

# Consequences of employing amino acid vs. bulk-tissue, stable isotope analysis: a laboratory trophic position experiment

R. E. BOWES<sup>1,2</sup> AND J. H. THORP<sup>1,2,3,†</sup>

<sup>1</sup>Kansas Biological Survey, Lawrence, Kansas 66047 USA

<sup>2</sup>Department of Ecology and Evolutionary Biology, University of Kansas, Lawrence, Kansas 66047 USA

**Citation:** Bowes, R. E., and J. H. Thorp. Consequences of employing amino acid vs bulk-tissue, stable isotope analysis: a laboratory trophic position experiment. *Ecosphere* 6(1):14. <http://dx.doi.org/10.1890/ES14-00423.1>

**Abstract.** An important metric of environmental health is food web structure because it reflects species richness, natural history diversity, and resource availability. While bulk-tissue stable isotope analysis has proven valuable for food web studies, field conditions may severely restrict its use and data can be quite variable. Amino acid stable isotope analysis potentially reduces this variability, in part by eliminating the need for signatures near the trophic base because a single top consumer contains both the primary producer signature (constant phenylalanine signature) and information reflecting number of trophic transfers (a progressively increasing  $\delta^{15}\text{N}$  signature of glutamic acid).

To evaluate the ecological sensitivity and cost/benefits of the techniques, we conducted a laboratory food chain experiment with four trophic levels. Water fleas (*Daphnia magna*) were cultured on a diet of powdered algae and then fed daily to guppies (*Poecilia reticulata*) for three months. These invertivorous fishes were then consumed by piscivorous bluegill sunfishes (*Lepomis macrochirus*) for a subsequent three months. All members of the food web were analyzed for  $^{15}\text{N}$  values and degree of fractionation using both bulk-tissue and amino acid stable isotope techniques.

Our experiment demonstrated that the amino acid technique more accurately identified the true trophic position (TP) and food chain length (FCL = maximum TP) with significantly less variability around mean values for each consumer trophic level. Moreover, use of amino acids requires significantly fewer replicates to identify TP. We discuss here the relative advantages and disadvantages of both approaches for determining TP and FCL and recommend that investigators switch as soon as possible to the amino acid isotope technique for determining FCL.

**Key words:** compound specific stable isotope analysis; food chain length; fractionation; nitrogen.

**Received** 8 November 2014; **accepted** 17 November 2014; **published** 27 January 2015. Corresponding Editor: D. P. C. Peters.

**Copyright:** © 2015 Bowes and Thorp. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited. <http://creativecommons.org/licenses/by/3.0/>

<sup>3</sup> Present address: Kansas Biological Survey, University of Kansas, 2101 Constant Avenue, Lawrence, KS 66047-3759 USA.

† **E-mail:** [thorp@ku.edu](mailto:thorp@ku.edu)

## INTRODUCTION

Trophic position (TP) and food chain length (FCL = maximum TP) have been assessed historically by several approaches, including behavioral observations, gut content analysis,

and chemical means. While all three approaches can contribute to a better understanding of aquatic food web relationships, behavioral approaches are not feasible in the field for most aquatic invertebrates and many fish. Moreover, gut contents can be difficult to identify and

count, they do not necessarily equate to assimilation, and they primarily indicate only what was ingested in the last 24 hr. Determining what organisms have assimilated over longer time scales requires chemical analyses, such as fatty acid/lipid analysis (Zelles 1999, Ruess et al. 2004, Haubert et al. 2011) and stable isotope analysis (Gannes et al. 1997, 1998, McClelland and Montoya 2002, Post 2002, Fry 2006, Popp et al. 2007, Crawford et al. 2008, Martinez del Rio et al. 2009, Chikaraishi et al. 2009, Jardine et al. 2013, Steffan et al. 2013, Bradley et al. 2014). A major advantage of fatty acid/lipid analysis is that the investigator can often distinguish between the consumption of closely related food sources (Ruess et al. 2004, 2005, Chamberlain and Black 2005). However, the field component is challenging especially in remote areas, the laboratory methods are complex, analysis still requires information on the signature of autotrophs or basal herbivores, and the analytical costs are high. In contrast, bulk-tissue stable isotope analysis is easier to use and cheaper in many food web studies, but it cannot distinguish as well among closely related food sources.

Stable isotope analysis using bulk-tissue techniques has been a widely employed and valuable technique for analyzing TP, FCL, and other food web metrics (Gannes et al. 1997, Post 2002, Fry 2006, Crawford et al. 2008, Layman and Post 2008, Martinez del Rio et al. 2009). For example, the ratio of heavy-to-light nitrogen ( $^{15}\text{N}/^{14}\text{N}$ ) in tissues in comparison to an atmospheric nitrogen standard ( $=\delta^{15}\text{N}$ ) can be used to estimate trophic position because the value of  $\delta^{15}\text{N}$  generally increases progressively up the food chain. This results because there is a tendency for selective retention of heavier isotopes and loss of lighter isotopes during physicochemical processes such as excretion, respiration, deamination, and transamination (Macko et al. 1986, 1987, Metges et al. 1996, Miura and Goto 2012). The  $\delta^{15}\text{N}$  of a consumer has historically been considered to increase or become enriched by 3–4‰ relative to its diet (Deniro and Epstein 1981), although some studies have indicated that 1.5‰ might be a more appropriate average fractionation level (Bunn et al. 2003, Hadwen and Bunn 2005). However, the use of bulk-tissue analysis has potential limitations in aquatic systems. Field conditions often restrict its use because: (1) autotrophic sources

may be unknown; (2) it is difficult to obtain clean epilithic and epiphytic algae (uncontaminated with other food items, including host vascular tissue) or suspended algae uncontaminated with dead organic matter (but see colloidal silica separation techniques in Hamilton et al. [1992]); and (3) algal signatures are often highly variable in time and space (Hamilton et al. 1992, Hayes 1993, Herman et al. 2000, Hadwen et al. 2010, Woodland et al. 2012, Jardine et al. 2013). In an attempt to circumvent this problem, some ecologists have used the isotopic signatures of primary (herbivorous) consumers. However, limited availability of these in some areas can pose significant problems (e.g., O'Reilly et al. 2002, Hamilton et al. 2005, Jardine et al. 2006, Wolf et al. 2009), and one never knows how representative that herbivore's diet is to the basal autotrophic source of the higher consumer. Because of this variability, investigators need to rely on large sample sizes of basal organisms and consumers to obtain a reasonable mean value.

A potential solution is to replace bulk-tissue analysis with amino acid compound specific isotope analysis (AA-CSIA). The analytical advantage is that the focal consumer contains information on both the basal signature of the primary producer and number of trophic transfers, thereby eliminating the need for separate signatures from a primary producer. This approach works because  $^{15}\text{N}$  isotopic signatures of some amino acids (e.g., glutamic acid) change substantially between trophic levels while others (e.g., phenylalanine) essentially remain the same (McClelland and Montoya 2002). By analyzing  $\delta^{15}\text{N}$  in both glutamic acid and phenylalanine, the algal signature and number of trophic transfers (e.g., Chikaraishi et al. 2007, Popp et al. 2007, Hannides et al. 2009) are revealed. While the cost of AA-CSIA is currently much higher, due to the complexity of the analytical methods and the paucity of labs performing these analyses, this disadvantage is lessened by elimination of autotroph/basal herbivore samples and by potentially fewer samples needed at upper consumer levels.

To evaluate the relative advantages and disadvantages of these two stable isotope methods for determining TP, we first conducted a laboratory food chain experiment with four trophic levels (autotroph, herbivore, invertivore, and pisci-

vore). Our null hypotheses were that bulk-tissue stable isotope analysis would not differ from amino acid analysis in calculated TP, variability around mean TP values, consistency of trophic fractionation, and number of replicates required for accurate prediction of TP values. Based on our results, we analyzed when and where the two techniques should be used and discussed other advantages and disadvantages of the two approaches.

## METHODS

### Laboratory feeding experiment

We maintained all test organisms in an environmentally controlled laboratory at 21°C on a 12 h light/12 h dark cycle. Water fleas (*Daphnia magna* (Straus, 1820)) were cultured in aerated, 13-L plastic containers and fed suspended, powdered Vegetable Calcium Flakes (Worldwide Aquatics, Arvin, CA, USA; see bestflake.com) twice weekly to apparent satiation. Guppies (*Poecilia reticulata* (Peters, 1859)) were raised through multiple generations in the lab and fed *Daphnia* daily for three months prior to being fed to a piscivorous fish (bluegill sunfish). Bluegills (*Lepomis macrochirus* (Rafinesque, 1819)) were kept individually in 95-L containers and fed one guppy per day for three months to allow for approximately complete turnover of the isotope signatures in their muscle tissues (Madigan et al. 2012). All guppies and sunfish added body weight during the experiment. This gain in mass indicates that they were replacing N isotopes during the experimental period and not starving, the latter of which would have increased their  $\delta^{15}\text{N}$  values (e.g., Bowes et al. 2014).

### Preparation for stable isotope analysis

All invertebrate and fish samples were washed with distilled water to remove contaminants, such as algal debris. *Daphnia* were then analyzed whole, whereas fish were dissected to isolate the muscle tissue. The tissue samples were then dried in an oven at 60°C for 48 h, ground to a fine, homogenized powder using a Wig-L-Bug Mixer/Amalgamator (Rinn/Crescent Dental Mfg., Elgin, IL, USA), and held in desiccators until submitted for analysis.

### Bulk-tissue stable isotope analysis

We evaluated the nitrogen isotopic composition of bulk-tissue (BT-isotope ratios) for the flake food, *Daphnia*, and muscle tissue of guppies and sunfish on half of our samples, but report here only the values for N. For this analysis we employed a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon, Cheshire, UK) at the University of California Davis (UC-Davis) Stable Isotope Facility. BT-isotope data for each sample included total N and  $\delta^{15}\text{N}$  values. The  $\delta^{15}\text{N}$  values were determined from the relative difference in isotopic ratio between the samples and known standards as represented by the following equation:  $\delta X = ((R_{\text{sample}}/R_{\text{standard}}) - 1) \times 1000$ , where X is  $^{15}\text{N}$ , and the corresponding ratio is  $R = ^{15}\text{N}/^{14}\text{N}$ . Atmospheric nitrogen was used as the N standard. All isotope ratios are given in per mil (‰).

### Amino acid stable isotope analysis

After drying, powdering, and homogenizing our samples, they were then analyzed for AA-isotope ratios at the UC-Davis Stable Isotope Facility. The general techniques for AA-isotope analysis are summarized below and described in greater detail in Walsh et al. (2014). Sample preparation involves acid hydrolysis for the liberation of amino acids from proteins and derivatization by methyl chloroformate to produce compounds amenable to GC analysis. Amino acid derivatives are injected in split ( $^{13}\text{C}$ ) or splitless ( $^{15}\text{N}$ ) mode and separated on an Agilent J&W factor FOUR VF-23ms column (30 m  $\times$  0.25 mm ID, 0.25 micron film thickness). Once separated, amino acid derivatives are quantitatively converted to  $\text{CO}_2$  and  $\text{NO}_x$  in an oxidation reactor at 950°C, and  $\text{NO}_x$  are subsequently reduced to  $\text{N}_2$  in a reduction reactor at 650°C. Following water removal through a nafion dryer,  $\text{N}_2$  or  $\text{CO}_2$  enters the IRMS. A pure reference gas ( $\text{CO}_2$  or  $\text{N}_2$ ) is used to calculate provisional  $\delta$ -values of each sample peak. Next, isotopic values are adjusted to an internal standard (e.g., norleucine) of known isotopic composition. Final  $\delta$ -values are obtained after adjusting the provisional values for changes in linearity and instrumental drift such that correct  $\delta$ -values for laboratory standards are obtained. The  $\delta^{15}\text{N}$  of two amino acids (glutamic acid and

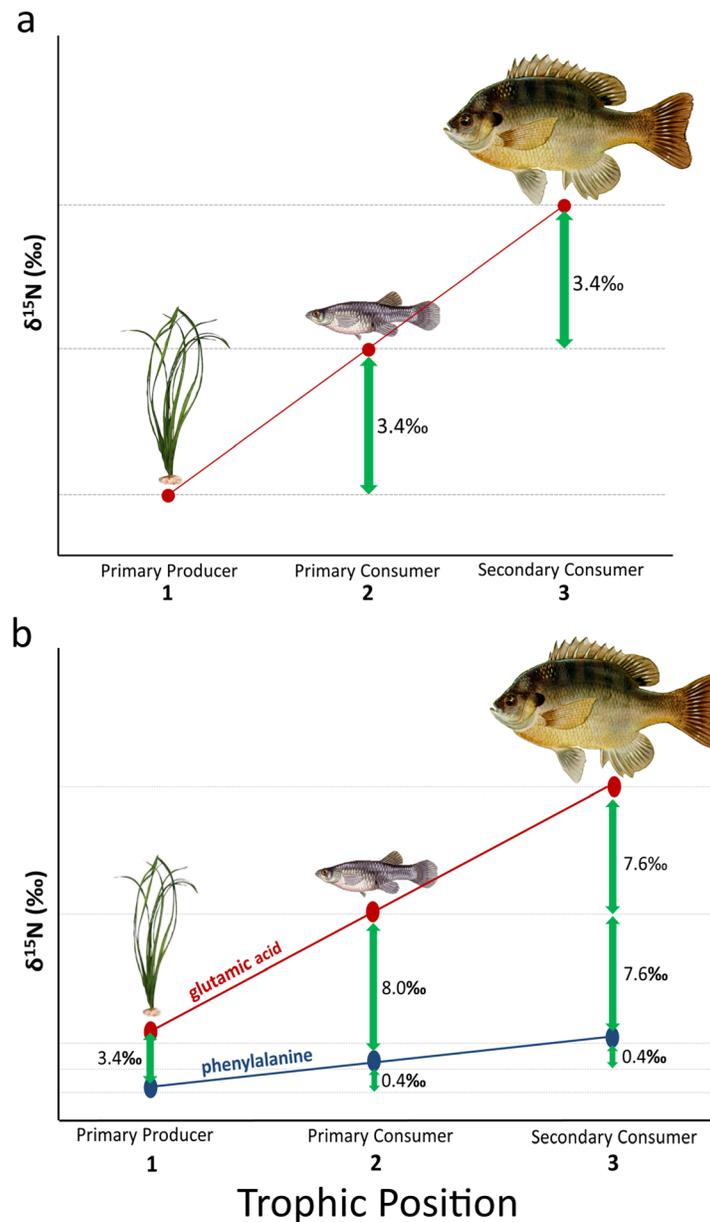


Fig. 1. (a) Trophic position is calculated from bulk-tissue stable isotope analysis using the equation:  $TP = [(\delta^{15}\text{N}_{\text{consumer}} - \delta^{15}\text{N}_{\text{producer}}) \div 3.4] + 1$ . (b) Trophic position using amino acid compound specific isotope analysis is calculated using the equation:  $TP = [((\delta^{15}\text{N}_{\text{glutamic acid}} - \delta^{15}\text{N}_{\text{phenylalanine}}) - 3.4) \div 7.6] + 1$ .

phenylalanine) were determined by this method.

#### Trophic position calculation

To calculate trophic position from B-T analyses, we used the following formula: Trophic Position =  $[(\delta^{15}\text{N}_{\text{consumer}} - \delta^{15}\text{N}_{\text{producer}}) \div 3.4] + 1$ , whereas for amino acid analyses, we em-

ployed the following modified formula: Trophic Position =  $[((\delta^{15}\text{N}_{\text{glutamic acid}} - \delta^{15}\text{N}_{\text{phenylalanine}}) - 3.4) \div 7.6] + 1$ . These two equations theoretically generate equivalent trophic position values, as illustrated in Fig. 1. We employed the historically and widely used value of 3.4‰ as the denominator value in our bulk tissue trophic

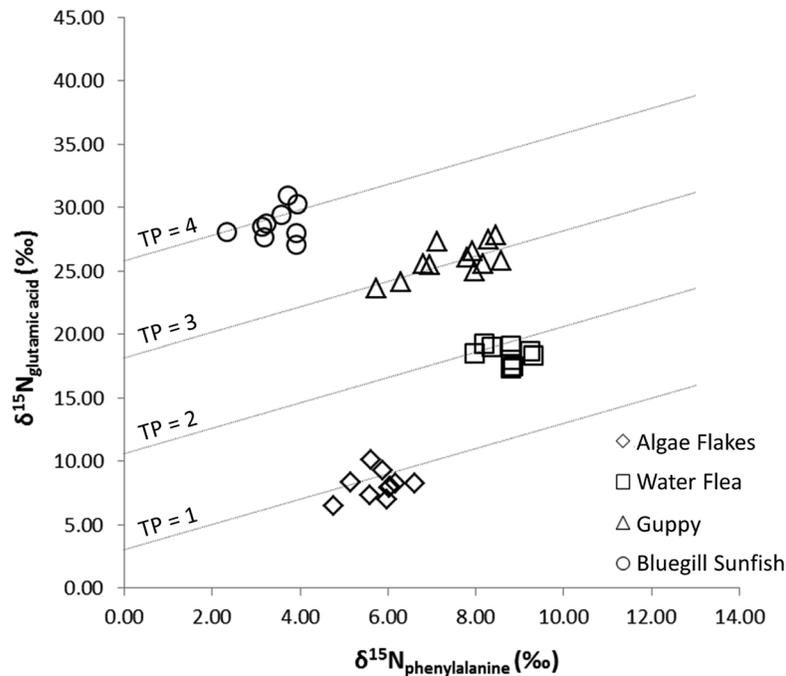


Fig. 2. Cross plot for  $\delta^{15}\text{N}$  values of glutamic acid and phenylalanine. Trophic isoclines are created for each trophic position using the equation  $\text{TP} = [(\delta^{15}\text{N}_{\text{glutamic acid}} - \delta^{15}\text{N}_{\text{phenylalanine}}) - 3.4] \div 7.6 + 1$ , each with a slope of 1.0 and between-line interval of 7.6.

position estimation equation, because it yielded more accurate estimates of the known trophic position. Furthermore, it was much more accurate in our calculations than the 1.5‰ value sometimes recommended (Bunn et al. 2003, Hadwen and Bunn 2005).

#### Statistical analyses

Differences between bulk-tissue and amino acid stable isotope estimates of mean TPs and FCL were tested using one-way ANOVA. The variances associated with TP values generated by each isotope technique were compared with an F-test and a Levene Test. Linear regressions were run for both methods comparing actual and projected TP values, with an accurate estimation having a high  $R^2$ , a y-intercept near 0, and a slope of  $\sim 1$ . A power and sample size analysis was performed to find the fewest required replicates each technique required for a 95% confidence level in a trophic position calculation. The pooled standard deviation generated from the original one-way ANOVA of each technique was used as the assumed standard deviation in

the power analysis for each technique (0.4906 and 0.1364 for bulk tissue and amino acid, respectively). In both cases  $\alpha = 0.05$  was used along with 4 trophic positions, with a maximum difference between trophic positions equal to 1. All data were checked for normality, unusual values, and heterogeneity of variances. All statistics were performed in Minitab 14 statistical software (Minitab, State College, PA, USA) with  $\alpha = 0.05$ .

#### RESULTS

Differences in the distribution and variability of individual amino acid analyses are evident in Fig. 2, in the form of a trophic isocline graph. Trophic isoclines define the trophic position of a food web in two-dimensional space (Chikaraishi et al. 2014). One of the advantages of this graphical method is that isotopic variability is readily perceptible (evident in the  $\delta^{15}\text{N}$  values of phenylalanine along the horizontal axis). No matter what the  $\delta^{15}\text{N}$  values of phenylalanine in an organism, the  $\delta^{15}\text{N}$  value of glutamic acid

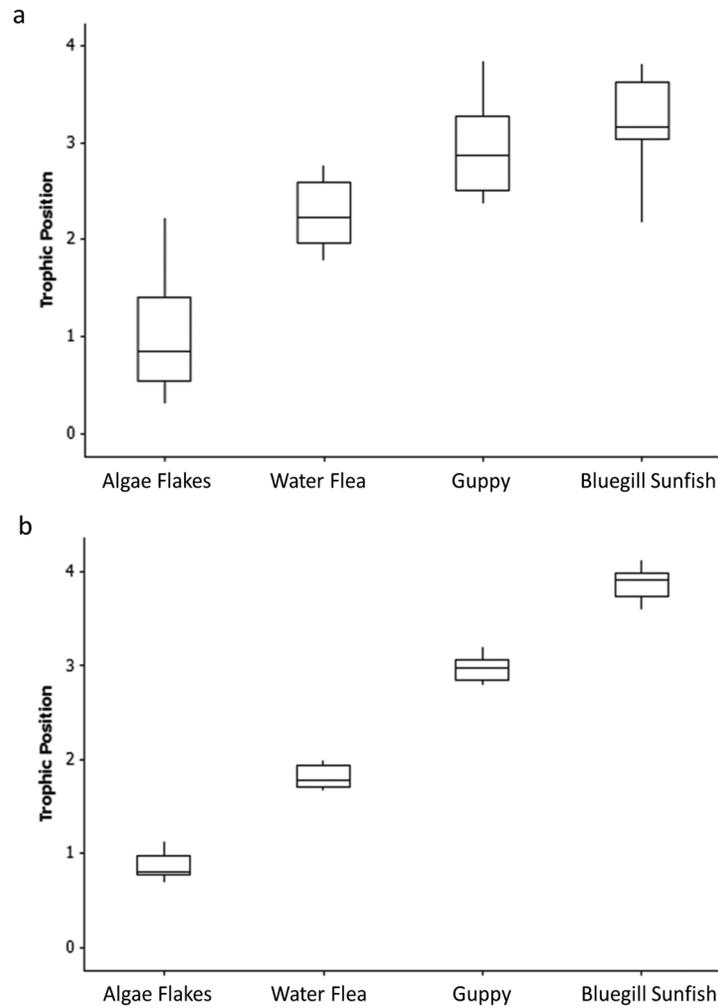


Fig. 3. Interquartile range box plots of calculated trophic position of each feeding group using (a) bulk-tissue stable isotope analysis; and (b) amino acid compound specific isotope analysis.

will reflect the organism's TP. When the  $TP_{Glu/Phe}$  values of organisms are displayed across trophoclines, it becomes apparent how populations simultaneously vary in trophic position and background heterogeneity of  $\delta^{15}N$  values (Chikaraishi et al. 2009, 2014, Steffan et al. 2013). The  $\delta^{15}N$  values of phenylalanine in a single consumer closely reflect the average of all the resources it assimilated (e.g., Chikaraishi et al. 2009, 2014, Steffan et al. 2013). This becomes important, in that the graphical representation of data points in space could reveal linear food chains within broader food web structure. All consumer species that fall within a range of  $\delta^{15}N$  values for phenylalanine may effectively be using

similar basal resources and fit into a distinct particular food chain, whereas a wide range of the  $\delta^{15}N$  values of phenylalanine could indicate that the consumer is a generalist that can exploit resources from multiple areas or communities. Fig. 2 clearly shows that the food source and three consumer species in our experiment closely aligned on their respective and distinct trophic isoclines. Our results also show a narrow range in phenylalanine values, which in the field could indicate that the organisms were all using the same basal carbon resource.

Although both isotope techniques generated estimates of TP and FCL, their mean values were significantly different (Figs. 3 and 4). Moreover,

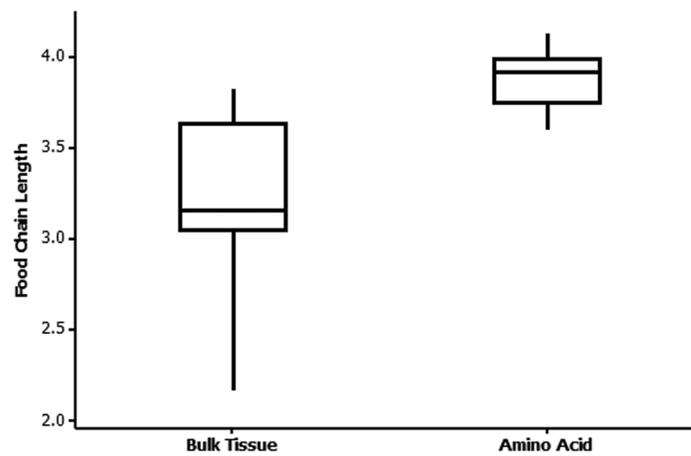


Fig. 4. Interquartile range box plot of the estimation of food chain length using the two techniques. There is a significant difference between the two techniques' calculation of food chain length ( $F_{1,16} = 16.15$ ,  $P = 0.001$ ). Amino acid analysis' estimation of food chain length is closer to the actual known food chain length of 4 for this laboratory experiment.

the variance in the bulk-tissue analysis estimations of trophic position was significantly greater than that generated by amino acid analysis (Fig. 3; Table 1).

Although both techniques revealed significant differences between all trophic positions, their estimates were not equivalent. Regression analysis of TP calculated by bulk-tissue analysis compared to the expected trophic positions ( $=eTP$ ; i.e., 1, 2, 3, and 4) produced the equation:  $TP_{bt} = 0.514 + 0.742 eTP$ , with an  $R^2 = 70.0\%$ . In contrast, the same regression analysis using amino acid analysis techniques yielded the equation:  $TP_{aa} = -0.173 + 1.02 eTP$ , with an  $R^2 = 98.5\%$ . AA-CSIA was, therefore, more accurate because it produced estimates with a higher  $R^2$  value, a y-intercept nearer to 0, and a slope of approximately 1. AA-CSIA technique also produced a more accurate estimate of FCL (Fig. 4;  $F_{1,16} = 16.15$ ,  $P = 0.001$ ).

Statistical power analyses revealed that more samples need to be analyzed using the bulk-tissue technique compared to the amino acid isotope analysis in order to achieve 95% confidence in TP. Based on the pooled standard deviation found for each technique (Table 2; bulk-tissue = 0.4906, amino acid = 0.1364), bulk-tissue analysis required a sample size of 10 (target = 95% power, actual = 96.5%) and amino acid analysis required only two samples (target = 95% power, actual = 95.9%).

## DISCUSSION

### *Ecological and metabolic comparisons of both methods*

Analysis of bulk-tissue stable isotopes is firmly established in the literature as a useful tool for exploring factors controlling food web complexity in both terrestrial and aquatic ecosystems.

Table 1. Test for equal variances between bulk-tissue and amino acid stable isotope analysis within each feeding group.

| Organism         | Sample size | F test         |       | Levene's test  |       |
|------------------|-------------|----------------|-------|----------------|-------|
|                  |             | Test statistic | P     | Test statistic | P     |
| Algae flakes     | 10          | 0.05           | 0.000 | 5.15           | 0.036 |
| Water flea       | 9           | 0.12           | 0.007 | 10.06          | 0.006 |
| Guppy            | 12          | 0.07           | 0.000 | 9.98           | 0.005 |
| Bluegill sunfish | 9           | 0.11           | 0.005 | 2.85           | 0.111 |

Table 2. Trophic position estimates for each feeding group using both bulk-tissue stable isotope analysis and amino acid compound specific isotope analysis. There were significant differences between all trophic positions using both techniques.

| Analysis and feeding group | Mean   | SD     | ANOVA      |       |        |           |
|----------------------------|--------|--------|------------|-------|--------|-----------|
|                            |        |        | $F_{3,36}$ | $P$   | $R^2$  | Pooled SD |
| Bulk-tissue analysis       |        |        | 40.33      | 0.000 | 77.07% | 0.4906    |
| Algae flakes               | 1.0000 | 0.5957 |            |       |        |           |
| Water flea                 | 2.2844 | 0.3555 |            |       |        |           |
| Guppy                      | 2.9550 | 0.4843 |            |       |        |           |
| Bluegill sunfish           | 3.9890 | 0.4849 |            |       |        |           |
| Amino acid analysis        |        |        | 904.68     | 0.000 | 98.69% | 0.1364    |
| Algae flakes               | 0.8542 | 0.1373 |            |       |        |           |
| Water flea                 | 1.8292 | 0.1234 |            |       |        |           |
| Guppy                      | 2.9691 | 0.1253 |            |       |        |           |
| Bluegill sunfish           | 3.8830 | 0.1603 |            |       |        |           |

Laboratory analytical techniques and mathematical procedures for determining trophic position (TP) and food chain length (FCL) are well accepted (Post 2002, Fry 2006, Crawford et al. 2008, Martinez del Rio et al. 2009). This is also a relatively inexpensive technique (\$8–12 as of August 2014) and can be analyzed at many laboratories around the world fairly rapidly on both an absolute time scale and relative to the current time required for amino acid techniques. For these and other reasons, we are “not” advocating total abandonment of the BT-isotope method at this time. Nonetheless, we maintain that bulk-derived TP estimates can be profoundly inaccurate in comparison to AA-CSIA (e.g., Steffan et al. 2013) and most research questions are best tested using compound-specific stable isotope techniques, as discussed below.

Interpretation of bulk-tissue isotope data is limited by ecological conditions and metabolic processes. From an ecological perspective, both  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  can vary spatially and temporally in even pristine ecosystems—a problem which seems especially acute in rivers. Variations in flow within small to large rivers can alter  $\delta^{13}\text{C}$  ratios laterally over distances of a few meters in some cases and vertically within millimeters in benthic algal layers (Hamilton et al. 1992, Hayes 1993, Herman et al. 2000, Hadwen et al. 2010, Woodland et al. 2012, Jardine et al. 2013). Likewise,  $\delta^{15}\text{N}$  ratios can vary from areas in the main channel with its mostly continuous flows to lateral backwater areas with minimal to zero flows, as both sources and N-processing pathways change (e.g., variable abundance of denitrifying bacteria) (Thorp et al. 2008). This poses

significant problems for the bulk-tissue technique if, as is almost certainly the case, the predator (invertivore to top predator) is either highly mobile relative to its prey or the basal signature used in calculating TP (autotrophs or herbivore) was determined from collections made at a different time or place from the predator. For example, ecologists typically collect grazing snails (feeding on benthic algae) and mussels (feeding on suspended algae) by hand from areas of small spatial extent but fish from large spatial extents by seining, trawling, or electroshocking from boats. If the investigator instead collects algae, the problem is magnified because the algal signature changes orders of magnitude faster than the predator’s signature and over very short distances. If the investigator instead opts to use the signature from basal herbivores to circumvent this problem, he/she must first have access to longer-lived herbivores (which are difficult to find in some ecosystems) and then collect both benthic and suspension feeding herbivores to get representative ecosystem autotrophic signatures. To gain a very long perspective on changes in food webs, one can use museum samples of fish (e.g., Delong et al. 2011). The problem here, however, is that: (1) long-term algal collections are typically unavailable; (2) you can often use the external periostracum of suspension-feeding unionid mussels for a protein signature (Delong and Thorp 2009), but gaining enough periostracum from grazing snails is more problematic and museum collections of gastropods are often not as complete as for mussels; and (3) the sampled fish and herbivores were almost certainly collected by different investigators in different places

and at different times (weeks to years).

These ecological problems are eliminated with AA-CSIA because the predator contains both the original basal nitrogen signature (from phenylalanine) and the trophically magnified nitrogen signatures (from glutamic acid).

From a metabolic perspective, the bulk-tissue technique suffers from at least two handicaps. First, the diet-to-tissue discrimination factors differ in both  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  among tissues in the same organism (e.g., blood, muscle, and bone) and are sometimes very large (Polito et al. 2009, Wyatt et al. 2010, Madigan et al. 2012, Xia et al. 2013). For this reason, researchers must be consistent in choice of tissues and recognize that the isotope replacement time in a consumer from a change in diet will vary with tissue type (Sakano et al. 2005, Miller 2006, Madigan et al. 2012). While dietary change time also needs to be considered when using amino acid isotope techniques, the isotopic relationship between glutamic acid and phenylalanine stays constant in different tissues of the consumer (Chikaraishi et al. 2007, 2009). Second, the degree to which  $^{15}\text{N}$  biomagnifies along trophic chains is poorly known for many ecological situations, and isotope signatures can fluctuate with variations in nutrient allocation within an organism, nutritional stress and body condition, and seasonal and temperature changes (Cherel et al. 2007, Kempster et al. 2007, Bowes et al. 2014). These changes will differ for each step in the food chain depending on the nutritional state of the consumer in each trophic level. As demonstrated in our laboratory experiment, the accumulated effects of metabolic variations among organisms as a whole and within tissues of a single organism will impair the investigator's ability to determine an accurate mean TP when using the number of replicates (3–5) typically employed in previous field studies. While nutritional condition affects the  $\delta^{15}\text{N}$  ratio in individual amino acids, the effects are comparable among similar amino acids, and thus the nitrogen relationship between glutamic acid and phenylalanine is unchanged.

#### *Statistical comparisons*

Our laboratory experiment showed that the amino acid stable isotope technique was a substantial improvement over the bulk-tissue

technique based on statistically significant differences in the calculations of TP and FCL. In comparison to the bulk-tissue calculations, the amino acid stable isotope technique more accurately calculated TP values for each trophic level from herbivore through piscivore. Moreover, our analyses of power and sample size (using the pooled standard deviation for each technique) found that to obtain 95% confidence in calculations of TP and FCL, an investigator using the bulk-tissue technique would require data from at least 10 consumers, whereas someone using the amino acid technique would need only 2 (but we suggest a minimum of 3 to obtain a statistical mean).

#### *Alternative choices*

If financial costs and laboratory time were not an issue is selection of isotope technique, the clear choice of analytical method would be AA-CSIA. As described above, it provides more accurate and precise estimates of trophic position and food chain length with fewer required sample replicates. Furthermore, the methods and time to process tissue samples in the field are the same with the two methods, but the overall time in the field is reduced with the amino acid method because you need to collect only the target consumers (and fewer of those) rather than those consumers plus either autotrophs or basal herbivores (assuming they are even easily available).

The main disadvantages of AA-CSIA at this time are: (1) the laboratory analytical methods are complex and the methods employed may not yet be consistent among laboratories; (2) because of this complexity, processing time and analysis turnaround is longer than the simpler and more traditional methods used in bulk-tissue analysis; (3) fewer isotope laboratories around the world offer such analyses, thereby increasing analytical time; and (4) the cost of the analysis is much greater than bulk tissue analysis. In the latter instance, the best of a wide range of analytical prices we found in the USA in late 2013 was \$65 plus shipping for one isotope and \$97 for two (based on complete chemical processing/analysis of weighed and dried tissue). However, each analysis includes values for 12 or more individual amino acids.

While a cursory look at the prices cited above

might lead one to automatically choose the bulk-tissue approach in trophic position studies if cost are a major concern, the choice is in fact more complex for financial as well as scientific reasons. In predicting expenditures, the investigator needs to account for the greater field costs (personnel, equipment, and time) and analytical fees from collecting basal organism signatures and extra consumer replicates. This assumes also that the autotrophs or substitute herbivores are available and representative of what ultimately ends up in the tissue of the higher consumers. If the study's focus is on food sources and/or food web complexity, then the bulk-tissue method requires collection of a large number of potential terrestrial and aquatic sources of whole, particulate, and dissolved organic sources from local and areas upstream areas. The cost for this does not rise on a per sample basis in B-T analysis because most labs provide isotope values for both C and N. In contrast, the costs rise by about 50% when analyzing two isotopes with AA-isotope procedures. However, the output from AA-isotope analysis includes a dozen or more amino acids, thereby allowing the investigator to more precisely identify food sources.

Based on our experimental results, we recommend that investigators switch as soon as they can from the bulk-tissue to the amino acid stable isotope technique to gain a much more accurate analysis of food chain length. This recommendation is consistent with recent conclusions of other scientists (Gannes et al. 1997, Martinez del Rio et al. 2009, Wolf et al. 2009). Although the relatively greater accuracy for evaluating food sources of carbon AA-isotope techniques over BT-isotopes is still waiting sufficient experimental confirmation, we strongly suspect that this technique will also prove superior to traditional approaches.

## ACKNOWLEDGMENTS

Research activities were supported by an NSF grant to J. H. Thorp (DEB 1249370). We appreciate the periodic assistance of the following students in organism care and laboratory work: M. Holliday Lafferty, Logan Luce, and Valerie Paul. Animals were collected, maintained, and sacrificed following the University of Kansas Animal Care Protocol 193-05 (IACUC).

## LITERATURE CITED

- Bowes, R. E., M. H. Lafferty, and J. H. Thorp. 2014. Less means more: nutrient stress leads to higher  $\delta^{15}\text{N}$  ratios in fish. *Freshwater Biology* 59:1926–1931.
- Bradley, C. J., D. J. Madigan, B. A. Block, and B. N. Popp. 2014. Amino acid isotope incorporation and enrichment factors in Pacific Bluefin Tuna, *Thunnus orientalis*. *PLoS ONE* 9:e85818.
- Bunn, S. E., P. M. Davies, and M. Winning. 2003. Sources of organic carbon supporting the food web of an arid zone floodplain river. *Freshwater Biology* 48:619–635.
- Chamberlain, P. M., and H. I. J. Black. 2005. Fatty acid composition of Collembola: unusually high proportions of  $\text{C}_{20}$  polyunsaturated fatty acids in a terrestrial invertebrate. *Comparative Biochemistry and Physiology* 140:299–307.
- Cherel, Y., K. A. Hobson, C. Guinet, and C. Vanpe. 2007. Stable isotopes document seasonal changes in trophic niches and winter foraging individual specialization in diving predators from the Southern Ocean. *Journal of Animal Ecology* 76:826–836.
- Chikaraishi, Y., Y. Kashiyama, N. O. Ogawa, H. Kitazato, and N. Ohkouchi. 2007. Metabolic control of nitrogen isotope composition of amino acids in macroalgae and gastropods: implications for aquatic food web studies. *Marine Ecology Progress Series* 342:85–90.
- Chikaraishi, Y., N. O. Ogawa, Y. Kashiyama, Y. Takano, H. Suga, A. Tomitani, H. Miyashita, H. Kitazato, and N. Ohkouchi. 2009. Determination of aquatic food-web structure based on compound-specific nitrogen isotopic composition of amino acids. *Limnology and Oceanography: Methods* 7:740–750.
- Chikaraishi, Y., S. A. Steffan, N. O. Ogawa, N. F. Ishikawa, Y. Sasaki, M. Tsuchiya, and N. Ohkouchi. 2014. High-resolution food webs based on nitrogen isotopic composition of amino acids. *Ecology and Evolution* 4:2423–2449.
- Crawford, K., R. A. McDonald, and S. Bearhop. 2008. Applications of stable isotope techniques to the ecology of mammals. *Mammal Review* 38:87–107.
- Delong, M. D., and J. H. Thorp. 2009. Mollusc shell periostracum as an alternative to tissue in isotopic studies. *Limnology and Oceanography: Methods* 7:436–441.
- Delong, M. D., J. H. Thorp, M. C. Thoms, and L. M. McIntosh. 2011. Trophic niche dimensions of fish communities as a function of historical hydrological conditions in a plains river. *River Systems* 19:177–187.
- Deniro, M. J., and S. Epstein. 1981. Influence of diet on the distribution of nitrogen isotopes in animals. *Geochimica et Cosmochimica Acta* 45:341–351.
- Fry, B. 2006. *Stable isotope ecology*. Springer, New

- York, New York, USA.
- Gannes, L. Z., D. M. Ó'Brien, and C. M. Rio del. 1997. Stable isotopes in animal ecology: assumptions, caveats, and a call for more laboratory experiments. *Ecology* 78:1271–1276.
- Gannes, L. Z., C. M. Rio del, and P. Koch. 1998. Natural abundance variations in stable isotopes and their potential uses in animal physiological ecology. *Comparative Biochemistry and Physiology* 119:725–737.
- Hadwen, W. L., and S. E. Bunn. 2005. Food web responses to low-level nutrient and  $^{15}\text{N}$ -tracer additions in the littoral zone of an oligotrophic dune lake. *Limnology and Oceanography* 50:1096–1105.
- Hadwen, W. L., M. Spears, and M. J. Kennard. 2010. Temporal variability of benthic algal  $\delta^{13}\text{C}$  signatures influences assessments of carbon flows in stream food webs. *Hydrobiologia* 651:239–251.
- Hamilton, S. K., and W. M. Lewis, Jr. 1992. Stable carbon and nitrogen isotopes in algae and detritus from the Orinoco River floodplain, Venezuela. *Geochimica et Cosmochimica Acta* 56:4237–4246.
- Hamilton, S. K., S. J. Sippel, and S. E. Bunn. 2005. Separation of algae from detritus for stable isotope or ecological stoichiometry studies using density fractionation in colloidal silica. *Limnology and Oceanography: Methods* 3:149–157.
- Hamilton, S. K., W. M. Lewis, and S. J. Sippel. 1992. Energy sources for aquatic animals in the Orinoco River floodplain: evidence from stable isotopes. *Oecologia* 89:324–30.
- Hannides, C. C. S., B. N. Popp, M. R. Landry, and B. S. Graham. 2009. Quantification of zooplankton trophic position in the North Pacific Subtropical Gyre using stable nitrogen isotopes. *Limnology and Oceanography* 54:50–61.
- Haubert, D., M. M. Pollierer, and S. Scheu. 2011. Fatty acid patterns as biomarkers for trophic interactions: changes after dietary switch and starvation. *Soil Biology and Biogeochemistry* 43:490–494.
- Hayes, J. M. 1993. Factors controlling  $^{13}\text{C}$  contents of sedimentary organic compounds: Principles and evidence. *Marine Geology* 113:111–125.
- Herman, P. M., J. J. Middelburg, J. Widdows, C. M. Lucas, and C. H. Heip. 2000. Stable isotopes' as trophic tracers: combining field sampling and manipulative labelling of food resources for macrobenthos. *Marine Ecology Progress Series* 204:79–92.
- Jardine, T. O., K. A. Kidd, and A. T. Fisk. 2006. Applications, considerations, and sources of uncertainty when using stable isotope analysis in ecotoxicology. *Environmental Science and Technology* 40:7501–7511.
- Jardine, T. D., R. J. Hunt, S. J. Faggotter, D. Valdez, M. A. Burford, and S. E. Bunn. 2013. Carbon from periphyton supports fish biomass in waterholes of a wet-dry tropical river. *River Research and Applications* 29:560–573.
- Kempster, B., L. Zanette, F. J. Longstaffe, S. A. MacDougall-Shackleton, J. C. Wingfield, and M. Clinchy. 2007. Do stable isotopes reflect nutritional stress? Results from a laboratory experiment in song sparrows. *Oecologia* 151:365–371.
- Layman, C. A., and D. M. Post. 2008. Can stable Isotope ratios provide for community wide measures of trophic structure? Reply. *Ecology* 89:2358–2359.
- Macko, S. A., M. L. F. Estep, M. H. Engel, and P. E. Hare. 1986. Kinetic fractionation of stable nitrogen isotopes during amino acid transamination. *Geochimica et Cosmochimica Acta* 50:2143–2146.
- Macko, S. A., M. L. Fogel, P. E. Hare, and T. C. Hoering. 1987. Isotopic fractionation of nitrogen and carbon in the synthesis of amino acids by microorganisms. *Chemical Geology: Isotope Geoscience* 65:79–92.
- Madigan, D. J., S. Y. Litvin, B. N. Popp, A. B. Carlisle, C. J. Farwell, and B. A. Block. 2012. Tissue turnover rates and isotopic trophic discrimination factors in the endothermic teleost, Pacific bluefin tuna (*Thunnus orientalis*). *PLoS ONE* 7:e49220.
- Martinez del Rio, C. M., N. Wolf, S. A. Carleton, and L. Z. Gannes. 2009. Isotopic ecology ten years after a call for more laboratory experiments. *Biological Reviews* 84:91–111.
- McClelland, J. W., and J. P. Montoya. 2002. Trophic relationships and the nitrogen isotopic composition of amino acids in plankton. *Ecology* 83:2173–2180.
- Metges, C. C., K. J. Petzke, and U. Henning. 1996. Gas chromatography-combustion-isotope ratio mass spectrometric comparison of n-acetyl- and n-pivaloyl amino esters to measure  $^{15}\text{N}$  isotopic abundances in physiological samples: a pilot study on amino acid synthesis in the upper gastrointestinal tract of minipigs. *Journal of Mass Spectrometry* 31:367–376.
- Miller, T. W. 2006. Tissue-specific response of  $\delta^{15}\text{N}$  in adult Pacific herring (*Clupea pallasii*) following an isotopic shift in diet. *Environmental Biology of Fishes* 76:177–189.
- Miura, K., and A. S. Goto. 2012. Stable nitrogen isotopic fractionation associated with transamination of glutamic acid to aspartic acid: implications for understanding  $^{15}\text{N}$  trophic enrichment in ecological food webs. *Researches in Organic Geochemistry* 28:13–17.
- O'Reilly, C. M., R. E. Hecky, A. S. Cohen, and P. D. Plisnier. 2002. Interpreting stable isotopes in food webs: recognizing the role of time averaging at different trophic levels. *Limnology and Oceanography* 47:306–309.
- Polito, M. J., S. Fisher, C. R. Tobias, and S. D. Emslie. 2009. Tissue-specific isotopic discrimination factors

- in gentoo penguin (*Pygoscelis papua*) egg components: implications for dietary reconstruction using stable isotopes. *Journal of Experimental Marine Biology and Ecology* 372:106–112.
- Popp, B. N., B. S. Graham, R. J. Olson, C. Hannides, M. J. Lott, G. A. López-Ibarra, F. Galván-Magaña, and B. Fry. 2007. Insight into the trophic ecology of yellowfin tuna, *Thunnus albacares*, from compound-specific nitrogen isotope analysis of proteinaceous amino acids. *Terrestrial Ecology* 1:173–190.
- Post, D. M. 2002. Using stable isotopes to estimate trophic position: models, methods, and assumptions. *Ecology* 83:703–718.
- Ruess, L., M. M. Häggblom, R. Langel, and S. Scheu. 2004. Nitrogen isotope ratios and fatty acid composition as indicators of animal diets in belowground systems. *Oecologia* 139:336–346.
- Ruess, L., K. Schütz, D. Haubert, M. M. Häggblom, E. Kandeler, and S. Scheu. 2005. Application of lipid analysis to understand trophic interactions in soil. *Ecology* 86:2075–2082.
- Sakano, H., E. Fujiwara, S. Nohara, and H. Ueda. 2005. Estimation of nitrogen stable isotope turnover rate of *Oncorhynchus nerka*. *Environmental Biology of Fishes* 72:13–18.
- Steffan, S. A., Y. Chikaraishi, D. R. Horton, N. Ohkouchi, M. E. Singleton, E. Miliczky, D. B. Hogg, and V. P. Jones. 2013. Trophic hierarchies illuminated via amino acid isotopic analysis. *PLoS ONE* 8:e76152.
- Thorp, J. H., M. C. Thoms, and M. D. Delong. 2008. *The riverine ecosystem synthesis: toward conceptual cohesiveness in river science*. Academic Press, Elsevier, New York, New York, USA.
- Walsh, R. G., S. He, and C. T. Yarnes. 2014. Compound-specific  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  analysis of amino acids: a rapid, chloroformate-based method for ecological studies. *Rapid Communications in Mass Spectrometry* 28:96–108.
- Wolf, N., S. A. Carleton, and C. M. Martinez del Rio. 2009. Ten years of experimental animal ecology. *Functional Ecology* 23:17–26.
- Woodland, R. J., P. Magnan, H. Glemet, M. A. Rodriguez, and G. Cabana. 2012. Variability and directionality of temporal changes in  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  of aquatic invertebrate primary consumers. *Oecologia* 169:199–209.
- Wyatt, A. S., A. M. Waite, and S. Humphries. 2010. Variability in isotope discrimination factors in coral reef fishes: implications for diet and food web reconstruction. *PLoS ONE* 5:e13682.
- Xia, B., Q. Gao, H. Li, S. Dong, and F. Wang. 2013. Turnover and fractionation of nitrogen stable isotope in tissues of grass carp *Ctenopharyngodon idellus*. *Aquaculture Environment Interactions* 3:177–186.
- Zelles, L. 1999. Fatty acid patterns of phospholipids and lipopolysaccharides in the characterization of microbial communities in soil: a review. *Biology and Fertility of Soils* 29:111–129.