13C values of glycolytic amino acids as indicators of carbohydrate utilization in carnivorous fish

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ABSTRACT

Background. Stable isotope analysis of single amino acids (AA) is usually applied in food web studies for tracing biosynthetic origins of AA carbon backbones and establishing trophic positions of consumers, but the method is also showing promise for characterizing quantity and quality of dietary lipids and carbohydrates.

Methods. To investigate whether changes in high- and low-digestible carbohydrates affect δ13C values of glycolytic AA, i.e., AA carbon backbones sourced from the glycolytic pathway, we compared Atlantic salmon (Salmo salar) from a feeding experiment with and without dietary inclusion of the red macroalga Palmaria palmata. The Control and experimental diets had similar relative proportions of macronutrients, but their ingredients differed; in the experimental treatment, 15% Palmaria inclusion substituted proteins from fishmeal and carbohydrates from corn starch.

Results. We found that 13C values of the glycolytic AA were highly sensitive to substitution of corn starch with Palmaria. The δ13C offsets of glycolytic AA between salmon and their diets were significantly greater in the Palmaria inclusion than Control treatment. This greater offset can be attributed to the different utilization of high- vs. low-digestible carbohydrate sources, i.e., corn starch vs. Palmaria, in the two treatments, and metabolic routing of dietary lipids. In addition, similar δ13C values of essential AA between treatments indicate similar nutrient assimilation efficiency for all terrestrial (pea protein concentrate and wheat gluten meal) and marine (fishmeal and red alga) derived protein sources. These results show that δ13C AA analysis is a promising tool for improving our understanding of how carnivorous fish utilize macronutrient and route metabolic intermediates to tissue.

INTRODUCTION

Compound specific stable isotope analysis (CISA) of proteinogenic amino acids (AA) is an emerging tool for dietary reconstruction of finfishes. While stable carbon isotope analysis
of AA is predominantly applied for tracing biosynthetic origins of AA (Larsen et al., 2009; Larsen et al., 2013; O’Brien, Fogel & Boggs, 2002; Scott et al., 2006), a number of recent studies show that the method has the potential to characterize a consumer’s intake of lipids and carbohydrates (Leigh, Papastamatiou & German, 2018; Newsome et al., 2011; Newsome et al., 2014; Whiteman et al., 2018). For example, CSIA has been applied to characterize assimilation and digestion of diets with varying macromolecular composition in e.g., tilapia and sharks, which in turn can be used to understand a species’ nutritional requirements and ability to use resources in its environment (Leigh, Papastamatiou & German, 2018; Newsome et al., 2011; Whiteman et al., 2018). The origins of AA building blocks are highly diverse. Of the 20 proteinogenic AA, half of them are classified as nutritionally essential since animals cannot synthesize their carbon backbones, depending instead on essential EAA from the diet itself (McMahon et al., 2010; Reeds, 2000). This also means that the δ13C values of these AA typically match those in source protein with little or no isotopic offsets (Jim et al., 2006; McMahon et al., 2010; O’Brien, Fogel & Boggs, 2002). Some aquatic consumers may also rely on EAA supplemented from gut microbes but this pathway is usually confined to herbivores feeding on nutritionally insufficient diets (Arthur et al., 2014; Newsome et al., 2011). Metabolic routing of the other 10 proteinogenic AA, the non-essentials (NEAA), are much more complex than for the EAA because their carbon skeletons may either be incorporated directly into proteinogic tissue or synthesized de novo from metabolic intermediates. While the term non-essential implies that animals can synthesize them at a rate that meets the cellular demand for protein synthesis, it is well documented that adequate amounts of dietary NEAA are required for maximum growth and optimum health (Horvath et al., 1996; Womack & Rose, 1947). Thus, post-ingestive de novo synthesis of NEAA carbon skeletons may vary according to quality and supply of dietary proteins (Newsome et al., 2011).

Animals synthesize NEAA carbon backbones from glycolytic or Krebs cycle intermediates (Berg et al., 2015). The carbon intermediates for the glycolytic AA, glycine (Gly), serine (Ser), and alanine (Ala), are thought to derive predominantly from carbohydrates and lipids (Fernandes, Nadeau & Grootes, 2012; Newsome et al., 2014; Wang et al., 2018). For example, naturally occurring stable carbon isotope (δ13C) variations of Ala are correlated to human sugar consumption (Choy et al., 2013), and δ13C values of Gly and Ser are highly sensitive biomarkers of aquatic and terrestrial lipid origins in captive Atlantic salmon (Wang et al., 2018). In contrast, the Krebs cycle intermediate AA, aspartic acid (Asx), glutamic acid (Glx), and proline (Pro), are thought to be sourced predominantly from dietary proteins and lipids (Fernandes, Nadeau & Grootes, 2012; Newsome et al., 2014). How species synthesize NEAA from carbon intermediates depends on their digestive physiology and nutritional requirements. A study on carnivorous leopard sharks showed that a dietary shift from squid to tilapia did not affect their AA metabolism despite the diets’ substantial differences in carbohydrate content (Whiteman et al., 2018). In contrast, a study with bonnethead shark showed that glycogen synthesized from dietary carbohydrates were used as intermediates for NEAA synthesis (Leigh, Papastamatiou & German, 2018). These different metabolic responses for sharks raise the question of how carnivorous bony fishes utilize dietary macronutrients, and whether we can use naturally occurring markers...
for assessing metabolic responses to dietary changes. It is becoming increasingly important to understand how carnivorous fish species respond to dietary change because both wild and domesticated fishes are experiencing dramatic changes in the food or prey they eat. For example, Atlantic salmon is strictly carnivorous in the wild, but the proportion of plant-based ingredients in salmon aquafeed is approaching 80% (Gatlin et al., 2007). No other fish have gone through comparable changes in feeding ecology in the last decades, thus, it is important understanding the metabolic fate of the plant carbohydrate as well as their effect on fish health (Hemre, Mommsen & Krogdahl, 2002).

Carbohydrates are not essential for Atlantic salmon since they are obligate carnivores (Krogdahl et al., 1999), but they are a cheap source of energy for aquafeed production, and have useful pellet binding properties (Hemre & Krogdahl, 1996). While excessive inclusion of digestible carbohydrates in the feed may cause glycogen accumulation in the liver and impair the salmon’s hepatic function (Aksnes, 1995; Brudeseth, 1996; Frøystad et al., 2006; Hemre, Mommsen & Krogdahl, 2002; Tan et al., 2009), no or very low carbohydrate inclusions will reduce protein retention (Hemre et al., 1995) in part because glucose formed from carbohydrates can divert AA away from oxidative pathways (Cowey, De la Higuera & Adron, 1977; Sanchez-Muros et al., 1995). The main carbohydrates sources in aquafeed for Atlantic salmon have, until now, derived from extruded maize, wheat and other plant-based starch in the forms of amylose and amylopectin (Krogdahl, Sundby & Bakke, 2011). These gelatinized starches are easier to digest compared to glucose and other mono- and polysaccharides (Bogevik, 2015). Furthermore, a high inclusion of complex carbohydrates may limit utilization of gelatinized starches (Hemre, Mommsen & Krogdahl, 2002). Despite these less desirable nutritional properties of complex carbohydrates, macroalgae meal is now under investigation as a potential feed additive to promote salmon health and digestion (Overland, Mydland & Skrede, 2017), and already exists in commercial feed products including OceanFeed™ (Ocean Harvest, Gortnaloura, Ireland) and DigestSea® and Algimun® (Olmix, Brittany, France). In a scientific feeding trial with Atlantic salmon, the inclusion of the red marine intertidal macroalgae Palmaria palmata, also known as red dulse, significantly decreased alanine transaminase activity, which indicated an improved liver health (Wan et al., 2016). However, since P. palmata is rich in hardly digestible carbohydrates such as cellulose, hemicellulose and xylans (up to 60%) (Jiao et al., 2012), it is unclear how the inclusion of macroalgae affect carbohydrate utilization, lipid and carbohydrate interaction as well as AA synthesis in Atlantic salmon.

In this study we investigate how varying proportions of high- and low-digestible carbohydrates affect $\delta^{13}C_{NEAA}$ values by analyzing Atlantic salmon and their diets in a controlled feeding experiment (Wan et al., 2016). The two diets in our study have similar relative proportions of macronutrients; i.e., the diets were isonitrogenous, isolipidic and isoenergetic (Table 1), but with different carbohydrate and protein sources. The control diet comprised of 19% corn starch and 41% fishmeal, and the experimental diet of 8% corn starch, 36% fishmeal and 15% $P$. palmata (hereafter Palmaria) meal (Table 1). While protein quality is similar between the diets, the carbohydrates are different because Palmaria consists mainly of mono- and polysaccharides, i.e., low-digestible carbohydrates (i.e., high in neutral detergent fibre) (Table 1). Neither feed intake nor growth performance
Table 1. Atlantic salmon (Salmo salar) diet composition and proximate composition for both control and Palmaria palmata inclusion experiment modified after Wan et al. (2016).

|                     | Control       | Palmaria inclusion |
|---------------------|---------------|--------------------|
| **Diet formulation; %** |               |                    |
| Fishmeal<sup>a</sup> | 40.74         | 35.75              |
| Fish oil<sup>a</sup> | 20.00         | 20.41              |
| *Palmaria palmata*  | –             | 15.00              |
| Extruded corn starch<sup>b</sup> (high-digestible carbohydrates) | 18.76 | 8.34 |
| Wheat gluten<sup>c</sup> | 9.00         | 9.00               |
| Pea protein concentrate<sup>c</sup> | 9.00 | 9.00 |
| Mineral & vitamin premix<sup>d</sup> | 2.00 | 2.00 |
| Antioxidant<sup>e</sup> | 0.50         | 0.50               |
| **Proximate composition; %** |       |                    |
| Moisture            | 6.3          | 6.6                |
| Crude protein       | 40.7         | 40.6               |
| Crude lipid         | 25.1         | 25.5               |
| Ash                 | 8.3          | 9.9                |
| Gross energy MJ Kg<sup>−1</sup> | 26.2 | 26.1 |

Notes.

*Palmaria* proximate composition: moisture 9%; crude protein 22%; crude lipid 1%, ash 25% and gross energy 15 MJ kg<sup>−1</sup>. And the estimated neutral detergent fibre, i.e., carbohydrates that resist to digestion and absorption, from *Palmaria* is 45%.

<sup>a</sup>United fish products Ltd., Donegal, Ireland.
<sup>b</sup>Laboratory grade, Sigma–Aldrich Company Ltd., UK.
<sup>c</sup>Purified feed ingredients, Roquette, France.
<sup>d</sup>Premier nutrition products Ltd., UK. (Manufacturers analysis: Ca-12.09%, Ash-78.71%, Na-8.86%, Vitamin A-1.0 µg kg<sup>−1</sup>, Vitamin D3 0.10%, Vitamin-E 7.0 g kg<sup>−1</sup>, Cu-250 mg kg<sup>−1</sup>, Mg 15.6 g kg<sup>−1</sup> and P 5.2 g kg<sup>−1</sup>.
<sup>e</sup>Barox plus liquid, Kemin Europa N.V., Belgium.
<sup>f</sup>n = 4.

...differed significantly between the two diet groups (Wan et al., 2016). We hypothesize that substitution of high- with low-digestible carbohydrates, i.e., substitution of corn starch with *Palmaria*, will decrease overall carbohydrate digestibility and availability, which in turn affect how metabolic intermediates are routed in the glycolysis pathway. The glycolytic AA are synthesized from two intermediates in glycolysis: 3-phosphoglyerate (Gly and Ser) and pyruvate (Ala) (Moran et al., 2012). We focus on the glycolytic AA because previous studies have shown that they are particularly sensitive biomarkers of carbohydrate and lipid sourcing and metabolism. These macronutrients also serve as the primary energy sources, and increased reliance of carbohydrates for energy may lead to a corresponding decrease in the demand of lipids for energy. Thus, shifts metabolic routing in the glycolytic pathway is likely to affect δ<sup>13</sup>C values of glycolytic AA because lipids are generally 5–8% depleted in <sup>13</sup>C compared to carbohydrates and protein. By decreasing the fraction of high-digestible carbohydrates, we posit that downstream products directly associated with the glycolysis pathway would become more <sup>13</sup>C enriched in the *Palmaria* than control treatment (Control) because lipid derived intermediates would be routed to energy rather than tissue formation. We also analyzed the EAA to test whether EAA assimilation efficiency and protein preference would be similar for terrestrial (i.e., wheat gluten, pea protein concentrate) and marine (i.e., fishmeal, *Palmaria*) derived protein sources. Finally,
to obtain the supporting information of the fate of dietary macromolecules, we also measured bulk carbon and nitrogen isotopes in the diet and fish.

**MATERIAL AND METHODS**

**Source of salmon fillet and diet protein ingredients**

The feeding trial was conducted at the Carna Research Station, Ryan Institute, NUI Galway, Ireland, where inclusion of red (*P. palmata*) macroalga was compared to the Control group fed on a basal diet formulation. The salmon were fed on the experimental diets for 14 weeks to ensure that at least half of the muscle carbon pool would reflect the new experimental diets (*Jardine* et al., 2004). At the end of trials, fish were euthanized by concussion, and pithing of the cranium. Feeding trial was carried out under the oversight of National University of Ireland Galway’s Animal Care Research Ethics. Details of the experimental design and diet compositions for the feeding study was previously published in (*Wan* et al., 2016). In short, the original feeding experiments were a factorial $4 \times 1$ design with varying macroalgae percentage (0%, 5%, 10% and 15%) as the main factor. For this study, we only included salmon from the Control group and 15% macroalga inclusion group. For each treatment, we analyzed three fish from each of three different tanks. The Control group were fed on $\sim 40\%$ of fishmeal as the main source of protein. Both diets were isonitrogenous (40%), isolipidic (25%) and isoenergetic (26 MJ kg$^{-1}$). The experimental group were fed alternative diet substituted with 15% of dried macroalgae into the diet by decreasing fishmeal by 5% and extruded corn starch by 10%, while the compositions of other ingredients remained unchanged (*Wan* et al., 2016). Both Control and experimental diets also comprised two commonly used terrestrial protein sources: pea protein concentrate and wheat gluten meal at the same inclusion rate (9%). No AA were added to the diet formula. A summarized description of the diet information and proximate composition for both Control and *P. palmata* inclusion experiment is presented in Table 1 (modified after *Wan* et al., 2016). The effect of different experimental diets on growth performance, morphometric indices, and feed parameters (i.e., weight gain, feed conversion ratio, and specific growth rate etc.) was monitored throughout the experiment to ensure satisfactory growth rate (Fig. 1). In short, there were no significant differences in the growth parameters (final weight and weight gain) and growth performance indices between the Control and the *Palmaria* inclusion diets (*Wan* et al., 2016). Fillet muscle samples were collected after the fish was euthanized by a sharp blow to cranium and followed by pithing of the brain. Mixed sex salmon smolt were sourced from Derrylea Holdings Ltd. (Lough Fee, Connemara, Ireland) and the detailed information of fish care was described in (*Wan* et al., 2016).

**Stable isotope analyses**

We measured the carbon stable isotope ratios of the individual amino acids ($\delta^{13}C_{AA}$) of salmon and diet at Leibniz-Laboratory for Radiometric Dating and Stable Isotope Research in Kiel, Germany. Freeze-dried fish fillet were homogenized and approximately 4 mg of each sample were analyzed for compound specific stable isotope analyses (*Wang* et al., 2018). For the diet samples, three replicate samples of each compound diet
and individual dietary components (each homogenized, ~10 mg each) were analyzed for CSIA on a Gas Chromatogram (GC) connected to a MAT 253 (Thermo-Fisher Scientific (Bremen) GmbH, Bremen, Germany) Isotope Ratio Mass Spectrometer (IRMS). The detailed procedure for AA hydrolyses and derivatization, carbon correction and data calibration as well as the GC temperature setting is described in Wang et al. (2018). In short, each sample was hydrolyzed in 1mL of 6 N HCl at 110 °C in a heating block for 20 h. To remove lipophilic compounds before derivatization, we vortexed the hydrolyzed samples with 2 ml n-hexane/DCM (6:5, v/v) for 30 s and subsequently discarded the solvent from the aqueous phase. The AA were derivatized to N-acetyl methyl esters following the protocols by Larsen et al. (2013) and Corr, Berstan & Evershed (2007). We also derivatized and analyzed a mixture of pure AA with known δ¹³C values under the same conditions as the samples to calculate correction factors specific to each AA to account for carbon addition and fractionation during derivatization (Larsen et al., 2013).

Isotope data are expressed in delta (δ) notation:

\[ \delta^iE_{\text{sample}} = \left( \frac{i}{j} \right)_{\text{sample}} - \left( \frac{i}{j} \right)_{\text{ref}}. \]

For the certain element E, the ratio of heavy (i) to light (j) isotope are measured in both sample and references (Coplen & Shrestha, 2016). Since isotope values are small numbers, they are multiplied by 1000 and expressed as per mil (‰). Calibration of our internal standard AA-mixture was carried out against the known δ¹³C values of A4 mixture (available from A. Schimmelmann, Biogeochemical Laboratories, Indiana.
University, USA). The isotope ratios are expressed relative to international standards Vienna Pee Dee Belemnite (VPDB) for carbon. In regard to analytical uncertainty, the average reproducibility for the internal reference standard nor-leucine (Nle) was 0.3%o ($n = 3$ for each batch) and the in-house AA standards ranged from 0.2%o for Pro to 0.6%o for Ala ($n = 4$–7 for each batch). We were able to analyze the following 15 AA: NEAA; alanine (Ala), asparagine/aspartic acid (Asx), glutamine/glutamic acid (Glx), glycine (Gly), proline (Pro), tyrosine (Tyr) and serine (Ser), and EAA; histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), threonine (Thr), and valine (Val). See Fig. S1 online for a chromatogram of salmon muscle generated from the GC-Combustion-Isotope Ratio Mass Spectrometer (GC-C-IRMS).

Duplicates of compound diets and individual protein ingredients were analyzed for bulk carbon and nitrogen at the Stable Isotope Facility of the Experimental Ecology Group, GEOMAR, Kiel, Germany. Approximately 100 µg dry mass of each sample was weighed into tin capsules and analyzed on a customised elemental analyzer (EA 1110, Fisons Instruments, Milan, Italy) connected to a ThermoElectron DeltaPlus Advantage IRMS (Hansen & Sommer, 2007). The isotope ratios are expressed relative to international standards; VPDB for carbon and atmospheric air for nitrogen. For the detailed description of the calibration of the data, see description previously in Wang et al. (2018). In short, calibration of internal standard was carried out against certified reference material (IAEA-N1, IAEA-N2, IAEA-N3 for $\delta^{15}$N and IAEA-CH-3, IAEA-CH6, IAEA-CH-7 for $\delta^{13}$C). Internal standard conducted for bulk $\delta^{13}$C and $\delta^{15}$N analyses during the sample sequence yielded $1\sigma = 0.2%o$ and $0.2%o$, respectively. Because lipids have more negative $\delta^{13}$C values than proteins, it is important to account for the large variability in lipid content, which can affect the $\delta^{13}$C values of bulk tissue and lead to false interpretation. Thus, the negative $\delta^{13}$C values are commonly corrected by extracting lipids from samples prior to isotope analyses, or applying a mathematical correction after isotope analyses based on sample C:N ratios (Logan et al., 2008; Logan & Lutcavage, 2008). We opted for the latter and applied posterior correction to the $\delta^{13}$C values if the fishmeal samples C:N ratio was greater than 3.5, following Logan et al. (2008). Given that the fishmeal is non-tissue specific and of marine origins, we used the all-tissue correction parameters for marine fish (Logan et al., 2008). A similar posterior correction was also applied to fish muscle as previously described and published in Wang et al. (2018).

Statistical analyses
All statistical analyses were performed using R version 3.4.3 (R Core Team, 2017) (Supplementary Information). All values in the text are given as mean and its corresponding standard deviation (SD). For each tank, the mean $\delta^{13}$C$_{AA}$ values of fish are based on triplicate fish analyses. Isotope offset between fish muscle (F) and diet ($\Delta^{13}$C$_{F-D}$) were calculated for all amino acid and bulk isotopes of each treatment as $\Delta^{13}$C$_{F-D} = \delta^{13}$C$_{F} - \delta^{13}$C$_{D}$, where the $\delta^{13}$C$_{F}$ and $\delta^{13}$C$_{D}$ represent the $\delta^{13}$C values of the fish (i.e., Atlantic salmon) and diet (both compound diet and individual protein ingredients), respectively. Assuming independent variables, the error term for $\Delta^{13}$C$_{F-D}$ are propagated according to $\sqrt{SD^2_F + SD^2_D}$ (Ku, 1966). We used univariate Analysis of Variance (ANOVA)
performed on the output from Multivariate Analysis of Variance (MANOVA) to access which dependent variables ($\delta^{13}C_{\text{AA}}$ values) are significantly different between groups (R function: `summary.aov`). The $P$-adjusted values from this ANOVA decomposition test was obtained using `p.adjust` function with FRD method (Benjamini & Hochberg, 1995). We did not perform student t-test for comparing the bulk isotope values of two compound diet as we only have duplicate runs for each diet. The student t-tests were performed to compare the mean EAA $\Delta^{13}C_{\text{F-D}}$ values of both treatments. Unless otherwise stated, statistical significance is assessed at $P < 0.01$. Due to our small sample numbers, we chose a 0.01 significance level ($\alpha$) to decrease the probability of Type I error. The statistical tests results are presented in the Supplementary Information.

RESULTS

Amino acid and bulk carbon isotope values of compound diets and protein ingredients

Among the four different dietary ingredients, $\delta^{13}C_{\text{AA}}$ values of fishmeal were the highest for all AA except for Lys and Met, which were slightly lower than those of Palmaria by 1–2‰. In general, $\delta^{13}C$ values (both AA and bulk) of marine sourced proteins (i.e., fishmeal and Palmaria) were much higher than the terrestrial protein sources (Fig. 2 and Tables S1 and S2) with the exception that the Gly $\delta^{13}C$ values of Palmaria is slightly lower than that of wheat gluten but higher than pea protein. Accordingly, $\delta^{13}C_{\text{AA}}$ and $\delta^{13}C_{\text{bulk}}$ values of the compound diets are in between those of the marine and terrestrial dietary sources (Fig. 2 and Table S1). For the NEAA, the $\delta^{13}C$ range was widest for Pro, Ser, and Gly ($\sim 13 \%$) and narrowest for Ala and Tyr ($\sim 6 \%$). For the EAA, the range was widest for Met ($\sim 12 \%$) and narrowest for Phe ($\sim 6 \%$). Among all AA in the individual ingredients, Ser, Gly and Thr were the most $^{13}C$-enriched in both treatments, whereas Tyr, Phe, and Leu were the most $^{13}C$-depleted (Fig. 2). In general, $\delta^{13}C_{\text{AA}}$ and $\delta^{13}C_{\text{bulk}}$ values of marine sourced protein fishmeal and Palmaria were more similar except for Ser and Gly (up to 8.9‰ difference). Likewise, individual AA $\delta^{13}C$ and $\delta^{13}C_{\text{bulk}}$ values of wheat gluten were similar to those of pea protein concentrate except for Gly and Ile (Fig. 2). Although the mean bulk $\delta^{13}C$ value of the Palmaria containing diet was 1.1‰ lower than that of the Control diet, $\delta^{13}C_{\text{bulk}}$ values of fish muscle for both Control and Palmaria inclusion groups are identical (both $\delta^{13}C = 20.2\%$) (Fig. 2 and Table S1).

$\delta^{13}C_{\text{NEAA}}$ offsets between fish muscle and compound diets

There was a large variability in $^{13}C_{\text{F-D}}$ offset ($\Delta^{13}C_{\text{F-D}}$) between fish and the compound diet for the individual NEAA. The $\Delta^{13}C_{\text{F-D}}$ values of all NEAA except Asx ($\sim 0\%$) showed much greater variability, ranging from Ala ($\sim 6.8\%$) to Ser ($5.9\%$) (Figs. 2A and 2B). Moreover, $\Delta^{13}C_{\text{F-D}}$ values of the NEAA were typically higher than their respective compound diet, with the exception of Ala in both treatments and Gly in the Control treatment. Furthermore, $\Delta^{13}C_{\text{F-D}}$ values of glycolytic amino acids and Tyr in Palmaria treatment were significantly different from those in Control diet treatment (ANOVA, $P$-adjusted < 0.01, Table S3). In contrast, there was no difference between two treatments for Krebs cycle AA Glx, Asx and Pro (ANOVA, $P$-adjusted=0.03, 0.153 and 0.25, respectively, Table S4). The NEAA
Δ^{13}C_{F−D} values in *Palmaria* fed fish were generally larger (e.g., up to 3.4‰ for Gly) than those of the Control fed fish. The patterns observed for Δ^{13}C_{F−D} values of all NEAA also mirrored the patterns observed in the Δ^{13}C_{F−D} of bulk samples (Figs. 3A and 3B).

**δ^{13}C_{EAA} offsets between fish muscle and compound diets**

The δ^{13}C_{EAA} patterns of fish muscle largely to reflect that of the compound diets instead of individual protein components for both treatments (Fig. 4A and Fig. S2). The δ^{13}C_{EAA} values of the fish muscle and compound diet are almost identical, resulting in a mean offset of 0.34‰ and 0.18‰, respectively for Control and *Palmaria* inclusion group (Figs. 4A and 4B). The mean EAA Δ^{13}C_{F−D} values of both treatments were not significantly different from 0‰ (one sample t-test, t = 1.90, df = 7, P = 0.099 for Control diet; t = 1.59, df = 7, P = 0.213 for *Palmaria* inclusion diet). There was no apparent isotopic offset between salmon muscle (fish) and diets (Δ^{13}C_{F−D}) for EAA (Figs. 4A and 4B) except for Met being 1.3 ± 0.7‰ more positive than the compound diet in the Control diet group. Also, Δ^{13}C_{F−D} values of individual EAA were not significantly different between *Palmaria* inclusion and Control treatment except for His and Met (ANOVA, both P-adjusted < 0.01, detailed ANOVA results at Table S4).

**DISCUSSION**

Our isotopic results show that Atlantic salmon from the two experiments utilized the macronutrients differently because substitution of high- with low-digestible carbohydrates led to more positive offsets between fish and their diets (Δ^{13}C_{F−D}) of the glycolytic AA, Ser, Gly and Ala. In our study, the starch fraction of the diets (i.e., 19% for Control vs. 8% for *Palmaria* inclusion experiment) was the greatest variable that influenced Δ^{13}C_{F−D} because both lipid sources were kept consistent and proteins were functionally similar between the two lipid sources (Wan et al., 2016). Therefore, we argue that the primary
cause for the greater discrimination factor of the three glycolytic AA can be ascribed to inclusion of low-digestible carbohydrates.

The more negative bulk δ13C values of the *Palmaria* than Control compound diet (Fig. 3A) can largely be attributed to two factors: (1) Gly and Ser are 13C depleted by 8–10‰ in *Palmaria* compared to fishmeal proteins (Fig. 2), and (2) bulk *Palmaria* is ca. 10‰ more 13C depleted (−20‰, this study, Table S1) than corn starch (−10‰, Tieszen & Fagre, 1993). Despite the more negative values of these compounds and ingredients in the *Palmaria* diet, the Δ13C_D values of bulk and the three glycolytic AA are larger for the *Palmaria* than the Control salmon group (Figs. 3A and 3B), indicating that the intermediates

Figure 3  Non-essential amino acid and bulk δ13C values (mean ± SD) of fish muscle and compound diets (A) and their offset (Δ13C_D (mean ± SD) for both Control and *Palmaria palmaria* inclusion experiments (B). Glycolytic amino acids and Tyr Δ13C_D values in *Palmaria* treatment were significantly different from those in Control diet treatment (ANOVA, P-adjusted < 0.01, Table S3). In contrast, there was no difference between two treatments for Krebs cycle amino acids Glx, Asx and Pro (ANOVA, P-adjusted = 0.03, 0.153 and 0.25, respectively, Table S3). We did not perform statistical comparison between the bulk samples as we only measured bulk isotopes of fish and diet twice each.

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used for synthesizing glycolytic AA were more $^{13}$C enriched for *Palmaria_inclusion* than Control salmon (Figs. 3A and 3B). Hence, it is unlikely that utilization of carbohydrates *per se* led to the more positive $\Delta^{13}$C$_{F-D}$ values in the glycolytic AA. Since lipids have $\delta^{13}$C values that are 3–8‰ lower than proteins and carbohydrates (*Cherry et al., 2011*), we posit that lack of easily-digestible starch carbohydrates in *Palmaria* (*Jiao et al., 2012*).
increased the demand for dietary lipids as an energy source and correspondingly decreased the fraction of lipids available for biosynthesizing NEAA intermediates.

A recent study comparing Atlantic salmon fed compound diets with isotopically distinct lipid sources found that lipids are sourced to glycolytic rather than Krebs AA (Wang et al., 2018). This result indicates that carbon for acetyl CoA, an upstream precursor of Krebs cycle AA, is sourced more from protein- than lipid-derived building blocks. In our study, we found no $\Delta^{13}C_{F-D}$ difference in Krebs AA between the two treatments further corroborating that Krebs AA are sourced more from proteins than lipid and carbohydrates. It is possible that the decrease in digestible starch content also increased gluconeogenesis, i.e., generation of glycolytic metabolites from AA oxidation. How this pathway affect the discrimination factor for the glycolytic AA in an obligate carnivore is unclear. A study with calorie restricted rats demonstrated that increased AA oxidation led to a $\Delta^{13}C_{F-D}$ decrease in proteins and their constituent NEAA and EAA (Huneau et al., 2019). As already mentioned above, we observe a general $\Delta^{13}C_{F-D}$ increase rather than decrease in the *Palmaria* treatment for the NEAA (significant for half of them) and no change for the EAA (Fig. 4 and Table S3). Thus, we find it less likely that Palmaria inclusion led to increased AA oxidation. Finally, we rule out the possibility that relative differences in the abundance of dietary AA contributed to the different discrimination factors between the two treatments because the molar balance between the two composite diets are almost identical (Table S5).

Interestingly, $\Delta^{13}C_{F-D}$ values were positive for Ser and Gly and negative for Ala indicating that pyruvate was more $^{13}$C depleted than 3-phosphoglycerate. Several processes could have contributed to this contrasting $^{13}$C-offset between the two glycolytic intermediates. Pyruvate is a major substrate for oxidative metabolism, and a branching point for synthesis of lactate, fatty acid, alanine and Krebs cycle intermediates (McCommis & Finck, 2015). While pyruvate in Atlantic salmon is mainly produced via the glycolytic pathway, it can also be produced by conversion of lactate and oxaloacetate (Kanehisa et al., 2016). How these multiple pathways contribute to isotopic discrimination of pyruvate relative to 3-phosphoglycerate is exceedingly complex because isotopic discrimination depends on the intrinsic fractionation of a reaction and the relative flow of intermediates through that reaction pathway. For Atlantic salmon, glycolysis appears to be the main pathway for synthesizing both pyruvate and 3-phosphoglycerate because change in the starch fraction of the diets from 19% to 8% and consequent change in lipid metabolism led to similar directional change in the isotope discrimination factor.

Enhanced gut microbial activity in the *Palmaria* treatment perhaps offers an alternative explanation for the more positive $\Delta^{13}C_{F-D}$ values of the glycolytic AA in the *Palmaria* treatment. Such activity would lead to increased loss of $^{13}$C depleted CO$_2$ resulting in more $^{13}$C enriched metabolic intermediates for NEAA biosynthesis (Larsen et al., 2016). This alternative possibility rests on the premise that the greater viscosity and lower digestibility of the seaweed carbohydrates compared to corn starch slowed down gut transit time and increased microbial digestion of dietary carbohydrates, i.e., seaweed inclusion changed the salmons’ gut microbiome to facilitate carbohydrate digestion and metabolism (Gajardo et al., 2016). However, we find the putative effect of microbial activity on the discrimination factor less likely because *Palmaria* inclusion did not appear to affect growth performance.
(Wan et al., 2016) indicating that the fish did not lack energy or metabolic intermediates. Finally, the offset in $\delta^{15}N$ values between fish muscle and diet between the two groups ($\Delta^{15}N_{F-D}$ values) are the same further suggesting no enhanced microbial activity in the Palmaria inclusion experiment (Table S1).

Unlike the other NEAA, the $\Delta^{13}C_{F-D}$ of Asx is $\sim0\%$ for both the Control and Palmaria salmon, which is an unusual finding in fish feeding studies (McMahon et al., 2010; Newsome et al., 2011; Whiteman et al., 2018). The virtually identical $\delta^{13}C_{Asx}$ values between salmon and their compound diets does not necessarily mean that Asx was routed exclusively from dietary sources to muscle tissue because Atlantic salmon do produce enzymes for catalysing the interconversion of oxaloacetate and glutamate to aspartate and $\alpha$-ketoglutarate (Kanehisa et al., 2016). If salmon indeed synthesized Asx de novo, $^{13}C$ fractionation during Asx synthesis coincidently led to similar $^{13}C$ values between diet and de novo synthesized Asx. Further feeding studies varying all three macronutrients (i.e., protein, lipid and carbohydrates) would be needed to explain the isotope discrimination factor of Asx.

We also compared $\Delta^{13}C_{F-D}$ values of EAA between Atlantic salmon and their protein sources to investigate EAA assimilation of terrestrial and marine protein sources. For the EAA, $\delta^{13}C_{EAA}$ values between fish muscle and their compound diets are mostly identical in both treatments except for Met (Figs. 4A and 4B). This finding corroborates with previous findings that EAA is passed on from dietary sources to fish without alternation of their carbon skeletons (McMahon et al., 2010). Our study indicates that there was no preferential EAA assimilation of any of the three protein source in the compound diets because $\delta^{13}C_{EAA}$ values in salmon were similar between the two treatments and to the compound diets. This finding was expected given that feed conversion ratios (FCR for Control, 1.28 ± 0.10; and for Palmaria diet, 1.32 ± 0.03) during the feeding trial were comparable across the macroalgal inclusion diet and Control diet (Wan et al., 2016). Our near zero $\Delta^{13}C_{F-D}$ values for all EAA in both feeding experiments rule out, unsurprisingly, EAA supplementation by gut microbes to host (McMahon et al., 2010).

Both protein digestibility and availability in commercial aquafeeds are designed to ensure maximum growth and feed utilization. This was also the case for the diets in our feeding trial (Wan et al., 2016); hence, both our diets had almost identical AA composition (see Table S5). It is also evident from the AA composition data that there are apparent AA mismatches between salmon and their diets. This raises the question whether these mismatches correlate with NEAA discrimination factors, because dietary deficiencies could lead to higher synthesis rates of particular NEAA to meet metabolic demands. We did not find such correlations ($R^2 = 0.0003$ for Control and $R^2 = 0.08$ for Palmaria diet, respectively), which underlines that discrimination factors are determined by multiple factors. Our study highlights that one of those factors is metabolic utilization of dietary macromolecules; in our case how carbohydrate availability presumably affect lipid utilization. Other factors that could affect metabolic demands are infection, recoveries from injury, physical activities and digestive processes (Dunstan et al., 2019). In addition, proteomics studies show that synthesis and degradation rates of individual proteins in fish vary greatly (Doherty et al.,
Since the AA composition vary between proteins, this is another factor that may lead to a disproportionally greater turnover of certain AA than others.

One potential bias in using \( \delta^{13}C_{\text{AA}} \) to assess dietary routing in salmon is whether the AA yield from acid protein hydrolysis in the laboratory is comparable to the yield from the salmon’s digestive system. Given that we have an analytical uncertainty of approximately 0.5\% and a dynamic range between terrestrial and marine derived AA of 7–12\%, our results suggests that this bias is very small because no EAA except Met have significantly different \( \delta^{13}C_{\text{EAA}} \) values between salmon muscle tissues and compound diets in the Control group. In this case, Met of fish muscle has slightly higher \( \delta^{13}C \) values than their compound diet by 1.3 ± 0.7\%. This result may be caused by methionine loss during acid hydrolysis (Jennings & Lewis, 1969) or analytical uncertainty due to the low abundance of Met (Fig. S1). Alternatively, \( ^{13}C \) fractionation of methionine may occur when it is used for other purposes than protein synthesis, e.g., for synthesizing cysteine used for producing pancreatic proteases (Holm, Fossum & Eide, 1973).

**CONCLUSIONS**

Our previous study (Wang et al., 2018) demonstrated that \( \delta^{13}C \) values of the glycolytic AA are sensitive markers of lipid origins (i.e., terrestrial vs. marine). By comparing treatments where corn meal was replaced by macroalga, we show that glycolytic AA are sensitive to the dietary carbohydrate sources and digestibility. The most parsimonious explanation for the more positive \( \Delta^{13}C_{F-D} \) values of the glycolytic AA in the *Palmaria* treatment is a decreased sourcing of lipid derived intermediates to AA synthesis because *Palmaria* carbohydrates are more \( ^{13}C \) depleted than corn starch. While more feeding studies are warranted for understanding metabolic routing of macronutrients, our findings show how characterization of \( \delta^{13}C_{\text{AA}} \) variability can be used to trace relative contributions of dietary carbohydrates, proteins and lipids for *de novo* NEAA biosynthesis for carnivorous bony fish raised under normal feeding and husbandry conditions. Furthermore, our results indicate \( \delta^{13}C_{\text{AA}} \) analysis can provide supplementary information on the gut microbiome’s role in carbohydrate digestion and metabolism. The wider adoption of compound specific isotope analysis, particularly for AA, can greatly improve our understanding of nutrient utilization during the fish growth of different aquaculture species and life stages (Le Vay & Gamboa-Delgado, 2011; Newsome et al., 2011; Whiteman et al., 2018). Feeding studies such as ours also help in validating assumptions and limitations on how to interpret \( \delta^{13}C_{\text{NEAA}} \) values in ecological studies.

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ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests
The authors declare there are no competing interests.

Author Contributions
• Yiming V. Wang and Thomas Larsen conceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
• Alex H.L. Wan performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, approved the final draft.
• Åshild Krogdahl approved the final draft, editorial view and critical comments and discussion on Atlantic salmon physiology.
• Mark Johnson performed the experiments, contributed reagents/materials/analysis tools, approved the final draft.

Animal Ethics
The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):
NUI Galway Animal Care Research Ethics Committee is the NUI Galway’s governing body in Institutional Animal Care and Use. The fish feeding trial reported in the manuscript does not require Animal Care Research Ethics Committee’s approval as it did not
conducted any procedures on the animals while they were alive, e.g., tissue harvest. This is a conventional feeding trial and only comprise conventional animal husbandry (feeding, growing and weighing) and were euthanised by trained individuals using approved methods under Irish/EU regulation, e.g., i.e., concussion to the cranium and pithing of the brain.

**Data Availability**
The following information was supplied regarding data availability:

The raw data is available at GitHub: [https://github.com/alsjmonsoon/Salmon-Feeding-Study](https://github.com/alsjmonsoon/Salmon-Feeding-Study). The code is available as Supplemental File.

**Supplemental Information**
Supplemental information for this article can be found online at [http://dx.doi.org/10.7717/peerj.7701#supplemental-information](http://dx.doi.org/10.7717/peerj.7701#supplemental-information).

**REFERENCES**

Aksnes A. 1995. Growth, feed efficiency and slaughter quality of salmon, *Salmo salar* L., given feeds with different ratios of carbohydrate and protein. *Aquaculture Nutrition* 1:241–248 DOI 10.1111/j.1365-2095.1995.tb00050.

Arthur KE, Kelez S, Larsen T, Choy CA, Popp BN. 2014. Tracing the biosynthetic source of essential amino acids in marine turtles using δ13C fingerprints. *Ecology* 95:1285–1293 DOI 10.1890/13-0263.1.

Benjamini Y, Hochberg Y. 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society Series B (Methodological)* 57:289–300 DOI 10.1111/j.2517-6161.1995.tb02031.x.

Berg JM, Stryer L, Tymoczko JL, Gatto GJ. 2015. *Biochemistry*. New York: W.H. Freeman and Company.

Bogevik AS. 2015. *Xylanase supplementation in fish feed*. Tromsø: Nofima, 15.

Brudeseth L. 1996. *Hydrolysis of carbohydrates in intestinal mucosa of atlantic salmon (Salmo salar) and mink (Mustela vision)* Dr. Scient. Oslo: Norwegian School of Veterinary Science.

Cherry SG, Derocher AE, Hobson KA, Stirling I, Thiemann GW. 2011. Quantifying dietary pathways of proteins and lipids to tissues of a marine predator. *Journal of Applied Ecology* 48:373–381 DOI 10.1111/j.1365-2664.2010.01908.x.

Choy K, Nash SH, Kristal AR, Hopkins S, Boyer BB, O’Brien DM. 2013. The carbon isotope ratio of alanine in red blood cells is a new candidate biomarker of sugar-sweetened beverage intake. *The Journal of Nutrition* 143:878–884 DOI 10.3945/jn.112.172999.

Coplen TB, Shrestha Y. 2016. Isotope-abundance variations and atomic weights of selected elements: 2016 (IUPAC Technical Report). *Pure and Applied Chemistry* 88:1203–1224 DOI 10.1515/pac-2016-0302.
Corr LT, Berstan R, Evershed RP. 2007. Development of N-acetyl methyl ester derivatives for the determination of δ^{13}C values of amino acids using gas chromatography-combustion-isotope ratio mass spectrometry. Analytical Chemistry 79:9082–9090 DOI 10.1021/ac071223.

Cowey C, De la Higuera M, Adron J. 1977. The effect of dietary composition and of insulin on gluconeogenesis in rainbow trout (*Salmo gairdneri*). British Journal of Nutrition 38:385–395 DOI 10.1079/BJN19770103.

Doherty MK, Brownridge P, Owen MAG, Davies SJ, Young IS, Whitfield PD. 2012. A proteomics strategy for determining the synthesis and degradation rates of individual proteins in fish. Journal of Proteomics 75:4471–4477 DOI 10.1016/j.jprot.2012.03.025.

Dunstan RH, Macdonald MM, Murphy GR, Thorn B, Roberts TK. 2019. Modelling of protein turnover provides insight for metabolic demands on those specific amino acids utilised at disproportionately faster rates than other amino acids. Amino Acids 51:945–959 DOI 10.1007/s00726-019-02734-1.

Fernandes R, Nadeau M-J, Grootes PM. 2012. Macronutrient-based model for dietary carbon routing in bone collagen and bioapatite. Archaeological and Anthropological Sciences 4:291–301 DOI 10.1007/s12520-012-0102-7.

Frøystad MK, Lilleeng E, Sundby A, Krogdahl Å. 2006. Cloning and characterization of α-amylase from Atlantic salmon (*Salmo salar* L.). Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 145:479–492 DOI 10.1016/j.cbpa.2006.08.003.

Gajardo K, Rodiles A, Kortner TM, Krogdahl Å, Bakke AM, Merrifield DL, Sørum H. 2016. A high-resolution map of the gut microbiota in Atlantic salmon (*Salmo salar*): a basis for comparative gut microbial research. Scientific Reports 6:30893 DOI 10.1038/srep30893.

Gatlin DM, Barrows FT, Brown P, Dabrowski K, Gaylord TG, Hardy RW, Herman E, Hu G, Krogdahl A, Nelson R, Overturf K, Rust M, Sealey W, Skonberg D, Souza EJ, Stone D, Wilson R, Wurtele E. 2007. Expanding the utilization of sustainable plant products in aquafeeds: a review. Aquaculture Research 38:551–579 DOI 10.1111/j.1365-2109.2007.01704.

Hansen T, Sommer U. 2007. Increasing the sensitivity of δ^{13}C and δ^{15}N abundance measurements by a high sensitivity elemental analyzer connected to an isotope ratio mass spectrometer. Rapid Communications in Mass Spectrometry 21:314–318 DOI 10.1002/rcm.2847.

Hemre G-I, Krogdahl Å. 1996. Effect of handling and fish size on secondary changes in carbohydrate metabolism in Atlantic Salmon, *Salmo salar* L. Aquaculture Nutrition 2:249–252 DOI 10.1111/j.1365-2095.1996.tb00067.

Hemre G-I, Mommsen TP, Krogdahl Å. 2002. Carbohydrates in fish nutrition: effects on growth, glucose metabolism and hepatic enzymes. Aquaculture Nutrition 8:175–194 DOI 10.1046/j.1365-2095.2002.00200.

Hemre G-I, Sandnes K, Lie Ø, Torrissen O, Waagbo R. 1995. Carbohydrate nutrition in Atlantic salmon, *Salmo salar* L.: growth and feed utilization. Aquaculture Research 26:149–154 DOI 10.1111/j.1365-2109.1995.tb00896.
Holm H, Fossum K, Eide WB. 1973. Chemical and biological evaluation of protein quality of locally produced and processed full-fat soya bean flour from three tanzanian villages. *Journal of the Science of Food and Agriculture* 24:333–341 DOI 10.1002/jsfa.2740240310.

Horvath K, Jami M, Hill I, Papadimitriou J, Magder LS, Chanasongcram S. 1996. Isocaloric glutamine-free diet and the morphology and function of rat small intestine. *Journal of Parenteral and Enteral Nutrition* 20:128–134 DOI 10.1177/0148607196020002128.

Huneau J-F, Mantha OL, Hermier D, Mathé V, Galmiche G, Mariotti F, Fouillet H. 2019. Natural isotope abundances of carbon and nitrogen in tissue proteins and amino acids as biomarkers of the decreased carbohydrate oxidation and increased amino acid oxidation induced by caloric restriction under a maintained protein intake in obese rats. *Nutrients* 11(5):1087 DOI 10.3390/nu11051087.

Jardine TD, MacLatchy DL, Fairchild WL, Cunjak RA, Brown SB. 2004. Rapid carbon turnover during growth of Atlantic Salmon (*Salmo salar*) smolts in sea water, and evidence for reduced food consumption by growth-stunts. *Hydrobiologia* 527:63–75 DOI 10.1023/B:HYDR.0000043182.56244.f6.

Jennings DM, Lewis OAM. 1969. Methionine loss during protein hydrolysis of plant material. *Journal of Agricultural and Food Chemistry* 17:668–669 DOI 10.1021/jf60163a019.

Jiao G, Yu G, Wang W, Zhao X, Zhang J, Ewart SH. 2012. Properties of polysaccharides in several seaweeds from Atlantic Canada and their potential anti-influenza viral activities. *Journal of Ocean University of China* 11:205–212 DOI 10.1007/s11802-012-1906.

Jim S, Jones V, Ambrose SH, Evershed RP. 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 DOI 10.1079/BJN20051685.

Kanehisa M, Furumichi M, Tanabe M, Sato Y, Morishima K. 2016. KEGG: new perspectives on genomes, pathways, diseases and drugs. *Nucleic Acids Research* 45:D353–D361 DOI 10.1093/nar/gkw1092.

Krogdahl Å, Nodrum S, Sørensen M, Brudeseth L, Rojsø C. 1999. Effects of diet composition on apparent nutrient absorption along the intestinal tract and of subsequent fasting on mucosal disaccharidase activities and plasma nutrient concentration in Atlantic salmon *Salmo salar* L. *Aquaculture Nutrition* 5:121–133 DOI 10.1046/j.1365-2095.1999.00095.

Krogdahl A, Sundby A, Bakke AM. 2011. Gut secretion and digestion. In: Farrell AP, ed. *Encyclopedia of fish physiology: from genome to environment*. London: Elsevier, 1301–1311.

Ku HH. 1966. Notes on the use of propagation of error formulas. *Journal of Research of the National Bureau of Standards* 70C:263–273 DOI 10.6028/jres.070c.025.
Larsen T, Taylor DL, Leigh MB, O’Brien DM. 2009. Stable isotope fingerprinting: a novel method for identifying plant, fungal or bacterial origins of amino acids. *Ecology* 90:3526–3535 DOI 10.1890/08-1695.1.

Larsen T, Ventura M, Andersen N, O’Brien DM, Piatkowski U, McCarthy MD. 2013. Tracing carbon sources through aquatic and terrestrial food webs using amino acid stable isotope fingerprinting. *PLOS ONE* 8:e73441 DOI 10.1371/journal.pone.0073441.

Larsen T, Ventura M, Maraldo K, Triado-Margarit X, Casamayor EO, Wang YV, Andersen N, O’Brien DM. 2016. The dominant detritus-feeding invertebrate in Arctic peat soils derives its essential amino acids from gut symbionts. *Journal of Animal Ecology* 85:1275–1285 DOI 10.1111/1365-2656.12563.

Le Vay L, Gamboa-Delgado J. 2011. Naturally-occurring stable isotopes as direct measures of larval feeding efficiency, nutrient incorporation and turnover. *Aquaculture* 315:95–103 DOI 10.1016/j.aquaculture.2010.03.033.

Leigh SC, Papastamatiou YP, German DP. 2018. Seagrass digestion by a notorious ‘carnivore’. *Proceedings of the Royal Society B: Biological Sciences* 285:20181583 DOI 10.1098/rspb.2018.1583.

Logan JM, Jardine TD, Miller TJ, Bunn SE, Cunjak RA, Lutcavage ME. 2008. Lipid corrections in carbon and nitrogen stable isotope analyses: comparison of chemical extraction and modelling methods. *Journal of Animal Ecology* 77:838–846 DOI 10.1111/j.1365-2656.2008.01394.

Logan JM, Lutcavage ME. 2008. A comparison of carbon and nitrogen stable isotope ratios of fish tissues following lipid extractions with non-polar and traditional chloroform/methanol solvent systems. *Rapid Communications in Mass Spectrometry* 22:1081–1086 DOI 10.1002/rcm.3471.

McCommis KS, Finck BN. 2015. Mitochondrial pyruvate transport: a historical perspective and future research directions. *The Biochemical Journal* 466:443–454 DOI 10.1042/BJ20141171.

McMahon KW, Fogel ML, Elsdon TS, Thorrold SR. 2010. Carbon isotope fractionation of amino acids in fish muscle reflects biosynthesis and isotopic routing from dietary protein. *Journal of Animal Ecology* 79:1132–1141 DOI 10.1111/j.1365-2656.2010.01722.

Moran LA, Horton RH, Scrimgeour G, Perry M, Rawn D. 2012. *Principles of biochemistry*. London: Pearson.

Newsome SD, Fogel ML, Kelly L, Del Rio CM. 2011. Contributions of direct incorporation from diet and microbial amino acids to protein synthesis in *Nile tilapia*. *Functional Ecology* 25:1051–1062 DOI 10.1111/j.1365-2435.2011.01866.x.

Newsome SD, Wolf N, Peters J, Fogel ML. 2014. Amino acid δ¹³C analysis shows flexibility in the routing of dietary protein and lipids to the tissue of an omnivore. *Integrative and Comparative Biology* 54:890–902 DOI 10.1093/icb/icu106.

O’Brien DM, Fogel ML, Boggs CL. 2002. Renewable and nonrenewable resources: amino acid turnover and allocation to reproduction in lepidoptera. *Proceedings
of the National Academy of Sciences of the United States of America 99:4413–4418 DOI 10.1073/pnas.072346699.

Øverland M, Mydland LT, Skrede A. 2019. Marine macroalgae as sources of protein and bioactive compounds in feed for monogastric animals. Journal of the Science of Food and Agriculture 99:13–24 DOI 10.1002/jsfa.9143.

R Core Team. 2017. R: a language and environment for statistical computing. Vienna: R Foundation for Statistical Computing. Available at https://www.R-project.org/.

Reeds PJ. 2000. Dispensable and indispensable amino acids for humans. The Journal of Nutrition 130:1835S–1840S DOI 10.1093/jn/130.7.1835S.

Sanchez-Muros MJ, Garcia-Rejon I, Lupianez JA, Higueral M. 1995. Long-term nutritional effects on the primary liver and kidney metabolism in rainbow trout, Oncorhynchus mykiss (Walbaum): adaptive response to a high-protein/non-carbohydrate diet and starvation of glucose 6-phosphate dehydrogenase activity. Aquaculture Nutrition 1:213–220 DOI 10.1111/j.1365-2095.1995.tb00046.

Scott JH, O’Brien DM, Emerson D, Sun H, McDonald GD, Salgado A, Fogel ML. 2006. An examination of the carbon isotope effects associated with amino acid biosynthesis. Astrobiology 6:867–880 DOI 10.1089/ast.2006.6.867.

Tan Q, Wang F, Xie S, Zhu X, Lei W, Shen J. 2009. Effect of high dietary starch levels on the growth performance, blood chemistry and body composition of gibel carp (Carassius auratus var. gibelio). Aquaculture Research 40:1011–1018 DOI 10.1111/j.1365-2109.2009.02184.

Tieszen LL, Fagre T. 1993. Carbon isotopic variability in modern and archaeological maize. Journal of Archaeological Science 20:25–40 DOI 10.1006/jasc.1993.1002.

Wan AHL, Soler-Vila A, O’Keeffe D, Casburn P, Fitzgerald R, Johnson MP. 2016. The inclusion of Palmaria palmata macroalgae in Atlantic salmon (Salmo salar) diets: effects on growth, haematology, immunity and liver function. Journal of Applied Phycology 28:3091–3100 DOI 10.1007/s10811-016-0821-8.

Wang YV, Wan AHL, Lock E-J, Andersen N, Winter-Schuh C, Larsen T. 2018. Know your fish: a novel compound-specific isotope approach for tracing wild and farmed salmon. Food Chemistry 256:380–389 DOI 10.1016/j.foodchem.2018.02.095.

Whiteman JP, Kim SL, McMahon KW, Koch PL, Newsome SD. 2018. Amino acid isotope discrimination factors for a carnivore: physiological insights from leopard sharks and their diet. Oecologia 188:977–989 DOI 10.1007/s00442-018-4276-2.

Womack M, Rose WC. 1947. The role of proline, hydroxyproline, and glutamic acid in growth. Journal of Biological Chemistry 171:37–50.