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Carbon isotope fractionation of amino acids in fish muscle reflects biosynthesis and isotopic routing from dietary protein

Kelton W. McMahon[1]*, Marilyn L. Fogel[2], Travis S. Elsdon[3] and Simon R. Thorrold[4]

[1]*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;* [2]*Carnegie Institution of Washington, 5251 Broad Branch Rd. N.W., Washington, DC 20015, USA;* [3]*Southern Seas Ecology Laboratories, School of Earth and Environmental Sciences, University of Adelaide, Adelaide, SA 5005, Australia; and* [4]*Biology Department, Woods Hole Oceanographic Institution, Woods Hole, MA 02543, USA*

Summary

1. Analysis of stable carbon isotopes is a valuable tool for studies of diet, habitat use and migration. However, significant variability in the degree of trophic fractionation ($\Delta^{13}C_{C\text{-}D}$) between consumer (C) and diet (D) has highlighted our lack of understanding of the biochemical and physiological underpinnings of stable isotope ratios in tissues.

2. An opportunity now exists to increase the specificity of dietary studies by analyzing the $\delta^{13}C$ values of amino acids (AAs). Common mummichogs (*Fundulus heteroclitus*, Linnaeus 1766) were reared on four isotopically distinct diets to examine individual AA $\Delta^{13}C_{C\text{-}D}$ variability in fish muscle.

3. Modest bulk tissue $\Delta^{13}C_{C\text{-}D}$ values reflected relatively large trophic fractionation for many non-essential AAs and little to no fractionation for all essential AAs.

4. Essential AA $\delta^{13}C$ values were not significantly different between diet and consumer ($\Delta^{13}C_{C\text{-}D} = 0{\cdot}0 \pm 0{\cdot}4‰$), making them ideal tracers of carbon sources at the base of the food web. Stable isotope analysis of muscle essential AAs provides a promising tool for dietary reconstruction and identifying baseline $\delta^{13}C$ values to track animal movement through isotopically distinct food webs.

5. Non-essential AA $\Delta^{13}C_{C\text{-}D}$ values showed evidence of both *de novo* biosynthesis and direct isotopic routing from dietary protein. We attributed patterns in $\Delta^{13}C_{C\text{-}D}$ to variability in protein content and AA composition of the diet as well as differential utilization of dietary constituents contributing to the bulk carbon pool. This variability illustrates the complicated nature of metabolism and suggests caution must be taken with the assumptions used to interpret bulk stable isotope data in dietary studies.

6. Our study is the first to investigate the expression of AA $\Delta^{13}C_{C\text{-}D}$ values for a marine vertebrate and should provide for significant refinements in studies of diet, habitat use and migration using stable isotopes.

Key-words: compound-specific stable isotope analysis, feeding experiment, food web, metabolic processing, trophic dynamics

Introduction

Stable isotope analysis (SIA) has become a routine tool in ecology for studies of diet and trophic dynamics (Peterson & Fry 1987; Gannes, Martínez del Rio & Koch 1998; Michener & Kaufmann 2007), habitat use (McMahon, Johnson & Ambrose 2005; Cherel *et al.* 2007) and animal migration (Hansson *et al.* 1997; Hobson 1999; Rubenstein & Hobson 2004). Bulk tissue SIA studies using carbon rely upon the assumption that the isotope composition of a consumer reflects the weighted average of the carbon isotope compositions of its diet with a small amount of diet (D) to consumer (C) fractionation, hereafter $\Delta^{13}C_{C\text{-}D}$ (typically 0–1‰, DeNiro & Epstein 1978; Fry, Joern & Parker 1978). Despite

*Correspondence author. E-mail: kmcmahon@whoi.edu

the prevalence of bulk SIA in ecological studies of diet and food webs, there are still a number of confounding factors that can complicate interpretations of bulk SIA data.

The carbon isotope composition at the base of the food web ($\delta^{13}C_{base}$) ultimately determines the $\delta^{13}C$ values of higher trophic level consumers. Without suitable estimates of $\delta^{13}C_{base}$, which can vary both spatially and temporally (Vander Zanden & Rasmussen 1999; Graham *et al*. 2009), it is difficult to interpret consumer $\delta^{13}C$ values using bulk SIA in light of potential changes in food web structure vs. variations in $\delta^{13}C_{base}$ (Post 2002). This can be particularly problematic when studying the diet and trophic dynamics of highly migratory marine organisms that move among isotopically distinct food webs (Estrada, Lutcavage & Thorrold 2005; Graham *et al*. 2009). There can also be significant variation in $\Delta^{13}C_{C\text{-}D}$, ranging from −3 to +5‰, depending on consumer taxa, diet and tissues analysed (Gannes, O'Brien & Martínez del Rio 1997; Vander Zanden & Rasmussen 2001; McCutchan *et al*. 2003; Olive *et al*. 2003). Furthermore, the $\delta^{13}C$ value of consumer tissue may not always follow bulk diet $\delta^{13}C$ values because the carbon skeletons of different dietary constituents (proteins, lipids and carbohydrates) can be routed to different tissue constituents ('isotopic routing'; Schwarcz 1991). Several studies have emphasized the problems that isotopic routing poses to the interpretation of stable isotope data in diet reconstructions (Parkington 1991; Schwarcz & Schoeninger 1991; Ambrose & Norr 1993). All of these factors can make interpretations of bulk tissue SIA challenging for studies of diet and migration, prompting a call for studies that examine the biochemical and physiological basis of stable isotope ratios in ecology (Gannes, O'Brien & Martínez del Rio 1997; Gannes, Martínez del Rio & Koch 1998; Karasov & Martínez del Rio 2007).

The opportunity now exists to increase the specificity of dietary studies by analysing $\delta^{13}C$ values of specific biochemical compounds, including amino acids (AAs), thanks to recent advances in gas chromatography-combustion-isotope ratio monitoring mass spectrometry (GC-C-irmMS) (Merritt, Brand & Hayes 1994; Meier-Augenstein 1999; Sessions 2006). Stable isotope analysis of individual AAs has the potential to provide more detailed information about diet (Fantle *et al*. 1999; Fogel & Tuross 2003; Popp *et al*. 2007) and the sources of complex mixtures of organic matter (Uhle *et al*. 1997; McCarthy *et al*. 2004) than conventional bulk tissue SIA.

There have been very few controlled feeding experiments examining the trophic fractionation of individual AAs between diet and consumer (Hare *et al*. 1991; Howland *et al*. 2003; Jim *et al*. 2006). Studies to date found modest bulk tissue $\Delta^{13}C_{C\text{-}D}$ values (~1‰) actually reflected an average of relatively large fractionations in many non-essential AAs and comparatively little fractionation in most essential AAs. However, there was considerable variation in $\Delta^{13}C_{C\text{-}D}$ across diets and individual AAs among studies. Furthermore, these studies all dealt with terrestrial vertebrates (pigs and rats), yet no controlled feeding experiments looking at compound-specific $\Delta^{13}C_{C\text{-}D}$ have been conducted on an aquatic vertebrate. Given the variability in bulk tissue $\Delta^{13}C_{C\text{-}D}$ across terrestrial and aquatic taxa (Vander Zanden & Rasmussen 2001; McCutchan *et al*. 2003), it is important to determine the mechanisms leading to variability in the fractionation of AA $\delta^{13}C$ values for aquatic taxa.

We reared common mummichogs (*Fundulus heteroclitus*, Linnaeus 1766) on four isotopically distinct diets to examine trophic fractionation ($\Delta^{13}C_{C\text{-}D}$) of individual AAs between diet and consumer. By choosing an herbivorous diet, two carnivorous diets and an omnivorous diet, we aimed to examine the potential variability in AA $\Delta^{13}C_{C\text{-}D}$. We addressed the specific question: What is the isotopic relationship between diet and consumer for individual AAs in fish muscle? We focused on muscle tissue, because it is one of the most commonly used tissues in ecological studies of diet and trophic dynamics. We hypothesized that non-essential AA $\delta^{13}C$ values would show evidence of both *de novo* biosynthesis and direct isotopic routing from dietary protein while essential AA $\delta^{13}C$ values would only reflect isotopic routing. Similar results were found for pigs (Hare *et al*. 1991; Howland *et al*. 2003) and rats (Jim *et al*. 2006), although the magnitude and direction of trophic fractionation was quite variable. We also hypothesized that fish fed a high protein content diet would exhibit a greater degree of isotopic routing because routing is thought to be more efficient than *de novo* biosynthesis when non-essential AAs are sufficiently available (Ambrose & Norr 1993; Tieszen & Fagre 1993; Jim *et al*. 2006). Finally, we predicted that a deficit in non-essential AA abundance in diet relative to consumer tissue would result in higher trophic fractionation than would be expected from diets with excess non-essential AAs due to enhanced biosynthesis. Our study is the first to investigate the expression of individual AA $\Delta^{13}C_{C\text{-}D}$ values for an aquatic, vertebrate and should provide significant refinements to studies of diet, habitat use and migration using stable isotopes.

Materials and methods

FEEDING EXPERIMENT

We conducted a controlled feeding experiment on juvenile mummichogs (*Fundulus heteroclitus*) reared at the Atlantic Ecology Division, U.S. Environmental Protection Agency, in Narragansett, Rhode Island, USA. Adult *F. heteroclitus* collected from a salt marsh in Sandwich, Massachusetts were held in flow through seawater at temperatures elevated above ambient to induce spawning. Eggs from two spawnings were collected and transferred to tanks and allowed to hatch. Juvenile fish were reared on an *Artemia* diet for 6 weeks (approximate length: 11 mm), after which they were transferred to experimental tanks.

Experimental tanks consisted of twelve 40 gallon aquaria with flow through seawater at ambient temperature (20 °C) in two randomly positioned rows under a 12 : 12 h light : dark cycle from fluorescent tubes. Twenty juvenile *F. heteroclitus* were placed in each tank. Dietary manipulations consisted of triplicate tanks of fish reared on one of four isotopically distinct diets. A plant-based commercial fish pellet (Vegi-Pro, Freedon Feeds Inc., Urbana, OH, USA) consisted primarily of wheat and soy with a small contribution from corn meal. A

second commercial fish pellet (Bio-Vita, Bio-Oregon, Westbrook, ME, USA) consisted of fish and krill meal, wheat gluten and whey protein. Two natural animal-based diets, squid and clam, were obtained from a local supermarket, homogenized and then freeze-dried before being fed to the experimental fish. Proximate analysis of moisture by loss on drying at 135 °C for 2 h (method 930·15; AOAC 2005), crude protein by combustion (method 990·03; AOAC 2005), crude fat by ether extraction (method 920·39; AOAC 2005), crude fibre (method 978·10; AOAC 2005) and ash (method 942·05; AOAC 2005) were conducted on all four diets at the New Jersey Feed Laboratory, Trenton, New Jersey, USA. Carbohydrate content was determined as 100% minus the sum of moisture, protein, fat and ash. Amino acid compositions (16 individual AAs) (method 994·12; AOAC 2005) of the four diets and fish muscle from each treatment were also determined at the New Jersey Feed Laboratory ($n = 3$ replicates).

SAMPLE PREPARATION AND ANALYSIS

Fish were fed to saturation once per day, and tanks were cleaned of excess food and bio-films every 3 days. Fish were reared on isotopically distinct diets for 8 weeks and more than doubled in biomass during that time. All fish were then sacrificed in an ice slurry, frozen and freeze dried for 72 h. White muscle was removed from each fish, homogenized using a mortar and pestle, and subdivided into two portions, one for bulk tissue SIA and the other for compound-specific SIA. Results from the bulk tissue analyses are presented elsewhere (Elsdon *et al.* 2010).

Approximately 2 mg of each sample, both diet and fish muscle, were acid hydrolysed in 1 mL of 6 N HCl at 110 °C for 20 h to isolate the total free AAs. Samples were neutralized with ultrapure water and evaporated to dryness via rotary evaporation to remove HCl before being resuspended in 1 mL of ultrapure water. Samples were then passed through solid phase extraction-C18 columns to remove particulates and melanoidins. After drying under a stream of N_2 gas, the total free AAs were derivatized by esterification with acidified isopropanol followed by acetylation with trifluoroacetic anhydride (Silfer *et al.* 1991). The resulting derivatized AAs were diluted to a concentration of 2 μg μL^{-1} in dichloromethane.

Approximately 2 μg of AAs (via 1 μL injection) were injected on column in splitless mode at 220 °C and separated on an HP Ultra-1 column (50 m length, 0·32 mm inner diameter and 0·52 μm film thickness; Hewlett Packard, Wilmington, DE, USA) in a Varian 3400 Gas Chromatograph (GC) at the Carnegie Geophysical Laboratory, Washington, DC, USA. 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 in a Finnegan GC continuous flow interface at 980 °C, then measured as CO_2 on a Finnegan MAT Delta^{Plus}XL or Delta V Advantage isotope ratio mass spectrometer. Twelve of the 16 individual AAs identified had sufficient baseline separation for stable carbon isotope analysis, accounting for *c.* 80% of the total AA per cent abundance. Glutamic acid and aspartic acid peaks contained unknown contributions from glutamine and asparagine, respectively, due to conversion to their dicarboxylic acids during acid hydrolysis. For consumer muscle, three replicate tanks were analysed per treatment, with three fish analysed per tank. Three replicate samples of each of the four diets were analysed following the same procedure as the fish muscle. All compound-specific SIA samples were analysed in duplicate along with AA standards of known isotopic composition.

DATA ANALYSIS

Stable isotopes are expressed in standard delta (δ) notation according to the equation

$$\delta^{13}C_{Sample} = [(\frac{^{13}C/^{12}C_{sample}}{^{13}C/^{12}C_{standard}}) - 1] \times 1000.$$

Trophic fractionation factors ($\Delta^{13}C_{C\text{-}D}$) were calculated for each treatment as $\Delta^{13}C_{C\text{-}D} = \delta^{13}C_C - \delta^{13}C_D$, where $\delta^{13}C_C$ and $\delta^{13}C_D$ represent the $\delta^{13}C$ values of the consumer and diet respectively. Standardization of runs was achieved using intermittent pulses of a CO_2 reference gas of known isotopic value.

To correct for the introduction of exogenous carbon and kinetic fractionation during derivatization (Silfer *et al.* 1991), AA standards of known isotopic value were derivatized and analysed concurrently with the samples. Error for determining the isotopic composition of the exogenous carbon added during derivatization averaged ± 0·4‰. Differences in bulk $\delta^{13}C$ of diet and fish muscle among treatments were assessed using separate one-way analyses of variance (ANOVAS) and Tukey's HSD *post-hoc* tests ($\alpha = 0{\cdot}05$). Differences in individual AA $\delta^{13}C$ values within and among treatments for both diet and fish muscle were determined using separate model I (treatment and AA factors fixed) two-way ANOVAS and Tukey's HSD *post-hoc* tests ($\alpha = 0{\cdot}05$). To examine differences in individual AA $\Delta^{13}C_{C\text{-}D}$ both within and among treatments, AAs were *a priori* subdivided into non-essential and essential AAs. Differences in non-essential and essential AA $\Delta^{13}C_{C\text{-}D}$ values were analysed using separate model I (treatment and AA factors fixed) two-way ANOVAS and Tukey's HSD *post-hoc* tests. Separate two-sided one sample *t*-tests were used to determine if AA $\Delta^{13}C$ values were significantly different from 0‰. Linear regressions were performed to compare AA $\delta^{13}C$ values in muscle ($\delta^{13}C_{muscle\_AA}$) to (i) their respective dietary AAs ($\delta^{13}C_{diet\_AA}$) and (ii) the bulk diets ($\delta^{13}C_{bulk\_diet}$). Using the AA composition data, we determined the difference in AA per cent abundance between diet and muscle, with negative values indicating a deficit in AA abundance in diet relative to muscle. To look for trends in trophic fractionation as a function of AA composition, we conducted a correlation analysis between the AA per cent abundance difference and AA $\Delta^{13}C_{C\text{-}D}$ for all AAs showing *de novo* biosynthesis ($\Delta^{13}C_{C\text{-}D}$ significantly different from 0‰). All statistics were performed in PRISM version 4.0.

Results

Bulk $\delta^{13}C$ values were significantly different among the diets (one-way ANOVA, $F = 717{\cdot}7$, $P < 0{\cdot}05$; Fig. 1) and fish muscle (one-way ANOVA, $F = 321{\cdot}8$, $P < 0{\cdot}05$; Fig. 1) across treatments. The Vegi-Pro diet had the highest carbohydrate content (73%) and lowest crude fat (6%) and protein (8%) content, while the squid and clam diets had the highest crude fat (18%) and protein (69% and 71% respectively) content and almost no carbohydrates (Table 1). Bio-Vita content was generally intermediate between the Vegi-Pro and the animal-based diets, with the exception of a high crude fat content (24%).

The mean range in $\delta^{13}C$ values across all 12 AAs analysed was 27·9 ± 6·9‰ for diet (Fig. 1a) and 23·6 ± 2·6‰ for fish muscle (Fig. 1b). We found significant differences in dietary $\delta^{13}C$ values (Fig. 1a) among individual AAs (two-way ANOVA, d.f. = 11, 96, $F = 1239{\cdot}0$, $P < 0{\cdot}05$) and among diet treat-

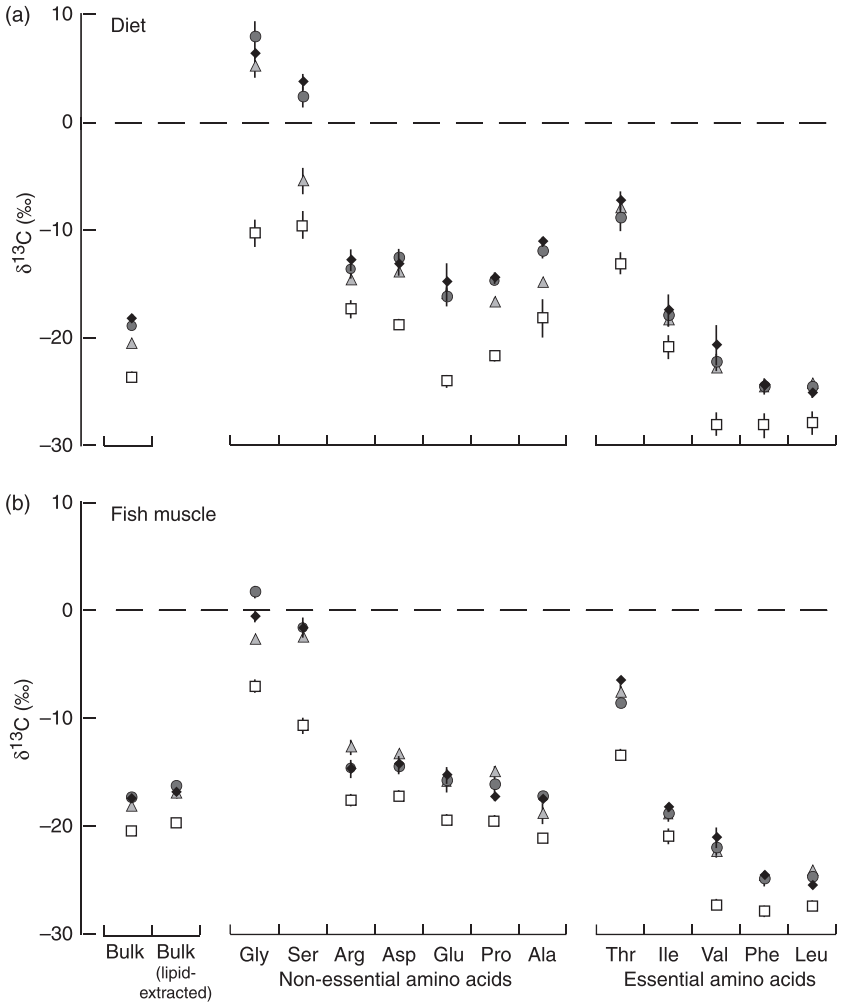

Fig. 1. Mean (±SD) bulk tissue and individual amino acid $\delta^{13}C$ values of (a) diet and (b) *Fundulus heteroclitus* muscle from four dietary treatments: Vegi-Pro (open squares), Bio-Vita (light gray triangles), squid (dark gray circles) and clam (black diamonds) ($n = 5$ replicates per treatment for diets and $n = 3$ tanks per treatment, consisting of three fish per tank for fish muscle).

ments (d.f. = 3, 96, $F = 552{\cdot}0$, $P < 0{\cdot}05$). However, variability in AA $\delta^{13}C$ values was not consistent among diet treatments, generating a significant diet × AA interaction (d.f. = 33, 96, $F = 21{\cdot}71$, $P < 0{\cdot}05$). Fish muscle AA $\delta^{13}C$ values (Fig. 1b) showed similar patterns to those of the diets, with significant differences among individual AAs (two-way ANOVA, d.f. = 11, 96, $F = 2681{\cdot}0$, $P < 0{\cdot}05$) and among diet treatments (d.f. = 3, 96, $F = 642{\cdot}7$, $P < 0{\cdot}05$), including a significant interaction term (d.f. = 33, 96, $F = 18{\cdot}72$, $P < 0{\cdot}05$).

Despite significant variability in individual AA $\delta^{13}C$ values in diet and muscle, there were several consistent patterns in our data. All AAs from the Vegi-Pro treatment, both diet and fish muscle, were the most ^{13}C-depleted, while AAs from the squid and clam treatments were typically the most ^{13}C-enriched. Glycine and serine were always the most ^{13}C-enriched AAs in all treatments for both diet and fish muscle, where as valine, phenylalanine and leucine were always the most ^{13}C-depleted AAs in all treatments. The $\delta^{13}C$ value of aspartic acid, glutamic acid and proline were generally similar to one another in each treatment for both diet and muscle, although the values diverged more so for the Vegi-Pro diet. Finally, the non-essential AAs were ^{13}C-enriched relative to the essential AAs by 7·5 ± 2·9‰ for diet and 7·3 ± 0·8‰ for fish muscle.

Table 1. Proximate analysis of moisture, crude protein, crude fat, crude fiber, ash and carbohydrate content (%) of four diets Vegi-Pro, Bio-Vita, squid and clam ($n = 1$)

Analysis	Vegi-Pro	Bio-Vita	Squid	Clam
Moisture	6·8	6·0	10·0	8·8
Protein (crude)	8·0	53·3	69·1	71·0
Fat (crude)	5·9	23·9	17·6	18·0
Fibre (crude)	2·0	0·3	0·3	0·2
Ash	6·7	10·9	2·9	2·1
Carbohydrates	72·6	6·0	0·3	0·2

Muscle essential AA $\delta^{13}C$ values showed stronger linear relationships to their respective dietary AA $\delta^{13}C$ values with slopes closer to unity ($m = 0{\cdot}9 \pm 0{\cdot}2$, $b = -2{\cdot}7 \pm 3{\cdot}8$,

Table 2. Slope (*m*), *y*-intercept (*b*) and R^2 values from linear regression analysis of *Fundulus heteroclitus* muscle amino acid $\delta^{13}C$ values compared to (a) dietary amino acid $\delta^{13}C$ values and (b) bulk diet $\delta^{13}C$ values

	(a) $\delta^{13}C_{muscle\text{-}AA}$ vs. $\delta^{13}C_{diet\text{-}AA}$			(b) $\delta^{13}C_{muscle\text{-}AA}$ vs. $\delta^{13}C_{bulk\text{-}diet}$		
Amino acid	*m*	*b*	R^2	*m*	*b*	R^2
Glycine[a]	0·40 ± 0·01	−3·09 ± 0·23	0·86 ± 0·07	1·43 ± 0·09	26·33 ± 1·74	0·86 ± 0·07
Serine[a]	0·62 ± 0·07	−2·92 ± 0·10	0·66 ± 0·01	1·73 ± 0·12	30·49 ± 2·26	0·89 ± 0·01
Arginine[a]	0·67 ± 0·21	−5·09 ± 3·22	0·41 ± 0·10	0·53 ± 0·16	1·05 ± 6·07	0·39 ± 0·09
Aspartic acid[a]	0·51 ± 0·11	−7·39 ± 1·67	0·77 ± 0·04	0·51 ± 0·12	−4·55 ± 2·54	0·55 ± 0·15
Glutamic acid[a]	0·45 ± 0·11	−8·66 ± 2·16	0·93 ± 0·05	0·75 ± 0·24	−1·72 ± 5·05	0·84 ± 0·21
Proline[a]	0·39 ± 0·10	−10·40 ± 1·81	0·46 ± 0·13	0·48 ± 0·13	−7·51 ± 2·80	0·35 ± 0·12
Alanine[a]	0·54 ± 0·06	−11·03 ± 0·97	0·92 ± 0·03	0·73 ± 0·08	−4·34 ± 2·04	0·93 ± 0·04
Threonine[b]	1·16 ± 0·10	1·82 ± 0·93	0·99 ± 0·01	1·15 ± 0·10	13·83 ± 1·94	0·83 ± 0·06
Isoleucine[b]	0·77 ± 0·24	−4·92 ± 4·37	0·91 ± 0·02	0·46 ± 0·13	−9·98 ± 2·54	0·87 ± 0·11
Valine[b]	0·87 ± 0·09	−2·84 ± 2·28	0·97 ± 0·05	1·10 ± 0·13	−1·14 ± 2·93	0·92 ± 0·11
Phenylalanine[b]	0·88 ± 0·16	−3·19 ± 3·95	0·96 ± 0·04	0·60 ± 0·11	−13·47 ± 2·31	0·80 ± 0·10
Leucine[b]	0·82 ± 0·17	−4·52 ± 4·35	0·95 ± 0·03	0·40 ± 0·10	−17·55 ± 1·99	0·47 ± 0·06
Mean NEAA[a]	0·51 ± 0·14	−6·95 ± 3·49	0·71 ± 0·21	0·88 ± 0·49	5·68 ± 15·28	0·69 ± 0·26
Mean EAA[b]	0·90 ± 0·20	−2·73 ± 3·84	0·95 ± 0·04	0·74 ± 0·35	−5·66 ± 11·71	0·78 ± 0·18

NEAA[a] = non-essential amino acids, and EAA[b] = essential amino acids (n = 3 tanks per treatment consisting of three fish per tank).

r^2 = 0·95 ± 0·04) than was found for non-essential AAs (m = 0·51 ± 0·14, b = −7·0 ± 3·5, r^2 = 0·71 ± 0·21) (Table 2). Muscle essential AA $\delta^{13}C$ values were also more closely related to their dietary AA $\delta^{13}C$ values than they were to bulk diet $\delta^{13}C$ values (m = 0·7 ± 0·4, b = −5·7 ± 11·7, r^2 = 0·78 ± 0·18) (Table 2). Non-essential AAs $\delta^{13}C$ values in muscle tissue typically showed stronger correlations to bulk diet $\delta^{13}C$ values (m = 0·9 ± 0·5, b = 5·7 ± 15·3, r^2 = 0·69 ± 0·26) than to their respective dietary AA $\delta^{13}C$ values (Table 2).

Bulk fish muscle showed positive, albeit diet-specific, trophic fractionation (Fig. 2) for all treatments, with Vegi-Pro having the highest $\Delta^{13}C_{C\text{-}D}$ values and the squid and clam treatments having the lowest $\Delta^{13}C_{C\text{-}D}$ values. There was a large range in $\Delta^{13}C_{C\text{-}D}$ (12·5‰) values across individual AAs and dietary treatments (Fig. 2), from −7·9 ± 0·7‰ for glycine in the Bio-Vita treatment to 4·7 ± 0·5‰ for glutamic acid in the Vegi-Pro treatment. Even within individual AAs, the mean range in $\Delta^{13}C_{C\text{-}D}$ across all four diets was large (5·8 ± 2·9‰), ranging from aspartic acid (3·5‰) to glycine (11·2‰). While there was significant variability in $\Delta^{13}C_{C\text{-}D}$ values among diets and individual AAs, we observed several consistent patterns in the data. Essential AA $\Delta^{13}C_{C\text{-}D}$ values were very consistent among individual AAs (two-way ANOVA, d.f. = 4, 40, F = 2·5, P > 0·05) and dietary treatments (d.f. = 3, 40, F = 1·7, P > 0·05). All essential AA $\Delta^{13}C_{C\text{-}D}$ values were not significantly different from 0‰ (mean $\Delta^{13}C_{C\text{-}D}$ = 0·02 ± 0·44‰) (one sample t-test, P > 0·05 for all essential AAs).

Conversely, $\Delta^{13}C_{C\text{-}D}$ values of non-essential AAs showed much larger deviations from 0‰ and considerably more variation among AAs (two-way ANOVA, d.f. = 5, 48, F = 165·0,

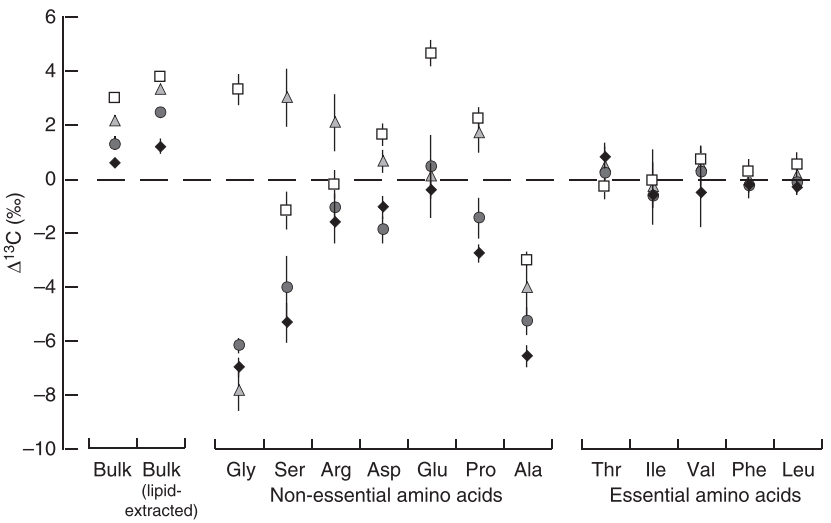

Fig. 2. Bulk tissue and individual amino acid stable carbon isotope trophic fractionation ($\Delta^{13}C_{C\text{-}D}$ ± SD) between diet and consumer for *Fundulus heteroclitus* fed four dietary treatments: Vegi-Pro (open squares), Bio-Vita (light gray triangles), squid (dark gray circles) and clam (black diamonds) (n = 3 tanks per treatment, consisting of three fish per tank).

$P < 0{\cdot}05$) and among treatments (d.f. = 3, 48, $F = 218{\cdot}4$, $P < 0{\cdot}05$), including a significant interaction term (d.f. = 15, 48, $F = 35{\cdot}1$, $P < 0{\cdot}05$). Only arginine in the Vegi-Pro treatment (one sample *t*-test, $t = 0{\cdot}8$, $P > 0{\cdot}05$) and glutamic acid in the Bio-Vita (one sample *t*-test, $t = 0{\cdot}3$, $P > 0{\cdot}05$), squid ($t = 0{\cdot}6$, $P > 0{\cdot}05$) and clam ($t = 0{\cdot}8$, $P > 0{\cdot}05$) treatments had $\Delta^{13}C_{C\text{-}D}$ values that were not significantly different from 0‰. Non-essential AA $\Delta^{13}C_{C\text{-}D}$ values generally followed the patterns observed in the bulk tissues $\Delta^{13}C$ values (Fig. 2). Non-essential AA $\Delta^{13}C_{C\text{-}D}$ values were typically the most positive in the Vegi-Pro treatment with the exceptions of serine and arginine, where Bio-Vita showed the highest $\Delta^{13}C_{C\text{-}D}$ values. Conversely, $\Delta^{13}C_{C\text{-}D}$ values of non-essential AAs from the squid and clam treatments were never significantly different from one another (Tukey's HSD *post-hoc* test, $P > 0{\cdot}05$) and were generally the lowest values.

There was notable variation in the per cent abundance of AAs for both diets (Fig. 3a) and fish muscle (Fig. 3b). In general, the non-essential AAs glutamic acid, aspartic acid and arginine were the most abundant AAs. Although lysine was also quite abundant in both diet and muscle, it was not analysed for $\delta^{13}C$ due to coelution with tyrosine. Leucine was the most abundant essential AA that was analysed for $\delta^{13}C$. The patterns of per cent abundance of AAs were very consistent across treatments for muscle (Fig. 2b), with a mean standard deviation of $0{\cdot}1 \pm 0{\cdot}1\%$ across all AAs. There was considerably more variation in AA per cent abundance across the four diets (Fig. 2a), with a mean standard deviation of $1{\cdot}0 \pm 0{\cdot}8\%$ across all AAs. In the Vegi-Pro and Bio-Vita treatments, all of the non-essential AAs analysed were less abundant in the diets than they were in the muscle (mean difference in per cent abundance, Vegi-Pro: $-2{\cdot}0 \pm 1{\cdot}6\%$ and Bio-Vita: $-1{\cdot}5 \pm 1{\cdot}0\%$). The squid and clam diets usually, but not exclusively, had a surplus of non-essential AAs ($0{\cdot}0 \pm 0{\cdot}9\%$ and $0{\cdot}4 \pm 1{\cdot}2\%$ respectively).

There was a significant negative correlation between the difference in non-essential AA per cent abundance in diet and muscle vs. AA $\Delta^{13}C_{C\text{-}D}$ (correlation coefficient, $r = -0{\cdot}43$, $P < 0{\cdot}05$; Fig. 4). Biosynthesized non-essential AAs tended to exhibit larger $\Delta^{13}C_{C\text{-}D}$ values when there was a greater deficit in AA per cent abundance in the diet relative

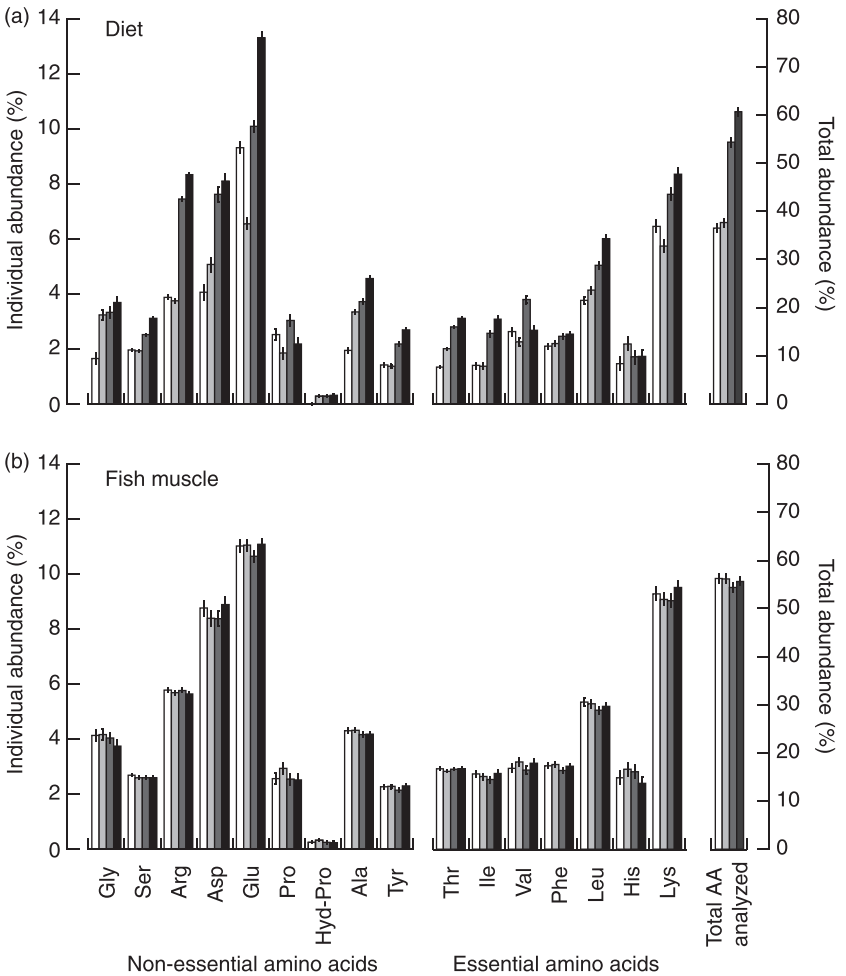

Fig. 3. Mean per cent abundance (% ±SD) of 16 individual amino acids (left axis) and the total per cent abundance of the 12 amino acids analysed for $\delta^{13}C$ values (right axis) in (a) diet and (b) *Fundulus heteroclitus* muscle from four dietary treatments: Vegi-Pro (open bars), Bio-Vita (light gray bars), squid (dark gray bars) and clam (black bars) ($n = 3$ replicates per treatment).

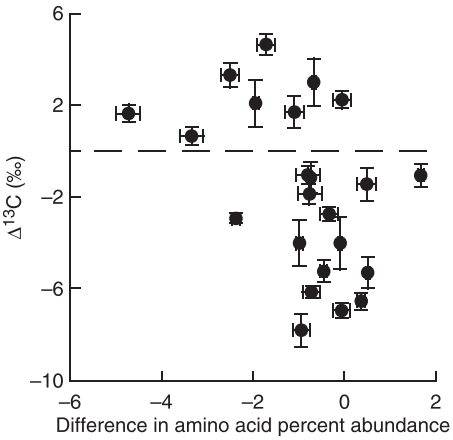

Fig. 4. Differences between amino acid per cent abundance in diet and muscle (mean % ± SD) vs. stable carbon isotope trophic fractionation ($\Delta^{13}C_{C\text{-}D}$ ± SD). Negative values signify a lower per cent abundance or $\delta^{13}C$ value in the diet relative to the muscle respectively ($n = 3$ for per cent abundance and $n = 3$ tanks per treatment, consisting of three fish per tank for $\Delta^{13}C_{C\text{-}D}$).

to fish muscle. The Bio-Vita treatment showed the most variability in $\Delta^{13}C_{C\text{-}D}$ values, with the $\Delta^{13}C_{C\text{-}D}$ values of aspartic acid and proline in the Bio-Vita treatment closer those in the Vegi-Pro treatment and the $\Delta^{13}C_{C\text{-}D}$ value of glycine more similar to those in the natural animal diet treatments (Fig. 2). Both aspartic acid and proline showed large deficits in the Bio-Vita diet compared to fish muscle, as was the case for Vegi-Pro (Fig. 3). Conversely, glycine was much closer to the per cent abundance in muscle for the Bio-Vita, squid and clam treatments (Fig. 3).

Discussion

We examined variability in carbon isotope fractionation of individual AAs in a common marine fish across a wide range of potential diets. Modest diet-specific $\Delta^{13}C_{C\text{-}D}$ values in bulk tissue reflected relatively large trophic fractionations for many non-essential AAs and little to no fractionation for all essential AAs. Essential AA $\delta^{13}C$ values reflected a purely dietary signature with $\Delta^{13}C_{C\text{-}D}$ values near 0‰, making them ideal tracers of carbon sources at the base of the food web. Consumer non-essential AAs showed a large range in $\Delta^{13}C_{C\text{-}D}$ across diets and a variable, diet-specific degree of isotopic routing from dietary protein, which together may contribute significantly to the high variability in bulk tissue $\Delta^{13}C_{C\text{-}D}$ observed in the natural environment. The diet-specific fractionation we found should promote discussion about the assumptions of minimal and invariant bulk tissue carbon isotope fractionation in dietary reconstructions.

The patterns observed in the bulk $\delta^{13}C$ values (Elsdon *et al.* 2010) were reflected in the $\delta^{13}C$ values of individual AAs (Fig. 1). For instance, AAs from the Vegi-Pro treatment (diet and muscle) were always the most ^{13}C-depleted, while those from the clam and squid treatments were typically the most ^{13}C-enriched. This is not surprising given that protein was a significant component (up to 71%) of the diets (Table 1) and fish muscle, making AAs a major contributor to bulk tissue $\delta^{13}C$ values. There were several consistent patterns in AA $\delta^{13}C$ values across all treatments. The large range in AA $\delta^{13}C$ values of diet (27·9 ± 6·9‰; Fig. 1a) and consumer muscle (23·6 ± 2·6‰; Fig. 1b) likely reflected the varied metabolic histories of these AAs. These ranges were similar to previous results on a variety of taxa including vertebrates (Hare *et al.* 1991; Fogel & Tuross 2003; Howland *et al.* 2003; Jim *et al.* 2006), invertebrates (Uhle *et al.* 1997; Fantle *et al.* 1999; O'Brien, Fogel & Boggs 2002) and plants (Fogel & Tuross 1999) from terrestrial and aquatic systems. This consistency likely reflects the influence of two main factors: (i) similarities in the major biosynthetic pathways that produce AAs in plants and animals and (ii) incorporation of dietary constituents directly into consumer tissue.

The patterns of $\Delta^{13}C_{C\text{-}D}$ of individual AAs generally mirrored those of the bulk tissue (Fig. 2), with $\Delta^{13}C_{C\text{-}D}$ values in the Vegi-Pro and Bio-Vita treatments significantly higher than those in the squid and clam treatments. A closer look at the $\Delta^{13}C_{C\text{-}D}$ values of individual AAs revealed some interesting insights into metabolic processes impacting the synthesis of muscle from dietary constituents. All essential AAs had $\Delta^{13}C_{C\text{-}D}$ values near 0‰ (Fig. 2), indicating no significant carbon isotope fractionation between diet and consumer muscle AAs. This observation was supported by the strong correlation and nearly 1 : 1 relationship between $\delta^{13}C_{diet\_essential\_AA}$ and $\delta^{13}C_{muscle\_essential\_AA}$ (Table 2). Small deviations from $\Delta^{13}C_{C\text{-}D} = 0‰$, and thus a slope of 1, most likely represented minor kinetic isotope fractionation during catabolism or conversion of essential AAs to other metabolites. If we interpret the slope of this regression to be roughly equivalent to the proportion of carbon routed into muscle directly from the diet, the results support our hypothesis of a high degree of isotopic routing of essential AAs into consumer muscle. Our data support previous work on a variety of taxa and tissues (Hare *et al.* 1991; Fantle *et al.* 1999; Howland *et al.* 2003; O'Brien, Boggs & Fogel 2003; Jim *et al.* 2006), indicating that these findings are generally applicable to a wide range of taxa and tissue types.

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, and thus must incorporate them directly from their diet (Borman *et al.* 1946; Reeds 2000). As a result, the $\delta^{13}C$ value of consumer essential AAs, such as phenylalanine and leucine, must represent the isotopic fingerprint of primary producers at the base of the food web ($\delta^{13}C_{Base}$). It should be noted that this relationship could be obscured when dealing with strict herbivores that receive a significant contribution of bacterially synthesized AAs from their symbiotic gut microbial community (Rimmer & Wiebe 1987). The fidelity with which essential AAs reflect dietary sources makes compound-specific SIA a powerful tool for foraging ecology and dietary reconstruction. Essential AA $\delta^{13}C$ values have provided insights into the diet of ancient humans and

herbivores (Stott *et al*. 1999; Fogel & Tuross 2003), the allocation of adult resources to eggs in butterflies (O'Brien, Fogel & Boggs 2002; O'Brien, Boggs & Fogel 2003, 2005), the contributions of carbon sources to marine dissolved organic matter (McCarthy *et al*. 2004), and the importance of marsh-derived diets in supporting the growth of juvenile blue crabs (Fantle *et al*. 1999). This approach may also provide a powerful new tool for reconstructing the diet of highly mobile consumers that move among isotopically distinct food webs. Certainly compound-specific SIA avoids the confounding variable of determining whether consumers with different bulk tissue $\delta^{13}C$ values represent feeding in the same food web but at different trophic levels, or feeding at the same trophic level but in isotopically distinct food webs (Post 2002).

Non-essential AAs showed significant deviations from $\Delta^{13}C_{C\text{-}D} = 0‰$, and much greater variability both among AAs and across diet treatments compared to essential AAs (Fig. 2). This variability most likely reflects the influence of the varied metabolic processes that shape the isotopic signatures of non-essential AAs during biosynthesis. The Vegi-Pro treatment exhibited primarily positive $\Delta^{13}C_{C\text{-}D}$ values while the natural diet treatments, squid and clam, typically showed large negative $\Delta^{13}C_{C\text{-}D}$ values (Fig. 2). The large $\Delta^{13}C_{C\text{-}D}$ values that shifted muscle non-essential AA $\delta^{13}C$ towards bulk diet $\delta^{13}C$ values suggest a high degree of *de novo* biosynthesis. This hypothesis was supported by linear regressions between $\delta^{13}C_{diet\_non\text{-}essential\_AA}$ and $\delta^{13}C_{muscle\_non\text{-}essential\_AA}$, where the mean slopes were far from unity (Table 2), indicating a disparity between the $\delta^{13}C$ values of dietary and muscle non-essential AAs.

The high degree of biosynthesis is surprising for the three diets containing animal matter, Bio-Vita, squid and clam, given the high protein content of those diets (53%, 69% and 71% respectively; Table 1). Previous research suggested that when fed high protein diets, organisms typically 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 & Norr 1993; Tieszen & Fagre 1993; Jim *et al*. 2006). Fish, however, use a significant portion of dietary protein for energetic purposes (Kim, Kayes & Amundson 1991; Dosdat *et al*. 1996), and thus it is possible that fish exhibit a lower degree of dietary routing than terrestrial vertebrates. Only the Vegi-Pro diet had a low protein content (8%) that would likely require biosynthesis, resulting in the high $\Delta^{13}C_{C\text{-}D}$ observed across most individual AAs in that treatment (Fig. 2). Hare *et al*. (1991) found that $\delta^{13}C$ of proline and glutamate differed by 5·7‰ in the bone collagen of pigs, suggesting that proline was being directly routed from diet into the consumer tissue. We found that proline had $\delta^{13}C$ signatures closer to those of glutamic acid rather than dietary proline (Fig. 1b) and had $\Delta^{13}C_{C\text{-}D}$ values significantly different from 0‰ (Fig. 2). This suggests that proline was biosynthesized from glutamic acid via reduction through a Schiff base intermediate (Baich & Pierson 1965) rather than being directly routed from the diet.

Arginine in the Vegi-Pro treatment and glutamic acid in the Bio-Vita, squid and clam treatments showed strong evidence of isotopic routing directly from dietary protein (Fig. 2), yet evidence of biosynthesis in the other dietary treatments. Arginine is synthesized from glutamate via glutamyl-γ-semialdehyde and thus if arginine and glutamic acid were both biosynthesized or both isotopically routed, we would expect them to have similar $\delta^{13}C$ values, as was the case for glutamic acid and proline discussed earlier. However, arginine and glutamic acid had different $\delta^{13}C$ values, reflecting the different pathways leading to arginine and glutamic acid incorporation into fish muscle. Glutamic acid and arginine account for over 18% of the AAs in fish muscle alone (Fig. 3), and it is probable that other AAs can be directly routed as well. We found that when glutamic acid was biosynthesized in the Vegi-Pro treatment, it exhibited relatively large $\Delta^{13}C_{C\text{-}D}$ values (~5‰) similar to the $\Delta^{13}C_{C\text{-}D}$ values Hare *et al*. (1991) and Howland *et al*. (2003) found for biosynthesized glutamic acid in pig bone collagen (~6–7‰). Thus varying degrees of isotopic routing vs. *de novo* biosynthesis for these abundant AAs could significantly alter consumer tissue $\delta^{13}C$ values relative to diet, further complicating the stable isotope relationship between diet and consumer.

We hypothesized that an underrepresentation of non-essential AAs in diet relative to muscle composition (Fig. 3) would necessitate a higher degree of biosynthesis than would be expected from diets with excess non-essential AAs. We found a significant correlation between diet and muscle AA per cent abundance and $\Delta^{13}C_{C\text{-}D}$ for biosynthesized non-essential AAs (Fig. 4). The AA composition of fish muscle is highly conserved (Wilson & Cowey 1985) as evidenced by the fact that muscle AA per cent abundance was very consistent across treatments (mean SD across treatments 0·1 ± 0·1%; Fig. 3b) despite feeding on diets with highly variable AA content (1·0 ± 0·8%; Fig. 3a). When there was a deficit in AA per cent abundance in the diet relative to the muscle, there tended to be greater trophic fractionation. This was particularly true for the Vegi-Pro and Bio-Vita treatments, where all non-essential AAs analysed were less abundant in the diets (Fig. 3a) than they were in fish muscle (Fig. 3b), and typically had the highest $\Delta^{13}C_{C\text{-}D}$ values. The disparity in AA per cent abundance was perhaps not surprising given that Vegi-Pro and Bio-Vita both contained plant matter, while the other diets were entirely animal protein.

Bio-Vita showed the most variability in $\Delta^{13}C_{C\text{-}D}$ values (Fig. 2), with some AAs trending towards Vegi-Pro and other towards the squid and clam treatments. For example, aspartic acid and proline had similar $\Delta^{13}C_{C\text{-}D}$ values in the Bio-Vita and Vegi-Pro treatments. Those AAs also showed large deficits in the Bio-Vita and Vegi-Pro diets compared to fish muscle. Conversely, glycine in the Bio-Vita treatment had a very negative $\Delta^{13}C_{C\text{-}D}$ value closer to those of the squid and clam treatments. In this case, the disparity in glycine per cent abundance between diet and fish muscle was small for the Bio-Vita, squid, and clam treatments. Differences in AA abundance in the diet relative to consumer muscle likely required varying the degree of biosynthesis and catabolism to meet the muscle composition demand, which may explain the corresponding shifts in AA trophic fractionation. However,

disparities in diet and muscle AA composition alone only explain a relatively small fraction ($r^2 = 0{\cdot}19$) of $\Delta^{13}C_{C\text{-}D}$ values.

The differences in $\Delta^{13}C_{C\text{-}D}$ values between the Vegi-Pro treatment and the squid and clam treatments may reflect differences in utilization of the bulk carbon pool from a plant-based diet vs. an animal-based diet. The Vegi-Pro diet had far more carbohydrates (73%) than lipids (6%), while the animal-based diets showed the opposite trend (18% lipid, < 1% carbohydrate (Table 1). The biosynthesis of non-essential AAs in the Vegi-Pro treatment appeared to rely on a more ^{13}C-enriched carbon pool than the other treatments, possibly indicating a greater contribution of carbohydrates to the bulk carbon pool (Teece & Fogel 2007). Howland *et al.* (2003) reared pigs on a plant-based diet with a $\delta^{13}C$ value close to the Vegi-Pro diet used in our study. Our results were similar to those of pig collagen $\Delta^{13}C_{C\text{-}D}$ values, showing large positive $\Delta^{13}C_{C\text{-}D}$ values for most non-essential AAs, particularly glutamic acid, proline and aspartic acid. Similar metabolic processes may, therefore, control $\Delta^{13}C_{C\text{-}D}$ values for many animals feeding on plant-based diets.

If lipids in the animal-based diets were being catabolized as a significant source of energy (Post *et al.* 2007), they would provide a very ^{13}C-depleted carbon pool relative to bulk diet values (6–8‰; DeNiro & Epstein 1977) from which non-essential AAs were biosynthesized. This may explain why the $\Delta^{13}C_{C\text{-}D}$ values in the animal-based dietary treatments were significantly more negative than in the Vegi-Pro treatment (Fig. 2). The divergence in $\Delta^{13}C_{C\text{-}D}$ between Vegi-Pro and the squid and clam treatments is greatest for glycine, serine and alanine, which are also the first AAs synthesized from carbohydrates entering the glycolysis as glucose. Glucose is converted to 3-phosphogylcerate, which is the precursor for both glycine and serine. Alanine is synthesized from pyruvate several steps after 3-phosphogylcerate and showed less of a difference in $\Delta^{13}C_{C\text{-}D}$ between the plant- and animal-based diets. The remaining non-essential AAs are synthesized from oxaloacetate and α-ketogluterate intermediates many steps later in the TCA cycle and showed the smallest differences in $\Delta^{13}C_{C\text{-}D}$ between the plant- and animal-based diets. If different carbon pools are in fact driving the diet-specific differences in $\Delta^{13}C_{C\text{-}D}$ values of non-essential AAs, the impact appears to be greatest near the source of carbon entering glycolysis and gets diluted or altered as carbon flows through the TCA cycle. Our work supports previous observations that organisms feeding on apparently homogeneous diets can show substantially different $\delta^{13}C$ values when routing of dietary components and alterations of available carbon pool $\delta^{13}C$ values become important (O'Brien, Fogel & Boggs 2002; O'Brien, Boggs & Fogel 2003; Jim *et al.* 2006).

The diets chosen for this study ranged from solely plant matter to solely animal matter to examine the potential variability in diet to consumer carbon isotope fraction. Without knowing the fractionation between steps as lipid and carbohydrate carbon enter the TCA cycle and get incorporated into AAs, we cannot accurately predict how the precursor $\delta^{13}C$ signatures will be manifested in the product AA $\delta^{13}C$ values. The next step will be to identify the mechanisms behind the high, diet-specific variability in $\Delta^{13}C_{C\text{-}D}$ and determine what information non-essential AA $\delta^{13}C$ values hold about consumer diet and metabolic history. This calls for targeted feeding experiments that track the fractionation of individual, potentially isotopically labelled dietary constituents as they are metabolically processed. While it is currently unclear how much useful information about diet and metabolic history is recorded in non-essential AA $\delta^{13}C$ values, the fact that the $\Delta^{13}C_{C\text{-}D}$ values in both animal diet treatments tracked very closely and were always significantly different from the plant-based Vegi-Pro diet (Fig. 2) holds promise that there may be some valuable underlying principles controlling consumer individual AA $\delta^{13}C$ values.

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References

Ambrose, S.H. & Norr, L. (1993) Carbon isotopic evidence for routing of dietary protein to bone collagen, and whole diet to bone apatite carbonate: purified diet growth experiments. *Molecular Archaeology of Prehistoric Human Bone* (eds J. Lambert & G. Grupe), pp. 1–37. Springer-Verlag, Berlin.

AOAC (2005) *Official Methods of Analysis*, 17th edn. AOAC International, Arlington, VA, USA.

Baich, A. & Pierson, D.J. (1965) Control of proline synthesis in *Escherichia coli*. *Biochimica et Biophysica Acta*, **104**, 397–404.

Borman, A., Wood, T.R., Black, H.C., Anderson, E.G., Oesterling, M.J., Womack, M. & Rose, W.C. (1946) The role of arginine in growth with some observations on the effects of argininic acid. *Journal of Biological Chemistry*, **166**, 585–594.

Cherel, Y., Hobson, K.A., Guinet, C. & Vanpe, C. (2007) Stable isotopes document seasonal changes in trophic niches and winter foraging individual specialization in diving predators from the Southern Ocean. *Journal of Animal Ecology*, **76**, 826–836.

DeNiro, M.J. & Epstein, S. (1977) Mechanism of carbon isotope fractionation associated with lipid synthesis. *Science*, **197**, 261–263.

DeNiro, M.J. & Epstein, S. (1978) Influence of diet on distribution of carbon isotopes in animals. *Geochimica et Cosmochimica Acta*, **42**, 495–506.

Dosdat, A., Servais, F., Metailler, R., Huelvan, C. & Desbruyeres, E. (1996) Comparison of nitrogenous losses in five teleost fish species. *Aquaculture*, **141**, 107–127.

Elsdon, T., Ayvazian, S., McMahon, K.M. & Thorrold, S.R. (2010) Experimental evaluation of stable isotope fractionation in fish muscle and otoliths. *Marine Ecology Progress Series*, **404**, 195–205.

Estrada, J.A., Lutcavage, M. & Thorrold, S.R. (2005) Diet and trophic position of Atlantic bluefin tuna (*Thunnus thynnus*) inferred from stable carbon and nitrogen isotope analysis. *Marine Biology*, **147**, 37–45.

Fantle, M.S., Dittel, A.I., Schwalm, S.M., Epifanio, C.E. & Fogel, M.L. (1999) A food web analysis of the juvenile blue crab, *Callinectes sapidus*, using stable isotopes in whole animals and individual amino acids. *Oecologia*, **120**, 416–426.

Fogel, M.L. & Tuross, N. (1999) Transformation of plant biochemicals to geological macromolecules during early diagenesis. *Oecologia*, **120**, 336–346.

Fogel, M.L. & Tuross, N. (2003) Extending the limits of paleodietary studies of humans with compound specific carbon isotope analysis of amino acids. *Journal of Archaeological Science*, **30**, 535–545.

Fry, B., Joern, A. & Parker, P.L. (1978) Grasshopper food web analysis: use of carbon isotope ratios to examine feeding relationships among terrestrial herbivores. *Ecology*, **59**, 498–506.

Gannes, L.Z., Martínez del Rio, C. & Koch, P. (1998) Natural abundance variations in stable isotopes and their potential uses in animal physiological ecology. *Comparative Biochemistry and Physiology*, **119A**, 725–737.

Gannes, L.Z., O'Brien, D.M. & Martínez del Rio, C. (1997) Stable isotopes in animal ecology: assumptions, caveats, and a call for more laboratory experiments. *Ecology*, **78**, 1271–1276.

Graham, B.S., Koch, P.L., Newsome, S.D., McMahon, K.W. & Aurioles, D. (2009) Using isoscapes to trace the movements and foraging behavior of top predators in oceanic ecosystems. *Isoscapes: Understanding Movement, Pattern and Process on Earth Through Isotope Mapping* (eds J. West, G.J. Bowen, T.E. Dawson & K.P. Tu). pp. 299–318. Springer, New York, NY, USA.

Hansson, S., Hobbie, J.E., Elmgren, R., Larsson, U., Fry, B. & Johansson, S. (1997) The stable nitrogen isotope ratio as a marker of food web interactions and fish migration. *Ecology*, **78**, 2249–2257.

Hare, P.E., Fogel, M.L., Stafford, T.W., Mitchell, A.D. & Hoering, T.C. (1991) The isotopic composition of carbon and nitrogen in individual amino acids isolated from modern and fossil proteins. *Journal of Archaeological Science*, **18**, 277–292.

Hobson, K.A. (1999) Tracing origins and migration of wildlife using stable isotopes: a review. *Oecologia*, **120**, 314–326.

Howland, M.R., Corr, L.T., Young, S.M., Jones, V., Jim, S., Van der Merwe, N.J., Mitchell, A.D. & Evershed, R.P. (2003) Expression of the dietary isotope signal in the compound-specific $\delta^{13}C$ values of pig bone lipids and amino acids. *International Journal of Osteoarchaeology*, **13**, 54–65.

Jim, S., Jones, V., Ambrose, S.H. & Evershed, R.P. (2006) Quantifying dietary macronutrient sources of carbon for bone collagen biosynthesis using natural abundance stable carbon isotope analysis. *British Journal of Nutrition*, **95**, 1055–1062.

Karasov, W.H. & Martínez del Rio, C. (2007) *Physiological Ecology*. Princeton University Press, Princeton, New Jersey, USA.

Kim, K., Kayes, T.B. & Amundson, C.H. (1991) Purified diet development and re-evaluation of the dietary protein requirement of fingerling rainbow trout (*Onchorynchus mykiss*). *Aquaculture*, **96**, 57–67.

McCarthy, M.D., Benner, R., Lee, C., Hedges, J.I. & Fogel, M.L. (2004) Amino acid carbon isotope fractionation patterns in oceanic dissolved matter: an unaltered photoautotrophic source for dissolved organic nitrogen in the ocean? *Marine Chemistry*, **92**, 123–134.

McCutchan, J.H. Jr, Lewis, W.M. Jr, Kendall, C. & McGrath, C.C. (2003) Variation in trophic shift for stable isotope ratios of carbon, nitrogen, and sulfur. *Oikos*, **102**, 378–390.

McMahon, K.W., Johnson, B.J. & Ambrose, W.G. (2005) Diet and movement of the killifish, *Fundulus heteroclitus*, in a Maine salt marsh assessed using gut contents and stable isotope analyses. *Estuaries*, **28**, 966–973.

Meier-Augenstein, W. (1999) Applied gas chromatography coupled to isotope ratio mass spectrometry. *Journal of Chromatography*, **842**, 351–371.

Merritt, D.A., Brand, W.A. & Hayes, J.M. (1994) Isotope-ratio-monitoring gas chromatography-mass spectrometry: methods for isotopic calibration. *Organic Geochemistry*, **21**, 573–583.

Michener, R.H. & Kaufmann, L. (2007) Stable isotope ratios as tracers in marine aquatic food webs: an update. *Stable Isotopes in Ecology and Environmental Science*, 2nd edn (eds K. Lajtha & R.H. Michener), pp. 238–282. Blackwell Publishing, Malden, MA, USA.

O'Brien, D.M., Boggs, C.L. & Fogel, M.L. (2003) Pollen feeding in the butterfly *Heliconius charitonia*: isotopic evidence for essential amino acid transfer from pollen to eggs. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, **270**, 2631–2636.

O'Brien, D.M., Boggs, C.L. & Fogel, M.L. (2005) The amino acids used in reproduction by butterflies: a comparative study of dietary sources using compound-specific stable isotope analysis. *Physiological and Biochemical Zoology*, **78**, 819–827.

O'Brien, D.M., Fogel, M.L. & Boggs, C.L. (2002) Renewable and nonrenewable resources: amino acid turnover and allocation to reproduction in Lepidoptera. *Proceedings of the National Academy of Sciences, USA*, **99**, 4413–4418.

Olive, P.J.W., Pinnegar, J.K., Polunin, N.V.C., Richards, G. & Welch, R. (2003) Isotope trophic-step fractionation: a dynamic equilibrium model. *Journal of Animal Ecology*, **72**, 608–617.

Parkington, J. (1991) Approaches to dietary reconstruction in the Western Cape: Are you what you have eaten? *Journal of Archaeological Science*, **18**, 331–342.

Peterson, B.J. & Fry, B. (1987) Stable isotopes in ecosystem studies. *Annual Review of Ecology and Systematics*, **18**, 293–320.

Popp, B.N., Graham, B.S., Olson, R.J., Hannides, C.C.S., Lott, M.J., López-Ibarra, G.A., Galván-Magaña, F. & Fry, B. (2007) Insight into the trophic ecology of yellowfin tuna, *Thunnus albacares*, from compound-specific nitrogen isotope analysis of proteinaceous amino acids. *Stable Isotopes as Indicators of Ecological Change* (eds T.D. Dawson & R.T.W. Siegwolf), pp. 173–190. Elsevier/Academic Press, Amsterdam.

Post, D.M. (2002) Using stable isotopes to estimate trophic position: models, methods, and assumptions. *Ecology*, **83**, 703–718.

Post, D.M., Layman, C.A., Arrington, D.A., Takimoto, G., Quattrochi, J. & Montaña, C.G. (2007) Getting to the fat of the matter: models, methods and assumptions for dealing with lipids in stable isotope analysis. *Oecologia*, **152**, 179–189.

Reeds, P. (2000) Dispensable and indispensable amino acids for humans. *Journal of Nutrition*, **130**, 1835S–1840S.

Rimmer, D.W. & Wiebe, W.J. (1987) Fermentative microbial digestion in herbivorous fish. *Journal of Fish Biology*, **31**, 229–236.

Rubenstein, D.R. & Hobson, K.A. (2004) From birds to butterflies: animal movement patterns and stable isotopes. *Trends in Ecology and Evolution*, **19**, 256–263.

Schwarcz, H.P. (1991) Some theoretical aspects of isotope paleodiet studies. *Journal of Archaeological Science*, **18**, 261–275.

Schwarcz, H.P. & Schoeninger, M.J. (1991) Stable isotope analyses in human nutritional ecology. *Yearbook Physical Anthropology*, **34**, 283–321.

Sessions, A.L. (2006) Isotope-ratio detection for gas chromatography. *Journal of Separation Science*, **29**, 1946–1961.

Silfer, J.A., Engel, M.H., Macko, S.A. & Jumeau, E.J. (1991) Stable carbon isotope analysis of amino-acid enantiomers by conventional isotope ratio mass spectrometry and combined gas-chromatography isotope ratio mass-spectrometry. *Analytical Chemistry*, **63**, 370–374.

Stott, A.W., Evershed, R.P., Jim, S., Jones, V., Rogers, J.M., Tuross, N. & Ambrose, S.H. (1999) Cholesterol as a new source of palaeodietary information: experimental approaches and archaeological applications. *Journal of Archaeological Science*, **26**, 705–716.

Teece, M.A. & Fogel, M.L. (2007) Stable carbon isotope biogeochemistry of monosaccharides in aquatic organisms and terrestrial plants. *Organic Geochemistry*, **38**, 458–473.

Tieszen, L.L. & Fagre, T. (1993) Effects of diet quality and composition on isotopic composition of respiratory CO_2, bone collagen, bioapatite, and soft tissues. *Prehistoric Human Bone Archaeology at the Molecular Level* (eds J. Lambert & G. Grupe), pp. 121–156. Springer-Verlag, Berlin.

Uhle, M.E., Macko, S.A., Spero, S.A., Engel, M.H. & Lea, D.W. (1997) Sources of carbon and nitrogen in modern plankton foraminifera: the role of algal symbionts as determined by bulk and compound specific stable isotope analysis. *Organic Geochemistry*, **27**, 103–113.

Vander Zanden, M.J. & Rasmussen, J.B. (1999) Primary consumer $\delta^{13}C$ and $\delta^{15}N$ and the trophic position of aquatic consumers. *Ecology*, **80**, 1395–1404.

Vander Zanden, M.J. & Rasmussen, J.B. (2001) Trophic fractionation: implications for aquatic food web studies. *Limnology and Oceanography*, **46**, 2061–2066.

Wilson, R.P. & Cowey, C.B. (1985) Amino acid composition of whole body tissue of rainbow trout and Atlantic salmon. *Aquaculture*, **48**, 373–376.

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