


ORIGINAL RESEARCH

Trophic discrimination of amino acid-specific nitrogen stable isotopes in raptor nestlings: implications for estimating trophic position

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Arctic Tundra; compound-specific stable isotope analysis; golden eagle; gyrfalcon; rough-legged hawk; trophic ecology; trophic discrimination factor.

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Abstract

Bulk stable isotope analysis of carbon and nitrogen is commonly used to assess trophic relationships. However, compound-specific stable isotope analysis of individual amino acids may be a more accurate approach for resolving food web structure. Nevertheless, recent studies suggest amino acid-specific nitrogen trophic discrimination factors (TDFs) can vary depending on the type of nitrogenous waste produced and the quality of the diet, potentially limiting inference. We compared the ability to discriminate between primary and secondary consumers using bulk and compound-specific methods in an Arctic tundra ecosystem. Specifically, we determined bulk $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values, as well as amino acid-specific $\delta^{15}\text{N}$ values, for red blood cells from nestling golden eagles (*Aquila chrysaetos*), rough-legged hawks (*Buteo lagopus*), and gyrfalcons (*Falco rusticolus*), as well as for muscle samples from common prey species. We subsequently used high-precision diet estimates from nest cameras to estimate TDFs for gyrfalcon nestlings and compare these against TDFs for other taxa from the literature. Although bulk $\delta^{15}\text{N}$ values of secondary consumers were enriched relative to primary consumers, overlap occurred across groups, and greater separation was apparent using $\delta^{15}\text{N}$ of amino acids. Comparing red blood cell $\delta^{15}\text{N}$ values to prey muscle $\delta^{15}\text{N}$ values, bulk TDFs and compound-specific TDFs for trophic amino acids were lower than values from the literature that have typically been used to estimate trophic position. Amino acid-specific TDFs for raptor nestlings may be particularly low due to their rapid growth, consumption of high-quality protein, and excretion of nitrogenous wastes as uric acid, which have previously been identified as factors influencing isotopic discrimination. Consistent with recent studies, our findings indicate that the use of 'universal' $\text{TDF}_{\text{Glu-Phe}}$ values will result in an underestimate of nestling trophic level, and more work is needed to establish appropriate TDFs that reflect the physiology and life-history stage of the consumer.

Introduction

Over the past several decades, stable isotope analysis has proven to be a highly effective technique for estimating diets, characterizing trophic relationships, and constructing food webs (Boecklen et al., 2011; Post, 2002). Unlike pellet analyses or DNA fecal analyses for raptor diet studies, which only provide a snapshot of what has recently been eaten, stable isotope analyses integrate diet over a longer period. The observation that nitrogen isotope values of bulk tissues (e.g., bulk $\delta^{15}\text{N}$ values

of blood or muscle) increase systematically by approximately 3.4‰ with trophic position has proven particularly useful for estimating absolute or relative trophic positions of animals within a food web (Post, 2002). Combining this approach with measurements of carbon stable isotopes in bulk tissues (bulk $\delta^{13}\text{C}$) has proven effective for evaluating the structure and dynamics of ecological communities (Kelly, 2000).

Nevertheless, the use of stable isotopes as dietary tracers is not without limitations (reviewed in Martínez del Río et al., 2009). In particular, while the average difference in

isotopic values between diet and tissue (i.e., diet–tissue discrimination) for nitrogen may be close to 3.4‰ (Post, 2002), the exact diet-tissue discrimination value can vary depending on a number of factors, including taxa (Caut *et al.*, 2009), tissue type (Kurle *et al.*, 2014), diet quality (Robbins *et al.*, 2010), nutritional stress (Hertz *et al.*, 2015; Williams *et al.*, 2007), and ontogeny (Micklelem *et al.*, 2021; Sears *et al.*, 2009). For these reasons, there has been increasing interest in developing compound-specific stable isotope approaches that take advantage of the fact that some monomers are predictably routed directly from the diet into animal tissue, whereas others are biochemically transformed during assimilation (Whiteman *et al.*, 2019). A key advantage of the compound-specific approach is that it is not necessary to measure amino acid nitrogen isotopes at the base of the food web, as these are estimated by the ‘source’ amino acids that show little enrichment with each trophic level (Fig. 1).

Use of amino acid nitrogen isotopes to estimate trophic position exploits the fact that ‘trophic’ amino acids [e.g., alanine (Ala), valine (Val), isoleucine (Ile), proline (Pro), and glutamic acid (Glu)] become substantially enriched in ^{15}N as a result of isotopic discrimination during the cleavage of the carbon–nitrogen (C–N) bond during metabolic transamination, whereas ‘source’ amino acids [e.g., methionine (Met), phenylalanine (Phe), and lysine (Lys)] exhibit little diet-tissue $\delta^{15}\text{N}$ discrimination (i.e., $\Delta^{15}\text{N}$ values are low for source amino acids), as C–N bonds are not formed or cleaved during dominant metabolic processes (Chikaraishi *et al.*, 2009). In practice, trophic level has typically been estimated based on the difference in $\delta^{15}\text{N}$ values of one trophic (Glu) and one source (Phe) amino acid according to the following equation (from Chikaraishi *et al.*, 2010):

$$\text{TL}_{\text{Glu/Phe (terrestrial)}} = \frac{\delta^{15}\text{N}_{\text{Glu}} - \delta^{15}\text{N}_{\text{Phe}} + \beta}{\text{TDF}} + 1 \quad (1)$$

In equation 1, within a given tissue type $\delta^{15}\text{N}_{\text{Glu}}$ and $\delta^{15}\text{N}_{\text{Phe}}$ represent the ratio of heavy (^{15}N) to light (^{14}N) nitrogen isotopes (relative to air) in Glu and Phe, respectively. Further, β (8.4 in the original Chikaraishi *et al.*, 2010 paper) indicates the differences in $\delta^{15}\text{N}$ between Glu and Phe at trophic level = 1 (i.e., at the base of the food web), and trophic discrimination factor (TDF) indicates the difference in the increase in $\delta^{15}\text{N}$ with each trophic level for Glu relative to Phe (i.e., $\text{TDF}_{\text{Glu-Phe}} = 7.6$ in Chikaraishi *et al.*, 2010). Note that other β and TDF values have been proposed, and studies increasingly use alternative or even multiple trophic and source amino acids (see Ramirez *et al.*, 2021).

Several studies have demonstrated that stable isotope analysis of amino acids can be effective for assessing trophic ecology in marine, freshwater, and terrestrial food webs (Besser *et al.*, 2022; Chikaraishi *et al.*, 2009, 2014). Nevertheless, studies in marine mammals, seabirds, and predatory fishes have shown that diet-tissue TDFs of trophic amino acids can be much lower than commonly applied values, resulting in inaccurate estimates of the trophic level of feeding using the original models proposed by Chikaraishi *et al.* (Germain *et al.*, 2013; Hoen *et al.*, 2014; Matthews *et al.*, 2020). For example, a meta-analysis of isotopic discrimination indicates that dietary quality and type of nitrogenous waste product produced affect TDFs for nitrogen in amino acids (McMahon & McCarthy, 2016). Indeed, the degree of amino acid similarity between diet and consumer can influence TDFs for trophic amino acids (McMahon, Thorrold, *et al.*, 2015). Similarly, changes in dietary protein content and/or changes in rates of growth alter

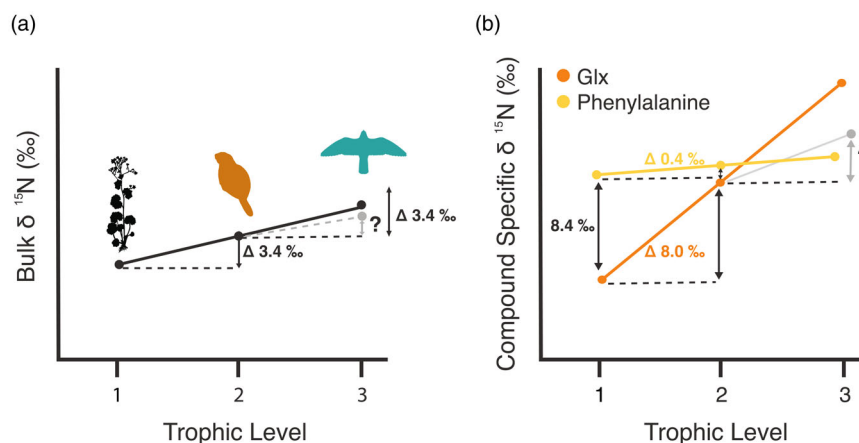


Figure 1 Visual representations of the theoretical changes in $\delta^{15}\text{N}$ in bulk-stable isotope analysis (left) and compound-specific stable isotope analysis of amino acids (right). (a) Trophic levels (TL; 1–3) from an Arctic tundra ecosystem are represented, with the most basal being represented by *Saxifraga spp.* as the primary producer (TL = 1). Primary consumers (TL = 2) are represented by a ground squirrel, which are then depredated by gyrfalcons (TL = 3) in this simplified food chain. Differences in TL $\delta^{15}\text{N}$ are represented by the arrows and accompanying TDF of $\Delta 3.4\text{‰}$ (Post, 2002). (b) In compound-specific stable isotope analysis of amino acids, TDFs are calculated by differences in source and trophic amino acids (glutamic acid/glutamine [Glx] and phenylalanine, respectively [$\text{TDF}_{\text{Glu-Phe}}$]). Hypothesized differences in our dataset deviating from traditional bulk and compound-specific stable isotope analysis of amino acids are denoted in gray with question marks. Adapted from Chikaraishi *et al.*, (2011).

nitrogen-use efficiency, which affects bulk nitrogen TDFs (Cantalapiedra-Hijar *et al.*, 2015; Sears *et al.*, 2009; Williams *et al.*, 2007) and is also predicted to impact nitrogen amino acid-specific TDFs (McMahon & McCarthy, 2016). To date, however, most TDFs have been developed for adult animals, and there is a need to derive amino acid-specific nitrogen TDFs for growing animals to fill this data gap.

Recently, we employed bulk stable isotope analyses to characterize trophic niche width (Johnson *et al.*, 2022) and estimated diets using Bayesian stable isotope mixing models (BSIMMs; Johnson *et al.*, 2020) in Arctic raptors. As part of these studies, we used camera traps at nests to quantify diets and estimate diet-tissue TDFs for bulk nitrogen and carbon in gyrfalcon (*Falco rusticolus*) nestlings. We found that bulk TDFs of nitrogen ($\Delta^{15}\text{N}$) for gyrfalcon nestlings were much lower than those previously estimated for adult raptors (Hobson & Clark, 1992) and that the use of gyrfalcon nestling-specific bulk TDFs greatly improved diet estimation based on BSIMMs across three different species of raptor nestlings (Johnson *et al.*, 2023). We attributed our much lower estimates of nitrogen TDFs to ontogenic effects, potentially associated with the occurrence of intense growth and increased nitrogen use efficiency during the nestling stage (Micklem *et al.*, 2021; Williams *et al.*, 2007).

In the current study, we measured amino acid nitrogen $\delta^{15}\text{N}$ values in the red blood cells of three raptor species [gyrfalcons, golden eagles (*Aquila chrysaetos*), and rough-legged hawks (*Buteo lagopus*)] and muscle tissue of their prey. Bulk $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values were also available for these raptor tissues from a previous study (Johnson *et al.*, 2020). We subsequently compared bulk and compound-specific approaches to determine whether amino acid $\delta^{15}\text{N}$ values improved our ability to discriminate between species within the Arctic raptor guild and/or between primary and secondary consumers within the Arctic food web. Raptors and potential prey species were identified as predominantly primary (herbivore) versus predominantly secondary (feeding on herbivores) consumers based on a literature review. We then capitalized on our camera trap data, which provides direct measures of prey intake by gyrfalcon nestlings, and estimated TDFs for nitrogen stable isotopes of amino acids. We estimated TDFs using a proportionally balanced equation that uses high-precision diet estimates from nest cameras in lieu of a controlled feeding study (see details in Johnson *et al.*, 2020, 2023). Given our prior finding that bulk nitrogen TDFs are smaller in raptor nestlings relative to adult raptors (Johnson *et al.*, 2023), we predicted TDFs for trophic amino acids would also be lower, potentially biasing trophic level estimates from models that assume trophic discrimination is constant across taxa.

Materials and methods

Study site and species

We conducted our study on the Seward Peninsula of western Alaska (64°N–65°N, 164°W–166° W) in 2016–2019 (Fig. S1). The Seward Peninsula consists of rolling tussock-shrub tundra with rocky outcroppings that provide nesting substrate for

cliff-nesting raptors, including gyrfalcons, golden eagles, and rough-legged hawks. Gyrfalcon nestlings on the Seward Peninsula are predominantly fed ptarmigan (*Lagopus lagopus* and *L. muta*) and arctic ground squirrels (*Urocyon parryii*) (Robinson *et al.*, 2019). In contrast, golden eagles are opportunistic dietary generalists whose diet includes arctic ground squirrels, ptarmigan, and waterfowl (Herzog *et al.*, 2019). Rough-legged hawks specialize predominantly in arvicoline rodents (Fufachev *et al.*, 2019).

Tissue sampling

We located occupied raptor nests and estimated the age of nestlings by conducting aerial surveys of >500 historically occupied raptor cliff-nest sites across the study site (Fig. S1; specific methods in Bente, 2011). We visited nest sites and collected blood samples midway through the brood-rearing period (golden eagles ~35 days old; gyrfalcons ~25 days old; rough-legged hawks ~20 days old). We conducted nest visits during the middle of the nestling phase so the isotopic composition of red blood cells would be predominantly determined by nestling diet (Williams *et al.*, 2007) and to minimize the risk of nestlings prematurely fledging in response to disturbance. Blood was collected from each nestling, deposited immediately into a heparinized vacutainer, and stored on ice for <6 h until plasma and cells could be separated. We analyzed red blood cells for stable isotopes because they have a longer turnover rate relative to plasma, and the proximate composition of plasma varies with time since the last meal. Diet-tissue discrimination may be highly variable for plasma depending on whether plasma amino acids predominantly reflect endogenous proteins or recently absorbed nutrients (Burke *et al.*, 2012). Red blood cells were frozen at -20°C until stable isotope analyses. Sample sizes for each group are shown in Table S1.

We used a variety of sampling approaches to collect representative prey known to be important to the Arctic raptor community. Prey samples were collected in 2018 and 2019, seasonally contemporaneous with the raptor brood-rearing period. We collected samples from only those species that constituted 95% of each prey category by biomass based on data from earlier years (Robinson *et al.*, 2019). We distributed our sampling efforts across the study site throughout the gyrfalcon brood-rearing period. Most tissue samples were collected opportunistically from prey remains at raptor nests, but some were collected actively via shotgun (9.1% of all samples) or snap-trap (12.9% of all samples) when necessary to account for underrepresented groups (see details in Table S1 of Johnson *et al.*, 2022). From each prey specimen, we dissected a ~1 g sample of muscle tissue (pectoralis for birds and quadriceps for mammals) and stored samples at -20°C until processed for stable isotope analysis.

Gyrfalcon camera traps

To characterize the diet of gyrfalcon nestlings, we installed motion-activated camera traps at gyrfalcon nest sites midway through the incubation period in 2016–2019 (details in Johnson

et al., 2020). Camera traps provided images of prey deliveries to nestlings from hatch until the time of blood sampling at age ~25 days. We identified each prey delivery to the lowest possible taxonomic level and estimated the proportion of prey consumed by nestlings to account for incomplete prey fed to young. We estimated the total consumed biomass for each delivery by multiplying the proportion of prey consumed by mass values from the literature for each prey species. For the development of trophic discrimination factors (see below), prey items were grouped into the following categories: ptarmigan (*Lagopus lagopus* and *L. muta*), arctic ground squirrels, insectivorous birds (shorebirds and passerines), and arvicoline rodents (*Microtus Lemmus* and *Myodes* spp.). Prey deliveries in other categories (e.g., ducks and raptors) constituted a small proportion of dietary biomass (<1% combined) and were not used for estimating trophic discrimination.

Classification of species trophic level

We conducted a literature search to classify raptors and their potential prey in our study system as primary consumers (trophic level = 2; animals that consume plants) or secondary-plus consumers (trophic level ≥ 3 ; animals that consume other animals; Table S1). Our search was conducted on Google Scholar and search terms included each species' scientific name and the words 'consum*', 'trophic', or 'diet*', and/or 'primary', 'secondary' or 'tertiary' + 'Arctic' or 'tundra'. We did not attempt to separate secondary and tertiary consumers. Species such as arctic ground squirrels, which will opportunistically, but irregularly, consume animal protein (Holmes, 1977), were classified as primary consumers (trophic level = 2).

Sample preparation and stable isotope analysis

Bulk stable isotopes of raptor and prey samples ($\delta^{15}\text{N}$ and $\delta^{13}\text{C}$) and corresponding trophic discrimination factors (TDFs) used in this paper have previously been described in (Johnson *et al.*, 2020, 2022). For raptors, we randomly selected a single raptor nestling from each nest for measurement of amino acid-specific $\delta^{15}\text{N}$ (sample sizes in Table S2). We freeze-dried all prey muscle samples and raptor red blood cells and then ground them into a fine powder with a mortar and pestle prior to analysis.

We measured bulk $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ stable isotopes using a Costech ECS 4010 (Costech Analytical Technologies) connected to a Delta V Plus XP Mass Spectrometer (IRMS; Thermo Fischer Scientific, Waltham, MA, USA) via a Finnigan Conflo III (Thermo Fischer Scientific) at the University of Alaska Fairbanks Stable Isotope Facility. Isotope ratios are expressed as $\delta X\text{‰}$, where $\delta X = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 1000$, and R is a ratio of heavy:light isotopes of a given element. Vienna PeeDee Belemnite and atmospheric nitrogen were used as standards for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, respectively. We calibrated our analyses using within-run alkaline peptone standards, achieving an analytical precision estimate of

$\pm 0.13\text{‰}$ (nitrogen) and $\pm 0.10\text{‰}$ (carbon) across all runs. The peptone standard was calibrated to NIST standards (REF 8544, 8550, 8551, 8549, 8542, USGS 40 and 41) twice annually for quality assurance. Accuracy was determined to be ± 0.08 for $\delta^{13}\text{C}$ and ± 0.14 for $\delta^{15}\text{N}$ on the basis of the difference between the observed and known δ values of the check standards.

Blood and muscle samples were analyzed in duplicate for amino acid-specific $\delta^{15}\text{N}$ values at the University of California Davis Stable Isotope Facility. Amino acids were liberated from sample material proteins by acid hydrolysis (6 M HCl, 70 min, 150°C under N_2 headspace) and were made suitable for gas chromatography by derivatization as N-acetyl methyl esters (Corr *et al.*, 2007). Derivatives were injected at 260°C (splitless; 1 min) and isolated on an Agilent DB 35 column at a constant flow rate of 2 mL/min under the following temperature program: 70°C (hold 2 min), 140°C (15°C/min, hold 4 min), 240°C (12°C/min, hold 5 min), and 255°C (8°C/min, hold 35 min). Gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS) was performed on a Thermo Trace GC 1310 gas chromatograph and a Thermo Scientific Delta V Advantage IRMS. Water was scrubbed from the gas via a Nafion dryer before entering the spectrophotometer. CO_2 was removed from the analyte via a liquid nitrogen trap in the post-combustion carrier stream. IRMS was performed using secondary reference materials calibrated against certified standard reference materials from USGS, NIST, and the IAEA (i.e., IAEA-600, USGS40, USGS41, USGS42, USGS43, USGS61, USGS64, and USGS65).

We determined $\delta^{15}\text{N}$ values for 14 amino acids. Due to cleavage of the terminal amine groups in glutamine (Gln) and aspartamine (Asn), Gln is converted to Glu, and Asn is converted to aspartic acid (Asp) during acid hydrolysis. This results in the measurement of combined Gln + Glu (referred to hereby as Glx) and Asn + Asp (referred to hereby as Asx). We then categorized amino acids into four groupings: Source amino acids—Lys, tyrosine (Tyr), Phe, and histidine (His); Trophic amino acids—Ala, Asx, Glx, Ile, leucine (Leu), Pro, and Val; Metabolic amino acids—threonine (Thr); Other amino acids—glycine (Gly), serine (Ser).

Trophic discrimination factors

TDFs ($\Delta X\text{‰}$) are typically estimated as the difference between tissue and dietary stable isotope values as assessed in captive animals fed controlled diets (i.e., $\Delta X = \delta X_{\text{consumer}} - \delta X_{\text{sources}}$; Stephens *et al.*, 2023). However, due to logistical and ethical considerations, these types of controlled experiments have not been conducted for raptor nestlings and, therefore, we previously implemented a novel approach for the estimation of TDFs between predator red blood cells and prey muscle tissue for bulk $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ (i.e., $\Delta^{13}\text{C}$ and $\Delta^{15}\text{N}$) in raptor nestlings (Johnson *et al.*, 2020, 2023). This approach involved estimating dietary proportions from high-precision nest camera data in lieu of the known proportions typical of a controlled feeding study and then applying a proportionally balanced TDF calculation:

$$\text{TDF} = \frac{\sum_{j=1}^m \left[\delta_j - \sum_{i=1}^n (\text{mean}(\delta_i) * P_{ij}) \right]}{m} \quad (2)$$

In equation 2, (*i*) represents one of (*n*) prey categories (see gyrfalcon camera traps section), (*j*) represents an individual consumer (e.g., a nestling raptor), (*m*) is the number of consumers in that subset, and (*P*) is a dietary proportion estimate based on the nest camera data for that nest. Diet estimates from nest cameras served as a proxy for known dietary percentages, which we used to weight the isotopic values of sources to compare consumer isotopic values to those of the prey they consume (i.e., trophic discrimination, which involves the isotopic shift that occurs when elements from food sources are incorporated into consumer tissue). Johnson *et al.* (2023) developed this approach for estimating TDFs for bulk stable isotopes that uses estimated dietary proportions based on camera trap data to estimate TDFs. In the present study, we applied a similar approach to estimate amino acid-specific TDFs between predator red blood cells and prey muscle samples for $\delta^{15}\text{N}$. We calculated the proportional contribution of each prey category to a nestling's diet at each nest by dividing the sum of that category's biomass by the sum of the total prey biomass consumed by nestlings over the observation period. These proportional contributions were subsequently used to estimate the average isotopic composition (either bulk or compound-specific) of the diet consumed by each nestling. We assume that the amino acid composition of tissue is consistent across prey types and that the stable isotope values of amino acids in the muscle of prey reflect what is incorporated into tissues (i.e., red blood cells). Further, we assume that the diet consumed by an individual nestling matched the diet fed to all nestlings within the nest, as measured using nest cameras. Finally, we calculated the trophic discrimination factor ($\text{TDF}_{\text{Glu-Phe}}$) as $\Delta\text{Glu} - \Delta\text{Phe}$, which is broadly used in calculations to estimate trophic position (Chikaraishi *et al.*, 2009; McClelland & Montoya, 2002).

Statistical analyses

We performed all statistical analyses in R (R version 4.2.1). We compared bulk $\delta^{15}\text{N}$ values between animals designated as primary versus secondary consumers using a mixed model including species as a random intercept. To reduce the dimensionality of the amino acid $\delta^{15}\text{N}$ dataset, we conducted a principal component analysis (PCA) and extracted the first and second principal components. We conducted a PCA on all species (i.e., raptors and their prey), as well as a PCA on a subset of individuals composed of only raptors (gyrfalcons, golden eagles, and rough-legged hawks). Additionally, because some studies exclude the metabolic amino acid Thr (Germain *et al.*, 2013), we repeated both PCAs with Thr excluded from the dataset. In the PCA that included all sampled animals and all amino acids, PC1 effectively separated primary from secondary consumers (see Results)—therefore, we compared bulk $\delta^{15}\text{N}$ to PC1 using a linear mixed model that included species as a random effect. For the raptor-only PCA, we compared PC1 between raptor groups using analysis of variance (ANOVA) and subsequently ran *post hoc* Tukey's HSD test for

multiple comparisons. We also compared Glu-Phe (an index of trophic level) between primary and secondary consumers using a mixed model that included random intercepts for species.

Results

Bulk and amino acid-specific stable isotope analysis

We obtained bulk $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values, as well as amino acid $\delta^{15}\text{N}$ values for 70 animals, including 30 raptors and 40 prey species (Table S2). As expected, species classified as secondary consumers based on the literature were significantly enriched in ^{15}N relative to individuals classified as primary consumers ($F_{1,6.6} = 7.7$; $P = 0.03$), though there was some overlap among consumer groups (Fig. 2). In particular, bulk $\delta^{15}\text{N}$ values for tundra voles and arctic ground squirrels overlapped with gyrfalcons. The inclusion of bulk $\delta^{13}\text{C}$ data created greater separation between primary and secondary consumers (Fig. 2). However, the bulk isotopic composition of one American golden plover fell well within the primary consumers despite this species being classified as a secondary consumer based on the literature review.

A PCA on the $\delta^{15}\text{N}$ values of the 14 amino acids revealed consistent differences between species classified as primary versus secondary consumers (Fig. 3). The first and second

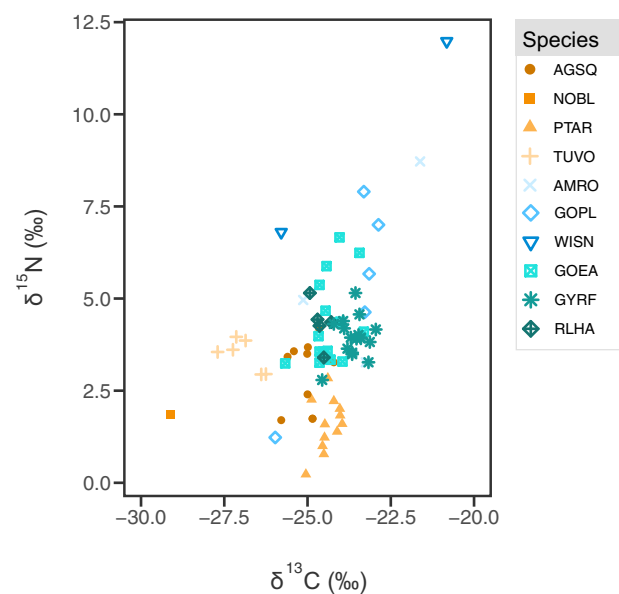


Figure 2 Bulk $\delta^{13}\text{C}$ versus $\delta^{15}\text{N}$ stable isotope values for red blood cell samples from raptor nestlings (GOEA = golden eagle, GYRF = gyrfalcon, RLHA = rough-legged hawk) and muscle samples from potential prey (AGSQ = arctic ground squirrel, NOBL = northern brown lemming, PTAR = rock and willow ptarmigan, TUVO = tundra vole, AMRO = American robin, GOPL = American golden plover, WISN = Wilson's snipe). Shades of orange represent primary consumers, whereas shades of blue represent secondary consumers, with cyan representing raptors.

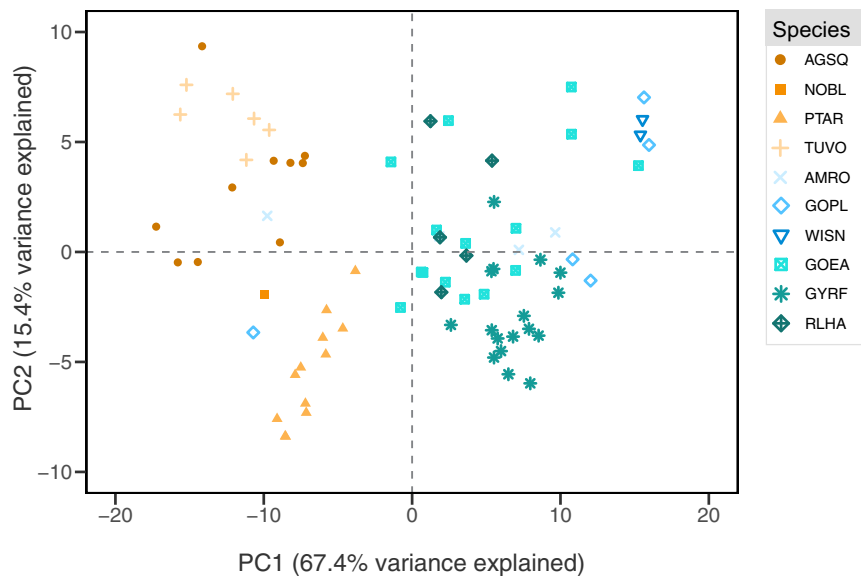


Figure 3 The first and second principal components (PC1 and PC2) for a principal component analysis (PCA) of 14 amino acid $\delta^{15}\text{N}$ values for all species. Shades of orange represent primary consumers, whereas shades of blue and cyan represent secondary consumers. See Fig. 2 for species abbreviations.

principal components accounted for 67.4 and 15.4% of the variance, respectively (see Table S3 for principal component loadings). Similar results were obtained when a PCA was conducted on 13 amino acids, excluding Thr from the dataset (Fig. S2). For both PCAs, PC1 values were significantly different between primary and secondary consumers (with Thr: $F_{1,5,4} = 50$, $P = 0.0006$; without Thr: $F_{1,3,1} = 36.5$, $P = 0.008$). Notably, the PCA that excluded Thr did a slightly poorer job of differentiating between species that the literature classifies as primary versus secondary consumers. Two outliers were notable in both PCAs—one American robin and one golden plover aggregated with the primary consumers (the golden plover was the same individual that had a bulk $\delta^{15}\text{N}$ value that fell within the range of the primary consumers). The $\delta^{15}\text{N}$ values of the ‘source’ amino acid Phe were lower in the Wilson’s snipe, and to a lesser extent, American golden plovers, compared with the raptors and other prey species (Table S2). Consistent with the utility of PC1 in separating between primary and secondary consumers, individual bulk $\delta^{15}\text{N}$ values were positively correlated with PC1 values, regardless of whether Thr was included in the PCA (with Thr: $F_{1,56,2} = 32.9$, $P < 0.0001$; without Thr: $F_{1,53,4} = 77$, $P < 0.0001$; Fig. 4).

Because we were also specifically interested in niche partitioning within the raptor guild, we subsequently conducted PCA analyses of $\delta^{15}\text{N}$ values of amino acids for just the three raptors: gyrfalcon, golden eagles, and rough-legged hawks. When the metabolic amino acid Thr was included, PC1 and PC2 accounted for 39.5 and 32.5% of the variability in amino acid $\delta^{15}\text{N}$ values, and there was a significant effect of species on PC1 values ($F_{2,34} = 18.3$, $P < 0.001$). *Post hoc* Tukey HSD tests revealed that gyrfalcons had significantly lower PC1 values than both golden eagles and rough-legged hawks

($P < 0.0001$), which were not different from one another. Similar results were obtained from a PCA when Thr was excluded (see Fig. S3).

Prior studies indicate that the subtraction of a source amino acid (typically Phe, used as a proxy for $\delta^{15}\text{N}$ values at the base of food webs) from a trophic amino acid (typically Glx) may provide greater insight into trophic position (Chikaraishi et al., 2009; McMahon, Thorrold, et al., 2015). Therefore, we examined how Glx-Phe values differed across species. Species classified as primary consumers had significantly lower Glx-Phe values than secondary consumers ($F_{1,6,5} = 15.7$, $P = 0.006$). However, we also found differences within the secondary consumer group, with American golden plovers and Wilson’s snipes having higher Glx-Phe values than the raptor nestlings (Fig. S4). Finally, while Glx-Phe values were generally more consistent among primary consumers, arctic ground squirrels exhibited a binomial distribution of Glx-Phe values.

Determination of amino acid $\delta^{15}\text{N}$ TDFs in raptor nestlings

Nest cameras revealed that gyrfalcon nestlings were fed a diet comprised primarily of ptarmigan (53.1% by mass) and arctic ground squirrels (22.6%), with lesser amounts of insectivorous birds (16.8%) and arvicoline rodents (4.5%; see Johnson et al., 2020 for details). We utilized our dataset of prey fed to gyrfalcon nestlings (as assessed using nest camera traps) in conjunction with our gyrfalcon and prey $\delta^{15}\text{N}$ amino acid datasets to calculate TDFs for each amino acid based on our mass-balance equation (equation 1) and compared these to prior estimates for avian species (Table 1). Estimates for three of four ‘source’ amino acids were close to zero (Fig. 5; Fig. S5); TDF estimates for histidine exceeded zero. In

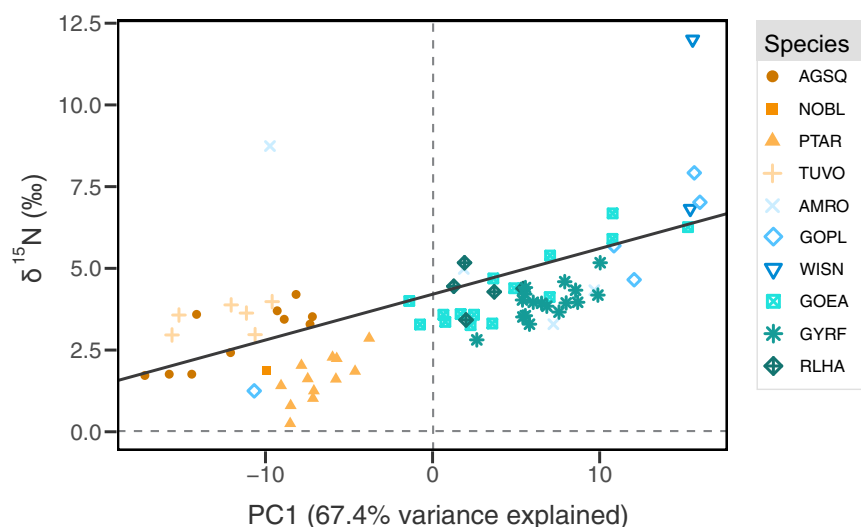


Figure 4 The first principal component (PC1) plotted against individual $\delta^{15}\text{N}$ values for all species included. Shades of orange represent primary consumers, whereas shades of blue and cyan represent secondary consumers. See Fig. 2 for species abbreviations.

Table 1 Trophic discrimination factors (mean \pm SD) for amino acids from four avian studies

Amino acid	Hebert et al. (2016) American Kestrel (<i>Falco sparverius</i>)	McMahon, Polito, et al. (2015) Gentoo Penguin (<i>Pygoscelis papua</i>)	Elliott et al. (2021) Thick-Billed Murre Chicks (<i>Uria lomvia</i>)	Present study Gyr Falcon nestlings (<i>Falco rusticolus</i>)
Bulk	Not reported	3.5 ± 0.4	3.1 ± 0.9	1.4 ± 0.2
Ala	2.3 ± 1.1	3.4 ± 0.5	2.9 ± 1.0	1.1 ± 1.1
Asx	7.0 ± 1.0	4.4 ± 0.6	5.4 ± 0.8	5.2 ± 0.7
Glx	4.6 ± 1.6	3.8 ± 0.6	4.2 ± 1.2	2.2 ± 1.1
Gly	0.4 ± 1.1	1.9 ± 0.9	1.3 ± 1.0	-0.1 ± 0.9
His	Not reported	Not reported	Not reported	2.0 ± 0.7
Ile	Not reported	5.4 ± 0.5	5.8 ± 1.5	2.2 ± 1.5
Leu	3.4 ± 0.8	4.9 ± 0.2	4.3 ± 0.6	1.2 ± 1.0
Lys	-1.1 ± 0.7	Not reported	-0.8 ± 0.9	-0.3 ± 1.0
Phe	1.3 ± 0.5	0.3 ± 0.5	-0.3 ± 0.4	-1.3 ± 0.8
Pro	6.2 ± 0.8	5.2 ± 0.4	6.1 ± 0.6	5.2 ± 1.5
Ser	1.9 ± 0.7	1.9 ± 0.4	1.8 ± 1.1	3.7 ± 1.1
Thr	-11.9 ± 1.5	-11.4 ± 1.1	-4.4 ± 0.9	-8.6 ± 1.9
Tyr	-3.8 ± 0.7	Not reported	Not reported	0.2 ± 1.6
Val	3.2 ± 1.4	4.0 ± 0.6	3.4 ± 0.9	2.0 ± 1.2

Species, as well as whether studies were performed in non-adult animals, are reported. All studies were performed on red blood cells, apart from McMahon, Polito, et al. (2015), which was performed on penguin feathers. Bulk $\Delta^{15}\text{N}$ for the present study is for the larger dataset of gyrfalcon nestlings, as reported in Johnson et al. (2020).

general, estimated TDFs for trophic amino acids exceeded zero (Fig. 5; Table 1). Nevertheless, some trophic amino acids were more enriched than others. For example, TDF estimates for both Asx and Pro exceeded estimates for Glx, even though Glx has been the most frequently used trophic amino acid for

estimating trophic position. Although TDFs for Asx and Pro were similar to what has previously been estimated for birds, TDFs for other trophic amino acids (e.g., Glx, Ile, Leu) were lower in our study than in other avian studies (Table 1). Finally, consistent with prior studies and its purported role as a metabolic amino acid, TDF estimates for Thr were negative (Fig. 5).

Discussion

Bulk and compound-specific stable isotope analyses have proven to be effective approaches for investigating trophic ecology in marine and terrestrial ecosystems, but there is growing concern that results can be misleading if appropriate TDFs are not employed, as a variety of factors can potentially influence isotopic trophic discrimination. We found that amino acid-specific nitrogen isotopes better separated primary and secondary consumers than did bulk nitrogen isotopes, but differences in source amino acids among species suggest some animals were feeding in different biomes prior to sampling. We then used camera traps at gyrfalcon nests to estimate nestling diets and calculate TDFs for nitrogen stable isotopes. We found that both bulk (Johnson et al., 2023) and amino acid-specific (current study) nitrogen TDFs for gyrfalcon nestlings were substantially lower than values from the literature (see Table 1).

TDF_{Glx-Phe} values, which are typically used to estimate the trophic level of feeding, were particularly low in our study ($3.45\% \pm 1.7\%$) relative to 'universal' values proposed in early studies (TDF_{Glu-Phe} $\approx 7.6\%$; Chikaraishi et al., 2010). Notably, our estimated TDF_{Glx-Phe} values were lower than prior estimates for adult raptors (TDF_{Glx-Phe} $\approx 5.82\%$; Hebert et al., 2016), but were similar to what has been reported for penguins (McMahon, Polito, et al., 2015). In combination with prior studies of trophic discrimination in avian and mammalian predators (Germain et al., 2013; Hebert et al., 2016;

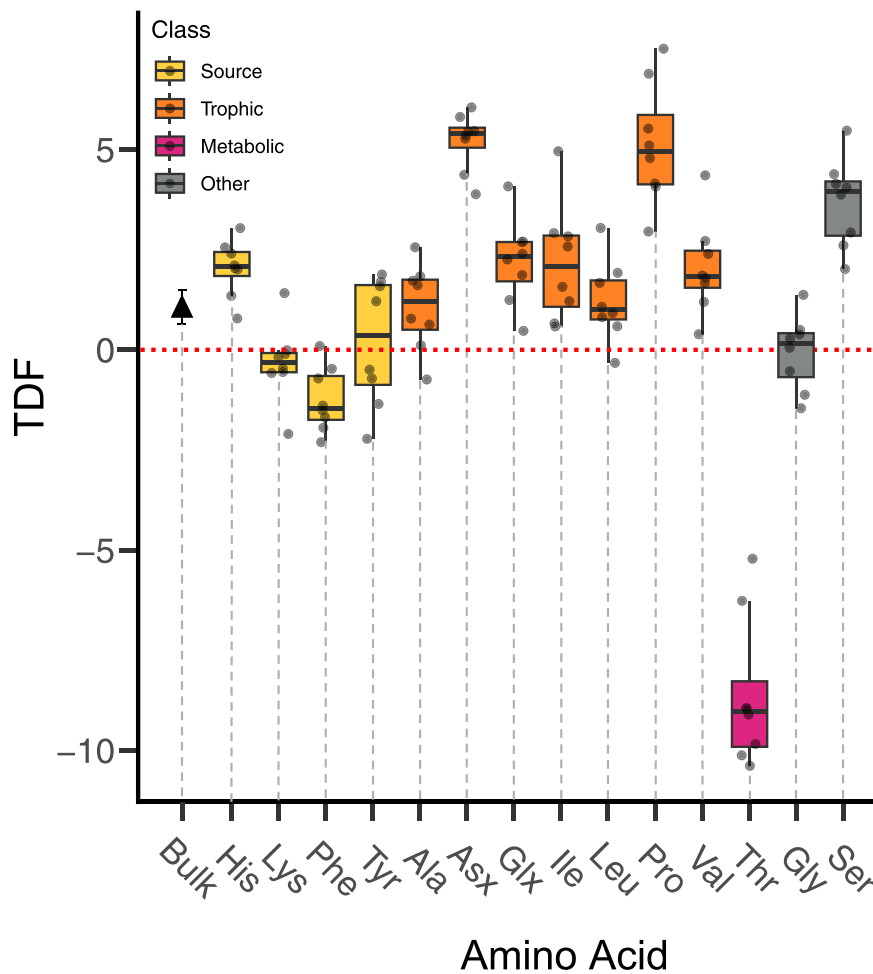


Figure 5 Boxplots of trophic discrimination factors (TDF) calculated for $\delta^{15}\text{N}$ values of individual amino acids across all gyrfalcon nestlings. Source, trophic, and metabolic amino acids are denoted by yellow, orange, and magenta boxplots, respectively. Lower and upper box boundaries delineate the 25th and 75th percentiles, respectively. The line inside the box denotes the median, and whiskers denote the lower and upper error lines, 10th and 90th percentiles, respectively. Bulk data (black triangle) represent previously reported values of trophic discrimination factors (TDF) calculated for bulk $\delta^{15}\text{N}$ values in gyrfalcon nestlings (Johnson *et al.*, 2022).

McMahon, Thorrold, *et al.*, 2015c), our results indicate that low TDF values for trophic amino acids are likely common outside of marine invertebrates and some fishes. Although the impacts of different putative cause(s) of particularly low TDF_{Glx-Phe} values in gyrfalcon nestlings cannot be ascertained with our study design, both the type of nitrogenous waste products produced by birds (*i.e.*, uric acid) and the quality and quantity of dietary protein can affect TDFs (McMahon & McCarthy, 2016). Given that TDFs for several trophic amino acids in our study are smaller than what has been previously reported for birds (see Table 1), we suggest that changes in nitrogen use efficiency due to rapid growth are likely an important additional contributing factor.

Bulk tissue $\delta^{15}\text{N}$ values are clearly useful in estimating trophic position (Boecklen *et al.*, 2011; Post, 2002), likely owing to enzymatic discrimination against the heavy isotope during the formation of nitrogenous wastes (Gannes *et al.*, 1998; Macko *et al.*, 1986). As expected, we found that animals

known to feed on other animals generally had higher bulk $\delta^{15}\text{N}$ values in their tissues compared with animals that feed on plants. However, a subset of Wilson's snipes, American golden plovers, and American robins had higher bulk $\delta^{15}\text{N}$ values than the three raptor species in our study, and we found some overlap between primary and secondary consumers. We were better able to separate primary and secondary consumers using PC1 from a principal component analysis of amino acid-specific $\delta^{15}\text{N}$ values, though the same subset of snipes, plovers, and robins that had high bulk $\delta^{15}\text{N}$ also had higher PC1 values than raptors. The cause of higher bulk and compound-specific $\delta^{15}\text{N}$ values of amino acids for insectivorous songbirds and shorebirds, relative to raptors, in our study is not clear but may stem from the use of non-terrestrial food resources (particularly for Wilson's snipes and American golden plovers) and/or feeding in other ecosystems prior to migrating to breeding grounds as $\delta^{15}\text{N}$ values for source amino acids of snipes, plovers, and robins were much lower than resident prey species

that are known to feed exclusively in the terrestrial biome (e.g., tundra voles, ptarmigan, and arctic ground squirrels). This highlights one difficulty with the use of amino acid $\delta^{15}\text{N}$ values to assess the trophic level of feeding, as current methods do not account for species that consume prey from multiple biomes. Higher trophic discrimination in songbirds and shorebirds may also occur because individuals sampled were adults, whereas raptors were nestlings, and growth is known to affect nitrogen-use efficiency.

While it is typically presumed that bulk $\delta^{15}\text{N}$ in tissues increases by $\sim 3\text{‰}$ – 3.4‰ with trophic level (Post, 2002), it has become increasingly clear that a variety of factors affect bulk TDFs (reviewed in Martínez del Rio *et al.*, 2009). Thus, while nitrogen stable isotopes provide a useful index of trophic position, they are subject to error. Amino acid-specific nitrogen stable isotopes have been proposed as a potentially more robust approach for estimating trophic level, though there is still a great deal that is unknown with regard to variability in nitrogen trophic discrimination of some amino acids (O'Connell, 2017), particularly Thr (Wallace & Hedges, 2016). The TDF_{Glu-Phe} estimate ($3.5\text{‰} \pm 1.7\text{‰}$) in our study was much lower than the previously estimated 7‰ – 8‰ difference in TDF_{Glu-Phe} of consumer versus prey that was originally developed to calculate trophic position (Chikaraishi *et al.*, 2010; McClelland & Montoya, 2002). Our findings are consistent with feeding studies in adult marine mammals (TDF_{Glu-Phe} = 4.3‰ ; Germain *et al.*, 2013), penguins (TDF_{Glu-Phe} = 3.5‰ ; McMahon, Polito, *et al.*, 2015), raptors (TDF_{Glu-Phe} = 5.4‰ ; Hebert *et al.*, 2016), and sharks (TDF_{Glu-Phe} = 2.1‰ ; Hoen *et al.*, 2014) that have revealed TDF_{Glu-Phe} values much smaller than the 'universal' values estimated from earlier ecosystem-wide studies. Though the exact causes of lower TDF_{Glu-Phe} in these groups are uncertain, they are likely at least partly related to the excretion of nitrogenous wastes as urea and uric acid, rather than ammonia (McMahon & McCarthy, 2016). However, low TDF_{Glu-Phe} has also been reported for a carnivorous teleost fish (TDF_{Glu-Phe} = 1.7‰ ; Hoen *et al.*, 2014), indicating other factors, such as diet quality (McMahon, Thorrold, *et al.*, 2015), can play an important role.

In our prior work, we used diet estimates from camera traps to calculate bulk TDFs in gyrfalcon nestlings and found that values were much lower than literature TDFs for raptors (Johnson *et al.*, 2023). Further, we demonstrated that applying bulk TDFs developed for gyrfalcon nestlings to BSIMMs greatly improved diet estimation compared with prior BSIMM studies of peregrine falcon (*Falco peregrinus*) and common buzzard (*Buteo buteo*) nestlings. Given our prior finding of low bulk $\delta^{15}\text{N}$ TDFs in gyrfalcon nestlings, we were interested in establishing whether amino acid TDF values were also low. Amino acid $\delta^{15}\text{N}$ TDF values for most trophic amino acids (i.e., Ala, Glx, Phe, Leu) were lower in our study than values previously reported for birds (Table 1). This may relate to the fact that raptor nestlings in our study were growing rapidly, as low TDFs have been reported for bulk $\delta^{15}\text{N}$ in seabird nestlings, which has been attributed to increased nitrogen-use efficiency during rapid growth (Sears *et al.*, 2009; Williams *et al.*, 2007). While recent studies support using additional trophic and

source amino acids in the estimation of trophic level (see Besser *et al.*, 2022), we suggest additional studies are needed to better establish the factors that influence amino acid-specific TDFs so that these can appropriately be accounted for.

Alternative approaches to captive feeding studies, which incorporate proportional diet estimates from a secondary high-accuracy method for a subset of the study population to calculate bulk or compound-specific TDFs, are becoming increasingly common (Elliott *et al.*, 2021; Johnson *et al.*, 2023; Newsome *et al.*, 2010). Increased use of these approaches may improve dietary estimates using stable isotope mixing models and allow for the identification of factors that alter TDFs. Nevertheless, there are some limitations of the approach. For example, it is important to generate proportional diet estimates that correspond to the temporal window of the isotopic turnover rate of the tissue in question. In our study, we collected diets from hatch until the time of sampling, but we do not have prey isotopic data across all years of the study and, therefore, must assume prey isotopes did not vary across years. Consistent with many isotope studies, we subsampled muscle tissue from prey rather than using a homogenate from whole prey samples. This has the benefit of excluding poorly digestible proteins (e.g., keratin in fur and feathers), but may result in bias if $\delta^{15}\text{N}$ values for amino acids in vital organs (e.g., liver, heart, GI tract) differ from skeletal muscle. We also were unable to determine which nestling within a nest consumed each prey item and, therefore, assumed that nestling diet was equivalent to the average diet of the nest, though this was supported by low levels of within-nest variability in bulk $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values (Johnson *et al.*, 2020). We also previously validated that the small bulk TDF values for gyrfalcon are more accurate than TDF values taken from the literature when applied using Bayesian stable isotope mixing models of free-living raptor nestlings (see Johnson *et al.*, 2023). At present, a lack of independent amino acid-specific $\delta^{15}\text{N}$ datasets for free-living raptor nestlings prevents validation of the amino acid-specific TDFs calculated in the present study, but we encourage future follow-up validation studies. Finally, we also encourage researchers to apply multiple approaches (e.g., nest cameras, fecal DNA metabarcoding, pellet analysis) to assess trophic ecology and/or diets whenever possible, as no one approach is infallible.

With continued technological improvements and reduced costs, we anticipate that the use of compound-specific stable isotope analyses will expand in animal ecology. However, our study, along with many others, indicates that the originally developed 'universal' TDF_{Glu-Phe} for estimating trophic level should not be applied to all species. Compound-specific approaches can also potentially improve diet estimation using BSIMMs. However, the use of stable isotopes to estimate diets and delineate food web structure requires appropriate TDFs for the taxa of interest (Kjeldgaard *et al.*, 2021). The use of methods that provide high-quality fine-scale dietary data for free-living animals, such as camera traps, provides an alternative to captive feeding trials that should help in generating better taxon-specific TDFs for amino acids. A greater recognition of the factors that affect trophic discrimination will improve the utility of stable isotope approaches for quantifying diets and understanding the processes that affect food web structure.

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Conflict of Interest

The authors confirm no conflict of interest.

Author contributions

DLJ and CTW conceived the ideas and designed the methodology; DLJ, DLA, MTH, and TLB conducted the fieldwork; DLJ conducted the lab work; AB, DLJ, and CTW analyzed the data; AB and CTW led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Data Availability Statement

Data are available from the Dryad Digital Repository (temporary URL for the purposes of review: <http://datadryad.org/stash/share/D0IITSbZjYAdsTVctcJfwKRK7kUBKluA5N0OSdBbxS4>).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Prey and predator species included in the study, along with their sample sizes and abbreviation as used in this study.

Table S2. Table denoting mean \pm SD $\delta^{15}\text{N}$ values of amino acids for each species, measured in parts per mil (‰).

Table S3. Depicts PCA loadings from PC1 and PC2 from analysis on all species of consumers included in the study.

Figure S1. Map of the Seward Peninsula, Alaska.

Figure S2. The first and second principal components (PC1 and PC2) for a principal component analysis (PCA) of 13 amino acid (excludes threonine) $\delta^{15}\text{N}$ values for all species.

Figure S3. The first and second principal components (PC1 and PC2) for principal component analyses (PCA) of the $\delta^{15}\text{N}$ values in raptor nestlings (GYRF = gyrfalcon, RLHA = rough-legged hawks, and GOEA = golden eagles).

Figure S4. Boxplots demonstrating the differences in compound specific $\delta^{15}\text{N}$ across species for glutamine and phenylalanine.

Figure S5. Density plots for TDFs calculated for all gyrfalcon nestlings included across all amino acids included in the study.