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Comparing compound-specific and bulk stable nitrogen isotope trophic discrimination factors across multiple freshwater fish species and diets

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Abstract: Compound-specific nitrogen stable isotope analysis provides an approach for estimating animal trophic position that may overcome key issues associated with stable isotope analysis of bulk tissue. Yet compound-specific trophic discrimination factors have not been estimated for a broad range of habitats, taxa, and diets. We conducted a controlled-feeding experiment to characterize the variation in compound-specific (TDF_{AA}) and bulk (TDF_{Bulk}) trophic discrimination factors of four freshwater fish species fed on three distinct diets. We also compared TDF_{AA} of fish muscle and scale to evaluate the viability of scales for making food web inferences. Mean ± 1 SD TDF_{Bulk} was 2.2‰ ± 0.9‰, and there were significant effects of species and diet trophic position on TDF_{Bulk}. Mean ± 1 SD TDF_{AA} was 6.9‰ ± 0.8‰. Although there was no effect of species on TDF_{AA}, there were significant differences in TDF_{AA} across the three diets. TDF_{AA} from fish scales were not significantly different from those of muscle. Our study illustrates the advantages of estimating trophic position using compound-specific stable isotopes and the need for continued investigation of factors resulting in variation in TDF values.

Résumé : L'analyse des isotopes stables d'azote de composés précis fournit une approche pour estimer la position trophique des animaux qui pourrait permettre de surmonter certains problèmes clés associés à l'analyse des isotopes stables de tissus en vrac. Cependant, les facteurs de discrimination trophique de composés précis n'ont pas été estimés pour un vaste éventail d'habitats, de taxons et de régimes alimentaires. Nous avons mené une expérience d'alimentation contrôlée afin de caractériser les variations des facteurs de discrimination trophique pour des composés précis (TDF_{AA}) et des échantillons en vrac (TDF_{Bulk}) pour quatre espèces de poissons d'eau douce nourries selon trois régimes alimentaires distincts. Nous avons également comparé les TDF_{AA} de muscles et d'écailles de poisson afin d'évaluer l'utilité des écailles pour faire des inférences concernant le réseau trophique. Le TDF_{Bulk} moyen ± 1 ET était de 2,2 ‰ ± 0,9 ‰, et il y avait des effets significatifs de l'espèce et de la position trophique du régime alimentaire sur les valeurs de TDF_{Bulk}. Le TDF_{AA} moyen ± 1 ET était de 6,9 ‰ ± 0,8 ‰. S'il n'y avait aucun effet de l'espèce sur les valeurs de TDF_{AA}, des variations significatives des TDF_{AA} entre les trois régimes alimentaires étaient présentes. Les TDF_{AA} des écailles de poisson n'étaient pas significativement différents de ceux des muscles. L'étude illustre les avantages d'estimer la position trophique à partir d'isotopes stables de composés précis et la nécessite de poursuivre l'étude des facteurs de variation des TDF. [Traduit par la Rédaction]

Introduction

The estimation of animal trophic position using stable nitrogen isotope analysis is an important approach in food web ecology (Cabana and Rasmussen 1996; Vander Zanden and Rasmussen 1999; Post 2002). The method has illuminated trophic relationships in countless organisms and ecosystems, informing natural resource preservation and management (Fry 2007). However, there are uncertainties associated with stable nitrogen isotope analysis that can lead to errors when estimating trophic position of organisms in the wild (Vander Zanden and Rasmussen 2001; Martínez del Rio et al. 2009). One source of uncertainty comes from difficulties associated with defining the isotopic baseline or the nitrogen isotope ratio ($\delta^{15}N$) of the system's primary producers or primary consumers (Vander Zanden and Rasmussen 1999; Post 2002). Another source of uncertainty associated with this approach is variation in trophic discrimination factors (TDF) — the change in $\delta^{15}N$ from prey to consumer ($\Delta^{15}N$), which is the basis for using stable nitrogen isotopes to estimate consumer trophic position (DeNiro and Epstein 1981; Minagawa and Wada 1984; Martínez del Rio et al. 2009). The following equation is commonly used to estimate trophic position using stable nitrogen isotopes of bulk tissue with a primary consumer baseline (Cabana and Rasmussen 1996):

$$(1) \qquad TP_{Bulk} = \frac{\delta^{15}N_{Consumer} - \delta^{15}N_{Prey}}{TDF_{Bulk}} + 2$$

Compound-specific stable isotope analysis of amino acids is a new approach for estimating animal trophic position, which may address uncertainties associated with traditional bulk tissue

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methods (Chikaraishi et al. 2009). The key advantage is that some amino acids have consistent and relatively large increases in isotopic values from resource to consumer ("trophic" amino acids), and others exhibit little to no change ("source" amino acids; McClelland and Montoya 2002; Chikaraishi et al. 2007; McCarthy et al. 2007; Popp et al. 2007). Because source amino acids exhibit little enrichment between resource and consumer, they can be used to establish an isotopic baseline for the study organism without having to rely on external isotopic baseline indicator taxa. Furthermore, the TDF between trophic and source amino acids is larger and less variable than bulk tissue trophic discrimination, making estimates less sensitive to error (Chikaraishi et al. 2009).

The following equation is used to determine an organism's trophic position using glutamic acid and phenylalanine as trophic and source amino acids, respectively (Chikaraishi et al. 2009):

$$(2) \qquad TP_{AA} = \frac{\delta^{15}N_{Glu} - \delta^{15}N_{Phe} - \beta}{TDF_{AA}} + 1$$

where TDF_{AA} is the glutamic acid – phenylalanine trophic discrimination factor, calculated as the difference between glutamic acid and phenylalanine trophic discrimination from resource to consumer, and β is the difference in $\delta^{15}N$ between glutamic acid and phenylalanine in primary producers. Chikaraishi et al. (2009) proposed TDF = 7.6‰ ± 1.3‰ and β = 3.4‰ ± 0.9‰ (for aquatic primary producers) based on controlled feeding studies of algae, zooplankton, and fish. This approach has been shown to result in accurate estimates of trophic position where the study organisms' diets are known (Steffan et al. 2013, 2015).

While stable isotope analysis of amino acids shows great promise, studies are needed to examine the consistency of TDF_{AA} across a range of taxa and diets (Choy et al. 2012). The method has been used to examine the trophic position of freshwater fishes in the wild (Ogawa et al. 2013; Ohkouchi et al. 2015) and has been shown to provide more accurate trophic position estimates than the bulk approach in a laboratory freshwater food chain experiment (Bowes and Thorp 2015). However, no laboratory studies have estimated TDF_{AA} for freshwater taxa. The majority of studies applying this approach have focused on marine organisms or terrestrial invertebrates. Some recent laboratory studies of marine vertebrates suggest that TDF_{AA} decreases with trophic position (Bradley et al. 2014; Hoen et al. 2014; McMahon et al. 2015), while others report no such effect for terrestrial and aquatic consumers (Chikaraishi et al. 2015; Steffan et al. 2013, 2015). Building on studies showing this trend, Hussey et al. (2014, 2015) showed a framework in which scaling of TDF_{Bulk} values based on dietary $\delta^{15}N$ values produced trophic position estimates in line with expected trophic relationships. Recent studies have attributed variation in TDF_{AA} with diet trophic position to the biochemical composition of the diet (Chikaraishi et al. 2015; McMahon et al. 2015). There is a clear need for controlled laboratory studies evaluating TDF_{AA} values across diets and taxa as well as the biochemical factors that may contribute to variation.

We sought to address this critical gap by conducting a laboratory experiment to characterize variation in TDF_{AA} and TDF_{Bulk} for several freshwater fish species fed exclusively on homogeneous, isotopically characterized, and distinct diets. Previous feeding experiments have typically been confined to a single diet or study organism. Our fully crossed factorial design allowed assessment of both the main and interactive effects of diet and taxa on trophic discrimination for compound-specific and bulk approaches. Additionally, we examined the relationship between dietary lipid content and TDF_{AA}. We also compared TDF_{AA} of fish muscle and scale to evaluate the viability of using scales in compound-specific stable isotope analysis. Such an understanding will serve as foundational research for using compound-specific stable isotope analysis of amino acids for estimating trophic position in future studies.

Methods

Experimental design

The experimental fishes used in this study were cared for in accordance with the *Guide to Care and Use of Experimental Animals*, and their use was reviewed and approved by the animal care review committee at University of Wisconsin–Madison, where the experiment was carried out. Four freshwater fish species (guppy (*Poecilia reticulata*), zebrafish (*Danio rerio*), fathead minnow (*Pimephales promelas*), and bluegill (*Lepomis macrochirus*)) were each reared on three distinctly different diets for a total of 12 unique consumer–diet combinations. These fish consumers and diets represent different taxonomy and life histories. Guppies, zebrafish, and fathead minnows were domesticated and bluegills were wild-caught. Similarly, the experimental diets were derived from shrimp (a marine crustacean), perch (a relatively small freshwater fish), or swordfish (*Xiphias gladius*, a large marine predatory fish). To create the diets, skinless fish fillets and peeled and deveined shrimp were mixed with all-purpose flour (no more than one part flour to three parts fish or shrimp or just enough to thicken the mixture, ensuring that nitrogen isotope ratios were largely unaffected given the low protein content of flour) and multivitamins (one capsule to 900 mL food). The mixture was homogenized and pureed in a food processor, spread onto baking sheets, baked at 177 °C for 15–25 min, and moved to a drying oven for ~48 h or until dry. Once dried, the pieces were ground to a powder using a food processor.

Fishes were housed at 25 °C in a flow-through aquarium system consisting of a series of 2.5 gal aquaria (1 gallon = 3.785 L). Each tank held up to four individual fish from the same diet treatment. Fish were fed one to three times daily until satiated. Any uneaten food was siphoned from the tanks within 12 h of feeding. Fish were sacrificed in clove oil solution when estimated to be in isotopic steady state with the experimental diets based on the equations for vertebrate ectotherm muscle (for bluegill) and whole body (for guppy, zebrafish, and fathead minnow) in Vander Zanden et al. (2015), by which time all individuals had grown to at least seven times their original body mass (refer to online Supplementary material, Table S1[1]). Specimens were rinsed in deionized water and frozen.

Guppy, zebrafish, and fathead minnow specimens were beheaded and gutted, while for bluegill, scales and dorsal muscle were extracted. Because guppy, zebrafish, and fathead minnow scales were small and difficult to extract, only bluegill scales were sampled for analysis. The samples were transferred to 3-dram glass vials (1 dram = 3.696 mL), dried in a drying oven at 48 °C for 2 weeks or until dried, and ground to a powder in the vials. Two individuals from each species–diet–tissue combination (N = 24) and three replicates of each experimental diet were sampled for compound-specific isotope analysis of amino acids. $N \geq 2$ individuals were sampled from each species–diet combination for bulk stable nitrogen isotope analysis except for guppy–swordfish and zebrafish–shrimp, which were not sampled for bulk due to a limited number of individuals (Table S2[1]).

Isotopic analysis, lipid analysis, and TDF calculation

Bulk stable nitrogen isotope analysis was performed at the University of California–Davis Stable Isotope Facility using a PDZ Europa 20–20 IRMS and Europa ANCA-GSL elemental analyzer

[1]Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/cjfas-2016-0420.

(Sercon Ltd., Cheshire, UK). The mean standard error for samples analyzed in duplicate (N = 8) was 0.09‰.

Stable nitrogen isotope analysis of amino acids was conducted at the Japan Agency for Marine–Earth Science and Technology (JAMSTEC) according to the procedures described in Chikaraishi et al. (2015). Samples were prepared for analysis by 12 mol·L^{-1} HCl hydrolysis at 110 °C overnight (>12 h). The hydrolysate was then washed with *n*-hexane:dichloromethane (3:2, *v*:*v*) to remove any hydrophobic constituents. After hydrolysis, samples were derivatized using thionyl chloride:2-propanol (1:4, *v*:*v*) at 110 °C for 2 h and then pivaloyl chloride:dichloromethane (1:4, *v*:*v*) at 110 °C for 2 h. The amino acid derivatives were then extracted with *n*-hexane:dichloromethane (3:2, *v*:*v*). Amino acid δ^{15}N values were determined by gas chromatography – combustion – isotope ratio mass spectrometry (GC–C–IRMS) using a 6890N GC instrument (Agilent Technologies, Palo Alto, California) coupled to a Delta^{plus}XP IRMS instrument via GC-combustion III interface (Thermo Fisher Scientific, Bremen, Germany).

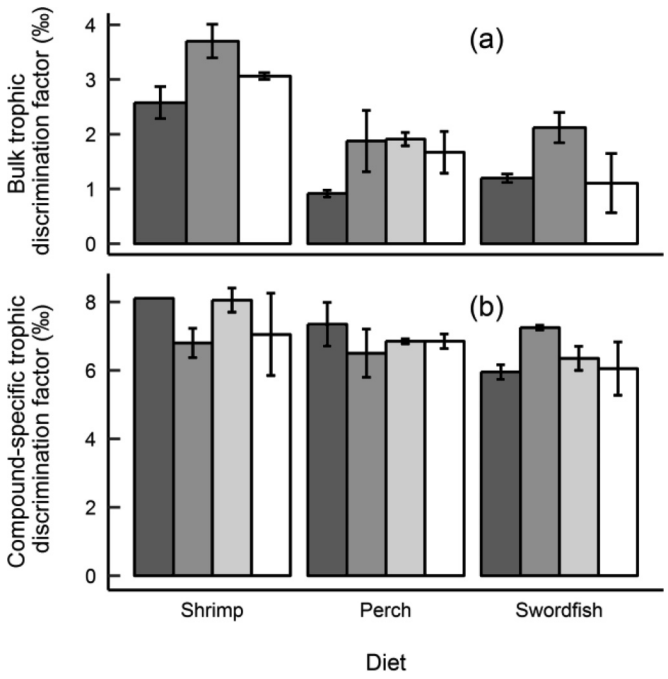
To assess isotope measurement reproducibility and obtain amino acid isotope composition, reference mixtures of nine amino acids (alanine, glycine, leucine, norleucine, aspartic acid, methionine, glutamic acid, phenylalanine, and hydroxyproline) with known δ^{15}N values (ranging from –26.4‰ to +45.6‰; Indiana University, Bloomington, USA; SI Science Co., Ltd., Sugito-machi, Japan) were analyzed after every five to eight sample runs, and three pulses of reference N_2 gas were discharged into the IRMS instrument at the beginning and end of each chromatography run for both reference mixtures and samples. The isotopic compositions of amino acids in samples are expressed relative to atmospheric nitrogen (air) and normalized to known δ^{15}N values of the reference amino acids. The accuracy and precision for the reference mixtures were always 0.0‰ (mean of Δ) and 0.3‰–0.6‰ (mean of 1 SD), respectively, for sample sizes of ≥0.5 nmol N. Amino acid δ^{15}N values were determined for alanine, glycine, valine, leucine, isoleucine, proline, serine, glutamic acid, and phenylalanine because their chromatographic peaks separate well with baseline resolution (Chikaraishi et al. 2009). The α-amino group of glutamine contributed to the δ^{15}N value calculated for glutamic acid due to quantitative conversion of glutamine to glutamic acid during HCl hydrolysis.

TDF_{Bulk} was calculated as the difference between consumer and diet bulk tissue δ^{15}N values. TDF_{AA} values were calculated as follows (Chikaraishi et al. 2009; Steffan et al. 2013):

$$(3)\quad TDF_{AA} = \left(\delta^{15}N_{Consumer} - \delta^{15}N_{Diet\ mean}\right)_{Glu} - \left(\delta^{15}N_{Consumer} - \delta^{15}N_{Diet\ mean}\right)_{Phe}$$

Lipid content was analyzed and quantified at JAMSTEC according to a modified procedure in Chikaraishi and Naraoka (2005). In brief, the dried sample was saponified by KOH in CH_3OH:H_2O (95:5, *w*:*w*) to hydrolyze ester bonds. Neutral lipids (including sterols) were partitioned with *n*-hexane:acetic acetate (4:1, *v*:*v*) from the alkaline solution, and subsequently acidic lipids (including *n*-fatty acids) were then extracted with *n*-hexane:acetic acetate (4:1, *v*:*v*) after addition of 12 mol·L^{-1} HCl (pH < 1). The neutral lipids were derivatized with *N*,*O*-bis(trimethylsilyl)acetamide at 75 °C for 30 min to form trimethylsilyl esters, and acidic lipids were esterified with HCl in CH_3OH at 75 °C for 8 h to form methylesters. Sterols and *n*-fatty acids were identified and quantified by gas chromatography – mass spectrometry (GC–MS) using an 7890A GC instrument connected to an 5975C MS instrument (Agilent Technologies, Palo Alto, California) by comparison with ion fragments and total ion area of external authentic standards (cholesterol, stigmasterol, and palmitic acid), respectively. The sum of sterol and *n*-fatty acid amounts was reported as lipid content.

Fig. 1. Mean trophic discrimination factors (TDF) of four freshwater fish species fed on each of three distinct diets calculated using stable isotope analysis of (*a*) bulk tissue and (*b*) amino acids (error bars = 1 SD). Bars (from dark to light) represent mean TDFs for guppy (*Poecilia reticulata*), zebrafish (*Danio rerio*), bluegill (*Lepomis macrochirus*), and fathead minnow (*Pimephales promelas*). $N \geq 2$ for each species–diet combination.



All analyses were conducted using the statistical programming language R (R Core Team 2014).

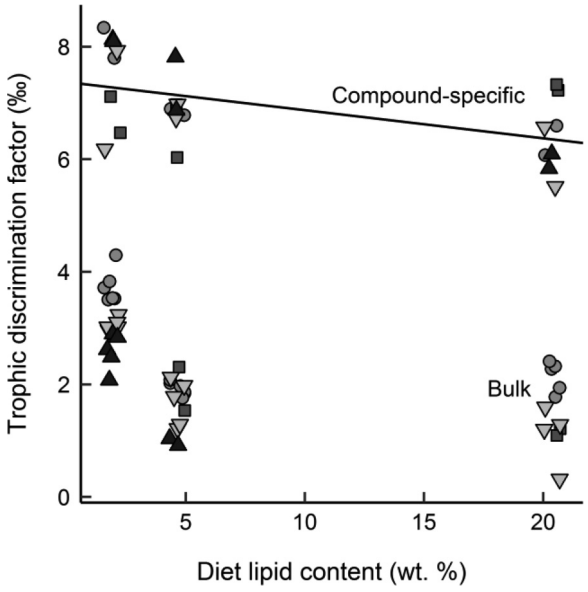
Results

Mean (±1 SD) TDF_{Bulk} from our experimental fish tissue was 2.2‰ ± 0.9‰ (N = 43 individual muscle TDF_{Bulk} values, range of individual TDF_{Bulk} values = 0.35‰–4.28‰; Fig. 1*a*). Diet, across all experimental taxa, had a significant effect on TDF_{Bulk}. Likewise, species had a significant effect on TDF_{Bulk} across all diet types. There was a marginally significant interactive effect between the experimental consumer species and diets (i.e., the effects of diet varied significantly among fish taxa) (ANOVA; Diet: $F_{[2,33]} = 137.8$, $p < 0.001$; Species: $F_{[3,33]} = 17.5$, $p < 0.001$; Species × Diet: $F_{[4,33]} = 2.7$, $p = 0.046$).

For the compound-specific isotope analysis, mean ± 1 SD TDF_{AA} for experimental fish muscle was 6.9‰ ± 0.8‰ (N = 24 individual muscle TDF_{AA} values, range of individual TDF_{AA} values = 5.5‰–8.3‰; Fig. 1*b*). There was a significant effect of diet on TDF_{AA}, but no species effect (ANOVA; Diet: $F_{[2,12]} = 8.37$, $p = 0.005$; Species: $F_{[3,12]} = 1.03$, $p = 0.414$; Species × Diet: $F_{[6,12]} = 2.67$, $p = 0.07$). Mean ± 1 SD TDF_{AA} was 7.5‰ ± 0.80‰ for the shrimp diet, 6.9‰ ± 0.49‰ for the perch diet, and 6.4‰ ± 0.64‰ for swordfish diet (N = 8 for each diet). The borderline significance of the interaction term appears to be driven by the zebrafish TDF_{AA} values, which did not follow the same trend (decreasing TDF_{AA} with trophic position, described below) as the other fish species. Significance of "diet" was maintained when the species × diet interaction was removed from the model (ANOVA; Diet: $F_{[2,18]} = 5.38$, $p = 0.015$; Species: $F_{3,18} = 0.663$, $p = 0.590$).

Our observed TDF_{AA} values (N = 24) were lower but not significantly different from the TDF_{AA} values in table 2 of Chikaraishi et al. (2009; two-sample t test; $t = 1.57$, df = 10.5, $p = 0.15$, N = 9). Mean ± 1 SD δ^{15}N enrichment from diet to consumer for glutamic

Fig. 2. The relationships between compound-specific ($y = -0.05x + 7.37$, $r^2 = 0.28$, $p = 0.007$) and bulk trophic discrimination factors (TDF) and experimental diet lipid content (wt. %, percentage of total by weight). Residuals of the relationship between bulk TDF and diet lipid content were not normally distributed. Each point represents a single consumer (from dark to light): guppy (triangles), zebrafish (squares), bluegill (circles), and fathead minnow (inverted triangles). Lipid content values were jittered to allow visualization.



acid and phenylalanine were 7.1‰ ± 0.75‰ and 0.17‰ ± 0.46‰, respectively.

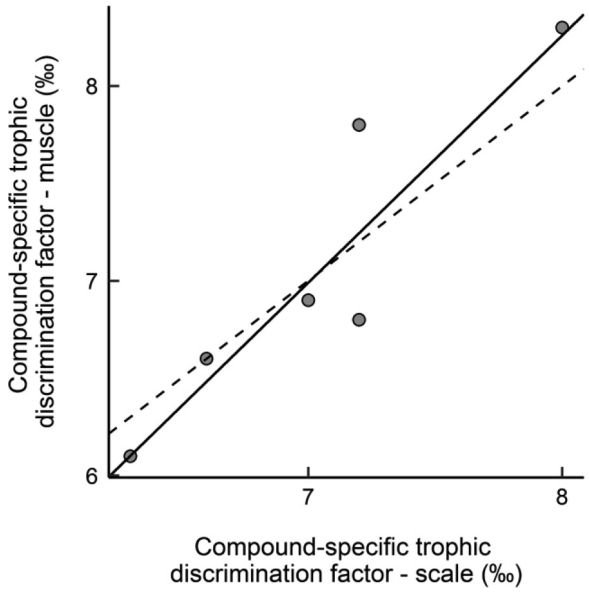
TDF_{AA} and TDF_{Bulk} decreased with diet trophic position (linear regression; compound-specific: $y = -0.715x + 9.58$, $R^2 = 0.35$, $t = -3.44$, df = 22, $p = 0.002$; Bulk: $y = -1.091x + 6.16$, $R^2 = 0.57$, $t = -7.40$, df = 42, $p < 0.001$; Table S2[1]). Similarly, TDF_{AA} and TDF_{Bulk} decreased with diet lipid content (percentage of total by weight: wt. %), though the residuals for the relationship between TDF_{Bulk} and lipid content were not normally distributed (linear regression; compound-specific: $y = -0.05x + 7.37$, $R^2 = 0.28$, $t = -2.95$, df = 22, $p = 0.007$; Fig. 2).

Mean ± 1 SD TDF_{AA} of bluegill scales was 7.05‰ ± 0.59‰ (N = 6 individual scale TDF_{AA} values). TDF_{AA} of fish scales and muscle tissue were not significantly different (paired t test; $t = -0.226$, df = 5, $p = 0.8$). There was a strong positive relationship between muscle and scale TDF_{AA} (linear regression; $y = 1.27x - 1.9$, $R^2 = 0.842$, $t = 4.61$, $p = 0.01$, $N = 6$; Fig. 3). Diet did not have a significant effect on bluegill scale TDF_{AA} (ANOVA; $F_{[2,3]} = 5.182$, $p = 0.106$). Consumer mean bulk and compound-specific TDF and $\delta^{15}N$ values and diet lipid content can be found in Table S2[1].

Discussion

We estimated a mean TDF_{Bulk} of 2.2‰ across our combination of three experimental diets and four fish taxa. This bulk TDF value is lower than the overall mean values (±1 SD) reported in previous literature syntheses (Vander Zanden and Rasmussen 2001, 2.9‰ ± 1.8‰; Post 2002, 3.4‰ ± 0.98‰; McCutchan et al. 2003, 2.3‰ ± 1.5‰; Vanderklift and Ponsard 2003, 2.54‰ ± 1.27‰; Sweeting et al. 2007, 2.8‰ ± 1.46‰; Caut et al. 2008, 2.75‰ ± 1.64‰), though we note that the TDF_{Bulk} estimates were consistently quite variable in these synthesis studies (mean SD = 1.4‰). Although our mean TDF_{Bulk} value falls within the 95% confidence interval of all of these syntheses, it illustrates that variability in TDF_{Bulk} values can lead to error in consumer trophic position estimates. Failure to correctly estimate bulk and compound-specific TDF values when estimating consumer trophic position with eqs. 1 and 2

Fig. 3. The relationship between experimental bluegill scale and muscle compound-specific trophic discrimination factors ($y = 1.27x - 1.9$, $r^2 = 0.842$, $p = 0.01$). Dashed line represents the 1:1 relationship.



results in biased trophic position estimates. For example, if we estimate (using eq. 1) the trophic position of a fish with a known trophic position of 4.0 and a "true" TDF_{Bulk} of 2.2‰, by assuming bulk TDF to be 3.0‰ across the entire food chain, we would underestimate trophic position by about 0.5 trophic levels. This example only addresses the issue of bias when estimating trophic position as a result of using a TDF value that differs from the true mean TDF (i.e., accuracy). Note that even in the case of an unbiased estimate of the mean TDF, the variability surrounding the mean also contributes to error in consumer trophic position. In contrast with the above, this is a matter of the precision of trophic position estimation (Vander Zanden and Rasmussen 2001).

Our fully crossed factorial controlled-feeding experiment produced TDF values for a suite of individual amino acids. We focused on the two amino acids that have become widely used for estimating trophic position — glutamic acid as a "trophic" indicator and phenylalanine as a "source" indicator (Popp et al. 2007; Chikaraishi et al. 2009). In our experiment the mean ± 1 SD TDF_{AA} was 6.9‰ ± 0.8‰. Our TDF_{AA} estimates were not significantly different from those of Chikaraishi et al. (2009), suggesting that 7.6‰ ± 1.3‰ is a reasonable TDF for estimating trophic position of freshwater fishes. A more recent synthesis of published laboratory-based estimates gave a mean ± 1 SD TDF_{AA} value of 7.2‰ ± 0.8‰ (P.S. Dharampal, C.M. Blanke, M.J. Vander Zanden, and S.A. Steffan, unpublished data). As with the bulk tissue approach, failure to use the correct TDF_{AA} can lead to error in trophic position estimation. Using the same reasoning as above, for a fish with a known trophic position of 4.0 and a "true" TDF_{AA} of 6.9‰, estimation of trophic position using eq. 2 assuming TDF_{AA} to be 7.6‰ would underestimate trophic position by about 0.3 trophic levels. These example calculations for bulk and compound-specific trophic position illustrate the importance of the assumed TDF value used in eqs. 1 and 2 and highlight the potential for error in trophic position as a result of misestimating the mean TDF value.

From our 12 experimental diet–fish taxa combinations, we estimated the variability for both bulk and compound-specific TDF values. 1 SD for TDF_{Bulk} was slightly higher than for TDF_{AA} estimates (0.9‰ and 0.8‰, respectively). When the variability is expressed relative to the mean (coefficient of variation, SD/mean), the variability of TDF_{Bulk} values was nearly four times higher than that for TDF_{AA} (40% and 11%, respectively). Low variability in TDF_{AA}

relative to TDF_{Bulk} translates directly into reduced error in trophic position estimation using the compound-specific method. This result is consistent with previous reports that the compound-specific approach provides more accurate estimates of trophic position as a result of decreased variability in TDF values (Bowes and Thorp 2015; Steffan et al. 2015).

The fully crossed factorial design of our controlled-feeding experiment allowed examination of variation in TDF values across experimental fish species and diets. TDF_{Bulk} varied significantly across both experimental diets and taxa. We found no difference in TDF_{AA} among the four fish species, but a significant difference among the three diets. The freshwater fishes used in our study represent three different taxonomic orders, originate from temperate, subtropical, and tropical regions, and represent diverse life history strategies (Dussault and Kramer 1981; Becker 1983; Engeszer et al. 2007). Though four fish species represent fairly limited taxonomic breadth in the broader scheme of the diversity of life, our study incorporates more taxonomic variation than other laboratory studies examining TDF values. It is encouraging that TDF_{AA} did not differ systematically among species, supporting the idea that the approach may be general across freshwater fishes.

We found that TDF_{AA} and TDF_{Bulk} decreased with the trophic position of the experimental diets. Other studies have observed similar trends and proposed lower TDF_{AA} values for higher-order marine consumers while showing that accounting for this variation can improve trophic position estimation (Lorrain et al. 2009; Dale et al. 2011; Choy et al. 2012; Germain et al. 2013; Hussey et al. 2014, 2015; Bradley et al. 2015; McMahon et al. 2015; Nielsen et al. 2015). However, the factors that drive this trend are still unknown, and further investigation into the mechanisms behind it is critical for the widespread application of compound-specific stable isotope analysis of amino acids for estimating trophic position. Compound-specific and bulk TDF values decreased with lipid content of the experimental diets in this study. The relationships between TDF and diet lipid content parallel the "diet" effects in the ANOVA model results (Fig. 2). For TDF_{AA}, there was a weak but significant effect of diet lipid content, and the slopes of the relationships differed among the four consumer species. TDF_{Bulk} decreased with diet lipid content across all four consumer species (N = 43) as well as for each individual consumer species (see Table S2[1] for N for each consumer species).

Diet quality has been proposed as a potential factor leading to variation in TDF_{AA} (Chikaraishi et al. 2015; McMahon et al. 2015). In Chikaraishi et al. (2015), the TDF_{AA} values of larval toads increased significantly with increasing dietary protein content. In contrast, McMahon et al. (2015) found that TDF_{AA} decreased significantly with decreasing disparity in amino acid concentrations between a consumer and its diet. These studies both included diet–consumer combinations that largely differed from what would be expected of the study organisms in the wild. One of the diets in McMahon et al. (2015), fed to the carnivorous mummichog (*Fundulus heteroclitus*), was fully plant-based, and the "protein-poor" boiled rice diet fed to tadpoles (*Bufo japonicus*) in Chikaraishi et al. (2015) consisted mostly of carbohydrates. In our study, multiple fish species were fed the same panel of diets, and all diets were animal-based and high in protein but differed in lipid content and trophic position. These diet biochemical compositions more closely resemble the diet biochemical compositions of the wild consumer species (despite obvious differences), with the aim of avoiding greater variation in TDF values due to unnatural differences in isotopic fractionation. Our results show that TDF values decreased with increasing lipid content, adding further to the investigation of potential factors behind the relationship between trophic position and TDF. TDF has been hypothesized to vary with the protein, fat, and carbohydrate balance within the diet. For example, it has been suggested that high lipid content could result in smaller TDF values, as reduced fractionation in trophic amino acids may occur if metabolic energy (for any physiological purpose) is derived from lipid metabolism rather than amino acid metabolism (Chikaraishi et al. 2015). Our results highlight the need for continued investigation of the effects of diet quality and biochemical composition on TDF_{AA}.

Recent studies have attempted to estimate compound-specific TDF values with wild-caught samples, using diet-based (stomach content analysis) trophic position estimation as a means of validation. Using this approach, Bradley et al. (2015) estimated TDF_{AA} for marine teleosts to be 5.7‰ ± 0.3‰ (mean ± 1 SD). Other field-based TDF values of this kind have also been consistently lower than laboratory estimates (Lorrain et al. 2009; Dale et al. 2011; Choy et al. 2012; Germain et al. 2013). Stable isotope and stomach content analyses are often employed in concert, which may reveal more about organismal trophic function than either approach alone — stomach contents offer a glimpse of immediate dietary components, while stable isotope analyses offer a more time-integrated understanding of the overall position within the food web (Wainright et al. 1993; Vander Zanden et al. 1997; Beaudoin et al. 1999; de la Morinière et al. 2003; Ho et al. 2007; Bradley et al. 2015). Because of the time-integrated nature of stable isotope analysis, laboratory-based TDF values derived from organisms in isotopic steady state with their diet are needed for examining mechanisms behind TDF variation. However, laboratory studies often employ diets that are not developed based on the study organism's natural food sources (this is certainly the case in our study), and future work should consider, along with dietary biochemical composition, more biologically realistic diets.

Fish scale TDF_{AA} values were comparable to those of muscle, suggesting that scales are a viable tissue in using compound-specific stable isotope analysis of amino acids to estimate fish trophic position. This relationship has been found for bulk stable isotopes and applied in ecological studies (Satterfield and Finney 2002; Rennie et al. 2009). Future studies should continue to evaluate fish scale TDF_{AA}, as our scale sample size was small (N = 6). Nevertheless, the close correlation between bluegill muscle and scale TDF values could expand the host of opportunities for employing compound-specific isotope analysis. The use of fish scales rather than muscle could allow use of less invasive sampling method, in which researchers could easily extract a few scales from wild fishes and release them without harm, making the method ideal for studies of ecologically threatened species. Additionally, long-term studies could make use of scale archives to examine how food webs have changed through time, as studies have done using the bulk approach (Wainright et al. 1993). Notably, in our study there was not a significant effect of diet on scale TDF_{AA}. If future studies continue to find that scales show more consistent trophic discrimination than muscle, scales may be a superior analysis tissue. However, nitrogen content, crude fat, and crude protein have been shown to vary in scales with growth stage (Saraswat 1978). These and other differences among tissue types may add to variation in trophic discrimination and should be carefully considered.

The use of compound-specific stable nitrogen isotope analysis of amino acids can improve accuracy and precision of trophic position estimates relative to the bulk tissue approach. Our fully crossed factorial controlled-feeding experiment allowed for assessment of both main and interactive effects on trophic discrimination, thereby illuminating sources of variation in bulk and compound-specific TDF values. We found that TDF_{AA} was relatively constant among four freshwater fish species, and the TDF_{AA} values in our study (mean ± 1 SD, 6.9‰ ± 0.8‰) were not significantly different from the TDF_{AA} values reported for marine (Chikaraishi et al. 2009) and terrestrial taxa (Steffan et al. 2013, 2015). However, TDF_{AA} did vary among the three experimental diets (16% difference between the smallest and largest diet mean TDF_{AA} values). While TDF_{AA} was less variable than bulk trophic discrimination factors, variation in compound-specific TDF values

still translates to error in trophic position estimates. Therefore, the driving mechanisms behind this variation require further investigation both in the laboratory and in the field, with careful consideration of potentially confounding factors. Continued examination and definition of compound-specific trophic discrimination factors is critical for advancing trophic position estimation using compound-specific stable isotope analysis.

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