macula (Murayama 2000; Murayama et al. 2004) was minor. As such, $\delta^{13}C$ analysis of otolith AAs should provide an archival record of fish diet that has previously been inaccessible with conventional inorganic otolith analysis.

Stable isotope analysis of individual AAs has greatly improved the study of diet (Fantle et al. 1999; Fogel and Tuross 2003), habitat use (Popp et al. 2007), and the sources of complex mixtures of organic matter (Uhle et al. 1997; McCarthy et al. 2004) for a number of terrestrial and aquatic taxa. It can be challenging with bulk SIA to distinguish differences in consumer $\delta^{13}C$ due to changes in trophic level versus changes in $\delta^{13}C_{Base}$. Most recently, in a controlled feeding experiment, McMahon et al. (2010) showed that essential AAs in fish muscle recorded the $\delta^{13}C$ values of diet with little or no trophic fractionation, thereby providing an accurate record of $\delta^{13}C_{Base}$ signature. We found that juvenile *L. ehrenbergii* in Al Lith Bay wetlands had essential AA $\delta^{13}C$ signatures that were very different from adult *L. ehrenbergii* on coastal and shelf reefs. The distinction was clear even when comparing *L. ehrenbergii* from Al Lith Bay wetlands and Coast Guard Reef, which were only 2 km apart. The unique habitat signatures in coastal wetlands and coral reefs can be traced to the local food web isotope values in those habitats (McMahon 2011). Our study suggests that otolith essential AA SIA should be particularly valuable for retrospectively assessing residence in and movement among habitats with distinct food web $\delta^{13}C_{Base}$ values.

O'Donnell et al. (2007) studied $\delta^{13}C$ values of AAs from modern and fossil *Mercenaria* shells to examine preservation of AAs in biominerals and regional and ontogenetic variability in $\delta^{13}C$ values. The authors concluded that the range of $\delta^{13}C$ values in AAs from modern *Mercenaria* collected from coastal Virginia and coastal Florida suggested the preservation of a dietary signal in the bivalve shells. In addition, the authors showed significant variation in *Mercenaria* shell AA $\delta^{13}C$ values within and among years, likely reflecting a shift in the relative contribution of primary producers at the base of the food web. Similar work by Johnson et al. (1998) provides another example of using AA SIA of biominerals to examine diet and local habitat use. These authors showed that AA $\delta^{13}C$ values from ostrich egg shells reflected the diet of ostrich at the time of egg formation and could identify local climate and vegetation conditions. Our data, in concert with these studies, suggest that compound-specific SIA of otoliths is a valuable approach to determining residence in habitats with distinct $\delta^{13}C_{Base}$ signatures.

In addition to determining $\delta^{13}C_{Base}$ signatures, AA $\delta^{13}C$ values in otoliths may record other valuable information about diet that was previously difficult to assess with conventional bulk otolith SIA. McMahon et al. (2010) found that $\delta^{13}C$ values of non-essential AAs in fish muscle showed diet-specific patterns of de novo biosynthesis and direct isotopic routing from dietary protein. The AA composition and lipid to carbohydrate ratio of the diet both appeared to play a role in determining the $\delta^{13}C$ value of muscle non-essential AAs. In our study, otolith non-essential AAs showed similar patterns to essential AAs, suggesting that they recorded similar shifts in $\delta^{13}C_{Base}$ among habitats. This observation suggests a high degree of direct isotopic routing of non-essential AAs from dietary protein into fish biomass. Previous research has shown that consumers feeding on high protein diets often route most AAs, including non-essentials, directly from diet as a means of energy conservation, because dietary routing is typically more efficient than de novo biosynthesis (Ambrose and Norr 1993; Tieszen and Fagre 1993; Jim et al. 2006). Although the mechanisms driving non-essential AA $\delta^{13}C$ values remain unknown, the findings of McMahon et al. (2010) suggest that valuable dietary information is stored in $\delta^{13}C$ values of non-essential AAs. Given the high correlation between muscle and otolith AA $\delta^{13}C$ values, it is likely that the otolith non-essential AAs will also provide valuable information about diet and food quality.

We calculated a minimum sample size of 500–1000 μg for AA $\delta^{13}C$ analysis of *L. ehrenbergii* otoliths. Although *L. ehrenbergii* have relatively large otoliths, the percentage organic matter in these otoliths is quite small (~0.6%). There is significant variability in the percentage organic matter in otoliths that may vary by as much as 10% depending on species and life history stage (Degens et al. 1969; Morales-Nin 1986; Jolivet et al. 2008). As a result, the minimum sample size necessary for compound-specific SIA of *L. ehrenbergii* otoliths is likely significantly larger than for many other species. The temporal resolution of our approach will depend largely on the size and organic content in otoliths of the study species. As instrument sensitivity improves and sample size requirements decrease, we remain confident that we will be able to address questions of diet and movement at higher temporal resolution and on smaller otoliths. The sample size necessary for compound-specific analyses using GC-C-irm-MS may never be as small as that necessary for bulk otolith $\delta^{13}C$ measurements; however, $\delta^{13}C$ values of otolith AAs provide a wealth of information not available from conventional bulk analyses.

In conclusion, $\delta^{13}C$ analysis of AAs in otoliths is a valuable new tool for retrospective analysis of diet and movement patterns of fishes. Otolith AA $\delta^{13}C$ signatures were highly correlated with muscle values and provided a purely dietary value that avoided the confounding factors of DIC dilution and variable metabolic carbon contribution found in bulk otolith $\delta^{13}C$ analysis. The otolith AA approach provides access to dietary information in otoliths that should compliment data from conventional bulk otolith SIA. As with any stable isotope study of diet and movement, if fish reside in isotopically indistinguishable habitats or move through habitats faster than the habitat signature is recorded, then the method that we describe here may not identify residence in all intermediate habitats. However, otolith essential AAs provide a valuable tracer of residence in isotopically distinct habitats that increases our ability to track the movement of migratory fish or determine the $\delta^{13}C_{Base}$ values for resident individuals. Conversely, non-essential AAs may provide important archived information about diet composition and metabolic processing. Further research will, however, be needed before we can confidently interpret the biological significance of $\delta^{13}C$ variations in non-essential AAs in otoliths or other tissues.

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