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On the Use of Stable Hydrogen Isotope Measurements (δ^2 H) to Discern Trophic Level in Avian Terrestrial Food Webs

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Abstract: The measurement of stable hydrogen isotope ratios (δ^2 H) in animal tissues is a popular means of inferring spatial origins and migratory connections. However, the use of this isotope to infer diet and potentially trophic position remains poorly understood, especially in non-aquatic terrestrial ecosystems. In many animal communities, tissue δ^{15} N values are strongly associated with trophic position. Correlations between tissue δ^2 H and δ^{15} N are expected, then, if δ^2 H is affected by trophic enrichment of ²H. In addition, within sites, we would expect higher tissue δ^2 H values in insectivorous species compared to granivores or nectarivores. We tested these hypotheses for two resident avian communities in Nigeria consisting of 30 species representing a range of dietary guilds (granivores, frugivores, nectarivores, omnivores, insectivores) by comparing feather δ^2 H, δ^{15} N and δ^{13} C values. We found considerable isotopic overlap among all guilds except granivores, with no clear pattern of enrichment in ²H with trophic position. However, at one of our sites (open scrubland), feather δ^2 H was positively correlated with feather δ^{15} N (R² = 0.30) compared to a closed canopy forest site (R² = 0.09). Our results indicate weak evidence for predictable trophic enrichment in ²H in terrestrial environments and indicate that controlled studies are now required to definitively elucidate the behavior of H isotopes in terrestrial food webs.

Keywords: deuterium; trophic enrichment; foraging guild; isotopic niche

1. Introduction

The measurement of naturally occurring stable isotope ratios of several elements in food webs has provided a wealth of ecological information over the last decades. Overwhelmingly, isotopic applications to date have been used in ecological studies to trace origins of primary production and consumer trophic position. Such approaches, in turn, have largely depended on the use of stable carbon (δ^{13} C) and nitrogen (δ^{15} N) isotope measurements based on key isotopic differences in photosynthetic pathways (C3, C4, CAM) and isotopic pools, as well as on the generally systematic increase in δ^{15} N with trophic level ([1], but see [2–5]). More recently, the use of stable hydrogen isotope (δ^2 H) measurements in plant and animal tissues has increased, especially as a tool to evaluate geographic origins of migratory animals [6] or as a forensic tool to evaluate authenticity of origin of several materials [7]. However, there remains great interest in using $\delta^2 H$ measurements to also glean information on energy fluxes within ecosystems as well as a potential indicator of trophic position of consumers in terrestrial and aquatic food webs [8]. Interest in using δ^2 H measurements as a trophic indicator stems from studies showing a weak correlation between δ^2 H and δ^{15} N values in food webs [9–11]. However, such trophic enrichment in δ^2 H can derive from exchange with ambient waters in aquatic food webs, causing a so-called apparent trophic enrichment [12], and processes that can lead to metabolic trophic



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Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). enrichment in terrestrial food webs per se are by no means clear. In fact, there seems to be almost no experimental support for metabolic processes as a mechanism for changes in δ^2 H values in terrestrial and aquatic consumers once other mechanisms are considered [12–16]. Nonetheless, data from bone collagen δ^2 H measurements [9,11] appear to reflect trophic level, such that δ^2 H values of carnivores are higher relative to those of other trophic groups.

Birchall et al. [9] is a much-quoted study reporting a trophic effect in δ^2 H in food webs. However, much of the uncertainty in that report was based in the fact that their analyses mixed aquatic and terrestrial organisms, and the methodology for the δ^2 H measurements did not account for exchangeable H in the collagen samples. Another study involving H isotopes in a controlled laboratory plant–insect system also found a significant trophic enrichment between plant and moth tissues in larvae and adults [17], which contradicts the findings of a separate controlled study that observed a negligible diet-tissue discrimination between monarch butterflies and host milkweed [14]. As noted by Vander Zanden et al. [8], it is still unknown whether the reported trophic enrichment occurs broadly in terrestrial organisms, and, if so, whether the mechanism is similar to the apparent trophic compounding effect found in aquatic organisms. Surprisingly, there are few published data corresponding to tissue δ^2 H values within local food webs that span trophic levels. Clearly, more data are needed to evaluate just how and when consumer tissue δ^2 H values can be used to infer trophic position.

Here, we examined feathers of resident terrestrial avian species known to have grown their feathers within a confined region of Nigeria, Africa. These species represented a range of trophic positions from granivores through to insectivores. In addition to measuring feather δ^2 H values, we also measured feather δ^{13} C and δ^{15} N values to evaluate possible trophic enrichment. We anticipated a positive relationship between feather δ^2 H and δ^{15} N values and highest δ^2 H values in insectivores if hydrogen isotopes were indeed linked to trophic level.

2. Materials and Methods

This study was conducted on the Jos Plateau (c. 1240 m a.s.l), within the northern Guinea savannah vegetation zone of north-central Nigeria. The general vegetation patterns in the savannah zone consist of predominantly tall grasses, shrubs, lianas and dry woodlands. Shrubs and trees such as *Rhus longipes*, *R. natalensis*, *Ochna schweinfurthiana*, *Isoberlinea doka*, *Parkia biglobosa*, *Uapaca somon*, *Vitex doniana*, *Olea capensis*, *Thesium leucanthum*, and evergreen trees such as *Syzyguim guineense*, *Ficus* spp., *Keetia venosa* and *Berlinea* spp. are abundant in this area. The area experiences between 30 and 1200 mm of rainfall in the wet season (May–October), with average daily temperatures ranging between 17 and 32 °C.

Feather sampling took place at two sites: the Amurum Forest Reserve (Site 1) and Naraguta (Site 2) (Figure 1). Amurum Forest Reserve (08°57′0″ E, 09°51′0″ N) is a 115 ha protected woodland interspersed by grassland and inselbergs. This area harbors over 300 different bird species, over 75 arthropod families, some mammals, reptiles and amphibians [18]. Amurum was previously a sacred community forest before being officially designated as a protected site. In the past seven years, however, there has been an increase in residential buildings and farmlands around the reserve. Naraguta (08°53′1″ E, 09°58′1″ N) is an unprotected site, characterized by short vegetation mainly made up of grass, shrubs and small bushes. This site has been influenced by many human activities, including grazing, mining and farming.

Birds were mistnetted at the two study sites between 23 February and 7 March 2016, between 06:00 h and 10:00 h. Birds were identified to species, aged, and various morphometric measurements taken, and three to four breast feathers were collected for isotopic analyses. Only resident species were used in this study. Birds were grouped into feeding guilds as nectarivores, frugivores, granivores, omnivores and insectivores (Supplementary Table S1).



Figure 1. Locations of the study sites within Nigeria (top right insert): Site 1 = Amurum Forest Reserve, Site 2 = Naraguta.

Feathers were cleaned in a fumehood using an overnight soak in 2:1 chloroform: methanol solvent and then rinsed in clean solvent. Once thoroughly dried in the fumehood (24 h), we used the distal vane material for isotopic assays. For carbon and nitrogen isotope analyses, we weighed 1 mg of feathers into pre-combusted tin capsules. Encapsulated feathers were combusted at 1030 °C in a Carlo Erba NA1500 or Eurovector 3000 elemental analyzer. The resulting N₂ and CO₂ were separated chromatographically and introduced to an Elementar Isoprime or a Nu Instruments Horizon isotope ratio mass spectrometer (both in our laboratory). We used two reference materials to normalize the results to VPDB and AIR: BWBIII keratin ($\delta^{13}C = -20.18$, $\delta^{15}N = +14.31\%$, respectively) and PRCgel ($\delta^{13}C = -13.64$, $\delta^{15}N = +5.07\%$, respectively). Within-run (n = 5) precisions as determined from both reference and sample duplicate analyses were $\pm 0.1\%$ for both $\delta^{13}C$ and $\delta^{15}N$.

For δ^2 H analyses, we weighed out 0.35 mg (±0.02 mg) of feathers into silver capsules. We used a Uni-Prep (Eurovector, Milan, Italy) temperature- and vacuum-controlled carousel set at 60 °C interfaced with a Eurovector 3000 elemental analyzer. Samples were combusted at 1350 °C on a glassy carbon reactor. Resultant H₂ gas was analyzed on a Thermo Delta V Plus mass spectrometer in continuous flow mode. We calibrated unknown samples to the VSMOW-SLAP scale using two keratin reference materials (5 of each per analytical run; CBS: -197‰ and KHS: -54.1‰) following the comparative equilibration technique of Wassenaar and Hobson [19]. We estimated our measurement error to be ±2‰.

In order to investigate the variance in stable isotope values among guilds, we fitted linear mixed-effects models for each of the isotopes using individual as a random factor and then performed post-hoc Tukey tests using R's build in *TukeyHSD* function from the *stats* package. We included all isotopes in the models because $\delta^{15}N$ was expected to show a strong trophic effect and $\delta^{13}C$ was expected to yield more clarification of any effects of diet or habitat, in addition to possible minor trophic effects. All numeric variables were rescaled for better comparison using the base function *scale* in R by centering values on the mean for each group of interest divided by the standard deviation. When the random effect of individual was found to be not relevant (i.e., variance of the estimate equal or close to 0), we fitted a maximal linear model including all explanatory variables (guild, site and their interaction, together with feather stable isotope values and their interaction (e.g., for δ^2 H, we included δ^{13} C, δ^{15} N and the interaction between δ^{13} C and δ^{15} N)). Subsequently, we ensured that the scaled residuals of this model were within acceptable limits using the *simulateResiduals* function from the DHARMa package in R [20]. To select the best model, we used the *dredge* function in R from the MuMIn package [21], which allowed us to compute all possible models and select the four best models based on their corrected Akaike's Information Criterion (AICc). Lastly, we calculated the R² value, and the relative amount of variance explained by each of the explanatory variables in the final model. If site of capture was kept in the final model, we redid the exercise described above with model selection on the data for each site separately to verify that our outcomes were consistent, regardless of capturing site.

We were interested in establishing isotopic niches to assist in understanding the relationships among the three stable isotope measurements. This was to examine possible trophic effects based on H and N but also to investigate evidence for microhabitat or diet effects using C. To visualize the isotopic niches of all the guilds, we created isotope biplots for all combinations: (δ^2 H vs. δ^{13} C, δ^2 H vs. δ^{15} N and δ^{13} C vs. δ^{15} N). As a measure of foraging niches for the different guilds, we calculated and plotted posterior ellipses using the functions *siberMVN* and *plotSiberObjects* from the SIBER package [22]. To calculate the posterior ellipses, we used 2 × 10⁶ iterations, a 1 × 10³ burning, thinned by 10 and over 3 chains. We used uninformed priors, as we had no prior knowledge of our expected results. We determined the size of the niche width of each guild for all three isotope combinations using Bayesian Standard Ellipse Areas (SEA), using the function *siberEllipses*. Subsequently, we calculated the relative percentage of niche overlap for the 40% ellipses (i.e., core niche areas) using *bayesianOverlap* for all guilds and each combination of isotopes.

3. Results

3.1. Model Selection

For the model evaluating variance in feather δ^2 H, the random effect of individual was not found to be relevant (variance ~ 0), so a linear model was fitted. Following model selection on our maximal linear model, guild, site and δ^{13} C were included in all top models and these variables were significant in all but one model (p < 0.05, Table 1). Overall, about 35% of the variance found in the data was explained by our explanatory variables (Table 1). Of this, 45% was explained by guild, 52% by residuals and the rest by site, δ^{13} C and δ^{15} N following the relative sum of squares in the best model. The best model for Site 1 retained δ^{13} C and guild (the next best model also included δ^{15} N, delta AICc 1.1), whereas Site 2 retained δ^{15} N and guild (the next best model also included δ^{13} C, delta AICc 0.9).

R ²	Overall AICc
0.37	219.2
0.34	220.6
0.37	221.5
0.37	221.5
0.45	203.1
0.47	204.0
0.48	204.6
0.49	204.9
0.41	212.4
0.39	213.7
0.42	214.4
0.37	214.8
	R ² 0.37 0.34 0.37 0.37 0.45 0.45 0.47 0.48 0.49 0.41 0.39 0.42 0.37

Table 1. Model selection for the various isotopes showing the best four models following the dredge function and their respective R squared and corrected Akaike's Information Criterion (AICc). Explanatory variables in bold were significant in the model, with asterisks representing the following *p*-values: * < 0.05, ** < 0.01, *** < 0.001.

We found clear differences in δ^2 H among guilds, with insectivores being most enriched while granivores were most depleted (Table 2). Omnivores were also more enriched in ²H compared to granivores, but there was no statistical difference between omnivores and insectivores (Table 2). For the model evaluating variance in feather δ^{13} C, the random effect of individual was not found to be relevant (variance ~ 0), so a linear model was fitted. Following model selection on our maximal linear model, values of δ^2 H and δ^{15} N and their interaction were included in all top models (Table 1). Overall, these models explained about 48% of the variance found in the data (Table 1). There was also a near significant effect of guild and site included in some of the top models (p > 0.05, Table 1). The variance was mostly explained by guild (79%) followed by residuals (20%).

Table 2. Median and interquartile ranges for the various isotopes by guild. The second panel summarizes between-group differences based on Tukey post-hoc tests, highlighting between-group differences in bold (see text).

Guild	Ν	δ ² Η (‰)	δ ¹³ C (‰)	δ ¹⁵ N (‰)
Nectarivores	8	-32.85 (-35.232.1)	-20.84 (-21.720.5)	10.46 (8.4–10.7)
Frugivores	14	-43.10(-48.238.8)	-22.67 (-23.322.3)	10.06 (7.9–11.0)
Granivores	25	-56.30(-60.644.1)	-10.38 (-10.99.8)	4.40 (4.1–5.1)
Omnivores	25	-31.00 (-38.822.5)	-19.88 (-21.617.2)	8.47 (7.5–9.7)
Insectivores	15	-24.30 (-29.67.9)	-20.13 (-22.017.5)	7.88 (6.7-8.9)

Frugivores were most enriched in ¹⁵N together with nectarivores, significantly more so than all other guilds, with granivores showing the highest δ^{13} C values (p < 0.001, Table 2). For the model evaluating variance in feather δ^{15} N, the random effect of individual was not found to be relevant (variance ~ 0), so a linear model was fitted. Site and δ^{13} C were included in all top models (Table 1). Overall, these models explained about 40% of the variance found in the data (Table 1). Variance was mostly explained by residuals (59%), but also to a lesser degree by δ^{13} C (16%) and δ^2 H (15%). The best model for Site 1 retained δ^2 H, whereas for site 2, δ^2 H and δ^{13} C were retained. Nectarivores and frugivores were most enriched in ¹⁵N, with granivores being most depleted, significantly so compared to all other guilds (p < 0.001, Tables 2 and 3).

Table 3. Between-guild differences in isotopes based on Tukey post-hoc tests, highlighting between-guild differences in bold (see text).

Guild	δ ² Η (‰)	δ ¹³ C (‰)	δ ¹⁵ N (‰)
Nectarivores–Frugivores Nectarivores–Granivores	n.s. p < 0.001	n.s. p < 0.001	n.s. <i>p</i> < 0.001
Nectarivores–Omnivores	n.s.	n.s.	n.s.
Nectarivores-Insectivores	n.s.	n.s.	n.s.
Frugivores–Granivores	n.s.	p < 0.001	<i>p</i> < 0.001
Frugivores-Omnivores	n.s.	<i>p</i> < 0.001	n.s.
Frugivores–Insectivores	p < 0.01	p < 0.01	n.s.
Granivores–Omnivores	p < 0.001	p < 0.001	<i>p</i> < 0.001
Granivores-Insectivores	<i>p</i> < 0.001	p < 0.001	<i>p</i> < 0.001
Omnivores-Insectivores	n.s.	n.s.	n.s.

3.2. Trophic Enrichment

Since site was retained in most models, we analyzed the linear regression between δ^{15} N and δ^{2} H separately for Site 1 and Site 2. We found only a weak regression for Site 1 (R² = 0.09), whereas Site 2 showed clearer signs of trophic enrichment (R² = 0.30, Figure 2).

3.3. Isotopic Niches

The Bayesian Estimate 40% Prediction Ellipse Area (i.e., core niche area) overlap analyses confirmed that granivores were clearly segregated from the other guilds for all isotopic niches (Figure 3a, Supplementary Table S2), and this finding was consistent across both sites (Supplementary Figures S1 and S2). Based on the size of the standard ellipse areas for the different isotopic niches and guilds, we can infer how specialized the different guilds are, with smaller SEAs representing more specialized guilds. For δ^2 H vs. δ^{13} C, nectarivores, frugivores and granivores had the most specialized trophic niche, for δ^2 H vs. δ^{15} N, granivores and insectivores were most specialized in their trophic niche and, finally, for δ^{13} C vs. δ^{15} N, again, nectarivores, frugivores and granivores had the smallest Bayesian Standard Ellipse Area (Figure 3b). Interestingly, in Site 1, granivores had the most specialized trophic niches across all isotopic niches, apart from δ^{13} C vs. δ^{15} N, where all but the omnivores seemed to be specialized (Supplementary Figure S1). In Site 2, these were nectarivores and frugivores, except for δ^2 H vs. δ^{15} N, where nectarivores were most specialized (Supplementary Figure S2).



Figure 2. Regression between δ^2 H vs. δ^{15} N by site, with colors representing different guilds. Regression lines are indicated in shaded grey, with the 95% confidence band of the regression estimate and its corresponding R² values.



Figure 3. (a) Independent calculated ellipses containing 40% of the isotopic data for the five different guilds for all combinations of isotopes. (b) Posterior distributions of isotopic niche size (Bayesian Standard Ellipse Areas) for the five different feeding guilds for all combinations of isotopes. Grey boxes represent 40% (dark shading), 75% (medium shading) and 95% (light shading) credible intervals, with red crosses designating the model values and black dots the median.

4. Discussion

By examining diverse resident avian communities at two sites in Nigeria, we were able to explore isotopic segregation among foraging guilds. The strength of our approach was that birds occupied a range of trophic levels and dietary specializations, representing local non-migratory populations. Our analyses revealed important information on ecological segregation among groups, but also provided a rare opportunity to examine evidence for a trophic effect in feather $\delta^2 H$ measurements. There are at least two ways in which the multiple isotope data can be examined in this context. One is direct examination of the standardized isotopic ellipse areas and overlap, and the other is to see if there is a positive relationship between feather δ^2 H and δ^{15} N, indicating that δ^2 H mirrors the trophic relationships captured in δ^{15} N. Except for granivores, there was considerable overlap among all guilds for all binary isotope plots. That suggests low support for a strong trophic effect for any isotope. Granivore δ^{13} C values were clearly indicative of C4 sources. However, granivores also had among the lowest $\delta^2 H$ values, potentially suggesting the use of irrigated crops. For the canopied forest site (Site 1), we found an extremely weak relationship (R² = 0.09) between feather δ^{2} H and δ^{15} N, but the relationship was stronger and significant ($R^2 = 0.30$) for the bird community occupying the more open Site 2. That finding suggests the possibility of trophic enrichment occurring in $\delta^2 H$ at the open site, an effect that might be associated with a simpler food web at that site. However, our results underline the fact that feather isotope values were clearly influenced by factors other than trophic level and suggest that key information is required from any given community before one can conclude a trophic driver in tissue $\delta^2 H$. Clearly, feather $\delta^2 H$ can be strongly influenced by local microclimate, water source and the ways in which different food web components are influenced by evapotranspiration [8]. We also note that feather δ^2 H values can be derived from both diet and drinking water and speculate that frugivore and nectarivore δ^2 H may be influenced by the high water content of their diets compared to granivores and insectivores. Differential effects of evapotranspiration in the food web could result in nectarivores and frugivores having higher feather $\delta^2 H$ than expected from trophic level per se. Similarly, even within a single site, prey δ^{15} N values can vary depending on whether or not plant N is fixed (as in the case of legumes) or reliant on symbiosis [23]. Regardless, our study emphasizes that, for food web applications, interpretation of feather δ^2 H in avian communities, especially in the tropics, requires significant ground-truthing of individual species' responses in concert with the use of a multi-isotope approach. Our study does not preclude the trophic effect in δ^2 H as postulated by Birchall et al. [9] but does reveal that each system will need to be examined individually in this respect, and a universal trophic enrichment factor for hydrogen isotopes in food webs is likely unrealistic [8].

As with all field studies, there are important caveats to our study. First, precise timing of body feather molt is unknown for most of the species we examined. It is possible, then, that the period of integration for each isotope differed among species. Any such differences in timing of molt might be a factor, especially in highly seasonal environments such as those studied here and could influence feather carbon and nitrogen isotopes in addition to those of hydrogen. Related to this point is the fact that it is often difficult to impossible to predict the precipitation hydrogen that is driving the food web or diets of interest. Again, it is possible that differential precipitation periods of hydrogen integration leading to subsequent differences in δ^2 H values of seeds, insects, fruits and so on, could contribute to misinterpretation of overlap in feather δ^2 H values among guilds and sites. Hydrogen in bird feathers can be derived from diet and drinking water, whereas nitrogen is exclusively mediated through dietary proteins. This can introduce an additional complicating factor in understanding the isotopic behavior of hydrogen in systems involving diverse metabolic pathways. Future studies should test the occurrence of a trophic effect in $\delta^2 H$ using a system already demonstrating a strong trophic effect based on δ^{15} N. Experimental studies that also control water and dietary δ^2 H values across trophic levels are clearly needed to evaluate trophic effects with δ^2 H in terrestrial food webs.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/ 10.3390/d13050202/s1, Table S1: Study species and corresponding information, Table S2: 40% SEA overlap analysis per site, Figure S1: Core niche areas for the different guilds in Site 1, Figure S2: Core niche areas for the different guilds in Site 2.

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Institutional Review Board Statement: Ethical guidelines regarding the capturing and handling of birds were followed in accordance with the guidelines set by the South African Ringing Scheme (SAFRING) which covered the ringing licenses needed for the study.

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