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Nitrogen isotope fractionation and amino acid turnover rates in the Pacific white shrimp *Litopenaeus vannamei*

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ABSTRACT: Fluctuations in an animal's trophic position (TP) can indicate changes within an ecosystem. Traditionally, bulk tissue or whole animal nitrogen (N) isotope analysis has been used to determine an animal's TP, but there are limitations to the application of this approach. Amino acid compound-specific isotope analysis (AA-CSIA) can be used to determine TP based on the difference between the $\delta^{15}N$ values of certain amino acids, commonly glutamic acid and phenylalanine. The rate at which ^{15}N is incorporated into amino acids is largely unknown, and this may affect the accuracy of relative TP estimates especially during periods of ecosystem change or ontogenetic changes in diet. To study amino acid turnover rates, the diet of the Pacific white shrimp *Litopenaeus vannamei* was changed in the laboratory from an unlabeled diet (7.2‰) to a ^{15}N-enriched diet (24‰), and animals were sampled over the course of 7 wk. AA-CSIA revealed that turnover times of amino acids vary considerably, with some amino acids having much more rapid turnover than others. The TP of *L. vannamei* (using $\delta^{15}N$ values of glutamic acid and phenylalanine) over the course of the experiment remained constant, and shrimp TP averaged 2.14 ± 0.15 (mean ± SD, n = 7) relative to its diet, which was as expected. Our results indicate that the $\delta^{15}N$ values of glutamic acid and phenylalanine can be used to accurately determine TP even while shrimp are undergoing a change to a diet with a different $\delta^{15}N$ value.

KEY WORDS: Compound-specific isotope analysis · Diet change · Trophic position · Crustacean

INTRODUCTION

Due to increasing concern about the effects of human behavior on species biomass, abundance, and diversity, efforts are being made to track natural and anthropogenic changes within ecosystems. However, it is difficult to quantify the effects of large-scale changes on ecosystems. Processes such as overfishing (Pauly et al. 1998) and the introduction of invasive species (Vander Zanden et al. 1999) can directly affect other organisms within a food web (Pace et al. 1999). An organism's trophic position (TP) reflects both its diet and position within a food web and the increase or decrease in an organism's TP can be indicative of change within an ecosystem (Peterson & Fry 1987, Gannes et al. 1997, Pauly et al. 1998, Pace et al. 1999, Vander Zanden et al. 1999, Fry 2006). Since TP can reflect life history characteristics of a species as well as monitor and detect changes within an ecosystem, it is necessary to establish accurate methods of estimating TP in field-caught samples.

Nitrogen (N) isotope analysis of whole organisms or their tissues ('bulk' analysis) in conjunction with stomach content analysis, has been used to determine an organism's TP, establish predator–prey relationships, and identify food sources (Peterson & Fry 1987, Gannes et al. 1997). N isotope analyses are a useful complementary tool for studying food web ecology as

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they can offer an integrated view of an organism's diet over a period of weeks or months, depending on turnover time of the tissue analyzed (Peterson & Fry 1987, Gannes et al. 1997, Schmidt et al. 2004). Bulk N isotope analyses can be used to determine the TP of a particular organism because $\delta^{15}N$ values of consumers increases (~3.0 ‰) with each increase in TP (Deniro & Epstein 1981, Post 2002). The accuracy of TP estimates determined using bulk $\delta^{15}N$ values can be confounded by other conditions that contribute to the enrichment or depletion of ^{15}N in whole tissues relative to the diet (Hobson et al. 1993, Best & Schell 1996, Gannes et al. 1997, Post 2002) including changes in $\delta^{15}N$ values at the base of the food web (Post 2002) and uncertainties in magnitude of ^{15}N enrichment in consumers relative to diet or the trophic discrimination factor (TDF) (Deniro & Epstein 1981, Post 2002).

Amino acid compound-specific isotope analysis (AA-CSIA) is a method that can identify the origins of variations in animal bulk tissue $\delta^{15}N$ values using differences in the N isotopic ratio of individual amino acids (McClelland & Montoya 2002). The $\delta^{15}N$ values of 'source' amino acids (sensu Popp et al. 2007) in a consumer (e.g. phenylalanine, glycine) appear to reflect and record the isotopic baseline, or the $\delta^{15}N$ values of primary producers integrated over the lifetime of the consumer, and these values change little as a function of TP. In contrast, the $\delta^{15}N$ values of some amino acids increase in a consistent manner as a function of TP (McClelland & Montoya 2002, McClelland et al. 2003, Schmidt et al. 2006, Chikaraishi et al. 2007, 2009, Popp et al. 2007, Hannides et al. 2009). These 'trophic' amino acids (sensu Popp et al. 2007) (e.g. glutamic acid, alanine) are consistently enriched in ^{15}N relative to source amino acids as TP increases. The difference in $\delta^{15}N$ values between trophic and source amino acids acts as an 'internal index of TP' (McClelland & Montoya, 2002) independent of fluctuations in the $\delta^{15}N$ values at the base of the food web.

Recent research using AA-CSIA to determine TP has focused on using the difference in the $\delta^{15}N$ values of glutamic acid and phenylalanine in a variety of organisms (McClelland et al. 2003, Chikaraishi et al. 2007, 2009, Hannides et al. 2009, Lorrain et al. 2009, Dale et al. 2011, Choy et al. 2012, Seminoff et al. 2012). However, other individual and combinations of several trophic and source amino acids have also been successfully used to determine the TP of organisms (Popp et al. 2007, Sherwood et al. 2011, Décima et al. 2013). It is however necessary to establish which source and trophic amino acids are most appropriate for estimating TP in multiple taxa. Furthermore, it is unclear whether a single pair of trophic and source amino acids or the average of multiple amino acids would produce the most accurate TP estimates (e.g. Décima et al. 2013).

The rate of incorporation of amino acids into protein and its effect on the difference in $\delta^{15}N$ values of trophic and source amino acids is largely unknown (Bradley et al. 2014). If rates of incorporation of source and trophic amino acids differ, amino acid based trophic status estimates could be affected in organisms undergoing dietary changes or migrating between environments with different isotopic baselines. Consequently, it is important to determine how quickly the $\delta^{15}N$ values of individual amino acids reach steady state in organism tissues. Many organisms undergo a change in diet or a change in the isotopic composition of a similar diet as a function of seasonality (Schmidt et al. 2006), migration (Best & Schell 1996, Popp et al. 2007), or overfishing (Pauly et al. 1998). The purpose of this study was to determine if trophic and source amino acids are incorporated into protein at the same rate. If source and trophic amino acids incorporate dietary N at different rates, then the difference in $\delta^{15}N$ values between them may not accurately estimate the TP of an organism undergoing a change in diet. Furthermore, if the turnover rates of source and trophic amino acids are different, useful but currently hidden ecological or physiological information may be encoded in the N isotopic compositions of amino acids.

We used AA-CSIA to study the N turnover rates of amino acids in the commercially important shrimp *Litopenaeus vannamei*. We sought to determine the rate of ^{15}N incorporation of individual amino acids into protein and to determine if rates are similar among amino acids. Furthermore, we evaluated which pairs of amino acids are able to estimate TP most accurately in *L. vannamei* and whether or not an average of multiple amino acids produces more consistent TP estimates than that of a single pair of trophic and source amino acids. Finally, we sought to evaluate if AA-CSIA can be used to accurately estimate the TP of an organism undergoing a change in diet that affects its $\delta^{15}N$ value.

MATERIALS AND METHODS

Diet shift

Juvenile *Litopenaeus vannamei* (4 to 5 g) used for this experiment were obtained from cultures maintained at the Oceanic Institute, Waimanalo, Oahu, Hawai'i. A total of 65 *L. vannamei* were cultured in

2 replicate flow-through seawater tanks. Shrimp were fed a pelleted diet (35% protein/5% squid, Zeigler Brothers) at a rate of 5.5 to 6.0% of their weight every 24 h. The $\delta^{15}N$ value of the diet was initially 7.2‰ for a 4 to 5 wk long acclimation period and was then changed to 24.3‰. The ^{15}N-enriched diet was made by reconstituting the acclimation diet with water and adding a small proportion (<1% of the dry weight of the diet) of *Chlorella*. This had been grown in batch culture on ^{15}N-labeled nitrate and subsequently freeze-dried and powdered prior to its addition to the acclimation diet. *L. vannamei* are known to consume heterotrophic bacterial biofloc to obtain supplemental nutrition (Xu & Pan 2012) and microalgae are widely used in the hatchery culture of penaeid shrimp as an important source of polyunsaturated fatty acids, carotenoids, sterols, vitamins, and minerals (Guedes & Malcata 2012). Consequently, *L. vannamei* readily consumes algae and we assume that even if the assimilation efficiency of *Chlorella* is very different from the rest of the protein in the feed, the ^{15}N signal in the very small amount of algae in the feed is transferred to the shrimp. The ^{15}N-enriched diet was then repelleted via extrusion at the Aquatic Feeds and Nutrition Laboratory at the Oceanic Institute and dried. To ensure isotopic homogeneity, the new diet was randomly subsampled and analyzed for bulk $\delta^{15}N$ values, both within the batch, 24.3 ± 0.3‰ (mean ± SD, n = 4), and along a single pellet, 24.4 ± 0.1‰ (mean ± SD, n = 3). The $\delta^{15}N$ value of the new diet ensured that the difference in $\delta^{15}N$ values between the 2 diets was enough to exceed natural variability yet the composition of the diet remained essentially constant.

Shrimp (n = 8) were collected before the change to the ^{15}N-enriched diet to determine the initial $\delta^{15}N$ values of the animals. Two shrimp (one from each tank) were subsequently sacrificed at each consecutive time point for the next 7 wk. The *L. vannamei* collected at each time point were weighed to determine growth rate over time, and the weights of all shrimp from a given time point were averaged. Shrimp were stored frozen (–20°C) until prepared for isotope analysis. Tissue samples from only the tail muscle were collected, dried, homogenized, and processed for analysis. In preparation for isotope analysis, the digestive tract was removed from each shrimp.

Isotope analysis

Bulk tail muscle isotope analysis was performed on all samples using a C-N analyzer coupled with an isotope ratio mass spectrometer (Finnigan Conflo II/Delta-Plus) at the Isotope Biogeochemistry Laboratory at the University of Hawai'i at Manoa. A subset of samples chosen for AA-CSIA were selected from throughout the experiment to determine if trends observed in the $\delta^{15}N$ value of amino acids were consistent with those observed in the bulk tissue. AA-CSIA focused on 7 selected samples from Tank 1 (0.5, 2, 48, 192, 288, 480, 1152 h) and both the baseline and the ^{15}N-enriched diet were analyzed.

Samples of dried and homogenized tissues were hydrolyzed (Macko et al. 1997) and the amino acids were subsequently derivatized to produce trifluoroacetic amino acid esters and then analyzed as described by Popp et al. (2007), Hannides et al. (2009), and Dale et al. (2011). During amino acid hydrolysis, asparagine is converted to aspartic acid and glutamine is converted to glutamic acid. Samples were purified by filtration (0.22 µm Millex-GP, Millipore) followed by cation exchange chromatography. The carboxyl termini of the amino acids were esterified using 2 ml 4:1 isopropanol:acetyl chloride. The amino acid esters were then acetylated by 3:1 methylene chloride:trifluoroacetic anhydride (TFAA). Amino acids were further purified by solvent extraction (Ueda et al. 1989). The acetylation step was repeated and samples were then stored in 3:1 methylene chloride:TFAA at 4°C until analyzed, usually within 1 mo of hydrolysis and derivatization. Just prior to analysis with isotope ratio monitoring gas chromatography-mass spectrometry (GC/C/IRMS), samples were dried under N_2 at room temperature then redissolved in ethyl acetate.

Amino acids were analyzed by GC/C/IRMS (Finnigan MAT253 mass spectrometer) coupled with a Trace GC chromatograph through a GC-CIII combustion furnace (980°C), reduction furnace (650°C), and a liquid N cold trap. Samples were analyzed in triplicate and each series of sample runs was bracketed by analysis of a suite of pure amino acids with known $\delta^{15}N$ values. L-2-aminoadipic acid and norleucine of known isotopic composition were co-injected into all samples and the laboratory reference materials analyzed, and all $\delta^{15}N$ values were normalized to those of the co-injected compounds. Samples and reference materials were injected onto a BPx5 *forte* capillary column (60 m × 0.32 m × 1.0 µm film thickness) at an injection temperature of 180°C using a split/splitless injector (in splitless mode) with a constant helium flow rate of 1.4 ml min^{-1}. The column was held at 50°C for 2 min, increased in stages to 190°C at a rate of 8°C min^{-1}. The temperature was then increased from 190°C to 300°C at a rate of 10°C

min^{-1} and then held at 300°C for 7.5 min. Isotope values are expressed in standard δ notation ($\delta^{15}N = ([R_{sample} - R_{standard} - 1] \times 10^3)$ relative to air.

Data analysis

Trophic discrimination factor (TDF). It was necessary to determine the TDF of the bulk tissue and that of the amino acids relative to the diet in order to estimate the new isotopic steady state following the diet shift. TDF was determined as follows:

$$TDF = \text{mean}\ (\delta^{15}N_{consumer} - \delta^{15}N_{food}) \quad (1)$$

TDF is the difference between the nitrogen isotopic value of the consumer's tissue ($\delta^{15}N_{consumer}$) and that of the diet ($\delta^{15}N_{food}$) in an organism that has reached an isotopic steady state with its diet. In order to determine more robust TDF values based on multiple *L. vannamei*, TDF values of individual amino acids were determined using data from a study conducted by C. A. Otoshi et al. (unpubl.), in which *L. vannamei* were cultured in clear water for 5 wk at the Oceanic Institute without a diet shift and the amino acids were analyzed at the University of Hawai'i using the same protocols as this study. The average difference between the $\delta^{15}N$ values of the shrimp and their feed obtained by Otoshi et al. was used to establish robust estimates of TDF for each amino acid in this study (see Fig. 5).

Reaction progress variable. The reaction progress variable model (Cerling et al. 2007) was applied to identify whether there were multiple N compartments present in the bulk tissue and constituent amino acids after a diet shift (Martínez del Rio & Anderson-Sprecher 2008):

$$(1 - F) = \frac{\delta X(\infty) - \delta X(t)}{\delta X(\infty) - \delta X(0)} = \sum_i p_i e^{-\lambda_i t} \quad (2)$$

where F is the fractional approach to steady state, $\delta X(\infty)$ is the δ value of the final isotopic steady state, $\delta X(0)$ is the initial δ value of the tissues before the diet shift, $\delta X(t)$ is the δ value at time t, p_i is the 'fractional size of each 'pool'', and λ_i is the rate constant of each 'pool' (Cerling et al. 2007, Martínez del Rio & Anderson-Sprecher 2008). Using this model, a linear fit to the isotopic data indicates that a single N turnover pool is present and the data can be sufficiently modeled as a single compartment such that a first order exponential model could be used to estimate N turnover. If the slope of the transformed data is nonlinear, this indicates the presence of multiple N turnover pools and a multiple compartment model should be used to calculate turnover (Cerling et al. 2007, Martínez del Rio & Anderson-Sprecher 2008). The estimated final, steady state $\delta^{15}N$ (or δ_f) values were derived from the empirically determined TDF values (see Fig. 5) and $\delta^{15}N$ values of the labeled diet. The estimated δ_f values were applied to both the exponential model and the reaction progress variable model in order to estimate turnover.

Exponential model. The turnover time of ^{15}N in bulk tissue and amino acids was determined using a first order exponential fit to the isotopic data (Fry & Arnold 1982, Tieszen et al. 1983, Hobson & Clark 1992, Podlesak et al. 2005):

$$\delta_t = a e^{-\lambda t} + c \quad (3)$$

where δ_t is the isotope value at time t, a is the difference between isotopic values of the initial and final steady states (δ_{i-f}), c is the isotopic value of the final steady state (δ_f), and λ is a first order rate constant (Fry & Arnold 1982, Tieszen et al. 1983, Hobson & Clark 1992, Podlesak et al. 2005, Madigan et al. 2012). The values of a and c were constrained to within 0.1‰ of the expected δ_{i-f} and δ_f. The N half-life ($t_{0.5}$) and $t_{0.95}$ of the bulk tissue and individual amino acids were determined using λ from Eq. (3) (modified from Buchheister & Latour 2010, Madigan et al. 2012):

$$t_{0.5} = \ln(2)/\lambda \quad (4)$$

$$t_{\alpha/100} = \ln(1 - \alpha/100)/\lambda \quad (5)$$

where $t_{\alpha/100}$ is the amount of time needed to reach α% turnover, i.e. $t_{0.5}$ and $t_{0.95}$ indicate the amount of time necessary to achieve 50 and 95% turnover, respectively.

Growth. Relative growth (W_R) was used to estimate the increase in biomass of *L. vannamei* over the course of this experiment:

$$W_R = (W_t / W_i) \quad (6)$$

where W_t is the average weight of all individuals sampled at time t and W_i is the average initial weight of shrimp collected prior to the diet shift (Herzka & Holt 2000). The weights of shrimp sampled at a single time point were highly variable, and as a result, relative growth estimates were highly variable. In many cases, relative growth was calculated to be below 1.0, which would suggest that the shrimp were losing weight. Due to this variability, a linear regression (change in the average mass as a function of time) was used to estimate the change in shrimp weight and recalculate relative growth.

Trophic position (TP). TP was estimated using 3 equations as informed by amino acid turnover and

the TDF values of individual amino acids in *L. vannamei*. The first was proposed by Chikaraishi et al. (2009):

$$TP_{x/y} = [(\delta^{15}N_x - \delta^{15}N_y - \beta_{x/y})/(\Delta_x - \Delta_y)] + 1 \quad (7)$$

where $\delta^{15}N_x - \delta^{15}N_y$ represents the difference in the N isotopic composition of the trophic (x) and source (y) amino acids in the consumer, $\beta_{x/y}$ is the difference between the isotopic values of the source (y) and trophic (x) amino acids in the diet, $\Delta_x - \Delta_y$ indicates the difference in ^{15}N enrichment (or TDF) of the respective source (y) and trophic (x) amino acids (Chikaraishi et al. 2009). The TDF values, Δ_x and Δ_y, were derived as described in Eq. (1) from *L. vannamei* that were at a steady state with their diet and did not undergo a diet shift (see Fig. 5). The $\beta_{x/y}$ was determined based on the difference between the trophic (x) and source (y) amino acids in our pelleted diet. The $\beta_{x/y}$ value applied to the TP equations listed below was an average of those derived for both artificial diets. These $\beta_{x/y}$ values are unique to this study and not meant to be applied to a natural system. The $\beta_{x/y}$, Δ_x, and Δ_y values were data-derived and used to produce the following equations:

$$TP_{Glu/Phe} = [(\delta^{15}N_{Glu} - \delta^{15}N_{Phe} - 6.5‰)/(7.1‰ - 0.6‰)] + 1 \quad (8)$$

where $\delta^{15}N_{Glu} - \delta^{15}N_{Phe}$ represents the difference in the N isotopic values of glutamic acid and phenylalanine in *L. vannamei*. This equation is modified from Chikaraishi et al. (2009).

$$TP_{Pro/Gly} = [(\delta^{15}N_{Pro} - \delta^{15}N_{Gly} - 8.4‰)/(7.0 - 1.1‰)] + 1 \quad (9)$$

where $\delta^{15}N_{Pro} - \delta^{15}N_{Gly}$ represents the difference in the N isotopic ratios of proline and glycine in *L. vannamei*.

$$TP_{TII\text{-}S} = [(\text{Average } \delta^{15}N_{TII} - \text{Average } \delta^{15}N_S - \text{Average } \beta_{TII/S})/(\Delta_{TII} - \Delta_S)] + 1 \quad (10)$$

$$TP_{TII\text{-}S} = [(\text{Average } \delta^{15}N_{TII} - \text{Average } \delta^{15}N_S - 10.0‰)/(2.4‰)] + 1 \quad (11)$$

where Average $\delta^{15}N_{TII}$ – Average $\delta^{15}N_S$ is the difference between the average $\delta^{15}N$ values of multiple trophic (alanine, aspartic acid, glutamic acid) and multiple source amino acids (glycine, lysine, serine) (modified from Chikaraishi et al. 2009 and Hannides et al. 2009).

Statistical analysis and modeling. The application of the time-based exponential model and growth-based exponential models to the data was completed using MATLAB R2012a (2012, The MathWorks). Statistical analysis was performed using JMP 10.0 (2012, SAS Institute) A non-parametric Wilcoxon test (rank sums) was used to assess if there was a significant difference in turnover time between trophic and source amino acids.

RESULTS

Bulk (whole) tissue

On average, the shrimp grew to approximately 1.5 times their initial weight. However, the weights of individual shrimp were highly variable (Fig. 1). In the first 48 h, when we expected minimal growth, the average weight of shrimp sampled varied between 4.28 g (0.5 h) and 7.93 g (48 h). Trends in the weight of individual shrimp sampled from Tanks 1 and 2 were similar, where approximately half of the shrimp with the highest weight were sampled from each tank. A linear regression was used to determine more accurate initial and final estimates of weight and estimate average growth (Fig. 1). Based on the linear regression, we estimate that the juvenile *Litopenaeus vannamei* grew approximately 0.0017 g h^{-1} or 0.041 g d^{-1} over the course of this experiment.

Despite the high amount of variability in the weight of individual shrimp, bulk $\delta^{15}N$ values showed little variability and the trend in the bulk $\delta^{15}N$ values between the tanks was consistent (Fig. 2). The $\delta^{15}N$ values of bulk tail muscle rapidly increased over time immediately following the change in diet (Fig. 2). However at ~200 h, the rate of increase in bulk $\delta^{15}N$ slowed (Fig. 2). Average bulk $\delta^{15}N$ values of shrimp sampled at t_0 were ~2.3‰ higher than in the initial diet, indicating a bulk TDF of ~2.3‰ in *L. vannamei*. This is consistent with the ^{15}N enrichment previously observed in *L. vannamei* relative to its diet (C. M. Holl unpubl. data). Assuming a constant TDF, we expected the shrimp to reach a final isotopic steady state $\delta^{15}N$ value of ~26.3‰ when grown on the ^{15}N-labeled diet (Fig. 2). Under these experimental conditions, *L. vannamei* only reached a bulk $\delta^{15}N$ of 17.1‰ in 1152 h.

When the bulk $\delta^{15}N$ data were transformed and linearized using the reaction progress variable model, the data did not fall in a single linear trend. The reaction progress variable model indicated that more than one N turnover pool was present in the bulk tissue (Fig. 3). Consequently N turnover was estimated assuming 2 pools using the 2-compartment model described by Martínez del Rio & Anderson-Sprecher (2008) (see Eq. 2). The $t_{0.5}$ of the first pool is

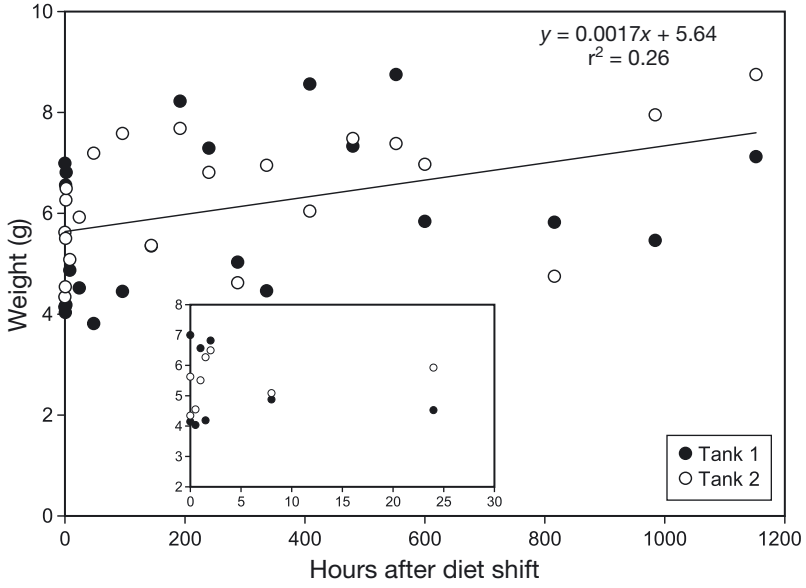

Fig. 1. Mean weight (g) of individual *Litopenaeus vannamei* over time (h) following the shift to the ^{15}N-enriched diet. The trend line represents the linear regression ($r^2 = 0.26$, $p < 0.05$). The graph inset is an enlargement of the first 24 h following the diet shift for detail

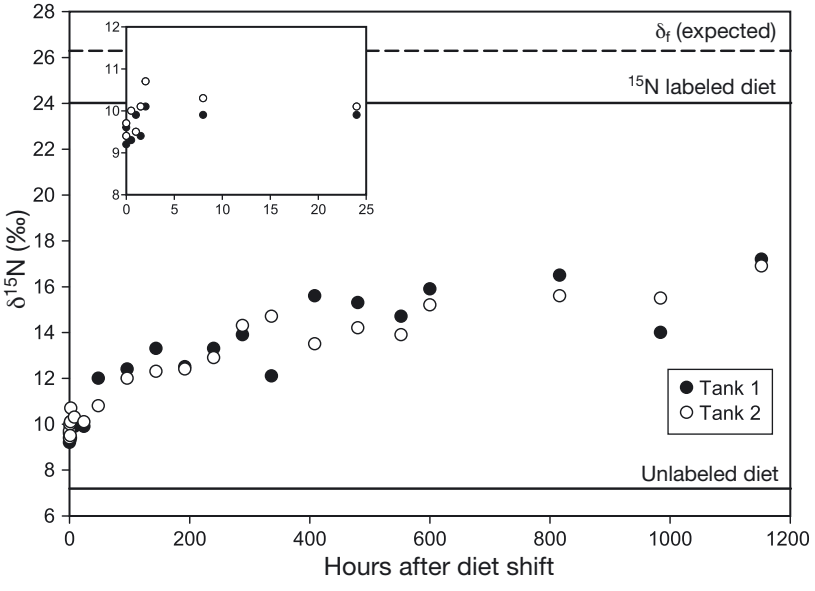

Fig. 2. Bulk δ^{15}N of *Litopenaeus vannamei* tail meat from individual shrimp over time (h) following the shift to a ^{15}N-enriched diet at t_0. Circles indicate individual shrimp sampled at each time point from (●) Tank 1 and (○) Tank 2. The graph inset is an enlargement of the first 24 h following the diet shift for detail. (– –) Expected final steady state (δ_f = 26.2‰); (—) bulk δ^{15}N values of the labeled and unlabeled diets (24.0‰ and 7.2‰, respectively)

approximately 39 h (95% CI = 22,146, $r^2 = 0.95$) and the $t_{0.5}$ (95% CI) of the second pool is approximately 1926 h (95% CI = 1685, 2248). As the bulk δ^{15}N values of the shrimp did not reach the expected isotopic steady state, we ran 2 versions of this model to confirm whether or not our assumed TDF of 2.3‰ was consistent with our data. In the first version of this model, we assumed a final steady state value of 26.3‰ and in the second version, we assumed a final steady state value of 17.1‰. The data was a good fit for both models with each model having an r^2 value greater than 0.9. In light of this fact and in combination with previous isotope work with *L. vannamei*, we believe that a final δ^{15}N value of 26‰ more accurately represents our data.

Amino acids

All amino acid isotopic results transformed using the reaction progress variable model fell along a single linear relationship, as exemplified for glutamic acid and phenylalanine (Fig. 4). There was insufficient evidence that any of the amino acids had more than one N turnover pool. As a result, we used a single compartment exponential model to determine the turnover of the amino acids.

The TDFs of individual amino acids were highly variable. The δ^{15}N values of trophic amino acids in the shrimp tissue were 1.9 to 7.1‰ higher than δ^{15}N values of the same amino acids in their diet (Fig. 5). The δ^{15}N values of source amino acids were similar to the δ^{15}N values of those same amino acids in the diet, except for methionine (TDF = 6.3‰) and threonine (TDF = –3.7‰). Of the trophic amino acids, glutamic acid and proline showed the greatest difference between the δ^{15}N values of the consumer and the food source. Of the source amino acids, δ^{15}N values of phenylalanine and lysine showed the least difference between the consumer and the food source. However, the δ^{15}N value of lysine was very difficult to determine and this compound was not present in more than half of the samples analyzed. Based on the conditions listed above, the following pairs of trophic and source amino acids seem most appropriate for estimating TP in *L. vannamei*: glutamic acid/glycine, glutamic acid/phenylalanine,

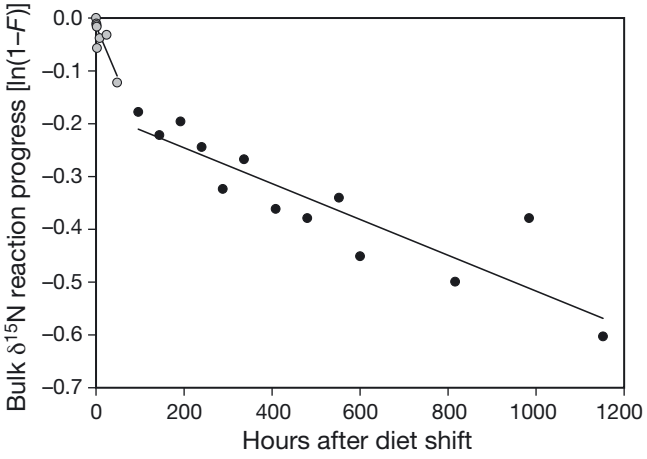

Fig. 3. Bulk $\delta^{15}N$ reaction progress [ln(1 – F)] (Eq. 2) over time (h) following diet shift. Values are indicative of (●) one turnover pool and (●) a second turnover pool

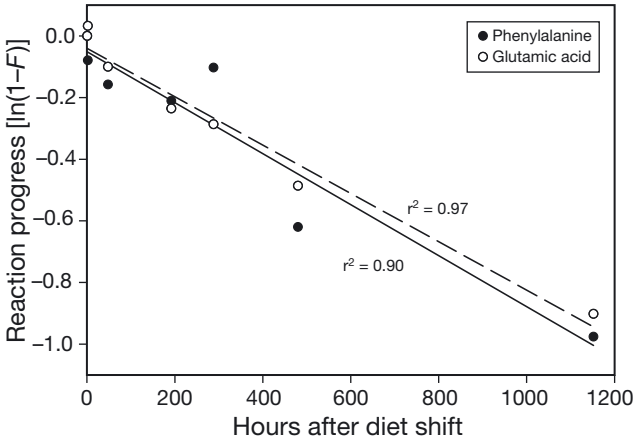

Fig. 4. Reaction progress [ln(1 – F)] of (○) glutamic acid and (●) phenylalanine over time (h) following diet shift in *Litopenaeus vannamei* from selected time points. (— —) Linear regression of the transformed glutamic acid isotopic data (r^2 = 0.97); (———) linear regression of the transformed phenylalanine isotopic data (r^2 = 0.90)

proline/glycine, and proline/phenylalanine. Since many of the amino acids did not reach steady state with the new diet, the TDF values of the individual amino acids determined using data from C. A. Otoshi et al. (unpubl.) (Fig. 5) were used to estimate the data-derived values a and c used in the first order exponential model.

Results of an exponential model fit to the amino acid isotopic data revealed a number of different trends concerning the turnover of amino acids (Table 1). Based on TDF and turnover time, glycine was the first and only amino acid to reach a new isotopic steady state. The amino acids with the most rapid turnover were glycine, a source amino acid ($t_{0.5}$ = 163 h, $t_{0.95}$ = 706 h), and proline, a trophic amino acid ($t_{0.5}$ = 369 h, $t_{0.95}$ = 1597 h). The amino acids with the slowest turnover were serine, a source amino acid ($t_{0.5}$ = 2280 h, $t_{0.95}$ = 9854 h), methionine, a source amino acid ($t_{0.5}$ = 2168 h, $t_{0.95}$ = 9370 h), and aspartic acid, a trophic amino acid ($t_{0.5}$ = 1530 h, $t_{0.95}$ = 6615 h). Phenylalanine ($t_{0.5}$ = 780 h, $t_{0.95}$ = 3371 h) turns over more rapidly than glutamic acid ($t_{0.5}$ = 940 h, $t_{0.95}$ = 4061 h). While there is some difference in the turnover times of glutamic acid and phenylalanine, this difference is not large when compared with the range in turnover rates of all other amino acids. Similar to the results of Bradley et al. (2014), who studied amino acid turnover in bluefin tuna, source and trophic amino acids in *L. vannamei* did not turnover at the same rates; there are no apparent

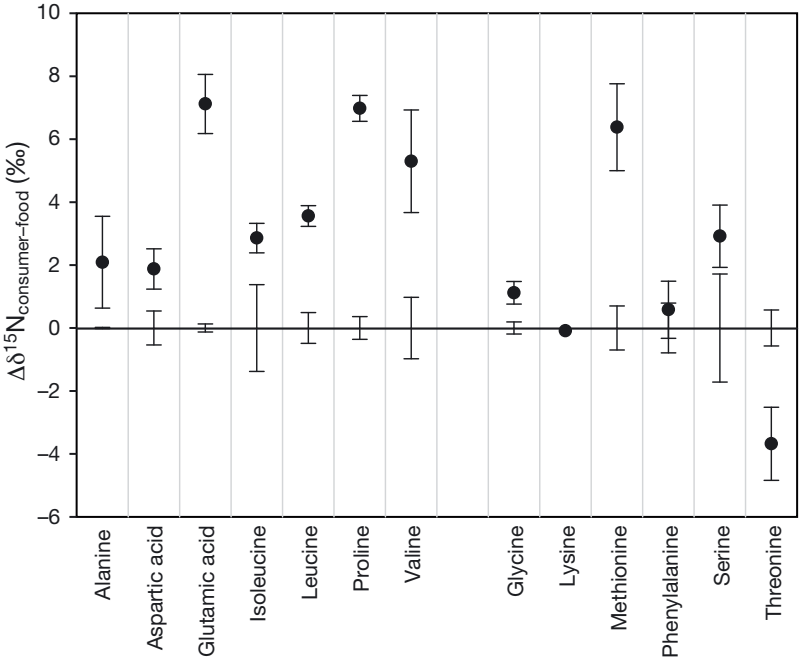

Fig. 5. Mean trophic discrimination factor (TDF) values of individual trophic (left) and source (right) amino acids calculated as $\Delta\delta^{15}N$ ($\delta^{15}N_{consumer} - \delta^{15}N_{food}$, ‰, tabulated from C. A. Otoshi et al. unpubl.). The black line indicates no difference between the $\delta^{15}N$ value of the consumer's tissue relative to its diet. The bars at each point indicate the SD of the $\delta^{15}N$ values of the consumer's tissue; the bars on the solid black line indicate the SD of the $\delta^{15}N$ values of the diet

Table 1. Rate constant (λ), and time needed (h) to achieve 50 and 95% N turnover ($t_{0.5}$ and $t_{0.95}$, respectively) based on a single compartment, first order exponential model (Eq. 2) applied to amino acids, including 95% confidence intervals

Amino acids	λ (95% CI)	r^2	$t_{0.5}$ (95% CI)	$t_{0.95}$ (95% CI)
Trophic amino acids				
Alanine	0.0011 (0.00047, 0.0017)	0.90	642 (411, 1467)	2774 (1775, 6338)
Aspartic acid	0.00045 (0.00014, 0.00076)	0.83	1530 (908, 4881)	6615 (3922, 21097)
Glutamic acid	0.00074 (0.00048, 0.0010)	0.95	940 (694, 1453)	4061 (3001, 6282)
Leucine	0.00077 (0.00032, 0.0012)	0.78	905 (572, 2163)	3910 (2472, 9350)
Proline	0.0019 (0.00022, 0.0035)	0.80	369 (196, 3151)	1597 (848, 13617)
Valine	0.00074 (0.00035, 0.0011)	0.84	942 (619, 1962)	4070 (2677, 8479)
Source amino acids				
Glycine	0.0042 (0.00069, 0.0078)	0.88	163 (89, 1004)	706 (384, 4339)
Lysine	0.00098 (0.000038, 0.0019)	0.58	706 (360, 18098)	3050 (1555, 78218)
Methionine	0.00032 (0.000073, 0.00057)	0.74	2168 (1223, 9562)	9370 (5284, 41326)
Phenylalanine	0.00089 (0.00027, 0.001509)	0.85	780 (459, 2576)	3371 (1985,11132)
Serine	0.00030 (0.00020, 0.00040)	0.95	2280 (1714, 3404)	9854 (7408, 1418)

general trends in turnover between trophic and source amino acids (Table 1) ($p > 0.05$). There are several pairs of source and trophic amino acids that turn over at similar rates including alanine/lysine, glutamic acid/phenylalanine, proline/glycine, and valine/phenylalanine.

Trophic position estimates

Based on both the TDF values and turnover times of the individual amino acids, glutamic acid/phenylalanine and proline/glycine appear to be most appropriate pairs of trophic and source amino acids for estimating the TP of *L. vannamei*. TP was also estimated using the multiple amino acid equation proposed by Hannides et al. (2009). As this was a controled laboratory experiment, and there were no other dietary N inputs, the 'TP' of the diet was necessarily 1, and as a result, we expected the shrimp to have a TP of 2 relative to that of the experimental diet.

Using the measured β values for the artificial diet and the TDF values calculated from the $\delta^{15}N$ values obtained by C. A. Otoshi et al. (unpubl.) (Table 2), the TP estimates using glutamic acid/phenylalanine were the closest to the expected TP of 2 (1.82 ± 0.17, mean ± SD, n = 7). TP estimates using multiple amino acids were considerably lower than the expected TP and showed the greatest variability over time. TP estimates produced using proline/glycine were lower than expected and showed the least variability over time. It is important to note that the TP estimates obtained using all 3 calculations remained fairly constant over the entire course of the diet shift experiment and showed little variability.

Table 2. Mean trophic position (TP) (±SD) and TP at individual time points following the diet shift estimating using glutamic acid and phenylalanine ($TP_{Glu/Phe}$), proline and glycine ($TP_{Pro/Gly}$), and multiple amino acids (TP_{TII-S}). $\beta_{x/y}$ is the average difference (‰) between the isotopic values of the trophic (x) and source (y) amino acids in the artificial diets; trophic discrimination factor (TDF) is the average difference (‰) between the $\delta^{15}N$ values of the *Litopenaeus vannamei* tissue and their diet at a steady state. $\beta_{x/y}$ and TDF values included in this table were used to calculate TP

Time (h)	$TP_{Glu/Phe}$ [a]	$TP_{Pro/Gly}$ [b]	Multiple amino acids (TP_{TII-S}) [c]
0.5	1.87	1.57	1.22
2	1.65	1.54	0.71
48	1.76	1.63	0.80
192	1.89	1.64	0.98
288	2.16	1.64	1.05
480	1.68	1.44	1.30
1152	1.75	1.94	1.72
Mean (±SD)	1.82 ± 0.17	1.63 ± 0.16	1.11 ± 0.34
$\beta_{x/y}$	6.5	8.4	10.0
TDF	6.5	5.9	2.4

[a]Chikaraishi et al. (2009)
[b]Modified from Popp et al. (2007)
[c]Hannides et al. (2009)

DISCUSSION

Effect of growth on nitrogen turnover

Growth and metabolism are the 2 major processes that contribute to the isotopic ratio of tissues in a given organism (Buchheister & Latour 2010, Madigan et al. 2012). The estimated average growth of

shrimp was much slower than expected (0.00017 g h^{-1} or 0.04 g d^{-1}). *Litopenaeus vannamei* grown in outdoor raceways at the Oceanic Institute typically grow at a rate of 0.15 g d^{-1} (Otoshi et al. 2007), approximately 4 times faster than the *L. vannamei* cultured for this experiment. The shrimp in outdoor raceways were grown at higher temperatures (26 to 31°C in outdoor raceways versus 20 to 22°C in indoor tanks), which affects both growth and metabolism in *L. vannamei* (Wyban et al. 1995). Furthermore, *L. vannamei* cultured in raceways have access to the microbial community, including algae, and fecal matter, which have been shown to act as supplemental sources of food including N (Otoshi et al. 2001). In order to constrain the sources of dietary N to only the pelleted diet, the shrimp in this experiment were kept in indoor tanks in filtered flow-through seawater. This allowed us to limit the sources of dietary N, but the cooler temperatures slowed the growth rate of the shrimp. Unfortunately, our results do not allow us to evaluate any potential effects of faster growth on amino acid turnover under warmer temperatures or with supplemental nutrition. Despite slow growth, ^{15}N from the labeled diet was observed in as little as 2 h in the tail muscle tissue and constituent amino acids.

TDF of bulk tissue and constituent amino acids

The ^{15}N enrichment of bulk tissue of *L. vannamei* relative to their diet in our experiments (2.2‰) was less than the average 3.0‰ but well within the range of −0.5 to +9.2‰ observed by Deniro & Epstein (1981) and equal to that observed in previous studies with *L. vannamei* (C. A. Otoshi et al. unpubl.). The TDF values of the individual amino acids were variable yet largely consistent with those observed in the literature. However, there were several notable exceptions. McClelland & Montoya (2002) and Chikaraishi et al. (2009) both reported a large difference between the $\delta^{15}N$ values of aspartic acid and alanine in the tissue and the diet, but in *L. vannamei*, the $\delta^{15}N$ values of these amino acids showed little difference between the consumer and diet (TDF of alanine = 2.1‰, TDF of aspartic acid = 1.9‰). Interestingly, the TDF values of alanine and aspartic acid were more consistent with TDF values observed in source amino acids. In contrast, there was an approximate 6‰ difference between the $\delta^{15}N$ values of methionine in *L. vannamei* tissue and its diet. We have no reason to believe *L. vannamei* would incorporate N differently than previously studied animals, but it is important to note that some of the discrepancies observed in the TDF values may be attributed to the fact that these *L. vannamei* were not living in a natural system and, in particular, were consuming an artificial, pelleted diet. This pelleted diet includes ingredients that *L. vannamei* and other penaeid shrimp would not consume in nature, such as corn meal. Despite variations in other amino acids, the TDF values of glutamic acid, glycine, phenylalanine, and proline in *L. vannamei* were highly consistent with values observed in previous studies (McClelland & Montoya 2002, Chikaraishi et al. 2009).

Amino acid turnover

Results from this study indicate that the turnover time of all amino acids was highly variable, that source and trophic amino acids did not turn over at the same rate, and that there was no trend between turnover time and an amino acid's classification as source or trophic. Similar observations have been made in amino acid turnover in bluefin tuna (Bradley et al. 2014). However, several pairs of trophic and source amino acids in *L. vannamei* had similar turnover times. Although the turnover time of phenylalanine was different from that of glutamic acid, when compared to the range of turnover rates of all amino acids, this difference was relatively small. This finding supports previous studies that have proposed the use of glutamic acid and phenylalanine to estimate TP (McClelland & Montoya 2002, McClelland et al. 2003, Chikaraishi et al. 2007, 2009, Hannides et al. 2009, Lorrain et al. 2009, Dale et al. 2011, Choy et al. 2012, Seminoff et al. 2012).

The variable turnover rates of individual amino acids may act as a biological clock (e.g. Fry 2006, Phillips & Eldridge 2006) with certain amino acids reaching a new isotopic steady state more rapidly than others during a natural diet shift, thus allowing a greater understanding of the processes that affect N incorporation into amino acids. Further work is necessary to determine if the turnover rates and trends observed in amino acids of *L. vannamei* are consistent with those observed in other animals.

Estimating trophic position

By determining both the TDF values and the turnover rates of the amino acids in *L. vannamei*, we have assessed which amino acids would be the most

appropriate for accurately estimating TP. While several pairs of trophic and source amino acids had similar turnover times in our study, only 2 pairs of trophic and source amino acids also showed little variability: proline/glycine and glutamic acid/phenylalanine. Glutamic acid and proline had the least variable TDF values and showed the most agreement with previous studies (McClelland & Montoya 2002, Chikaraishi et al. 2009). Glycine and phenylalanine had TDF values closest to zero, making them suitable amino acids to represent the isotopic composition of N at the base of the food web.

We estimated the TP of *L. vannamei* throughout the course of the diet shift using 3 separate calculations that have been previously proposed for other organisms. TP of *L. vannamei* were estimated using glutamic acid and phenylalanine (Chikaraishi et al. 2009, Seminoff et al. 2012), proline and glycine (modified from Popp et al. 2007) and the mean $\delta^{15}N$ values of multiple amino acids (alanine, aspartic acid, glutamic acid, glycine, lysine, and serine) (Hannides et al. 2009). Glutamic acid and phenylalanine produced TP estimates that were closest to our expected TP of 2 (1.82 ± 0.17, mean $TP_{Glu/Phe}$ ± SD, n = 7). This is consistent with work by McClelland & Montoya (2002) and Chikaraishi et al. (2009), which suggested that glutamic acid and phenylalanine was the best pair of amino acids for estimating TP. While all 3 calculations varied little over the course of the diet shift, only one, glutamic acid and phenylalanine, produced accurate TP estimates. TP estimates using proline and glycine produced a TP lower than our expected TP of 2 (1.63 ± 0.16, mean $TP_{Pro/Gly}$ ± SD, n = 7), but it showed the lowest amount of variability of all 3 methods. The inaccuracy of the TP estimates using proline and glycine may be due to the difference in the $\beta_{x/y}$ values of the labeled and unlabeled diet. There was little difference in the $\beta_{x/y}$ values for glutamic acid and phenylalanine ($\beta_{x/y}$ Labeled Diet = 6.21, $\beta_{x/y}$ Unlabeled Diet = 6.77) whereas there was a ~3‰ difference in the $\beta_{x/y}$ values for proline and glycine ($\beta_{x/y}$ Labeled Diet = 6.95, $\beta_{x/y}$ Unlabeled Diet = 9.84). Such differences are rarely found in algae at the base of marine food webs (e.g. Chikaraishi et al. 2009, McCarthy et al. 2013). Our calculations assumed that the $\beta_{x/y}$ of each of these diets contributed equally to the isotopic composition of the tissue, and therefore may have contributed to our TP estimates using proline and glycine being lower than expected. TP estimates using multiple amino acids were too low (1.11 ± 0.34, mean TP_{TII-S} ± SD, n = 7) and suggest that the TP of *L. vannamei* was an entire TP lower than their expected TP of 2. The inaccuracy of the TP estimates using multiple amino acids can largely be attributed to the disparate turnover rates of the amino acids. The highly variable turnover rates of some of the amino acids suggests that multiple amino acid models may only be appropriate for estimating the TP of organisms that are at an isotopic steady state, and are, therefore, inappropriate for organisms that may have undergone a diet shift.

CONCLUSIONS

Diet shift experiments in the laboratory are an important complement to isotope studies performed on wild caught samples. In this study, juvenile *Litopenaeus vannamei* underwent a controlled change from a diet with a low $\delta^{15}N$ value to a diet relatively enriched in ^{15}N by 16.8‰. An exponential model and reaction progress variable model were used to quantify N incorporation, determine the N isotope turnover rate in amino acids, and to determine if there were multiple N turnover pools present in either the bulk tissues or the amino acids. We determined that glutamic acid and phenylalanine turned over at similar rates, which supports the hypothesis that the relationship between these trophic and source amino acids in field-caught crustaceans could be used to accurately estimate TP even if the organisms were undergoing a diet shift. In short, laboratory studies such as this allow us to determine the rate at which ^{15}N is incorporated in bulk tissue and the constituent amino acids and, therefore, calculate the turnover rate of those amino acids; an important 'unknown' to date. Our TDF and turnover rate results confirm that glutamic acid and phenylalanine are the most appropriate pair of trophic and source amino acids for estimating TP, at least in *L. vannamei*. Finally, our results substantiate the use of this new isotopic technique with field-caught samples whose diet and natural history is unknown and allows us to accurately place them in complicated and shifting marine food webs.

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