Contributions of direct incorporation from diet and microbial amino acids to protein synthesis in Nile tilapia

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Summary

1. Further advancement in the use of stable isotope analysis in animal ecology and physiology requires a better understanding of how organisms incorporate the macromolecular elements they consume into the tissues they biosynthesize.

2. Mixing models used to infer diets from isotopic data assume that assimilated macromolecules are dissembled into elements and then reassembled in animal tissues.

3. To test this assumption, we fed Nile tilapia diets with contrasting levels of protein and in which the carbon isotopic composition of protein differed from that of other macronutrients (carbohydrates and lipids). We then analysed the $\delta^{13}C$ values of individual tilapia amino acids using compound-specific stable isotope analysis (CSIA).

4. The incorporation of dietary protein carbon was dependent on dietary protein content and on each amino acid's biosynthesis pathway. The $\delta^{13}C$ values of glycolytic amino acids, such as glycine, serine and alanine, had roughly constant values that reflected a large contribution of dietary carbohydrates and a small contribution of dietary protein. The $\delta^{13}C$ values of aspartate and glutamate that are biosynthesized from Krebs cycle intermediaries paralleled bulk diet.

5. The $\delta^{13}C$ values of indispensable amino acids resembled that of carbohydrates at low protein intakes but tended asymptotically to that of the $\delta^{13}C$ values of their corresponding dietary amino acid as protein intake increased. This pattern is consistent with assimilation of indispensable amino acids of microbial origin by tilapias fed low protein food.

6. Our results suggest that the assumptions of mixing models are sound in situations where omnivores consume protein-deficient diets, as the elemental constituents used to biosynthesize amino acids in tissues may be derived from various ingested macromolecules (e.g. protein, carbohydrates, or lipids) and their elemental components. In contrast, for omnivores that consume sufficient amounts of protein, macromolecular (e.g. protein) routing likely occurs and mixing model assumptions are violated.

7. Our study shows that CSIA is a novel method to quantify the contribution of symbiotic microbes to the amino acid homeostasis of animals.

Key-words: amino acids, isotopic routing, Nile tilapia, protein synthesis, stable isotopes

Introduction

Stable isotope mixing models are often used to determine the relative contribution of various dietary sources to an animal's diet (reviewed by Martínez del Rio *et al.* 2009). These models assume that ingested macromolecules (e.g. protein, lipids and/or carbohydrates) are dissembled into their elemental components (e.g. carbon and nitrogen) and then reassembled into the molecules used in tissue synthesis (Van der Merwe 1982; Martínez del Rio *et al.* 2009). This 'scrambled egg' assumption of mixing models is particularly unrealistic for amino acids because they are biosynthesized not from single carbon atoms but from various carbon skeletons. For example, the carbon skeletons of indispensable (i.e. essential) amino acids must be derived intact from assimilated nutrients as they cannot be biosynthesized *de novo* by many eukaryotes. Even for dispensable (i.e. non-essential) amino acids, the preferential inclusion of

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assimilated dietary amino acids in the consumer's proteins is expected, as the use of premanufactured carbon skeletons reduces the costs of synthesis (Horton *et al.* 2002). An implication of these observations is that contrary to the predictions of mixing models, the carbon isotope ($\delta^{13}C$) value of animal protein in tissues should resemble *not* that of bulk diet but to be closer to that of dietary protein (Ambrose & Norr 1993). Direct incorporation of an amino acid from dietary protein into animal tissue is often referred to as isotopic routing (Schwarcz & Schoeninger 1991). The routing hypothesis has received support in bulk tissue experiments on birds (Podlesak & McWilliams 2006), mammals (Ambrose & Norr 1993; Voight *et al.* 2008), and fish (Kelly & Martinez del Rio 2010). While these experiments demonstrate that routing produces results that deviate from expectations based on mixing models, they were not able to characterize the isotopic patterns among specific amino acids that contribute to routing because they were conducted at the bulk tissue level.

Compound-specific stable isotope analysis (CSIA) offers an informative method for tracing the elemental origins of biomolecules used to biosynthesize tissues, but has been used sparingly to investigate routing in controlled experiments in comparison with bulk tissue analysis. The $\delta^{13}C$ values of individual amino acids in a single protein can vary by $> 20‰$, but similarity in the overall pattern of amino acid $\delta^{13}C$ values among animals with different diets suggests that biosynthetic pathway is an important factor in driving the observed differences in $\delta^{13}C$ values among amino acids in a single tissue (Hare *et al.* 1991). Recent controlled feeding experiments on vertebrates show that indispensable amino acids were routed directly from diet with little isotopic fractionation, whereas the $\delta^{13}C$ values of dispensable amino acids can differ from those in diet by $> 20‰$ (Howland *et al.* 2003; Jim *et al.* 2006; McMahon *et al.* 2010). Jim *et al.* 2006 estimated a minimum estimate of *c.* 50% routing of dietary amino acid carbon into rat bone collagen, with indispensable amino acids accounting for nearly half (*c.* 22%) of this total. Determining which dietary macromolecules provide the source of the other *c.* 50% of carbon in an animal's proteinaceous tissues is an open question. The $\delta^{13}C$ values of dispensable amino acids biosynthesized from glycolytic (e.g. glycine), or Krebs cycle (e.g. glutamate) precursors, however, are not closely correlated with their respective dietary amino acid but rather with bulk diet $\delta^{13}C$ value (Howland *et al.* 2003; Jim *et al.* 2006). Moreover, in an experiment designed to assess resource allocation to reproduction in Lepidopterans, O'Brien, Fogel & Boggs (2002) found that dispensable amino acids in eggs were biosynthesized from ingested nectar sugars, which resulted in a large fraction (*c.* 50%) of total amino acids in butterfly protein being derived from non-protein sources.

To test the routing hypothesis in an omnivorous fish, we fed Nile tilapia (*Oreochromis niloticus*) a combination of diets in which the $\delta^{13}C$ value of dietary protein (C_3 casein, $-27‰$) was different from that of dietary carbohydrates and lipids (C_4 corn, $-11‰$). To examine how routing responds to dietary protein quantity, we varied casein content from 3·75% to 30·0% (by mass). As predicted by the routing hypothesis, the isotopic value of bulk fish muscle was significantly different from that of bulk diet (and hence different from that predicted by a mixing model) when the protein content of diet was higher than 3·75% (Kelly & Martinez del Rio 2010). At the lowest protein content (3·75%), however, the $\delta^{13}C$ value of bulk muscle was statistically indistinguishable from that of bulk diet ($-13‰$). This result was surprising because it suggested that the carbon in indispensable amino acids must be derived from non-protein dietary sources. Approximately 25% of the carbon in fish muscle protein is comprised of indispensable amino acids (Ozyurt & Polat 2006). Thus, if we assume that casein is the only source for indispensable amino acids and that the remaining carbon in dispensable amino acids was derived from the non-protein dietary sources (e.g. carbohydrates and lipids), tilapia muscle should have a $\delta^{13}C$ value of *c.* $-18‰$, which is *c.* 5‰ lower than the observed $\delta^{13}C$ value of tilapia muscle ($-13‰$) fed a low protein (3·75%) diet. It is possible that the source of this ^{13}C-enriched carbon in tilapia muscle was protein biosynthesized by the fishes' gastrointestinal microbiota from carbon derived from non-protein dietary sources. To test this hypothesis, we used CSIA to determine the isotopic composition of individual amino acids in the same tilapia muscle analysed by Kelly & Martinez del Rio (2010).

Previous studies and theoretical considerations suggest that the isotopic composition of amino acids of tilapia muscle in our experiment would be driven by two main factors: (i) the amino acid biosynthetic pathway (Hare *et al.* 1991; O'Brien, Fogel & Boggs 2002; Howland *et al.* 2003; Jim *et al.* 2006) and (ii) the protein content of diet. Other factors such as growth rate and metabolic state are also important considerations, because several amino acids (e.g. proline) can be conditionally indispensable when *de novo* synthesis does not meet requirements for growth (Ball, Atkinson & Bayley 1986; Chung & Baker 1993; Kirchgessner, Fickler & Roth 1995; Dabrowski *et al.* 2005); such factors are addressed in the Discussion. We established three amino acid categories *a priori* (Fig. 1). The first category includes dispensable amino acids biosynthesized from precursors derived during the first steps of glycolysis (Horton *et al.* 2002), including glycine (Gly), serine (Ser) and alanine (Ala). Alanine can also be biosynthesized in a second pathway from aspartate via oxaloacetate, an intermediate in the Krebs cycle. Because our experimental diets had a relative excess of carbohydrates relative to proteins or lipids, the isotopic composition of 'glycolytic' amino acids should reflect that of carbohydrates and be relatively independent of the amino acid composition of the diet given the small range (0–30%) in diet protein content (Fig. 1). The second category includes the amino acids such as aspartate (Asp), glutamate (Glu), arginine (Arg), proline (Pro) and possibly alanine (see earlier) that are biosynthesized from precursors derived from Krebs cycle reactions (Horton *et al.* 2002). Because these inputs into the Krebs cycle originate from dietary carbohydrates, lipids and protein, the carbon isotope composition of this second class of dispensable amino acids should reflect a mixture of dietary sources and be similar to
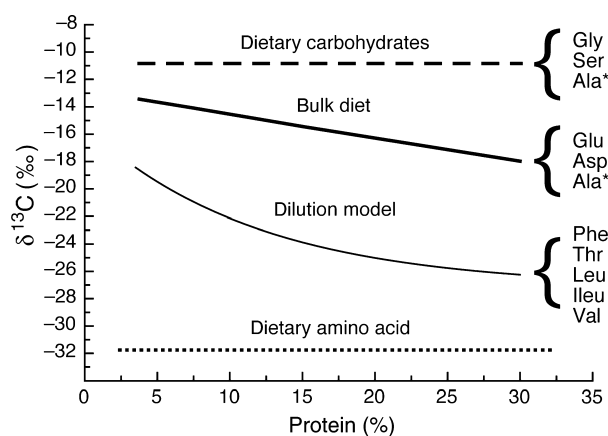
Fig. 1. Conceptual diagram of how the $\delta^{13}C$ value of three categories of amino acids were expected to change with increasing dietary protein content. Asterisk for alanine (Ala) denotes that this amino acid can be biosynthesized from glycolytic and/or Krebs cycle precursors. Glycolytic amino acids (Ser, Gly, Ala) were expected to mirror the isotopic composition of dietary carbohydrates irrespective of dietary protein content. Amino acids derived from Krebs cycle intermediaries (Glu, Asp, Ala) were expected to track the isotopic composition of bulk diet. The $\delta^{13}C$ value of indispensable amino acids (Phe, Thr, Leu, Ileu, Val) were expected to show a nonlinear response with increasing dietary protein because they could be sourced directly from dietary amino acids and/or indispensable amino acids biosynthesized by gut microbes that utilize bulk diet as a carbon source.

the $\delta^{13}C$ value of bulk diet (Fig. 1). Finally, the third category includes the indispensable amino acids – isoleucine (Ileu), leucine (Leu), phenylalanine (Phe), threonine (Thr) and valine (Val). If indispensable amino acids biosynthesized by microbes in the gut provided a significant source to the consumer, then indispensable amino acids in consumer tissues would have intermediate $\delta^{13}C$ values between those of the corresponding dietary amino acid and the isotopic composition of bulk diet. Conversely, if microbial contribution was

minimal, the $\delta^{13}C$ value of indispensable amino acids should tend towards values similar to that of the amino acid in dietary protein (casein) with increasing dietary protein content. Figure 1 illustrates these expectations graphically. In the Models and Statistical Analyses section in Materials and Methods, we transform the expectations described previously into mathematical models.

Materials and methods

TILAPIA EXPERIMENTAL DESIGN AND SAMPLE COLLECTION

Fingerling tilapias were purchased in June 2007 (AmeriCulture, Inc., Animas, NM, USA) and were housed in the University of Wyoming's Red Buttes Animal Care Facility. Fish with an average initial mass ($\pm SD$) of 0.51 g (± 0.07) were randomly assigned to one of four different experimental groups and placed individually in tanks ($25.5 \times 25.5 \times 15$ cm) at 24 °C (± 1 °C) and a 12L : 12D hour photoperiod. The throughput rate of the holding tanks was 80–90 mL min^{-1}. The four experimental groups were fed diets that varied in (i) relative protein content (from 3.75% to 30% dry weight) and (ii) isotopic composition (Table 1); refer to Kelly & Martinez del Rio (2010) for details regarding the composition of each diet treatment. We varied the protein content from 3.75% to 30% in an attempt to capture the natural variation in the diet of wild omnivorous tilapia, which are known to vary seasonally (Lim & Webster 2006). The mean $\delta^{13}C$ value ($\pm SD$) of the protein source (casein) was different ($-27 \pm 0.1‰$) from that of all other dietary components – corn starch, corn syrup, corn oil, cellulose, methyl cellulose – that had mean $\delta^{13}C$ values of $-11‰$ (± 0.1). Fish were fed once daily at a ration that exceeded their maximal intake. CO_2 asphyxiation was used to euthanize five fish per treatment (AVMA 2007), chosen at random when the average mass of all fish in the treatment was equal to *c.* 300% of starting mass. Because growth rate differed among fish, the time at which each fish was euthanized varied between 28 and 133 days. This protocol was approved by the University of Wyoming's IACUC committee (#A-3216–01). Euthanized fish were

Table 1. Mean $\delta^{13}C$ values of individual amino acids measured in each of the four protein treatments (3.75%, 7.5%, 15.0%, 30.0%) and dietary protein source (casein)

Amino acid	Pathway	Casein	Protein content			
			3.75% (5)	7.5% (4)	15.0% (4)	30.0% (4)
Glycine (Gly)	Glycolytic	−7.8 (6.4)	−2.3 (2.2)	−7.5 (1.8)	−7.3 (3.0)	−8.2 (4.2)
Serine (Ser)	Glycolytic	−26.3 (4.0)	−5.6 (7.1)	−10.4 (3.2)	−11.4 (3.3)	−13.5 (2.7)
Alanine (Ala)	Glycolytic/Krebs	−19.7 (0.8)	−9.1 (2.2)	−9.9 (2.3)	−12.0 (0.7)	−12.0 (2.0)
Aspartate (Asp)	Krebs	−23.4 (0.9)	−10.2 (1.9)	−12.2 (1.2)	−13.6 (1.4)	−15.3 (1.9)
Glutamate (Glu)	Krebs	−25.4 (0.3)	−12.1 (2.0)	−13.6 (1.6)	−14.6 (1.4)	−16.5 (1.5)
Arginine (Arg)	Krebs*	−18.3 (1.4)	−10.3 (1.3)	−13.5 (2.1)	−13.1 (1.7)	−14.4 (1.7)
Proline (Pro)	Krebs*	−25.2 (0.6)	−17.0 (3.1)	−19.7 (1.5)	−20.7 (2.4)	−22.7 (0.8)
Isoleucine (Ileu)	Indispensable	−27.6 (2.1)	−33.9 (3.6)	−36.1 (1.6)	−36.1 (1.6)	−40.2 (1.5)
Leucine (Leu)	Indispensable	−34.8 (0.7)	−22.5 (3.7)	−23.6 (2.9)	−25.8 (1.7)	−29.2 (1.6)
Phenylalanine (Phe)	Indispensable	−32.0 (0.3)	−24.0 (3.1)	−25.6 (2.0)	−27.2 (1.4)	−29.6 (1.1)
Threonine (Thr)	Indispensable	−18.4 (2.2)	−10.3 (2.6)	−12.8 (1.8)	−14.3 (1.5)	−16.6 (2.3)
Valine (Val)	Indispensable	−31.8 (1.9)	−19.6 (3.3)	−21.7 (1.8)	−23.4 (2.3)	−26.7 (2.1)

Numbers in parentheses adjacent to $\delta^{13}C$ values represent standard deviation; numbers in parentheses adjacent to protein contents represent the number of tilapia analysed in each treatment. Amino acid pathways as described in the text are labelled; asterisk denotes that arginine and proline can be conditionally indispensable in rapidly growing animals such as the tilapia examined in our experiments.

immediately stored frozen at −20 °C. Dissected lateral myotome muscles from each fish were dried at 40 °C and ground into a homogenous mixture. Lipid was then extracted from the mixture with three sequential petroleum ether extractions.

COMPOUND-SPECIFIC (AMINO ACID) STABLE ISOTOPE ANALYSIS

We measured the $\delta^{13}C$ value of individual amino acids in various tissues using gas chromatograph combustion isotope ratio mass spectrometry. Approximately 1·5 mg of dried tilapia muscle or casein was hydrolysed to amino acids in 1 mL of 6N hydrochloric acid at 110 °C for *c.* 20 h. Hydrolysed amino acids were then derivatized to *N*-trifluoroacetic acid isopropyl esters (Fantle *et al.* 1999; O'Brien, Fogel & Boggs 2002) in several batches and run in triplicate. Derivatized samples were injected into a Varian 3400 gas chromatograph for separation, converted to CO_2 gas via a combustion interface and analysed with a Finnigan Delta Plus XL (Thermo Scientific's mass spectrometry division is in Bremen, Germany) isotope ratio mass spectrometer. Isotopic results are expressed as δ values, $\delta^{13}C = 1000*$ $[(R_{sample} − R_{standard})/R_{standard}]$, where R_{sample} and $R_{standard}$ are the $^{13}C/^{12}C$ ratios of the sample and standard, respectively. The internationally excepted standard is Vienna-Pee Dee Belemnite limestone for carbon, and the units are expressed as parts per thousand or per mil (‰).

Using this derivatization method, measurements of $\delta^{13}C$ value for a given amino acid include carbon from both the amino acid and the derivatization reagents (e.g. isopropanol), which can be heavily fractionated depending on the synthesis pathway and on the number of exchangeable vs. non-exchangeable carbon atoms in each amino acid. Amino acid standards of known $\delta^{13}C$ composition were derivatized and analysed along with each batch of samples. The results of the standards reflect the effect of carbon added by the derivatization reagents and provided the information required to calculate the $\delta^{13}C$ value of amino acid carbon in the samples. Individual amino acid $\delta^{13}C$ values ($\delta^{13}CAA_{sample}$) were calculated using the equation:

$$\delta^{13}CAA_{sample} = \frac{\delta^{13}CAA_{dsa} − \delta^{13}CAA_{dst} + \delta^{13}CAA_{std} * p_{std}}{p_{std}} \quad (eqn\ 1)$$

The terms $\delta^{13}C_{dsa}$ and $\delta^{13}C_{dst}$ refer to the derivatized sample and standard, respectively, and std refers to the standard. p_{std} is equal to the proportion of carbon in the derivative that was sourced from the amino acid (Fantle *et al.* 1999; O'Brien, Fogel & Boggs 2002). We resolved 12 amino acids using these methods, and seven of which are typically considered dispensable for most eukaryotes – glycine, serine, alanine, aspartate, glutamate, arginine and proline – and five of which are considered indispensable, including threonine, valine, leucine, isoleucine and phenylalanine. A set of amino acid standards were analysed every three unknown samples. The standard deviation of $\delta^{13}C$ values among standards ranged from 0·8‰ for phenylalanine to 2·6‰ for glycine ($n = 15$). The average $\delta^{13}C$ standard deviation for all amino acids measured was 1·3‰.

MODELS AND STATISTICAL ANALYSES

The relationship between amino acid $\delta^{13}C$ value and per cent protein (%) in diet was fitted to models that represent three possible origins (e.g. glycolytic, Krebs or microbial dilution):

Model 1

For dispensable amino acids biosynthesized from glycolytic precursors, we used the constant function:

$$\delta^{13}C(\%) = \beta_0, \quad (eqn\ 2)$$

where β_0 represents the $\delta^{13}C$ value of carbohydrates plus a discrimination factor because of potential fractionation during biosynthesis (i.e. $\beta_0 = \delta^{13}C_{carbohydrates} + \Delta$).

Model 2

For dispensable amino acids derived from Krebs cycle intermediaries, a linear function was used:

$$\delta^{13}C_{muscle} = \beta_0 + \beta_1\%, \quad (eqn\ 3)$$

which represents the assumption of mixing in cataplerotic reactions. If we interpret eqn 3 as a linear mixing model, β_0 is as defined earlier, whereas β_1 is the change in $\delta^{13}C$ value per unit increase in the percentage of protein in the diet (with units ‰ per % protein).

Model 3

The $\delta^{13}C$ value of indispensable amino acids was estimated from a mixing model in which the contributions of amino acids from direct incorporation from the diet are related to potential microbial inputs:

$$\delta^{13}C = p(\%)\delta^{13}C_{AA} + (1 − p(\%))\delta^{13}C_{M}, \quad (eqn\ 4)$$

where $\delta^{13}C_{AA}$ is the isotopic composition of the dietary amino acid, and $p(\%)$ is the fraction of the amino acid derived directly from diet. The $\delta^{13}C_{M}$ is the isotopic composition of the amino acid biosynthesized by gut microbes, which we assume is more similar to the isotopic composition of the bulk diet than to the corresponding dietary amino acid. We use the symbol (%) to signify that the value of p is a function of the protein percentage in diet (see eqn 2). In this experiment, we have hypothesized that the fraction p depends on the protein content in the diet (%). The simplest form for the dependence of p on % assumes that the relative amount of the amino acid derived directly from diet is proportional to the % of protein in diet (i.e is equal to $\alpha\%$, where α is a proportionality constant), whereas the contribution of microbially derived carbon in these amino acids is constant (λ). Thus,

$$p(\%) = \frac{\alpha\%}{\alpha\% + \lambda} = \frac{\%}{\% + \frac{\lambda}{\alpha}}, \quad (eqn\ 5)$$

The parameter λ/α in eqn 5 represents the ratio of the contribution of microbes relative to that of the corresponding dietary amino acid when the protein content of the diet is very small. Therefore,

$$\delta^{13}C_{muscle} = \delta^{13}C_{M} + \left(\frac{\%}{\% + \frac{\lambda}{\alpha}}\right)\left(\delta^{13}C_{AA} − \delta^{13}C_{M}\right). \quad (eqn\ 6)$$

Equation 6 describes a process in which the isotopic signal of microbially biosynthesized amino acids become diluted as the contribution of dietary indispensable amino acids increases. Thus, for

indispensable amino acids derived from a mixture of microbial synthesis and diet, we used a re-parameterization of eqn 6 that leads to Model 3:

$$\delta^{13}C_{muscle} = \beta_0 + \beta_1 \left(\frac{\%}{\beta_2 + \%} \right), \quad \text{(eqn 7)}$$

where $\beta_0 = \delta^{13}C_M$, $\beta_1 = \delta^{13}C_{AA} - \delta^{13}C_M$, and $\beta_2 = \lambda/\alpha$. To fit the three models (eqns 2, 3, and 7), we used standard linear squares methods, including a Levenberg-Marquardt method in the case of eqn 7 (Gill & Murray 1978). We used small sample Akaike Information Theoretical Criteria (AICc) to assess the relative support for each model given our data (Burnham & Anderson 2002). If two models (*i* and *j*) differed in AICc by more than 3 (i.e. if $\Delta_{ij} = AICc_i - AICc_j$), we deemed the model with the lowest AICc as better supported (Burnham & Anderson 2002). If the difference in AICc between two models was ≤ 3 (i.e. if the evidence ratio between two models was $< 2·7$), we considered that both models are similarly supported and used other criteria to evaluate the merit of each competing model.

Results

As reported in Kelly & Martinez del Rio (2010), dietary protein content had a significant effect on fish growth rates, but only for tilapia in the 30% protein treatment. Growth rates for tilapia in the 3·75%, 7·5% and 15% dietary protein treatments were not different from one another.

The $\delta^{13}C$ value of individual amino acids varied with the content of protein in diet (Table 1). The relationship between an amino acid's $\delta^{13}C$ value and the protein content in diet revealed three distinct patterns. At low dietary protein concentrations, glycine, serine and alanine had $\delta^{13}C$ values that were more positive than the $\delta^{13}C$ value of dietary carbohydrates, but rapidly decreased to relatively constant values (Fig. 2). The difference between the $\delta^{13}C$ value of these amino acids and of carbohydrates at low dietary protein content (3·75%) is approximately equal to the discrimination factor (Δ) in the equation ($\beta_1 = \delta^{13}C_{carbohydrates} + \Delta$) that results from isotopic fractionation during biosynthesis. Our model selection procedure (Table 2) ranked the linear model (Model 2) as superior to the constant model (Model 1). Because it is evident that the relationship between $\delta^{13}C$ value and dietary protein in this situation was nonlinear (Fig. 2), however, we used an *ad hoc* exponential model that provides a more flexible fit than the linear model:

$$\delta^{13}C (\%) = \beta_0 + \beta_1 (e^{-\beta_2 \%}). \quad \text{(eqn 8)}$$

This equation (Model 4) was selected as the model with the strongest support in all the amino acids assumed *a priori* as having glycolytic precursors (Table 2). For alanine and serine, the asymptote (β_1) of this model did not differ significantly ($t = 1·4$ and 0·69, $P > 0·1$, respectively) from the $\delta^{13}C$ value of carbohydrates (−11‰). The β_1 value for glycine was significantly more positive than that of carbohydrates in the diet ($\beta_1 \pm SE = -7·7 \pm 1·0$, $t = 3·3$, $P < 0·05$). For consistency, we applied this *ad hoc* model to all amino acids (Table 2).

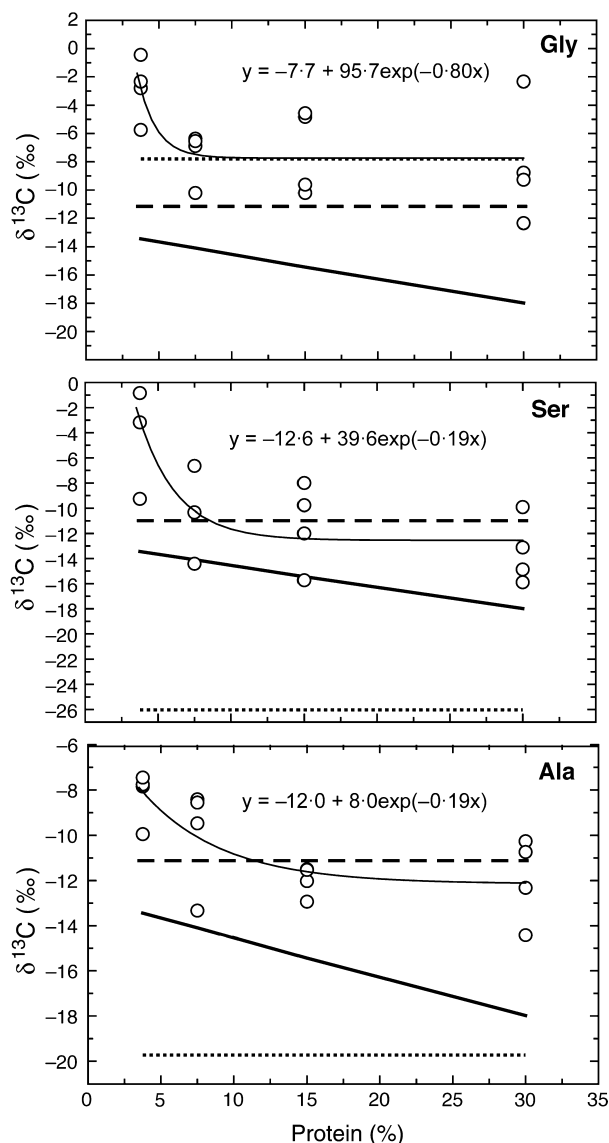**Fig. 2.** Change in the $\delta^{13}C$ value of glycolytic amino acids (Gly, Ser, Ala) in tilapia muscle as a function of dietary protein content. As in Fig. 1, the coarse dashed line is the $\delta^{13}C$ value of dietary carbohydrates (−11‰); the fine dashed line is the $\delta^{13}C$ value of the amino acid in dietary protein; the solid line represents the $\delta^{13}C$ value of bulk diet carbon. In all cases, the nonlinear model (eqn 8) provided the best fit to the change in $\delta^{13}C$ values of glycolytic amino acids with increasing dietary protein content (Table 2).

Aspartate and glutamate, which have Krebs cycle intermediary precursors, had similar, albeit not identical patterns (Fig. 3). In both cases, $\delta^{13}C$ values decreased monotonically as dietary protein content increased, and the $\delta^{13}C$ value of each amino acid was intermediate between the $\delta^{13}C$ value of dietary carbohydrates and the carbon isotope value of bulk diet (Fig. 3). Model 2 (linear function, eqn 3), however, was better supported by data for glutamate, whereas Model 3 (microbial dilution, eqn 7) was better supported for aspartate (Table 2). It is notable that for both aspartate and glutamate, the slope of the linear model was similar to that of the relationship between the $\delta^{13}C$ value of bulk diet and dietary

Table 2. We used Akaike Information Theoretic Criteria corrected for small samples (AICc $= n\log(\sum \hat{\varepsilon}_i/n) + 2K(n/(n - K - 1)$ to compare the support of four alternative models given the data (Burnham & Anderson 2002)

Amino acid	Model 1 β_0	Model 2 $\beta_0 + \beta_1\%$	Model 3 $\beta_0 + \beta_1\left(\frac{\%}{1+\beta_2}\right)$	Model 4 $\beta_0 + \beta_1 e^{(-\beta_2\%)}$	Best model r^2
Glycine (Gly)	44·0	43·2	No Fit	**42·9**	0·40
Serine (Ser)	56·4	51·4	No Fit	**48·0**	0·61
Alanine (Ala)	26·1	24·6	No Fit	**23·2**	0·54
Arginine (Arg)	31·6	26·6	No Fit	**23·1**	0·57
Aspartate (Asp)	34·6	20·5	**17·5**	19·2	0·76
Glutamate (Glu)	30·7	**16·3**	16·6	16·7	0·63
Proline (Pro)	40·1	29·1	**26·5**	27·0	0·70
Isoleucine (Ileu)	41·9	**26·7**	30·2	30·4	0·64
Leucine (Leu)	41·9	28·2	**27·1**	27·7	0·76
Phenylalanine (Phe)	39·0	36·5	**25·0**	26·2	0·79
Threonine (Thr)	41·8	27·1	**23·6**	24·2	0·72
Valine (Val)	45·4	31·3	**30·4**	31·0	0·71

Numbers in bold represent the AICc value of the model with strongest support. If two models differed in AICc by more than 3, we deemed the model with the lowest AICc as better supported (Burnham & Anderson 2002). If the difference in AICc between two models was ≤3, we considered both models are similarly supported and used other criteria to evaluate the merit of each competing model. Note that β_0 and β_1 are defined differently for each model; see text (Materials and Methods and Results) for details. Note that whereas Models 1, 2 and 3 have clear mechanistic interpretations, Model 4 is used only for descriptive purposes. We used $r^2 = 1 - \frac{SSE}{SST}$ to describe the goodness-of-fit of the model best supported by data.
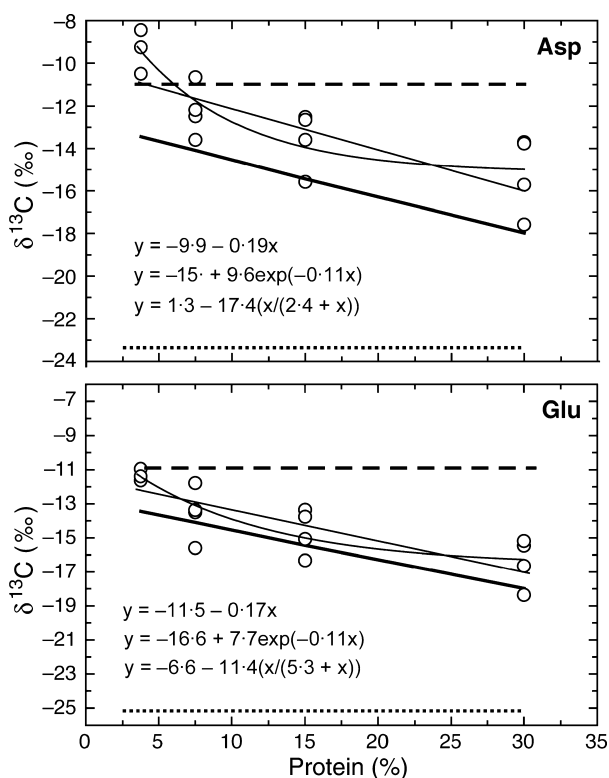

Fig. 3. Change in the δ^{13}C value of Krebs cycle amino acids (Glu and Asp) in tilapia muscle as a function of dietary protein content; see caption for Fig. 1 for definitions of the solid and dashed lines. A linear function (eqn 3) provided the best fit for glutamate with increasing dietary protein content, whereas the microbial dilution model (eqn 7) was better supported by the data for aspartate (Table 2).

protein content denoted by the bold solid line in Fig. 3. The slopes (±0·0·) of the linear model for aspartate and glutamate were 0·19 (±0·04) and 0·17 (±0·03) per mil, respectively, for

every 1% increase in the amount of dietary protein. Although Model 4 (exponential function, eqn 8) and Model 3 (microbial dilution, eqn 7) described the data for aspartate and glutamate well (Table 2), the asymptotic values that these models predict were higher than the δ^{13}C value of these amino acids in diet. Asymptotic values (±SE) were −15·7 ± 8‰ and −18·1 ± 9·0‰ for aspartate and glutamate, respectively, and both were significantly higher ($t > 8·0$, $P < 0·001$) than the respective δ^{13}C value of the amino acid in diet (Fig. 3; Table 1).

With the exception of isoleucine, the data supported Model 3 (microbial dilution, eqn 7) over the constant and linear models for all indispensable amino acids (Table 2). For isoleucine, Model 2 (linear function, eqn 3) received the strongest support (Table 2). All indispensable amino acids had δ^{13}C values that were intermediate between that of the value of the corresponding dietary amino acid and the isotopic composition of bulk diet (Fig. 4). To further confirm the adequacy of the microbial dilution model, we estimated the δ^{13}C value of the amino acid found in diet from the asymptotic value reached by eqn 7 when the protein content in diet is high (i.e. the limit of eqn 7 when protein % tends to infinity, which equals $\beta_0 + \beta_1$). The model very accurately predicted that the δ^{13}C value of the amino acid in tissues would become equal to that in diet when protein content is > 30%.

Arginine and proline had patterns that linked them with different categories (Fig. 5). Arginine had a pattern that resembled that of glycolytic amino acids (Gly, Ser, Ala), and Model 4 (exponential function, eqn 8) provided the best fit to the data (Table 2). Unlike glycine, serine and alanine, the asymptotic value (β_0) for arginine was significantly lower (−13·8 ± 0·6‰) than the δ^{13}C value of dietary carbohydrates. In contrast, the dependence of proline's δ^{13}C value on dietary protein content (Fig. 5) strongly resembled that of
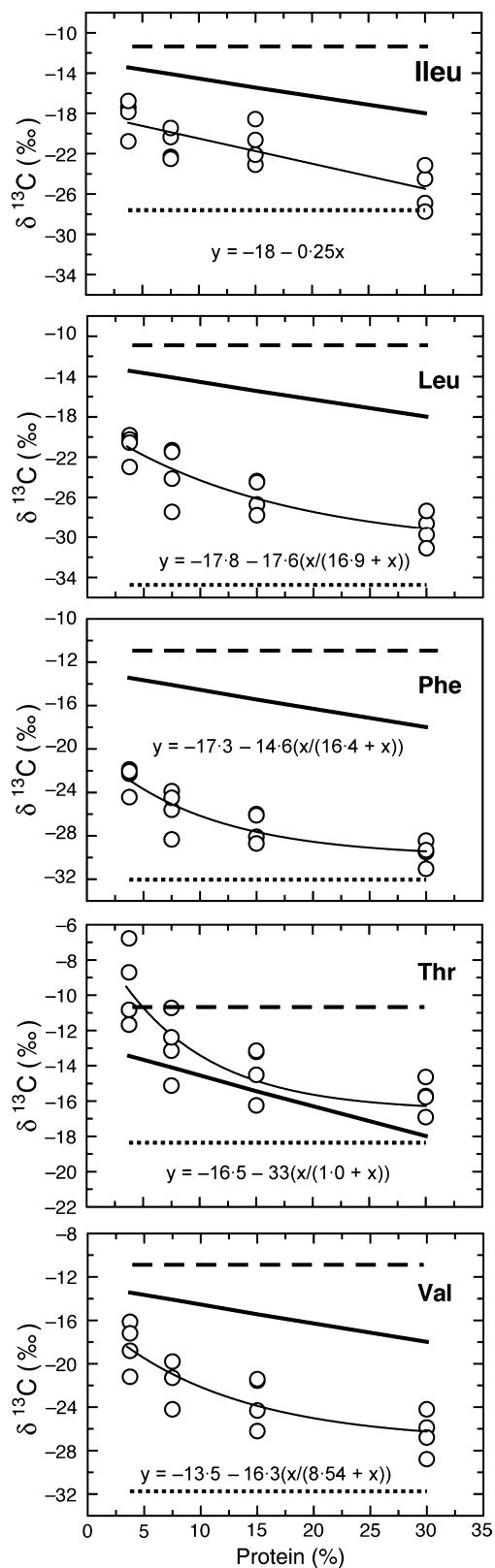
Fig. 4. Change in the $\delta^{13}C$ value of indispensable amino acids (Phe, Thr, Leu, Ileu and Val) in tilapia muscle as a function of dietary protein content; see caption for Fig. 1 for definitions of the solid and dashed lines. In all cases, the nonlinear microbial dilution model (eqn 7) provided the best fit to the change in $\delta^{13}C$ values of indispensable amino acids with increasing dietary protein content (Table 2).
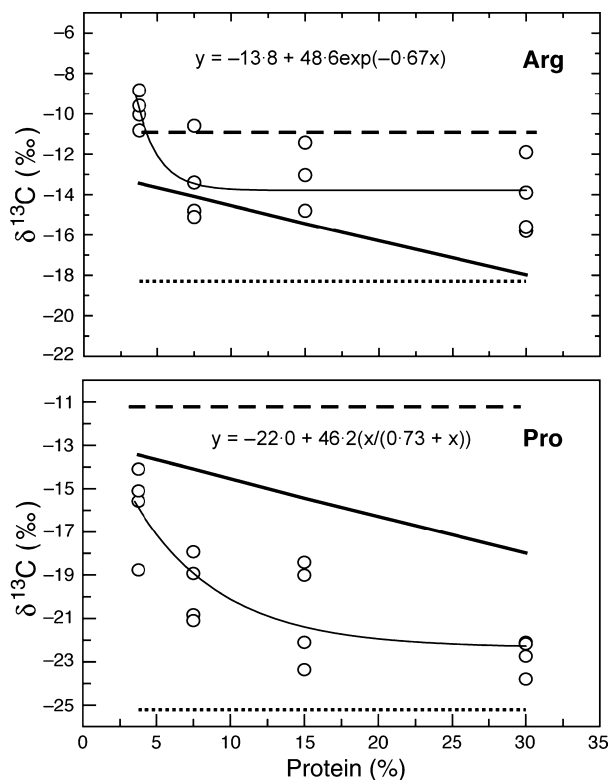

Fig. 5. Change in the $\delta^{13}C$ value of amino acids arginine and proline in tilapia muscle as a function of dietary protein content; see caption for Fig. 1 for definitions of the solid and dashed lines. For arginine, the nonlinear model (eqn 8) provided the best fit to the change in $\delta^{13}C$ value with increasing dietary protein content (Table 2). For proline, the nonlinear microbial dilution model (eqn 7) provided the best fit to the change in $\delta^{13}C$ value with increasing dietary protein content (Table 2).

indispensable amino acids. For proline, Model 3 (microbial dilution, eqn 7) was marginally better supported by the data (Table 2) than Model 4 (exponential function, eqn 8) and the asymptotic value ($\pm$ SE) of the model ($-24\cdot2 \pm 5\cdot0$) was statistically indistinguishable ($t = 0\cdot2$, $P > 0\cdot5$) from that of the $\delta^{13}C$ value ($-25\cdot2 \pm 1\cdot0$) of dietary proline (Table 1).

Discussion

With a few exceptions, the relationship between an amino acid's $\delta^{13}C$ value and protein was that expected *a priori* from the amino acid's biosynthetic pathway. In some cases, however, our simplistic models predicted the form of the relationship poorly. For example, it is clear that a constant function is a poor descriptor for the patterns exhibited by the amino acids derived from glycolytic precursors. In other cases, however, our models provided a good fit, especially for indispensable amino acids.

GLYCINE, SERINE AND ALANINE

Because glycine, serine and for the most part alanine are biosynthesized from glycolytic precursors and our experimental

diets were rich in carbohydrates, we (incorrectly) hypothesized that their isotopic composition would resemble that of diet and that it would be relatively independent of dietary protein content. In contrast, we found that the $\delta^{13}C$ values of all these amino acids were more positive at the lowest protein content (3·75%), and then became relatively constant with increasing dietary protein (Fig. 2). Model 4 (exponential function, eqn 8) with a relatively high rate constant best described this pattern (Table 2). Two factors are likely driving the observed pattern. First, the $\delta^{13}C$ value of these amino acids represents the mixing of a large fraction of carbon derived from carbohydrates and a small contribution from dietary amino acids. The amino acid contribution from diet is negligible at very low protein intakes but increases to a small constant fraction that makes a measurable difference if the dietary protein content exceeds *c*. 7·5%. Second, the biosynthesis of glycolytic amino acids from carbohydrates likely has a positive discrimination factor as described in the following paragraphs (Fig. 2).

Alanine can be biosynthesized via two distinct pathways – one uses pyruvate in glycolysis as the precursor and the other utilizes aspartate derived from oxaloacetate, an intermediate in the Krebs cycle. Our data suggests that the former pathway was most prevalent in our experiment because the asymptotic $\delta^{13}C$ value of tilapia muscle alanine more closely resembled the $\delta^{13}C$ value of dietary carbohydrates than bulk diet (Fig. 2). In the glycolytic pathway, a positive discrimination factor between carbohydrates and alanine is likely as it is biosynthesized in one step by the transamination of pyruvate. DeNiro & Epstein (1977) have shown that de-carboxylation of pyruvate to coenzyme-A discriminates against ^{12}C (see also Melzer & Schmidt 1987), and as a consequence, the remaining pyruvate pool must be enriched in ^{13}C relative to assimilated carbohydrates.

Further enrichment of glycine could result from the transfer of ^{12}C from serine to tetrahydrofolate (Horton *et al.* 2002). The ^{13}C-enrichment of glycine relative to diet is a well-documented observation (Tuross, Fogel & Hare 1988; Howland *et al.* 2003; Jim *et al.* 2006) that seems to explain why the $\delta^{13}C$ value of bone collagen, composed of *c*. 33% glycine (Eastoe 1957), is *c*. 4‰ more positive than that of other tissues commonly analysed in ecology (Koch 2007). The high $\delta^{13}C$ values of serine and glycine at low protein intakes could result from enrichment in the isotopic composition of the carbohydrate pool, which at low protein concentrations would experience great metabolic demand. These amino acids are derived primarily from 3-phosphoglycerate, which can be biosynthesized directly from glucose or from the, presumably ^{13}C-enriched, pyruvate pool (Walsh & Sallach 1966).

ASPARTATE AND GLUTAMATE

The carbon isotope composition of aspartate and glutamate exhibited similar patterns with increasing protein intake. These patterns qualitatively resembled those predicted by our *a priori* linear model (Model 2, eqn 3) and are close to those expected from amino acids biosynthesized from Krebs cycle precursors (Fig. 3). A linear model (Model 2) was best supported by the glutamate data, but a nonlinear microbial dilution model (eqn 7) was better supported in the case of aspartate (Table 2). There are two reasons to hesitate to accept the nonlinear model as the best fit for aspartate. First, the difference in the AICc values between the linear and nonlinear model was small (Table 2), indicating at least partial support for the linear model. Second, the slope of the linear model relating $\delta^{13}C$ value with % protein in diet was not significantly different from that of bulk diet with % protein in diet (Fig. 3).

Aspartate and glutamate are biosynthesized from oxaloacetate and α-ketoglutarate, respectively. These two compounds are generated by a variety of precursors in anaplerotic reactions within the Krebs cycle. Multiple cycling of metabolites through the Krebs cycle could result in carbon skeletons are that have been 'shuffled', because several of the intermediates in the Krebs cycle (e.g. succinate and fumarate) are symmetrical molecules. This observation suggests that the isotopic analysis of aspartate and glutamate in various tissues may be particularly useful in studies attempting to reconstruct bulk diet (Howland *et al.* 2003).

INDISPENSABLES: ISOLEUCINE, LEUCINE, PHENYLALANINE, THREONINE AND VALINE

With the exception of isoleucine, our results for the indispensable amino acids were best characterized by Model 3, the nonlinear microbial dilution model (Table 2 and Fig. 4). Isoleucine was best characterized by a linear function (Table 2), but its overall carbon isotope composition was more similar to that of other indispensable amino acids than those associated with glycolytic or Krebs precursors (Fig. 4). At high dietary protein contents, the asymptotic value of all indispensable amino acids closely resembled that of the corresponding amino acid in dietary protein (Fig. 6), suggesting
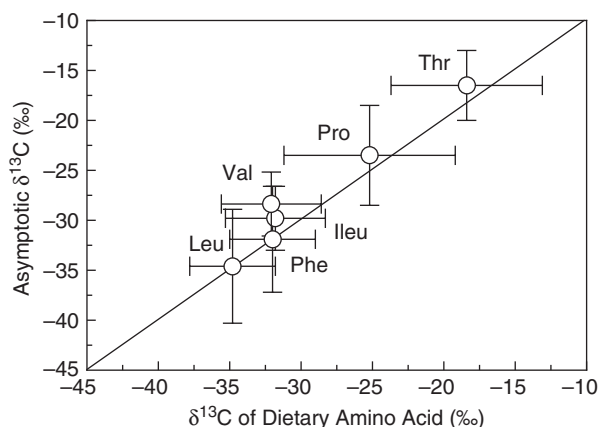

Fig. 6. Relationship between the asymptotic $\delta^{13}C$ value predicted by the microbial dilution model (eqn 7) and $\delta^{13}C$ value of the dietary amino acid for the indispensable amino acids such as Phe, Thr, Leu, Ileu, Val, and conditionally indispensable Pro.

that indispensables are being directly routed from diet (Fig. 4).

The isotopic composition of the indispensable amino acids at low dietary protein contents, however, was significantly higher than the corresponding amino acid $\delta^{13}C$ value in dietary protein. For example, the mean $\delta^{13}C$ value ($\pm SD$) of tilapia threonine ($-10\cdot3 \pm 2\cdot6‰$) was indistinguishable from that of dietary carbohydrate at low dietary protein content ($3\cdot75\%$). Likewise, the mean $\delta^{13}C$ value of tilapia valine ($-19\cdot6 \pm 3\cdot3‰$) at low dietary protein content was more similar to that of bulk diet than the $\delta^{13}C$ value of valine ($-31\cdot8 \pm 1\cdot9‰$) in dietary protein (Table 1). The other three indispensable amino acids (Ileu, Leu and Phe) had $\delta^{13}C$ values intermediate between bulk diet and dietary protein. There are several potential reasons for this pattern, because all of these amino acids (Thr, Val, Ileu, Leu and Phe) are known to be indispensable to rapidly growing tilapia (Santiago & Lovell 1988). First, isotopic fractionation can occur during the catabolism of amino acids from available bodily tissues (e.g. muscle, bone) leading to higher $\delta^{13}C$ values in tilapia muscle vs. dietary amino acids (Macko & Estep 1985). Tilapia were fed a daily ration that exceeded their maximal intake to maintain rapid growth and tripled their mass during the course of the experiment (Kelly & Martinez del Rio 2010); thus, it is unlikely that tissue amino acids were catabolized for energy. Second, it is possible that tilapia were acquiring some of their indispensable requirements by grazing algae, microbial biofilms, phytoplankton, or other detritus in the holding tanks (Bowen 1980); however, this source was likely minimal because we frequently cleaned the tanks and the throughput rate was high (80–90 mL min^{-1}) in comparison with the size of the tanks ($25\cdot5 \times 25\cdot5 \times 15$ cm). Lastly, this pattern could be caused by the *in vivo* synthesis of amino acids from the bulk diet (i.e. carbohydrates) by gut microflora to provide indispensable amino acids to tilapia when they are fed protein-deficient diets (Kelly & Martinez del Rio 2010).

One of the many functions of the gastrointestinal microbiota is to provision hosts with essential molecules (e.g. vitamins). The microbial contribution to the host's nutrition in terms of amino acids has been relatively well studied in ruminants (Kung & Rode 1996; Karasov & Carey 2008); however, whether a majority of amino acids can originate from microbiota for non-ruminants like tilapia remains much less clear (Fuller & Reed 1998). A number of studies have relied on the oral administration of non-specific ^{15}N as urea or ammonium chloride to label lysine biosynthesized by microbes and trace its flux into the host because animals do not transaminate lysine (D'Mello 2003). The method has the serious shortcoming of being limited to lysine. Nonetheless, it has revealed a significant contribution of microbial lysine to the lysine in the host's body protein (reviewed by Metges 2000).

HOW MUCH DOES MICROBIOTA CONTRIBUTE TO THE INDISPENSABLE AMINO ACID BUDGET OF TILAPIA?

Our results point to a novel method to quantify the contribution of the microbiota to the indispensable amino acid intra-cellular pool. The nonlinear microbial dilution model (Model 3) seems to describe adequately well the variation of $\delta^{13}C$ values of most of the indispensable amino acids with changes in dietary protein intake (Fig. 4). The terms in the equation that represent Model 3 are biologically relevant and simple to interpret: $\beta_0 = \delta_M$, $\beta_0 + \beta_1 = \delta_{AA}$ and $\left(\frac{\%}{\beta_2+\%}\right)$ equal the fraction of carbon in an amino acid derived directly from diet (compare eqns 6 and 7). Thus, the fraction of carbon in an amino acid derived from microbes, which we will designate by $p_M(\%)$, is equal to $1 - \left(\frac{\%}{\beta_2+\%}\right)$.

Inspection of the estimates that result from fitting isotopic values for each amino acid to eqn 7 revealed that this equation accurately estimates δ_{AA} (Fig. 6). The estimated isotopic values of the amino acids biosynthesized by microbes from carbohydrate precursors (δ_M), however, are unrealistically enriched in ^{13}C (Fig. 4). These estimated δ_M values ranged from $13\cdot5‰$ to $22\cdot0‰$. Considering that the non-protein carbon sources had a $\delta^{13}C$ value of $-11‰$, the observed $\delta^{13}C$ values would require an unreasonably large discrimination against ^{12}C during microbial biosynthesis. Previous work suggests that microbial synthesis of amino acids by heterotrophic microbes has modest discrimination values and often discriminates against ^{13}C (Blair *et al.* 1985; Macko *et al.* 1987). Because an incorrect estimate of δ_M will lead to biased estimates of $p_M(\%)$, the model needs to be constrained with realistic values for δ_M.

To estimate $p_M(\%)$, we constrained the value of $\beta_0 = \delta_M$ in the microbial dilution models using $\Delta_{\text{source amino acid}}$ values estimated by Larsen *et al.* (2009) for an Actinobacterium and *Rhodococcus* spp. grown on a medium with sucrose as the sole carbon source (Table 3). Table 3 lists the δ_M values that we used to constrain the microbial dilution model and the AICc values for this model. Despite the assumption that these microbes are similar to gut microbiota, the AICc values revealed that this new constrained model performed well relative to the unconstrained model. Indeed, the estimated δ_{AA} value was very similar to that estimated by the unconstrained model and to the value of the amino acid in diet. Furthermore, the curves generated by both models were indistinguishable over the range of dietary protein content ($3\cdot75$–30%) in our experiments. More strikingly, the pattern of change in amino acids derived from microbes, $p_M(\%)$, was remarkably similar among all indispensable amino acids

Table 3. We used $\Delta^{13}C_{\text{source amino acid}}$ values for two heterotrophic bacteria (Larsen *et al.* 2009) to constrain the $\delta_M = \beta_0$ value included in the microbial dilution model. Judging from its lower AICc values, this new model performed well relative to the 'unconstrained' model. Constraining the value of δ_M permitted fitting the microbial dilution model for isoleucine

Amino acid	$\Delta^{13}C_{\text{amino acid source}}$	δ_M	AICc
Isoleucine (Ileu)	$-2\cdot4$	$-13\cdot4$	$30\cdot4$
Leucine (Leu)	$-1\cdot1$	$-12\cdot1$	$26\cdot7$
Phenylalanine (Phe)	$-4\cdot0$	$-15\cdot1$	$16\cdot0$
Threonine (Thr)	$7\cdot0$	$-4\cdot0$	$20\cdot7$
Valine (Val)	$-2\cdot0$	$-13\cdot0$	$27\cdot5$

(Fig. 7). The model estimated substantial microbial contributions ranging from *c*. 55% to 70% of indispensable amino acids when fish were fed diets with low (3·75%) protein content, but declined rapidly as dietary protein content increased. The model suggests that when growing tilapia were fed diets with optimal protein content (*c*. 45%, Lim & Webster 2006), the contribution of microbes to amino acids was small (10–20%) but significant (Fig. 7). Again, we emphasize the tentative and speculative nature of this analysis. Only direct measurement of $\Delta_{\text{source amino acid}}$ for gastrointestinal microbiota will permit quantitative estimates of the contribution of the gut microbiota to the assimilation of indispensable amino acids.

OUTLIERS: ARGININE AND PROLINE

Our model approach linked arginine and proline to different precursor categories. Arginine is considered to be a conditionally indispensable amino acid because it can be biosynthesized *de novo* via conversion of citrulline, but this pathway is energetically expensive and may not produce sufficient arginine, especially in rapidly growing (i.e. young) animals. Citrulline can be derived from multiple sources, but one of the most common precursors is orthinine, which is produced via the catabolism of glutamate, a Krebs cycle intermediate. For arginine, however, our modelling approach identified its isotopic pattern as being more similar to that of the dispensable glycolytic amino acids (Gly, Ser, Ala) than indispensable amino acids. Arginine δ^{13}C values were slightly higher than dietary carbohydrates at low dietary protein content but then decreased to values intermediate between bulk diet and dietary carbohydrates as dietary protein content was increased (Fig. 5). This suggests that sufficient amounts of arginine can be biosynthesized *de novo* from glutamate (Caldovic & Tuchman 2003) derived from bulk diet when diets are protein deficient. As dietary protein was increased to moderate levels (*c*. 7·5%), tilapia arginine δ^{13}C values dropped below that of dietary carbohydrates and remained relatively constant with increasing dietary protein content. This pattern is similar to
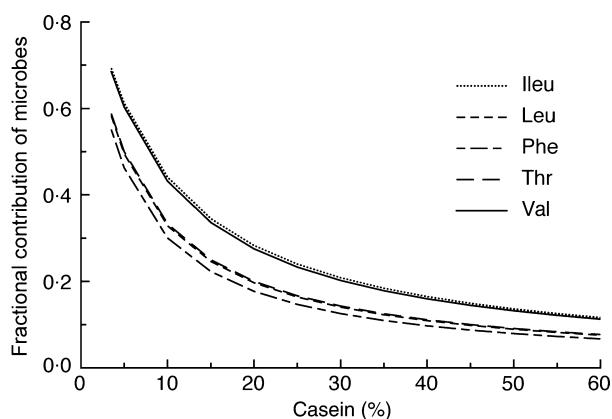
that observed for the glycolytic amino acids (Gly, Ser and Ala) and suggests that some dietary arginine was being routed into tilapia muscle when moderately available in diet (Fig. 5).

Like arginine, proline can be biosynthesized *de novo* from glutamate, a dispensable amino acid biosynthesized from precursors derived from Krebs cycle reactions that closely tracked the δ^{13}C value of bulk diet in our experiments (Fig. 3). Our modelling approach (Table 2), however, identified Model 3 (microbial dilution, eqn 7) as having a similar amount of support as Model 4 (exponential function, eqn 8). In addition, proline's overall carbon isotope composition with increasing dietary protein content was more similar to that of indispensable amino acids than amino acids biosynthesized from Krebs cycle precursors. If proline was being biosynthesized from glutamate at low dietary protein contents (3·75%), then tilapia proline δ^{13}C values would be higher than the bulk diet δ^{13}C value because of a positive fractionation during *de novo* biosynthesis, which is similar to the pattern observed for aspartate and glutamate (Fig. 3). At low dietary protein contents, however, tilapia proline δ^{13}C values were intermediate between that of bulk diet and dietary protein sources (Fig. 5 and Table 1). This pattern is more similar to that of indispensable amino acids (Fig. 4) than dispensable amino acids biosynthesized from Krebs cycle precursors (Fig. 3). Furthermore, proline δ^{13}C values decreased significantly with increasing protein content such that its asymptotic δ^{13}C value at high dietary protein content (30%) was indistinguishable from that of proline in dietary protein (Fig. 5). Thus, there was little carbon isotope fractionation of proline from diet to tissue at high dietary protein contents, which is a pattern expected of indispensable amino acids when readily available in diet. In agreement with data from recent experiments (Ball, Atkinson & Bayley 1986; Chung & Baker 1993; Kirchgessner, Fickler & Roth 1995; Dabrowski *et al*. 2005), our results suggest that proline is conditionally indispensable for tilapia fed protein-deficient diets (3·75%) and is directly routed with minimal isotopic fractionation at high dietary protein contents (30%).

IMPLICATIONS FOR ISOTOPIC ECOLOGY: PROTEIN ROUTING AND MICROBIAL CONTRIBUTIONS

Overall, our results have implications for protein routing and the interpretation of isotopic data in ecology and more broadly for the contribution of gut microbiota to the budget of indispensable amino acids in animals. Our results show that routing of dietary protein into consumer proteinaceous tissues occurs in a rapidly growing omnivorous ectotherm. When coupled with recently published data for bulk muscle tissue from the same fish (Kelly & Martinez del Rio 2010), the amino acid-specific patterns reported here show that 'scrambled egg' assumption made by stable isotope mixing models is not valid for omnivores, such as tilapia, that consume moderate amounts of protein (≥10%). At lower protein concentrations, the isotopic composition of tilapia resembled that of diet. This resemblance at the level of bulk tissue, however, was the result of complex underlying patterns at the level of

Fig. 7. Relationship between the fractional contribution (%) of indispensable amino acids derived from gastrointestinal microbiota with increase in casein (protein) content of diet.

individual amino acids. The finding of isotopic routing has significant implications for any applied study that uses isotopic data to characterize omnivore diets, especially for animals that often experience seasonal or spatial changes in protein availability. In agreement with available evidence from experiments focused on bulk tissue analysis (Podlesak & McWilliams 2006; Voight *et al.* 2008; Kelly & Martinez del Rio 2010), our results suggest that for animals fed diets with moderate to high amounts of protein, mixing models can *overestimate* the contribution of dietary protein sources to diet. At moderate to high protein diet levels, the proteinaceous tissues (e.g. muscle, feather, blood) routinely analysed in ecology tend to preferentially reflect the isotopic composition of dietary protein. Martínez del Rio & Wolf (2005) constructed models that assume perfect routing in which for animals that consume moderate to high amounts of protein, the isotopic composition of proteinaceous tissues exclusively reflects that of dietary protein. Our analysis suggests that these models may be incorrect. Even at high protein levels, the biosynthesis of dispensable amino acids might add carbon from non-protein dietary sources (carbohydrates and lipids) to the proteinaceous tissues biosynthesized by the animal. Ultimately, future experiments that utilize compound-specific stable isotope analysis (CSIA) could lead to a new generation of mixing models for omnivores that include more mechanistic detail and hence lead to more accurate diet reconstructions.

Tilapia appeared to be able to acquire their indispensable amino acid requirements directly from gastrointestinal microbiota when dietary sources were insufficient. This observation highlights yet another critically important process mediated by the symbiosis between monogastric omnivorous animals and their gut microbiota. Our approach also shows that CSIA of individual amino and/or fatty acids is a powerful proxy (Evershed *et al.* 2007) for exploring questions that lie at the intersection between microbiology and animal nutrition.

Acknowledgements

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