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Amino Acid Specific Stable Nitrogen Isotope Values in Avian Tissues: Insights from Captive American Kestrels and Wild Herring Gulls

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ABSTRACT: Through laboratory and field studies, the utility of amino acid compound-specific nitrogen isotope analysis (AA-CSIA) in avian studies is investigated. Captive American kestrels (*Falco sparverius*) were fed an isotopically characterized diet and patterns in $\delta^{15}\text{N}$ values of amino acids (AAs) were compared to those in their tissues (muscle and red blood cells) and food. Based upon nitrogen isotope discrimination between diet and kestrel tissues, AAs could mostly be categorized as source AAs (retaining baseline $\delta^{15}\text{N}$ values) and trophic AAs (showing ^{15}N enrichment). Trophic discrimination factors based upon the source (phenylalanine, Phe) and trophic (glutamic acid, Glu) AAs were 4.1 (muscle) and 5.4 (red blood cells), lower than those reported for metazoan invertebrates. In a field study involving omnivorous herring gulls (*Larus argentatus smithsonianus*), egg AA isotopic patterns largely retained those observed in the laying female's tissues (muscle, red blood cells, and liver). Realistic estimates of gull trophic position were obtained using bird Glu and Phe $\delta^{15}\text{N}$ values combined with β values (difference in Glu and Phe $\delta^{15}\text{N}$ in primary producers) for aquatic and terrestrial food webs. Egg fatty acids were used to weight β values for proportions of aquatic and terrestrial food in gull diets. This novel approach can be applied to generalist species that feed across ecosystem boundaries.

INTRODUCTION

Monitoring and research programs using top-trophic-level predatory birds have been conducted for decades. These species integrate lower food web processes, making them useful indicators of ecosystem conditions. Integral to these programs is the ability to accurately estimate organism trophic position. For example, changes in bird trophic position can provide insights into alterations in food web structure.¹ Trophic position is also an important factor to be considered when interpreting spatial and temporal trends in concentrations of contaminants that biomagnify in birds.^{2–4} One method with which to estimate trophic position is through the application of stable nitrogen isotope ratios ($^{15}\text{N}/^{14}\text{N}$ relative to a standard expressed as $\delta^{15}\text{N}$ values). Many studies have demonstrated that higher trophic level organisms have greater $\delta^{15}\text{N}$ values.⁵ In the past, most nitrogen isotopic studies of birds have been based upon the analysis of bulk matter in tissues, e.g., generation of one $\delta^{15}\text{N}$ value from the analysis of a tissue sample analyzed as a whole. However, spatial and temporal comparison of $\delta^{15}\text{N}$ values may not be appropriate because of potential differences in tissue composition and baseline $\delta^{15}\text{N}$ values. With respect to the latter issue, interpreting $\delta^{15}\text{N}$ values in a trophic context may only be appropriate when comparing individuals or species at one location during the same time

period. When comparing organism $\delta^{15}\text{N}$ values over time and across space, it is necessary to consider spatial differences and temporal changes in $\delta^{15}\text{N}$ values associated with nitrogen being incorporated into the base of food webs,⁶ even at one site. Many studies do this by measuring $\delta^{15}\text{N}$ values in primary invertebrate consumers, such as filter-feeding molluscs.⁷ These primary consumer values are used as baselines from which trophic position estimates can be generated for higher-order consumers.⁷ However, it is often not possible to collect such food web samples, either because of logistical difficulties associated with working in isolated locations (e.g., Arctic sites) or because samples are being studied, e.g., archived samples, for which no contemporary food web samples exist. Furthermore, such an approach to baseline correction assumes that the higher trophic level organisms being evaluated are solely linked to the food web integrated in time and space by the primary consumer. This may or may not be the case for organisms that exhibit individual specialization in feeding habits, e.g., wildlife such as birds.^{8,9}

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Many research and monitoring studies involving wildlife focus on birds and their eggs. Egg collections often form the basis of such studies.¹⁰ While egg collections may not be possible for all species, e.g., species at risk, they have a minimal impact on bird populations. Eggs can be used to measure a variety of end points (e.g., diet composition, chemical contaminants, and stress hormones) and are an effective way to evaluate how changes in the environment may be affecting top predators.^{4,11–14} When egg samples are the only sample matrix available, it is necessary to utilize a method that will generate both a baseline $\delta^{15}\text{N}$ value as well as a trophic position estimate solely from the wildlife sample being studied. Such an approach is feasible through compound-specific stable isotope analysis (CSIA).^{15–18} More specifically, the measurement of $\delta^{15}\text{N}$ values in individual amino acids (AA-CSIA) in egg samples offers a possible method to generate both baseline (or source) $\delta^{15}\text{N}$ values and trophic position. Most amino acids (AAs) can be categorized as either source or trophic AAs (sensu Popp et al.¹⁹). Source AAs have $\delta^{15}\text{N}$ values that retain the baseline or source $\delta^{15}\text{N}$ values in the food webs utilized by higher-order consumers.¹⁹ This is thought to reflect the fact that the dominant metabolic pathways for these AAs do not involve transamination reactions. Conversely, the amine groups on trophic AAs undergo extensive deamination and transamination, resulting in trophic discrimination of nitrogen isotopes.^{20,21} As a result, the $\delta^{15}\text{N}$ values of trophic AAs are greater in consumers than in their diet.^{18,19} To date, most studies applying the AA-CSIA approach to estimate organism trophic position have focused on using the difference in the $\delta^{15}\text{N}$ values of glutamic acid (Glu) and phenylalanine (Phe) as trophic and source AAs, respectively.^{18,19} A variety of studies involving a diverse range of taxa have consistently shown their utility in this context.^{17–24} Significant work has laid the foundation for applying the AA-CSIA approach to the evaluation of trophic position in aquatic systems. However, intertaxa differences in the degree of trophic discrimination of amino acids require further study, especially for groups such as birds, for which few studies have been conducted (but see refs 25 and 26). Results from Lorrain et al.²⁵ (penguin blood) and McMahon et al.²⁶ (penguin feathers) point to the likelihood that AA nitrogen isotope fractionation in birds is different than in lower-trophic-level metazoan invertebrates. However, the paucity of bird studies highlights the need for further avian research. In particular, captive work is required to evaluate the validity of the AA-CSIA approach for avian species in a controlled setting. To date, there has only been one such study.²⁶ Also required are studies examining a wider variety of tissue types, e.g., eggs, muscle, liver, and species, e.g., terrestrial carnivores and aquatic and terrestrial generalists. With respect to the latter, little effort has been devoted to applying the AA-CSIA approach to species that consume food originating from more than one type of ecosystem (e.g., aquatic and terrestrial). Here, we use an aquatic and terrestrial omnivore to describe a method for estimating trophic position in species that utilize resources from multiple ecosystems.

The issues outlined above were addressed through two studies. Study 1 was a diet to bird tissue comparison. Captive adult American kestrels (*Falco sparverius*), a terrestrial carnivore, were fed an isotopically characterized diet, tissue (muscle and red blood cell) samples were collected, and AA-specific $\delta^{15}\text{N}$ values were generated, and patterns in kestrel tissues were compared to their food to determine whether different AAs exhibited patterns in $\delta^{15}\text{N}$ values consistent with

source and trophic AAs. Study 2 was an adult tissue to egg comparison. Wild adult female herring gulls were captured in the field and sacrificed, and AA-specific $\delta^{15}\text{N}$ values were measured in tissues (muscle, red blood cell, and liver) and compared to values in eggs laid by the same female. Herring gulls are colonial waterbirds that generally focus on aquatic prey, but they are also opportunists that will shift their diets to whatever foods may be locally abundant, e.g., terrestrial foods. These two studies allow us to assess the AA-CSIA approach for evaluating trophic position in birds and their eggs, an important component of many avian-based environmental monitoring programs.

MATERIALS AND METHODS

Diet to Bird Tissue Comparison. Captive American kestrels were individually housed in outdoor flight pens at the United States Geological Survey Patuxent Wildlife Research Center (Laurel, MD). The five male American kestrels used in the captive feeding study were fully grown 1 year old adult birds. Kestrels fledge from the nest 28–31 days post-hatch, at which time they are fully grown. Hence, at the time of sampling, the kestrels used in this study would have been at adult mass for the previous 11 months. Birds were fed a constant ration during the study and maintained a similar level of activity in their flight pens. Therefore, with the exception of the first month post-hatch, the kestrels in this study would not have changed much in size or condition. Isotopic turnover in bird tissues is rapid compared to ectotherms but is tissue-specific, with isotopic half-lives ($t_{1/2}$) differing among tissues ($t_{1/2}$ is the time required for an isotope ratio to change 50% from its initial steady-state value to a new steady-state value). In birds, $t_{1/2}$ in red blood cells > muscle > liver, but due to the rapid metabolic rates of homeothermic birds, the $t_{1/2}$ of all these tissues is relatively rapid (<20 days).⁵ Therefore, the birds in this study should have been in an isotopic steady state with their diet. Kestrels were fed an isotopically characterized diet for months prior to sacrifice. Diets of these birds consisted primarily of day-old cockerels (domestic chicken, *Gallus gallus domesticus*) (90%) with some small rodents, i.e., mice (*Mus musculus*) (10%). The $\delta^{15}\text{N}$ values of these foods were determined, and using the above proportions, the isotopic composition of the kestrel diet was estimated arithmetically using a simple two-component isotope mass balance. Two weeks prior to sacrifice, red blood cell (RBC) samples were taken from the kestrels weekly (three time points) as a measure of whether their body tissues were in isotopic steady state with their diet. At sacrifice, RBC and pectoral muscle (1 g) samples were collected. Other body tissues were not available for isotopic examination as they were used to measure other end points as part of another study.²⁷ AA-specific stable nitrogen isotope analysis was performed on diet samples (whole homogenized cockerels and whole homogenized mouse) and kestrel tissues, i.e., RBC and muscle. This allowed an evaluation of the degree to which different AAs showed patterns in $\delta^{15}\text{N}$ values that were reflective of source or trophic AAs. Trophic discrimination factors (TDF) were calculated using $\delta^{15}\text{N}$ values of Phe and Glu in RBC and in muscle according to the following:

$$\begin{aligned} \text{TDF}_{\text{Glu-Phe}} &= \Delta\text{Glu} - \Delta\text{Phe} \\ &= (\delta^{15}\text{N}_{\text{Glu,kestrrel}} - \delta^{15}\text{N}_{\text{Glu,food}}) \\ &\quad - (\delta^{15}\text{N}_{\text{Phe,kestrrel}} - \delta^{15}\text{N}_{\text{Phe,food}}) \end{aligned} \quad (1)$$

Adult Tissue to Egg Comparison. A total of four wild female herring gulls were captured in May 2010, at Chantry Island, Lake Huron, Ontario, Canada, during the early incubation of eggs. Birds were sacrificed and tissues (liver, muscle, and RBC) collected. In addition, the entire clutch of fresh (i.e., no apparent embryonic development) eggs (2–3 eggs depending upon the nest) corresponding to each female was collected. These collections were done in accordance with Environment and Climate Change Canada's animal care protocols and under the authorization of appropriate collection permits. Upon collection, adult tissues were frozen in liquid nitrogen vapor, and eggs were placed in foam-lined cases. These samples were then transported to Environment and Climate Change Canada's National Wildlife Research Centre (NWRC) in Ottawa, ON. Upon arrival, tissue samples were maintained in liquid nitrogen vapor. Eggs were opened and egg contents, i.e., albumen and yolk, were homogenized together. Homogenized egg contents were stored at -40°C until they were analyzed. Prior to analysis, samples were freeze-dried. All adult tissues and two of the eggs from each nest were used for AA-specific stable nitrogen isotope analysis. Patterns of AA-specific $\delta^{15}\text{N}$ values in adult maternal body tissues and eggs were compared. Significant differences in $\delta^{15}\text{N}$ values among tissue types were envisioned given that gulls were not studied under controlled conditions. Furthermore, seabirds, such as gulls, are thought to be income breeders; that is, they primarily use exogenous resources for egg formation.²⁸ Hence, the degree to which tissues were in isotopic steady state could have influenced differences in tissue $\delta^{15}\text{N}$ values.

Trophic Position Estimates. Trophic position estimates stemming from the application of the AA-CSIA approach rely to a significant degree on defining the differences in $\delta^{15}\text{N}$ values between Glu and Phe in primary producers. These differences in $\delta^{15}\text{N}$ values between Glu and Phe in primary producers (termed beta (β) values) vary across ecosystems.²⁹ Herring gulls will consume both aquatic and terrestrial food; therefore, it was necessary to develop an approach to estimate the proportion of the gull diet that originated from aquatic and terrestrial sources. This was done through the measurement of egg fatty acids. Albumen and yolk were homogenized together and used for fatty acid analysis, as previously described.^{9,30,31} Higher levels of omega-3 fatty acids in body tissues and eggs of birds have been associated with increased omega-3 (O3) fatty acid consumption,^{32,33} and long-carbon-chain omega-3 FA are found at higher concentrations in phytoplankton-based food webs compared to terrestrial systems.³⁴ In herring gulls, the ratio of egg omega 3 to omega 6 (O6) fatty acids (O3/O6) increases with the proportion of aquatic food in the gull diet.¹ To define aquatic and terrestrial O3/O6 end-members, we used egg values from an obligate aquatic feeding species, i.e., the double-crested cormorant (*Phalacrocorax auritus*), and an obligate terrestrial feeder, the domestic chicken, respectively. Dietary protein for chickens is primarily from plant sources (i.e., soybean);³⁵ fish meal, if used, is only incorporated in small amounts because of possible flavor tainting.³⁵ O3/O6 ratios were measured in cormorant eggs collected from Lake Huron (mean O3/O6 (± 1 standard deviation (std dev)) = 0.72 ± 0.18 , $n = 15$). O3/O6 fatty acid values in chicken eggs were

taken from the literature³⁶ (O3/O6 = 0.06). As part of other research,¹ O3/O6 ratios were measured annually in herring gull egg contents collected through time at Chantry Island (1981–2014 O3/O6 range = 0.24–0.63; in 2010, O3/O6 = 0.24). An estimate of the herring gull diet originating from aquatic and terrestrial sources in 2010 was done according to Phillips and Gregg.³⁷ The 2010 Chantry Island herring gull O3/O6 egg value and the aquatic and terrestrial O3/O6 end-members as defined above were input into the model. The relative amounts of aquatic (27%, 95% CI: 19–36%) and terrestrial (73%, 95% CI: 64–81%) foods in the gull diet in 2010 were then incorporated into the following equation (modified from ref 17) estimating gull trophic position (TP):

$$\text{TP} = \left\{ [\Delta\delta^{15}\text{N}_{\text{GluPhe}} + f_{(\text{aq})} \times \beta_{(\text{aq})} + (1 - f_{(\text{aq})}) \times \beta_{(\text{terr})}] / \text{TDF}_{\text{bird}} \right\} + 1 \quad (2)$$

where $\Delta\delta^{15}\text{N}_{\text{GluPhe}}$ is the difference in egg $\delta^{15}\text{N}$ values between Glu and Phe, $f_{(\text{aq})}$ is the fractional abundance of aquatic food in the diet, $\beta_{(\text{aq})}$ represents the difference in $\delta^{15}\text{N}$ between Glu and Phe of primary producers associated with aquatic foodwebs (-3.4‰ for aquatic cyanobacteria and algae^{18,38,39}), and $\beta_{(\text{terr})}$ represents the difference in $\delta^{15}\text{N}$ between Glu and Phe of primary producers associated with terrestrial food webs ($+8.4\text{‰}$ for terrestrial C3 plants;³⁸ C4 plants are rare in the local, natural plant community).⁴⁰ TDF_{bird} is the trophic discrimination factor for Glu and Phe in kestrel red blood cells.

Amino-Acid-Specific Nitrogen Isotope Analysis. Isotopic analyses were performed on lyophilized samples at the University of Hawaii using established methods.^{19,41} Briefly, tissue samples were hydrolyzed and AAs were derivatized prior to nitrogen isotope analysis. Dried, ground tissue (1–5 mg) was hydrolyzed (6 N HCl) at 150°C for 70 min.⁴² Acid hydrolysis under these conditions cleaves the terminal amide groups in glutamine (Gln) and asparagine (Asn), resulting in the conversion of these AAs to Glu and aspartic acid (Asp), respectively. Thus, the isotope value of a combined Glu + Gln is measured (termed Glu here), and the isotope value of a combined Asn + Asp is measured (termed Asp here). Acid hydrolysis results in the destruction of tryptophan and cystine. Hydrolysates were dissolved in 0.01 N HCl and then passed through a 0.2 μm poly(ether sulfone) membrane (VWR), followed by cation-exchange chromatography using a vacuum manifold with commercial cartridges (GracePure SPE Cation-X). Samples were esterified by heating at 110°C for 60 min in 4:1 isopropanol–acetyl chloride, after which AAs were acylated by heating at 100°C for 15 min in 3:1 methylene chloride–trifluoroacetic anhydride (TFAA). Using a 2:1 P-buffer–chloroform mix (1 M P-buffer: $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ in Milli-Q water, pH 7), AAs were further purified by partitioning into chloroform, discarding any compounds dissolved in the aqueous phase.⁴³ A final acylation step was repeated, and samples were stored in 3:1 methylene chloride–TFAA at -20°C for up to 6 months. Samples were dried under N_2 at ambient temperature and dissolved in ethyl acetate just prior to isotope analysis. $\delta^{15}\text{N}$ values (‰ , relative to atmospheric N_2) of derivatized samples were determined using an isotope ratio mass spectrometer (either a Thermo Scientific Delta V Plus or a Thermo Scientific MAT 253) interfaced to a trace GC gas chromatograph (Thermo Finnigan) via a GC-C III combustion interface (Thermo Finnigan 980°C). Samples were injected (split–splitless injector in splitless mode) onto a forte BPX5 capillary column (60 m $\times$ 0.32 mm $\times$ 1.0 μm film thickness) at

an injector temperature of 180 °C with a constant helium flow rate of 1.4 mL/min. The column was initially held at 50 °C for 2 min and then increased to 120 °C at a rate of 15 °C/min. Once at 120 °C, the temperature was increased at a rate of 4 °C/min to 195 °C, increased by 5 °C/min to 255 °C, and then increased by 15 °C/min to 300 °C, where it was held for 8 min. All samples were analyzed at least in triplicate. Between triplicate runs of each sample, a suite of AAs (alanine, threonine, isoleucine, proline, Glu, and Phe) of known isotopic composition were analyzed. In addition, internal reference compounds, norleucine and aminoadipic acid, were coinjected with these AAs and all samples. For isotopic correction of unknown AAs when co-injected standards were unusable due to co-elution effects, a linear correction was derived from the AA suites run immediately before and after the triplicate sample analysis and applied to measured isotope ratios. The standard deviation of $\delta^{15}\text{N}$ values derived from replicate analyses averaged 0.52‰ and ranged from 0.03‰ to 1.2‰. In the bird samples analyzed here, $\delta^{15}\text{N}$ values of 14 AAs were quantified: alanine (Ala), Asp, glycine (Gly), Glu, isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), Phe, proline (Pro), serine (Ser), threonine (Thr), tyrosine (Tyr), and valine (Val). AAs were categorized as source, trophic or metabolic AAs according to previous studies:^{19,44} trophic AAs (Ala, Asp, Glu, Ile, Leu, Pro, and Val), source AAs (Gly, Lys, Ser, Tyr, Phe, and Met), and metabolic AA (Thr). It is important to note that these AA categorizations were only used to facilitate the assessment of whether bird isotopic values followed patterns previously reported for lower trophic level metazoan organisms. However, deviations from these categories were observed in birds (see the Results section) and other taxa.²¹

In the kestrel study, repeated measures ANOVA followed by Tukey's HSD test were used to test for statistically significant differences in amino acid-specific $\delta^{15}\text{N}$ values in RBC collected across the three periods. Statistical differences in amino acid-specific $\delta^{15}\text{N}$ values in RBC versus muscle at the time of sacrifice were examined using two-tailed *t* tests. Separate one-sample *t* tests were used to determine whether differences between $\delta^{15}\text{N}$ values in kestrel diet and tissues (RBC ($\Delta\delta^{15}\text{N}_{\text{R-D}}$) and muscle ($\Delta\delta^{15}\text{N}_{\text{M-D}}$)) were significantly different from zero ($\alpha = 0.05$). Single-sample *t* tests were also used to determine whether differences in $\delta^{15}\text{N}$ values between herring gull tissues (RBC, muscle, and liver) and eggs ($\delta^{15}\text{N}_{\text{tissue}} - \text{average } \delta^{15}\text{N}_{\text{egg}}$) were significantly different from zero ($\alpha = 0.05$). AA isotopic data used in the tests described above met parametric assumptions, i.e., normal distribution (Kolmogorov–Smirnov test) and homogeneity of variance (Brown–Forsyth test). All tests were performed with $\alpha = 0.05$ using Statistica version 12.⁴⁵

RESULTS

Diet to Bird Tissue Comparison. For 11 AAs, sufficient data were available allowing statistical comparisons of AA-specific $\delta^{15}\text{N}$ values in RBC across periods. Only Asp ($F(2, 8) = 5.0$, $P = 0.04$) and Pro ($F(2, 8) = 15.5$; $P = 0.002$) showed statistically significant interperiod differences in AA-specific $\delta^{15}\text{N}$ values (Figure 1 and Table 1). At the time birds were sacrificed, there were significant differences in $\delta^{15}\text{N}$ values between RBC and muscle for five AAs: Asp ($t(8) = 4.8$, $P = 0.001$), Gly ($t(8) = 6.0$, $P < 0.001$), Leu ($t(8) = 2.5$, $P = 0.04$), Pro ($t(8) = 3.0$, $P = 0.02$), and Thr ($t(8) = 2.5$, $P = 0.04$; Figure 1 and Table 1). No statistically significant differences were observed between tissues for Ala, Glu, Lys, Phe, Ser, Tyr, or Val

Figure 1. Mean $\delta^{15}\text{N}$ values (± 1 std dev) of individual amino acids in male American kestrel tissues and their food (triangles). Tissue samples consisted of red blood cells (RBC; open circles) and muscle (squares). RBC were collected at three time points: at time of sacrifice and at 1 and 2 weeks prior to euthanasia. Categorizations of amino acids as trophic, source, or metabolic (MB) amino acids are from refs 19 and 44.

($P > 0.05$). Relative patterns of AA-specific $\delta^{15}\text{N}$ values in RBC and muscle were similar (Figure 1). The degree to which there were differences between $\delta^{15}\text{N}$ values of food and kestrel tissues was AA-specific (Figure 1). In general, these differences reflected previous categorizations of AAs as source, trophic, or metabolic AAs.^{19,44} However, there were some notable differences. In particular, previously categorized source AAs, Gly and Ser, showed significant trophic discrimination in bird tissues (Figures 1 and 2). Of the trophic AAs, Pro exhibited the greatest increase in $\delta^{15}\text{N}$ values from food to bird tissues (Figure 2), while the source AAs showing the least degree of change in $\delta^{15}\text{N}$ between food and kestrel tissues were Phe, Lys, and Met (Figure 2). Mean (± 1 std dev) TDF_{Glu-Phe} in RBC and muscle were $5.39 \pm 1.26\text{‰}$ and $4.07 \pm 1.03\text{‰}$, respectively. TDF_{Glu-Phe} derived from RBC was statistically greater than that derived from muscle ($t(18) = 2.12$, $P = 0.048$).

Adult Tissue to Egg Comparison. Relative patterns of AA-specific $\delta^{15}\text{N}$ values in adult female herring gull tissues (i.e., liver, muscle, and RBC) were similar to those observed in kestrels (Figure 3). Patterns generally followed those expected for previously categorized source, trophic, and metabolic AAs.^{19,44} However, similar to kestrels, Gly and Ser showed deviations from $\delta^{15}\text{N}$ values expected for source AAs signaling significant trophic discrimination of nitrogen isotopes in body tissues and eggs of herring gulls (Figure 3). AA-specific patterns in $\delta^{15}\text{N}$ values of herring gull body tissues and eggs were largely similar (Figure 3). However, differences in tissue and egg $\delta^{15}\text{N}$ values did show deviations from zero for seven of the 14 muscle–egg AA comparisons, five of the liver–egg AA comparisons, and three of the RBC–egg comparisons (Figure 4, Table 2). In general, the trophic AA displayed greater differences between tissue and egg $\delta^{15}\text{N}$ values. For example, Ala, Asp, and Glu displayed relatively high differences in tissue–egg $\delta^{15}\text{N}$ values. Conversely, other trophic AAs, such as Pro, showed no differences in tissue–egg $\delta^{15}\text{N}$ values. Intertissue differences in $\delta^{15}\text{N}$ values were least apparent for comparisons between RBC and eggs.

Table 1. Mean ($\pm$ 1 std dev) $\delta^{15}\text{N}$ Values in Amino Acids Measured in Tissues of Captive American Kestrels^a

tissue sampling period	RBC 1	RBC 2	RBC 3	muscle 3	diet NA	$\Delta\delta^{15}\text{N}_{\text{R-D}}$	$\Delta\delta^{15}\text{N}_{\text{M-D}}$
Ala	7.24	7.48	8.06	8.98	5.77	2.29**	3.21***
<i>n</i>	5	5	5	5	3		
SD	0.33	0.29	1.07	0.70	0.73	1.07	0.70
Asp	11.66 ^B	12.20 ^{AB}	12.49^A	9.99	5.45	7.04***	4.54***
SD	0.43	0.88	1.04	0.51	0.43	1.04	0.51
Glu	10.73	11.04	11.40	9.87	6.85	4.55**	3.02***
SD	0.61	0.84	1.56	0.47	0.11	1.56	0.47
Gly	6.02	5.99	5.96	9.41	5.57	0.39 ^{NS}	3.84***
SD	0.70	0.61	1.08	0.68	0.34	1.08	0.68
Ile	NA	NA	NA	11.38	5.31	NA	6.07*
<i>n</i>				5	3		5
SD				3.23	0.75		3.23
Leu	6.19	6.48	6.89	7.95	3.53	3.36***	4.42***
SD	0.48	0.47	0.80	0.48	0.60	0.80	0.48
Lys	−1.06	−1.04	−1.27	−0.72	−0.17	−1.10*	−0.55 ^{NS}
SD	1.32	0.82	0.71	0.95	0.30	0.71	0.95
Met	NA	NA	NA	1.31	1.13	NA	0.18 ^{NS}
<i>n</i>				5	3		
SD				1.12	1.53		
Phe	3.51	3.15	3.21	3.44	4.48	−1.26**	−1.04*
SD	0.25	0.47	0.53	0.81	0.81	0.53	0.81
Pro	12.98 ^B	13.38 ^B	13.97^A	15.23	7.79	6.18***	7.45***
SD	0.68	0.89	0.83	0.43	0.43	0.83	0.43
Ser	8.70	9.04	8.66	8.79	6.79	1.87**	2.00*
SD	0.24	0.55	0.70	1.24	0.67	0.70	1.24
Thr	−14.42	−14.74	−14.72	−16.90	−2.86	−11.86***	−14.04***
SD	0.75	0.86	1.54	1.25	0.76	1.54	1.25
Tyr	1.62	2.08	1.18	2.71	4.97	−3.79 ^{NS}	−2.26**
<i>n</i>	1	2	2	5	3	2	5
SD	NA	0.86	0.71	0.84	0.26	0.71	0.84
Val	7.88	8.52	8.87	8.29	5.66	3.21**	2.63*
SD	0.54	0.28	1.40	1.62	0.71	1.40	1.62

^aRed blood cells (RBC) were collected at weekly intervals beginning 2 weeks prior to birds being sacrificed (period 1, 2 weeks prior to sacrifice; period 2, 1 week prior to sacrifice; period 3, time of sacrifice). At sacrifice, muscle was also collected. AA-specific $\delta^{15}\text{N}$ values in the kestrel diet are also shown as is the change in $\delta^{15}\text{N}$ values between diet and tissue at the time of sacrifice (RBC vs diet: $\Delta\delta^{15}\text{N}_{\text{R-D}}$; muscle vs diet: $\Delta\delta^{15}\text{N}_{\text{M-D}}$). Sample size for all AAs in kestrels and in food is the same as Ala except where indicated. Statistically significant inter-period differences in AA $\delta^{15}\text{N}$ values in RBC are indicated by superscript letters beside means. Statistically significant differences in mean $\delta^{15}\text{N}$ values of RBC and muscle at time of sacrifice are indicated in bold for individual AA (Asp, Gly, Leu, Pro, and Thr). The results of one-sample *t* tests ($\alpha = 0.05$) to determine whether individual AA $\Delta\delta^{15}\text{N}_{\text{R-D}}$ and $\Delta\delta^{15}\text{N}_{\text{M-D}}$ values were significantly different from 0‰ are indicated (not significant (NS), $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Trophic Position Estimates. Egg $\delta^{15}\text{N}$ values for Glu and Phe were used in conjunction with the equation defined in the [Materials and Methods](#) section (red blood cell TDF, 5.4) to estimate herring gull trophic position. Trophic position estimates ranged from 2.8 to 3.7 with a mean ($\pm$ 1 std dev) value of 3.0 ($\pm$ 0.3). If TDF_{bird} was the trophic discrimination factor for Glu/Phe in kestrel muscle (4.1), then trophic position estimates ranged from 3.3 to 4.6 with a mean ($\pm$ 1 std dev) value of 3.6 ($\pm$ 0.4).

DISCUSSION

Results from this study and another recent controlled feeding study involving penguins²⁶ indicate that, in general, birds exhibit similar patterns in AA-specific $\delta^{15}\text{N}$ values as those reported for other metazoan taxa.^{17–19} For example, all of the AAs previously defined as trophic AAs¹⁹ showed ^{15}N enrichment in birds compared to levels in their diets. There were, however, some important intertaxa differences in those

AAs previously identified as source AAs. AAs exhibiting ^{15}N trophic discrimination in kestrels included Ala, Asp, Glu, Ile, Leu, Pro, and Val as well as the “source” AAs, Gly and Ser. Of all of these AAs, Pro showed the greatest trophic discrimination between diet and consumer tissues (6–7‰), similar to what has been reported for other high-trophic-level marine endotherms.^{25,44} This differed from what has been observed in zooplankton and fish in which Glu or Ala showed the greatest trophic enrichment of ^{15}N .^{15,17,18,23,24} McMahon et al.⁴⁶ found that Pro was the least-sensitive trophic AA to changes in trophic discrimination associated with diet quality. Furthermore, trophic discrimination factors for Pro–Phe have been found to be relatively similar among consumer taxa, trophic levels, and diet types.^{17,18,20,26,44,46} However, there was little correlation between trophic discrimination factors for Pro–Phe and trophic position in marine teleosts,²³ and in a meta-analysis of AA-CSIA results, including those from penguins, trophic discrimination factors for Pro–Phe were quite different in herbivores compared to those in omnivores

Figure 2. Mean difference in $\delta^{15}\text{N}$ values (± 1 std dev) of American kestrel food and kestrel tissue, i.e., muscle and red blood cells. Categorizations of amino acids as trophic, source, or metabolic amino acids are from refs 19 and 44.

and carnivores.²⁴ Hence, these findings may point to the potential utility of Pro as an alternative to Glu in some trophic position calculations, although with caution. Significant differences in $\delta^{15}\text{N}$ values of Pro between RBC and muscle were found for the kestrels but not for the gulls studied. It is interesting to note that the only two AA (Asp and Pro) that showed statistically significant temporal differences in RBC $\delta^{15}\text{N}$ values were also the two that exhibited the greatest degree of trophic discrimination in kestrels. We can only speculate on the reasons for this, but one possibility is that change in internal

Figure 4. Mean difference in $\delta^{15}\text{N}$ values (± 1 std dev) in adult female herring gull body tissues and their eggs. Categorizations of amino acids as trophic, source, or metabolic amino acids are from refs 19 and 44.

isotopic fractionation of these compounds was occurring rapidly in kestrels. Further research, including controlled feeding studies, are needed to address this issue.

In kestrels, tissue $\delta^{15}\text{N}$ values of Phe, Lys, and Met showed negligible trophic enrichment of ^{15}N ; however, Phe and Lys did exhibit small decreases in $\delta^{15}\text{N}$ values relative to diet. As mentioned above, evidence of trophic discrimination of ^{15}N in Gly and Ser indicated they should not be considered as source AAs for birds (also see refs 16, 26, and 44). $\delta^{15}\text{N}$ values of Ser were greater in both muscle and RBC than in kestrel food,

Figure 3. $\delta^{15}\text{N}$ values in body tissues (stars: red blood cells; triangles: liver; inverted triangles: muscle) from wild-caught adult female herring gulls and eggs (circles) laid by the same bird. Each panel shows results for one female and her eggs. Categorizations of amino acids as trophic, source, or metabolic amino acids are from refs 19 and 44.

Table 2. Mean (± 1 std dev, $n = 4$ Individuals) Differences between $\delta^{15}\text{N}$ Values in Adult Female Herring Gull Tissues (i.e., Liver, Muscle, and Red Blood Cells) and Average Values in Their Eggs ($n = 2$ from Each Bird)^a

amino acid	$\Delta\delta^{15}\text{N}_{\text{L-E}}$	$\Delta\delta^{15}\text{N}_{\text{M-E}}$	$\Delta\delta^{15}\text{N}_{\text{R-E}}$
alanine	3.2 ± 0.6 (10.5**)	2.8 ± 1.0 (5.5*)	1.3 ± 1.6 (1.6)
aspartic acid	3.6 ± 0.5 (15.1***)	2.4 ± 1.1 (4.4*)	1.7 ± 1.4 (2.5)
glutamic acid	3.3 ± 0.8 (7.9**)	2.5 ± 1.3 (3.9*)	2.2 ± 1.5 (3.0)
glycine	3.8 ± 0.8 (9.3**)	-2.1 ± 2.1 (2.0)	-2.7 ± 1.3 (4.2*)
isoleucine	-0.8 ± 2.4 (0.6)	0.4 ± 1.6 (0.4)	
leucine	0.4 ± 0.9 (1.0)	2.7 ± 1.1 (5.0*)	1.1 ± 1.6 (1.4)
lysine	-0.4 ± 0.9 (0.9)	0.9 ± 0.6 (3.3*)	0.1 ± 1.1 (0.1)
methionine	0.1 ± 0.5 (0.4)	0.8 ± 2.0 (0.8)	
phenylalanine	-0.1 ± 1.4 (0.1)	0.9 ± 0.5 (3.3*)	0.7 ± 0.9 (1.6)
proline	0.4 ± 1.0 (0.7)	1.1 ± 1.5 (1.5)	1.8 ± 2.1 (1.7)
serine	1.7 ± 1.1 (3.1)	-0.5 ± 1.0 (1.1)	-1.0 ± 1.1 (1.8)
threonine	-4.5 ± 1.3 (6.7**)	-6.9 ± 1.5 (9.1**)	-3.8 ± 2.1 (3.7*)
tyrosine	-0.5 ± 0.9 (1.1)	0.1 ± 1.4 (0.2)	-1.9 ± 0.4 (7.9*)
valine	1.2 ± 0.8 (2.9)	2.1 ± 1.4 (3.0)	0.9 ± 1.0 (1.8)

^aResults for individual amino acids are shown. $\Delta\delta^{15}\text{N}_{\text{L-E}}$, $\Delta\delta^{15}\text{N}_{\text{M-E}}$, and $\Delta\delta^{15}\text{N}_{\text{R-E}}$ are differences between liver and egg, muscle and egg, and RBC and egg, respectively. Separate one-sample t tests were used to determine whether differences in $\delta^{15}\text{N}$ values between tissue and egg were significantly different from zero (t test results and significance [$*$, $P < 0.05$; $**$, $P < 0.01$; $***$, $P < 0.001$] are in parentheses).

while muscle Gly $\delta^{15}\text{N}$ values were greater than in food. Although we did not have specific information regarding the diets of the wild herring gulls studied here, results for that species showed elevated $\delta^{15}\text{N}$ values in Gly and Ser compared to those of other candidate source AAs. This provided further support arguing against the use of these AAs as source AAs in birds.

Threonine $\delta^{15}\text{N}$ values measured in kestrels and gulls displayed extreme ^{15}N depletion relative to all other AAs. This finding was consistent with previous reports.^{16,19,23,44,47–49} Factors proposed for low Thr $\delta^{15}\text{N}$ values included the possibility of progressive lowering of $\delta^{15}\text{N}$ values with trophic position,^{23,50} with longevity, due to nutritional stress,¹⁶ or because the organisms were associated with marine environments. Depletion of ^{15}N in terrestrial and freshwater (gulls in this study were from freshwater) organisms examined here and elsewhere^{16,50} indicate that patterns in Thr $\delta^{15}\text{N}$ values cannot be explained by connections to marine food webs. Also, the kestrels in our study were fed ad libitum, so they should not have been experiencing nutritional stress. Clearly, the factors underlying $\delta^{15}\text{N}$ values in Thr require further study.

Amino acid specific patterns in $\delta^{15}\text{N}$ values of tissues in adult female herring gulls and their eggs were similar, indicating that eggs largely retained the isotopic compositions of adult tissues. This finding is important as it provides a foundation for applying the AA-CSIA approach to evaluate bird trophic position using eggs. Differences in $\delta^{15}\text{N}$ values between eggs and other tissues were least apparent for eggs and RBC. Therefore, nondestructive sampling of RBC could provide similar trophic position information to that obtained through the collection of eggs. Both of these sampling matrices are preferable to the lethal sampling required to collect muscle or liver tissue. Furthermore, differences in tissue and egg $\delta^{15}\text{N}$ values were more apparent in muscle and liver. Differences in Glu $\delta^{15}\text{N}$ values between eggs and muscle or liver could result in trophic position estimates stemming from AA-CSIA analysis of eggs being lower than those based upon the analysis of liver or muscle. A possible mechanism contributing to tissue–egg isotopic differences could stem from the fact that gulls are primarily income breeders, with egg isotope patterns reflecting less dietary isotopic discrimination than body tissues.

Furthermore, differences between body tissues and eggs could have reflected intertissue differences in isotopic routing and rates of isotopic turnover. All of these possibilities require further investigation. It is interesting to note that Pro showed no differences in tissue–egg $\delta^{15}\text{N}$ values, indicating that its use as a trophic AA to assess trophic position would result in consistent trophic position estimates regardless of the type of tissue analyzed. The use of Pro as an alternative to Glu in trophic position calculations has been suggested previously⁴⁶ and merits further study, provided that Pro $\delta^{15}\text{N}$ values show consistent enrichment in ^{15}N across taxa.

In AA-CSIA studies to date, Glu and Phe have been most widely used as trophic and source AAs, respectively. Recent studies have highlighted the potential value of utilizing multiple source and trophic AAs when estimating organism trophic position,^{23,24} but it is evident that care must be taken to ensure that AAs designated as source and trophic AAs consistently fall into those categories across taxa. Further research should investigate the advantages of using a multiple AA approach for estimating trophic position. For our estimates of trophic position, we focus on the use of the canonical AA, i.e., Phe and Glu, which have consistently demonstrated source (i.e., Phe) and trophic (i.e., Glu) AA characteristics across a variety of taxa.

Accurate trophic position estimates depend on the application of taxa-specific trophic discrimination factors (TDF) for Phe and Glu. For lower trophic level metazoan aquatic organisms, $\text{TDF}_{\text{Glu-Phe}}$ has traditionally been thought to be on the order of 7.6‰,^{17,18} but recent research has indicated that a lower value ($\sim 5.7 \pm 0.3\text{‰}$) may be more appropriate for teleost fish.²³ Furthermore, even lower $\text{TDF}_{\text{Glu-Phe}}$ values have been measured in marine mammals (i.e., seal serum and muscle)⁴⁴ and birds (penguin feathers)²⁶ or estimated (based upon expected trophic position) in birds (i.e., penguin whole blood)²⁵ and elasmobranchs (i.e., stingray and shark muscle).⁵¹ In the kestrels studied here, $\text{TDF}_{\text{Glu-Phe}}$ derived from RBC was statistically greater than that derived from muscle. This pattern was consistent with results for harbor seals (*Phoca vitulina*) that showed greater $\text{TDF}_{\text{Glu-Phe}}$ values in blood serum compared to those in muscle.⁴⁴ Kestrel muscle $\text{TDF}_{\text{Glu-Phe}}$ was very similar to that reported for penguin whole blood. Regardless of intertissue differences in $\text{TDF}_{\text{Glu-Phe}}$ values, both $\text{TDF}_{\text{Glu-Phe}}$ derived from

kestrels were consistent with lower values reported in seals, penguins, and elasmobranchs. Different hypotheses have been put forward to explain these lower $TDF_{Glu-Phe}$, including growth status, feeding ecology, and differences in nitrogen metabolism and excretion.^{24,44,46} In our study, the growth status of the kestrels examined would not have been important as the birds were fully grown. However, the latter two factors could have contributed to the lower $TDF_{Glu-Phe}$ values in kestrels. Diet quality and degree of carnivory^{24,46} could have been important in contributing to the lower $TDF_{Glu-Phe}$ values observed here for kestrels. Similarly, differences in nitrogen processing and production of waste products, i.e., urea and uric acid in mammals and birds versus ammonium in fish (see refs 21 and 44) could have played a role in generating lower $TDF_{Glu-Phe}$ values in kestrels. It is worth noting that the birds used in captive feeding studies to date, i.e., penguins and kestrels, are divergent evolutionarily yet show similar source and trophic AA patterns and similar $TDF_{Glu-Phe}$ values. This may indicate that the patterns observed here and in McMahon et al.²⁶ may be broadly applicable across carnivorous avian species (see ref 24). To better understand the mechanism underlying lower $TDF_{Glu-Phe}$ values in birds, it will be necessary to conduct controlled feeding studies using omnivorous and herbivorous species. Regardless of the mechanisms regulating $TDF_{Glu-Phe}$ values, it is apparent that there are consistent differences in $TDF_{Glu-Phe}$ values among taxa that point to the need of adopting a multi-TDF approach when calculating trophic position across entire food webs (as advocated in refs 44 and 52). The trophic position estimates generated here, based upon AA-CSIA analysis of gull eggs, resulted in estimates of gull trophic position at the Chantry Island colony that were consistent with the known feeding habits, i.e., considerable quantities of terrestrial prey, of birds nesting at that location. Application of the multisource trophic position equation complicates the estimation of trophic position because it requires knowledge of the proportions of aquatic versus terrestrial food in the consumer diet. However, not considering the potential for terrestrial foods to be incorporated into Chantry Island gull diets results in unrealistically low trophic position estimates for these birds (eq 2 100% aquatic diet; mean ± 1 std dev = 1.4 ± 0.3). The novel method applied here for assessing trophic position in a species that crosses ecosystem boundaries to feed resulted in more-realistic estimates of trophic position in this omnivorous predator. This finding suggests that in birds in which trophic position is reasonably well-known, the proportions of aquatic and terrestrial foods might be estimated from AA-CSIA by rearranging eq 2.

There is great potential to apply the AA-CSIA approach to generate baseline and trophic level $\delta^{15}N$ values in eggs collected as part of avian monitoring programs. Further AA-CSIA analysis of samples from these programs, e.g., egg contents, possibly eggshell, could improve our understanding of intertissue similarities and differences in nitrogen isotope discrimination. Such information is critical for expanding the scope of avian CSIA research into fields such as paleoecology. Eggshells obtained from archeological sites and housed in museum collections could be a rich source of material for AA-CSIA studies investigating changes in bird trophic position over long periods of time. AA-CSIA approaches could also assist with the interpretation of other types of information generated from avian research and monitoring programs. Frequently, these involve the collection and analysis of eggs to assess spatial

and temporal patterns in levels of biomagnifying contaminants in the environment. Organism trophic position is a critical factor regulating chemical exposure and uptake, but most work has focused on using bulk $\delta^{15}N$ values to assess organism trophic position. Application of the AA-CSIA approach offers a new method to estimate organism trophic position while adjusting “internally” for possible baseline shifts in nitrogen isotope values. Trophic position-normalized contaminant levels are more suitable for deducing the factors, e.g., proximity to sources and changes in contaminant emissions as a result of mitigation activities that regulate spatial and temporal differences in contaminant levels in wildlife. Clearly, opportunities to apply AA-CSIA continue to expand; here, we strengthen the foundation upon which to apply this approach in avian research and monitoring studies.

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Notes

The authors declare no competing financial interest.

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