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Amino Acid-Specific Nitrogen Isotope Discrimination in Raptor Nestlings: Advancing Trophic Position Estimation

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ABSTRACT

Background: Stable isotope analysis is a cornerstone of trophic ecology, yet estimating trophic position (TP) accurately is often hindered by variable bulk tissue nitrogen trophic discrimination factors (TDFs). Amino acid-specific compound-specific isotope analysis (AA-CSIA) offers enhanced resolution by differentiating metabolically distinct amino acids, providing a more precise tool for tracing nitrogen flow through food webs. Empirical data on AA-specific TDFs, particularly in developing avian predators, remains limited.
Objectives: This study aimed to determine amino acid-specific nitrogen stable isotope trophic discrimination factors (Δ15NAA) in raptor nestlings under controlled dietary conditions. We evaluated the variability of these TDFs among different amino acids and assessed their implications for refining trophic position estimation in these ecologically significant predators.
Methods: We reared raptor nestlings on a precisely controlled diet with a known isotopic signature. Tissues (e.g., blood, feathers) were collected at various developmental stages. Amino acids were extracted, derivatized, and analyzed for their δ15N values using gas chromatography/combustion/isotope ratio mass spectrometry. Δ15NAA values were calculated by comparing nestling tissue and diet amino acid δ15N values. Trophic positions were then estimated using established AA-CSIA models.
Results: Significant variability in Δ15NAA was observed across different amino acids, with 'trophic' amino acids showing consistent enrichment relative to 'source' amino acids. These empirically derived TDFs allowed for more accurate and less variable trophic position estimates in nestlings compared to those derived from bulk tissue analysis. Our findings provide species-specific TDFs critical for robust trophic modeling.
Conclusion: This study establishes precise amino acid-specific nitrogen trophic discrimination factors for raptor nestlings, significantly enhancing the accuracy of trophic position estimation in these crucial avian predators. Integrating these refined TDFs into ecological studies will improve our understanding of raptor foraging ecology, food web dynamics, and conservation strategies in a changing environment.

KEYWORDS: Trophic position, stable isotopes, amino acid, raptors, Gyrfalcon, trophic discrimination factor, Falco rusticolus.

INTRODUCTION

Stable isotope analysis (SIA) has emerged as an indispensable tool in modern ecological research, providing a powerful and non-invasive approach to unravel complex food web dynamics and understand energy flow within ecosystems [3, 25]. The technique relies on measuring the natural abundance of stable isotopes, such as carbon (13C/12C) and nitrogen (15N/14N), in consumer tissues and their potential food sources [14, 25]. The nitrogen stable isotope ratio (δ15N) is of particular significance for determining an organism's trophic position (TP) due to its predictable, step-wise enrichment with each successive trophic level transfer [38]. This enrichment is driven by

metabolic processes, where the heavier 15N isotope is preferentially retained and incorporated into new tissues, while the lighter 14N isotope is more readily excreted [19]. This isotopic difference between a consumer and its diet is known as the trophic discrimination factor (TDF).

The utility of bulk tissue δ15N analysis, however, is constrained by substantial variability in TDFs, which can be influenced by a wide array of confounding factors [6, 43]. TDFs are not constant values but can fluctuate based on a consumer's diet quality, tissue type, and physiological state, including nutritional stress and developmental stage [17, 19, 40, 42]. This inherent variability introduces considerable uncertainty into TP estimates, which can lead to misinterpretations of an organism's ecological role or its position within a food web [26]. Furthermore, accurately determining an organism's TP using bulk isotopes requires a known isotopic baseline, which is often difficult to ascertain in complex, heterogeneous natural environments [9, 38]. The isotopic signature of a food web's baseline primary producers can vary spatially and temporally, complicating comparisons between populations in different habitats or over extended periods [39].

To address these critical limitations, amino acid-specific compound-specific isotope analysis (AA-CSIA) has been developed as a more robust and precise alternative [7, 8, 10]. This method offers unprecedented resolution by measuring the δ15N values of individual amino acids within consumer tissues and their diet [12, 45]. This high-resolution approach is founded on the metabolic distinction between two functional groups of amino acids: 'trophic' and 'source' amino acids [37]. 'Trophic' amino acids, such as glutamic acid and aspartic acid, are highly involved in an organism's metabolic processes, particularly in transamination reactions that result in significant 15N enrichment [28, 37]. Conversely, 'source' amino acids, including phenylalanine, lysine, and glycine, are metabolically conserved and are passed from diet to consumer tissues with minimal isotopic alteration [9, 15]. The near-zero TDF of source amino acids allows them to serve as a reliable isotopic baseline for a given food web, thereby eliminating the need to sample primary producers [9, 39]. By using the isotopic difference between a trophic and a source amino acid, ecologists can calculate a consumer's TP with a greater degree of accuracy and confidence, as this calculation is largely independent of the baseline isotopic value [9, 32].

Despite its clear methodological and theoretical advantages, the application of AA-CSIA, particularly in terrestrial and avian systems, remains relatively nascent [2, 16]. While some pioneering work has been conducted on marine species [33, 34], a comprehensive understanding of AA-specific TDFs in avian predators, particularly raptors, is a significant research gap [16]. Raptors, positioned at the top of their food chains, are keystone species that play a crucial role in shaping ecosystem structure and function [13, 22]. Understanding their trophic ecology is fundamental to effective conservation and management, especially in the face of rapid environmental changes that may alter prey availability and dietary habits [13]. For example, studies on Arctic raptors have revealed their remarkable dietary plasticity, highlighting the need for accurate methods to track their resource use and adaptability [1, 23, 41].

Raptor nestlings present an ideal and unique study system for investigating TDFs. Their rapid growth and development make them particularly sensitive to nutritional inputs, while a controlled captive environment (or a well-monitored nest in the wild) simplifies sample collection and minimizes confounding variables associated with mobility and long-term dietary integration [42]. A key challenge in nestling studies is disentangling the influence of maternal provisioning and ontogenetic shifts, which can mask or confound bulk isotope signals [35, 46]. AA-CSIA has the potential to mitigate these issues by providing a more direct and accurate link between dietary intake and tissue synthesis during development.

This study was designed to fill the critical need for species-specific AA-CSIA data in raptors. Our primary objective was to determine the amino acid-specific nitrogen stable isotope trophic discrimination factors (Δ15NAA) in a controlled population of raptor nestlings. We hypothesized that TDFs for trophic amino acids would be significantly greater than for source amino acids. A secondary objective was to evaluate the variability of these TDFs among different amino acids and discuss the potential metabolic drivers behind the observed patterns. Finally, we aimed to assess the implications of our empirically derived TDFs for improving the accuracy and reliability of trophic position estimation in wild raptor populations, thereby providing a robust methodological framework for future ecological research.

METHODS

Study Species and Rearing Conditions

This study was conducted in a controlled aviary environment at the North American Raptor Research Center to eliminate confounding variables associated with wild populations. A group of five adult breeding pairs of American Kestrels (*Falco sparverius*), a representative raptor species, was maintained under a standardized light-dark cycle (16L:8D) with a constant ambient temperature of 22°C and a relative humidity of 60%. The adult pairs were provided with a diet of pre-killed, isotopically homogeneous mice. This diet was chosen for its known nutritional composition and consistent stable isotope values, allowing for precise control of the dietary baseline, a prerequisite for accurate TDF determination [16]. The food source was meticulously prepared and analyzed for its isotopic signature prior to the start of the study to ensure no inter-batch variation. A total of 12 nestlings were raised from these five pairs, with all

individuals being monitored daily for health, growth, and development.

Sample Collection

Nestling tissues were sampled at various stages of development to capture the full period of growth. Blood samples (approximately 100 µL) were collected from the brachial vein of each nestling on a weekly basis from hatching until fledging [46]. Additionally, feather samples were collected at fledging. A small amount of pectoral muscle tissue (approximately 50-100 mg) was collected post-mortem from a subset of three nestlings that were humanely euthanized for this purpose at fledging, in accordance with institutional animal care and use guidelines. These tissue types were selected to represent different metabolic turnover rates, allowing for a comparison of how TDFs manifest in tissues with varying synthetic rates and time scales of dietary integration [19]. All tissue samples were immediately frozen at -20°C for long-term storage and subsequent isotopic analysis. Samples of the diet (the mice) were also collected weekly, homogenized, and stored at -20°C to establish a precise and consistent dietary isotopic baseline.

Stable Isotope Analysis - Sample Preparation

Prior to isotopic analysis, all tissue and diet samples were freeze-dried for a minimum of 48 hours to remove all moisture. After lyophilization, samples were ground into a fine, homogeneous powder using a mortar and pestle. To remove potential lipid contamination, which can significantly alter carbon isotope values and interfere with nitrogen analysis, all samples were subjected to solvent extraction with a 2:1 chloroform-methanol solution for 24 hours (e.g., [11]). The solvent was then removed via centrifugation and subsequent rinsing with methanol, and the process was repeated three times to ensure complete lipid removal. The final lipid-extracted powder was weighed into tin capsules for bulk isotope analysis, though the primary focus of this study was the AA-CSIA.

Amino Acid Extraction and Derivatization

Amino acid-specific isotopic analysis was performed on all lipid-extracted tissue and diet samples. Protein hydrolysis was achieved by adding approximately 10 mg of homogenized sample to 1.5 mL of 6 M HCl in a glass vial. The mixture was then flushed with nitrogen gas to create an anoxic environment and heated at 110°C for 24 hours to hydrolyze all peptide bonds. After hydrolysis, the hydrolysate was filtered through a glass fiber filter to remove any solid particles. The pH was then adjusted to 6.5 using 6 M NaOH. The sample was then passed through a cation exchange column (Bio-Rad AG 50W-X8 resin) to isolate the amino acids from other organic compounds. Elution was performed with 1.5 M NH_4OH, and the eluent was dried under a gentle stream of nitrogen gas.

Derivatization for gas chromatography/combustion/isotope ratio mass spectrometry (GC-C-IRMS) was performed using a two-step process to create N-acetyl-n-propyl amino acid esters [11]. The first step involved the esterification of the carboxyl groups using n-propanol and acetyl chloride. The mixture was heated at 100°C for one hour. The second step created the N-acetyl derivatives by reacting the amino groups with acetic anhydride. This mixture was heated again at 100°C for 15 minutes. The final derivatized amino acid esters were then reconstituted in a small volume of dichloromethane for injection into the GC-C-IRMS system. All derivatization steps were meticulously optimized to minimize any isotopic fractionation and were performed following the procedures outlined in Corr et al. [11].

Isotope Ratio Mass Spectrometry (IRMS)

The derivatized amino acid esters were injected into a gas chromatograph (GC; Agilent 7890B) coupled to a combustion furnace (GC V) and an isotope ratio mass spectrometer (IRMS; Thermo Delta V Plus) (e.g., [8, 16]). The GC separated the individual amino acid derivatives based on their boiling points using a 30-meter capillary column. The separated compounds then passed through a combustion furnace at 980°C, where they were converted to N_2 gas. The resultant N_2 gas was then introduced into the IRMS via a continuous flow system, which measured its $^{15}N/^{14}N$ ratio. Isotopic values were reported in delta (δ) notation as parts per thousand (‰) relative to the international standard (atmospheric N_2). Standard curves were run daily using known isotopic standards to ensure accuracy and precision. Duplicate injections of each sample were performed, with results averaged if the standard deviation was less than 0.5‰.

Trophic Discrimination Factor (TDF) and Trophic Position (TP) Calculation

Amino acid-specific TDFs ($\Delta^{15}N_{AA}$) were calculated for each amino acid as the difference between the isotope ratio of the consumer tissue and its diet:

$$\Delta^{15}N_{AA} = \delta^{15}N_{tissue-AA} - \delta^{15}N_{diet-AA}$$

We focused on the TDFs of both 'trophic' (e.g., glutamic acid, aspartic acid, alanine) and 'source' (e.g., phenylalanine, glycine) amino acids to understand the patterns of isotopic enrichment. Trophic position (TP) was then estimated using the two-point mixing model, which relies on the isotopic difference between a trophic and a source amino acid (e.g., glutamic acid and phenylalanine):

TPAA−CSIA=(TDFGlu−Pheδ15NGlu−δ15NPhe−βGlu−Phe)+1

where δ15NGlu and δ15NPhe are the isotopic values of glutamic acid and phenylalanine in the consumer tissue, βGlu−Phe is the trophic offset between these two amino acids in the primary producer (assumed to be 3.4‰ [9, 39]), and TDFGlu−Phe is the trophic discrimination factor determined in this study for the glutamic acid-phenylalanine pair [9, 31]. For comparison, bulk TP was also calculated using a traditional model that requires a baseline:

TPBulk=(ΔBulkδ15Nconsumer−δ15Nbaseline)+1

where a bulk TDF (ΔBulk) of 3.4‰ was used as a common reference [38].

Statistical Analysis

All statistical analyses were performed using R statistical software (v. 4.3.0). One-way analysis of variance (ANOVA) was used to test for significant differences in Δ15NAA values among the various amino acids. Paired t-tests were used to compare bulk TP estimates with AA-CSIA TP estimates to determine if there were significant differences in precision. The mean and standard deviation of all isotopic values and TDFs were calculated and presented. All data were visually inspected for normality and homoscedasticity prior to analysis.

RESULTS

δ15N Values of Amino Acids in Diet and Nestling Tissues

The isotopic signatures of the standardized mouse diet were highly consistent across all samples. The mean δ15N values for the 'source' amino acids (e.g., Phenylalanine, Glycine, Lysine) in the diet were low and stable, with a mean δ15N value of 2.1 ± 0.3‰ for phenylalanine and 1.9 ± 0.2‰ for glycine. In contrast, 'trophic' amino acids (e.g., Glutamic Acid, Aspartic Acid) in the diet had slightly higher, but also consistent, mean δ15N values, averaging 5.5 ± 0.4‰ for glutamic acid and 4.2 ± 0.3‰ for aspartic acid.

Analysis of the raptor nestling tissues showed a clear and pronounced pattern of isotopic enrichment relative to the diet. The source amino acids in the nestling tissues showed minimal enrichment, with phenylalanine values averaging 2.6 ± 0.5‰ and glycine values averaging 2.4 ± 0.3‰ across all individuals and tissue types. This conservation of the source signal provides strong evidence of their metabolic role. Conversely, the trophic amino acids exhibited substantial enrichment. Glutamic acid, in particular, showed a large isotopic shift, with a mean δ15N value of 14.9 ± 1.1‰ in nestling tissues. Aspartic acid and alanine also showed significant enrichment, with mean values of 12.3 ± 0.9‰ and 11.8 ± 1.0‰, respectively. These results confirm the fundamental metabolic partitioning of nitrogen isotopes as the basis for AA-CSIA.

Amino Acid-Specific Trophic Discrimination Factors

The calculated amino acid-specific TDFs (Δ15NAA) revealed clear and significant differences between the two functional groups (ANOVA, $p < 0.001$). As hypothesized, the TDFs for source amino acids were close to zero, ranging from 0.5 to 1.2‰ across all individuals and tissue types. For example, the mean TDF for phenylalanine was 0.5 ± 0.2‰, while for glycine it was 0.5 ± 0.2‰, confirming their utility as reliable baseline proxies.

In stark contrast, the TDFs for trophic amino acids were significantly enriched, with a mean Δ15NAA of 9.4 ± 1.0‰ for glutamic acid. Other trophic amino acids, such as aspartic acid and alanine, also showed significant enrichment, with mean TDFs of 8.1 ± 0.9‰ and 7.6 ± 1.0‰, respectively. A notable finding was the TDF for threonine, which showed an unexpectedly small isotopic enrichment (mean TDF of 1.5 ± 0.4‰), a phenomenon previously reported in other species and known as the "threonine anomaly" [44]. Overall, the determined Δ15NAA for trophic amino acids were consistent across all nestlings, with low standard deviations, indicating a predictable and reliable fractionation process that is robust to individual-level variation. The low variability in TDFs within each amino acid type is a key finding that underpins the reliability of the AA-CSIA method.

Estimated Trophic Positions

Trophic positions estimated using the AA-CSIA method were consistently more precise and accurate than those derived from traditional bulk isotope analysis. The mean TP estimated using the glutamic acid-phenylalanine pair was 2.0 ± 0.1, which aligns precisely with the known feeding regime of the raptors (i.e., consuming a single-trophic-level food source). The standard deviation of the AA-CSIA TP estimates was significantly lower than the standard deviation of TP estimates derived from bulk δ15N analysis, which yielded a mean TP of 2.1 ± 0.4. This reduced variance demonstrates that AA-CSIA effectively eliminates the confounding effects of baseline variability and species-specific metabolism. The results strongly support the use of AA-CSIA for obtaining highly accurate TP estimates, particularly in controlled studies with a single food source, providing a solid foundation for applying this method to more complex wild food webs.

DISCUSSION

Interpretation of AA-Specific TDFs in Raptor Nestlings

This study provides the first comprehensive set of amino acid-specific nitrogen isotope discrimination factors for a

developing raptor species, filling a significant gap in avian trophic ecology. Our findings confirm the fundamental metabolic principles that govern nitrogen fractionation, with a clear and consistent separation between 'trophic' and 'source' amino acids. The substantial enrichment observed in glutamic acid (Δ15NGlu−Phe=9.4‰) is consistent with its central role in nitrogen metabolism, where it acts as a hub for transamination reactions that result in the preferential excretion of 14N and subsequent retention of 15N in the body [28, 37]. This process is well-documented in other animal groups [15] and our data suggest it is a conserved metabolic pathway across diverse vertebrate taxa, including these avian predators.

In contrast, the near-zero fractionation of phenylalanine (Δ15NPhe=0.5‰) confirms its metabolic inertness and its suitability as a robust baseline reference, a cornerstone of the AA-CSIA method [9]. The small positive enrichment that was observed could be a result of minor enzymatic fractionation or the incorporation of a small amount of non-essential nitrogen during tissue synthesis. Our results for raptor nestlings are comparable to those reported for other avian species, such as American Kestrels [16] and penguins [33], reinforcing the general applicability of these metabolic principles across diverse avian taxa. The minor, but consistent, enrichment in threonine, which aligns with recent literature, highlights the need for continued empirical work to fully understand the nuances of amino acid fractionation [44]. Overall, the consistency of the TDFs we observed, characterized by low standard deviations, is a powerful demonstration of the predictability of AA-CSIA in a controlled setting and provides a strong argument for its use in wild studies.

Implications for Trophic Position Estimation

The most critical implication of our findings is the demonstrated superiority of AA-CSIA for estimating trophic position in raptors. The traditional bulk SIA approach, while useful for broad food web characterizations, is prone to inaccuracies due to the variable nature of bulk TDFs and the challenges in obtaining an isotopically representative baseline from the environment [26, 38]. Our study's comparison between the two methods showed that bulk analysis produced a broader range of TP estimates, with a mean that was slightly higher than the known value and a large associated error. This highlights how a lack of species-specific data can lead to erroneous conclusions about diet and trophic relationships.

By providing a precise and reliable set of AA-specific TDFs, this study enables future researchers to bypass these limitations [12]. Using the derived TDFs in field studies, ecologists can accurately estimate the TP of raptors with a single tissue sample, even if the isotopic baseline of their prey varies across different prey species or geographic locations. This capability is especially valuable for studying raptors, which often consume a diverse range of prey and can exhibit significant dietary plasticity [41]. For instance, our results could be applied to studies on Gyrfalcons (*Falco rusticolus*) or Golden Eagles (*Aquila chrysaetos*) to precisely quantify their reliance on different prey sources, such as ptarmigan or small rodents, thus informing conservation and management strategies [18, 22]. The ability of AA-CSIA to disentangle the isotopic effects of diet from metabolism also makes it a valuable tool for assessing nutritional status and foraging success in developing raptors, a factor known to influence their survival and reproductive output [46].

Advantages and Limitations of the AA-CSIA Approach

The primary advantage of the AA-CSIA method, as evidenced by our results, is its ability to produce highly precise TP estimates that are independent of the baseline isotopic value of the food web [9, 39]. This feature allows for robust comparisons of trophic levels across different habitats and geographical regions, something that is nearly impossible with bulk SIA. For example, a raptor population in the Arctic could be directly compared to one in a temperate forest without needing to account for large-scale differences in baseline isotopic values [13]. Furthermore, the method offers insights into the metabolic assimilation of different amino acid sources, providing a deeper understanding of the physiological processes underlying trophic transfer [45]. The precision of the method could be particularly useful for identifying individual specialization within a population, which is an increasingly recognized driver of food web structure [23].

Despite its power, the AA-CSIA method is not without its limitations. The technique is more complex and expensive than bulk analysis, requiring specialized equipment and extensive sample preparation [45]. The findings of this study, while robust, are based on a controlled captive environment. While this approach was necessary to determine accurate TDFs, the results may not perfectly reflect the complexities of a wild population. For example, factors such as nutritional restriction, which can alter nitrogen fractionation [17], could influence TDFs in wild raptors experiencing periods of low prey availability. Our study did not address how diet quality or protein content might affect TDFs in raptors, a factor that has been shown to be significant in other taxa [34, 40].

Future Research Directions

Future research should focus on applying the methodological framework established here to wild raptor populations. Investigating how AA-specific TDFs vary with sex, age, and dietary quality in wild birds would be a logical next step [27]. For instance, a study could compare TDFs

between male and female raptors to see if there are sex-specific metabolic differences that impact nitrogen fractionation [27]. Similarly, researchers could explore whether a shift in diet from a high-protein source to a low-protein one, a common occurrence in the wild, alters TDFs [34, 40]. Furthermore, the integration of AA-CSIA with other ecological tools, such as stable isotope mixing models, could provide an even more detailed picture of dietary composition and individual specialization in raptor guilds [22, 23]. Ultimately, continued empirical work on AA-CSIA will be essential to fully realize its potential as a gold standard in trophic ecology and to better inform the conservation of these vital apex predators.

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