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LETTER

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Variation in gut microbial contribution of essential amino acids to host protein metabolism in a wild small mammal community

Alexi C. Besser[1,2] | **Philip J. Manlick**[1,3] | **Christina M. Blevins**[1] | **Cristina D. Takacs-Vesbach**[1] | **Seth D. Newsome**[1]

[1]Department of Biology, University of New Mexico, Albuquerque, New Mexico, USA

[2]School of Earth and Space Exploration, Arizona State University, Tempe, Arizona, USA

[3]Pacific Northwest Research Station, USDA Forest Service, Juneau, Alaska, USA

Correspondence
Alexi C. Besser, School of Earth and Space Exploration, Arizona State University, Tempe, Arizona, USA.
Email: acbesser@asu.edu

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Abstract

Herbivory is a dominant feeding strategy among animals, yet herbivores are often protein limited. The gut microbiome is hypothesized to help maintain host protein balance by provisioning essential macromolecules, but this has never been tested in wild consumers. Using amino acid carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope analysis, we estimated the proportional contributions of essential amino acids (AA_{ESS}) synthesized by gut microbes to five co-occurring desert rodents representing herbivorous, omnivorous and insectivorous functional groups. We found that herbivorous rodents occupying lower trophic positions (*Dipodomys* spp.) routed a substantial proportion (~40%–50%) of their AA_{ESS} from gut microbes, while higher trophic level omnivores (*Peromyscus* spp.) and insectivores (*Onychomys arenicola*) obtained most of their AA_{ESS} (~58%) from plant-based energy channels but still received ~20% of their AA_{ESS} from gut microbes. These findings empirically demonstrate that gut microbes play a key functional role in host protein metabolism in wild animals.

KEYWORDS

16S rRNA, 18S rRNA, carbon isotope fingerprinting, Chihuahuan Desert, compound-specific stable isotope analysis, Sevilleta LTER, trophic position

INTRODUCTION

Nearly a third of extant animals are herbivores (Román-Palacios et al., 2019) that consume low-quality diets containing toxic plant secondary compounds, recalcitrant carbohydrates and little protein (Dearing et al., 2005; McArt et al., 2009; Mithöfer & Boland, 2012; White et al., 2014). Recent evidence suggests gut microbiota play crucial roles in the nutrient acquisition of their hosts (e.g. Regan et al., 2022) and likely enabled the independent evolution of herbivory across several mammalian lineages (Ley et al., 2008; Moeller & Sanders, 2020; Muegge et al., 2011; Price et al., 2012). Given the vast metabolic potential encoded in the genes of diverse gut microbial assemblages (Holman et al., 2022; Qin et al., 2010; Xiao et al., 2015), it is unsurprising that the gut microbiota of mammalian herbivores perform diverse functions related to host nutritional physiology (Milani et al., 2020). The gut microbiome's roles in the degradation of complex carbohydrates (den Besten et al., 2013; White et al., 2014) and detoxification of plant toxins (Kohl et al., 2014; Miller et al., 2014, 2016) have been documented in both domesticated (Dodd et al., 2011; Matthews et al., 2019) and wild (Hanya et al., 2020) mammals; however, far less is known about the gut microbiome's role in host protein metabolism despite the fact that most mammalian herbivores and many omnivores are protein limited.

Proteins are comprised of amino acids (AAs) and are the main structural components of animal tissues (e.g. skeletal muscle); yet, animals lack the metabolic machinery needed to synthesize essential amino acids (AA_{ESS}) de novo and instead must route them from dietary protein or acquire them from symbiotic gut microbes (Bergen, 2015; Metges, 2000; Wu, 2009, 2010). Given the scarcity of protein in herbivore diets, symbiotic gut microbiota may be a significant source of AA_{ESS} (Bergen, 2015; Muegge et al., 2011), particularly during periods of physiological (e.g. rapid growth, hibernation) or environmental (e.g. drought) stress. Symbiotic gut microbiota have been shown to supplement AA_{ESS} to their

hosts in controlled feeding experiments on Nile tilapia (*Oreochromis niloticus*; Newsome et al., 2011), eastern subterranean termites (*Reticulitermes flavipes*; Ayayee et al., 2015), Asian long-horned beetles (*Anoplophora glabripennis*; Ayayee et al., 2016) and house mice (*Mus musculus*; Newsome et al., 2020), but the contributions of gut microbiota to the AA_{ESS} budgets of wild animal populations remains unexplored.

AA_{ESS} $\delta^{13}C$ 'fingerprinting' (Larsen et al., 2009, 2013; Scott et al., 2006) is a promising approach for quantifying gut microbial AA_{ESS} contributions to host tissues in wild animals. Organisms capable of AA_{ESS} synthesis (e.g. plants, algae and bacteria) exhibit varied isotopic discrimination during de novo AA_{ESS} synthesis, which imprints on their AA_{ESS} $\delta^{13}C$ values to create distinct multivariate 'fingerprints' (Besser et al., 2022). These isotopic fingerprints can be used to trace AA_{ESS} sources, as the $\delta^{13}C$ values of the AA_{ESS} in an animal's tissues will reflect those of the organism that synthesized them (Manlick & Newsome, 2022; McMahon et al., 2015). Using this approach, Arthur et al. (2014) found that herbivorous green sea turtles (*Chelonia mydas*) likely relied on facultative hindgut fermentation by symbiotic bacteria for a significant proportion of the AA_{ESS} they used to synthesize muscle. Terrestrial herbivores consume even lower quality diets than their marine counterparts due to low protein content (McArt et al., 2009) coupled with the presence of complex structural carbohydrates and toxic secondary compounds synthesized by terrestrial plants to deter herbivory (Dearing et al., 2000; Dearing & Kohl, 2017). In contrast, omnivores and carnivores generally consume diets containing enough protein to maintain homeostasis, grow and reproduce. However, our understanding of the mammalian gut microbiome's role in maintaining host energy and nitrogen balance has largely been limited to captive and domesticated animals (e.g. Dodd et al., 2011; Matthews et al., 2019; White et al., 2014). To date, no study has isolated and analysed the AA_{ESS} $\delta^{13}C$ fingerprints of gut microbes or explored AA_{ESS} provisioning by gut microbiota across trophic levels within a community of wild animals.

Here, we investigate the importance of gut microbes as a potential source of AA_{ESS} to mammalian hosts across trophic levels and functional groups. We focus on five co-occurring small mammal taxa, including three granivorous kangaroo rat species in the genus *Dipodomys*, a complex of omnivorous deer mice (*Peromyscus* spp.), and an insectivorous grasshopper mouse (*Onychomys arenicola*) in the northern Chihuahuan Desert of New Mexico, USA. Using AA_{ESS} $\delta^{13}C$ fingerprinting, we quantify proportional contributions of three AA_{ESS} sources—C_3 plants, C_4 plants and gut microbes—to the red blood cells of small mammals. We then couple these estimates with AA nitrogen isotope ($\delta^{15}N$) analysis to investigate the relationship between host trophic position (TP) and the proportion of AA_{ESS} derived from gut microbiota. We predicted that gut microbial contributions to host AA_{ESS} budgets would increase with decreasing TP, such that individuals occupying the lowest TPs (granivores) would receive the highest contributions of AA_{ESS} synthesized by gut microbes. We expected omnivores to receive intermediate contributions and carnivores to receive insignificant contributions of gut microbe synthesized AA_{ESS}.

MATERIALS AND METHODS

Study site, monthly animal trapping and sample collection

Small mammals were live-trapped and their blood was sampled monthly from March to November 2017 on two adjacent trapping webs at the Sevilleta National Wildlife Refuge, Socorro County, New Mexico, USA (Protocol S1; Noble et al., 2019; Manlick et al., 2021). For this study, we analysed a subset of 50 red blood cell (RBC) samples from *D. merriami* ($n=10$), *D. ordii* ($n=10$), *D. spectabilis* ($n=10$), *O. arenicola* ($n=10$) and *Peromyscus* spp. ($n=10$) from a total of 41 unique individuals. *Peromyscus* represents a complex of cryptic species that we were unable to identify to the species level (Bradley et al., 2007; Miller & Engstrom, 2008; Platt II et al., 2015), but morphological measurements and known occurrences suggest that *P. boylii*, *P. leucopus* and *P. truei* were the most likely taxa captured (Frey, 2007). We analysed RBCs because they are a metabolically active proteinaceous tissue that turns over continuously and has an isotopic incorporation rate of ~60 days (Miller et al., 2008). Further, blood can be collected via minimally invasive procedures that reduce harm to the animal and allow for the repeated sampling of individuals over time. We contend that isotopic measurements of RBCs provide a good proxy for those of skeletal muscle because the two tissues have comparable trophic discrimination factors (Caut et al., 2009), likely because they have similar AA compositions (Wolf et al., 2015). All animal handling protocols were approved by the University of New Mexico Institutional Animal Care and Use Committee (IACUC #19-200,940-MC) and adhered to current guidelines on the use of wild mammals in research (Sikes, 2016). Lastly, we also captured grasshoppers from the same site in October 2017; grasshoppers were frozen, lyophilized and ground to a fine powder using a mortar and pestle.

Isolation and genetic sequencing of microbial cells from faeces

To characterize gut microbes, we isolated microbial cells from 20 faecal samples from *D. merriami* ($n=6$), *D. ordii* ($n=6$), *D. spectabilis* ($n=6$) and *Peromyscus* spp. ($n=2$) collected from a total of 19 unique individuals; faecal samples were not collected from individuals from which

we analysed RBCs. Microbial cells were isolated from faeces using a Nycodenz density gradient and centrifugation following procedures outlined by Amalfitano and Fazi (2008) and Hevia et al. (2015) with some modifications (Protocol S2). The final microbial pellet was resuspended in 100 µL of PCR water, from which 90 µL was aliquoted for AA isotope analysis and 10 µL was aliquoted for 16S and 18S rRNA gene sequencing to identify the microbes contributing to AA isotopic patterns (Protocol S3).

Amino acid $\delta^{13}C$ and $\delta^{15}N$ sample preparation and analysis

Approximately, 3–8 mg of RBCs and homogenized (whole) grasshoppers or all 90 µL of resuspended faecal microbial cells were hydrolysed and derivatized to *N*-trifluoroacetic acid isopropyl esters alongside an in-house AA reference material containing a mixture of commercially available AA powders (Besser et al., 2022; Silfer et al., 1991). The $\delta^{13}C$ and $\delta^{15}N$ values of 13 AAs were measured separately on a Thermo Scientific Trace 1310 outfitted with a 60 m × 0.32 mm ID BPX5 × 1.0 µm column and GC Isolink II combustion interface coupled to a Delta V Plus isotope ratio mass spectrometer at the University of New Mexico Center for Stable Isotopes. Each sample was injected in duplicate, and the in-house AA reference material was analysed every two samples for $\delta^{13}C$ analysis and bracketed every sample for $\delta^{15}N$ analysis. Isotope values are expressed in delta (δ) notation and reported in parts per thousand or per mil (‰): $\delta = [(R_{sample} - R_{reference})/R_{reference}]$, where $R = {}^{13}C/{}^{12}C$ or ${}^{15}N/{}^{14}N$; the internationally accepted reference standards are Vienna Pee Dee Belemnite (VPDB) for $\delta^{13}C$ analysis and atmospheric N_2 (AIR) for $\delta^{15}N$ analysis. Mean within-run standard deviation (SD) of the in-house AA reference material ranged from 0.2‰ (Ile) to 0.4‰ (Tyr) for $\delta^{13}C$ and 0.3‰ (Phe) to 0.7‰ (Tyr) for $\delta^{15}N$ (Table S1). Mean $\delta^{13}C$ values of each AA were calculated across injections for every sample and corrected to account for the carbon added during derivatization, while $\delta^{15}N$ corrections on the mean values of each AA were made using offsets between the measured and known $\delta^{15}N$ values of the in-house AA reference material (Besser et al., 2022; Newsome et al., 2011; Whiteman et al., 2018).

Statistical analysis

We focused statistical analyses on the $\delta^{13}C$ values of five AA_{ESS} (Ile, Leu, Phe, Thr and Val). Lys $\delta^{13}C$ values were excluded because only 11 of the 20 faecal microbe samples analysed contained measurable amounts of Lys. We also analysed the $\delta^{15}N$ values of 'trophic' AAs (Glx, Pro, Ala, Asx, Ile, Leu, Val and Thr) that undergo frequent transamination during consumer metabolism thereby increasing their $\delta^{15}N$ values by ~3–8‰ each trophic step, and 'source' AAs (Phe and Lys) that undergo very little transamination during consumer metabolism such that their $\delta^{15}N$ values reflect those at the base of the food web (McMahon & McCarthy, 2016; O'Connell, 2017). Shapiro–Wilk and Levene's tests (R packages *stats* and *car*) were used to test for normality and homogeneity of variance before we assessed differences in AA $\delta^{13}C$ and $\delta^{15}N$ values among small mammal taxa and sources using Kruskal–Wallis and pairwise Wilcoxon rank-sum tests (R package *stats*).

To estimate the TP of each individual, we used the equation in Chikaraishi et al. (2009): $TP = 1 + [(\delta^{15}N_{ConsumerTrophicAA} - \delta^{15}N_{ConsumerSoureAA} - \beta)/TDF]$, where $\beta = \delta^{15}N_{TrophicAA} - \delta^{15}N_{SourceAA}$ in local primary producers and TDF (trophic discrimination factor) $= \Delta^{15}N_{TrophicAA} - \Delta^{15}N_{SourceAA}$ between the consumer and its diet. Most studies utilizing AA $\delta^{15}N$ analysis to estimate TP have used Glx and Phe in marine pelagic food webs (McMahon & McCarthy, 2016; Matthews et al., 2020; Ramirez et al., 2021), but given the wide ranges of $\Delta^{15}N_{TrophicAA\text{-}Phe}$ observed in our rodent samples, and the variability (SD) of Phe $\delta^{15}N$ values previously reported for C_3 (6.0‰) and C_4 plants (4.1‰) from our field site (Besser et al., 2022), we used Lys as a source AA to estimate TP. We considered trophic AAs that are also AA_{ESS} to ensure any differences observed among taxa are due to internal nitrogen cycling (i.e. transamination reactions) rather than de novo synthesis (O'Connell, 2017). Few estimates of AA $\delta^{15}N$ TDFs for rodents are available, so we estimated TDFs for four trophic AA_{ESS} (Ile, Leu, Thr, Val) and the source AA Lys by defining *D. spectabilis* as a primary consumer (TP = 2), given data from previous stomach content analysis conducted on this species at our field site (Hope & Parmenter, 2007) and low $\delta^{15}N$ values relative to other *Dipodomys* in the community. We used the mean (± SD) β-values for C_3 and C_4 plants ($n = 19$) from Besser et al. (2022) and rearranged the TP equation to solve for TDF. From this, we selected Val as our trophic AA ($\beta_{Val\text{-}Lys} = 1.1 \pm 2.1$‰; $TDF_{Val\text{-}Lys} = 3.3 \pm 0.6$‰) because it generated the best constrained TP estimates within an ecologically realistic range from primary consumer (TP = 2) to tertiary consumer (TP = 4). Our $TDF_{Val\text{-}Lys}$ estimate agreed well with the mean $TDF_{Val\text{-}Lys}$ (3.4 ± 2.8‰) estimated across diets with varied protein content from a recent controlled feeding experiment on house mice (*Mus musculus*; Whiteman et al., 2021). Error associated with $\beta_{Val\text{-}Lys}$ and $TDF_{Val\text{-}Lys}$ was determined using second-order Taylor Expansion (*propagate* package in R).

We used AA_{ESS} $\delta^{13}C$ data for C_3 and C_4 plants collected from the two trapping webs in the same year ($n = 60$; Besser, 2022) and reported in Besser et al. (2022) and faecal AA_{ESS} $\delta^{13}C$ data ($n = 20$) from this study (Table 1) to characterize the $\delta^{13}C$ fingerprints of potential AA_{ESS} sources with linear discriminant analysis (LDA; R package *MASS*) following methods described in previous studies (e.g. Elliott Smith et al., 2021; Larsen et al., 2013;

TABLE 1 Breakdown of samples with associated statistical parameters and major results. C_3 and C_4 plant data were previously published in Besser et al. (2022). All other samples were analysed in this study.

		AA $\delta^{13}C$			AA $\delta^{15}N$
AA_{ESS} sources	*n*	*LDA*	*MixSIAR*	*n*	*Trophic position estimates*
C_3 plants	40	100% success. reclass.	Potential source	9	$\beta_{Val\text{-}Lys} = 1.1 \pm 2.1‰$
C_4 plants	20	100% success. reclass.	Potential source	10	
Gut microbes	20	100% success. reclass.	Potential source	7	Pairwise comparisons with plants and small mammals
16S rRNA gene seq.	15	Major Bacterial Phyla: Firmicutes (42.1–71.7%) and Bacteroidetes (18.9–55.5%)			
18S rRNA gene seq.	15	Major Eukaryotic Phyla: Basidiomycota (0.1–85.8%), Ascomycota (0.0–21.6%), Preaxostyla (0.0–98.6%)			
Insects					
Grasshoppers	13	15% classify with gut microbes	3–8% gut microbial contribution	0	NA; intermediary consumer
Small mammals					
Dipodomys merriami	10	80% classify with gut microbes	43% gut microbial contribution	10	TP = 2.2 ± 0.7
Dipodomys ordii	10	70% classify with gut microbes	50% gut microbial contribution	10	TP = 2.3 ± 0.7
Dipodomys spectabilis	10	60% classify with gut microbes	40% gut microbial contribution	10	TP = 2.0 ± 0.7
Peromyscus spp.	10	50% classify with gut microbes	16% gut microbial contribution	10	TP = 2.4 ± 0.7
Onychomys arenicola	10	40% classify with gut microbes	25% gut microbial contribution	10	TP = 3.3 ± 0.8

Manlick & Newsome, 2022). Three statistical approaches were used to quantify the proportional contributions of AA_{ESS} sources to small mammal RBCs. First, LDA allows for the classification of unknown samples (i.e. consumers) with a potential source based on the unknown sample's proximity to the centroids of each source. If a consumer sample plots directly on top of a source group, it can be reasonably assumed that the consumer obtained nearly all its AA_{ESS} from this group. However, this strict classification system does not allow for mixtures. To better quantify proportional contributions for samples plotting in between different source ellipses, we also ran two Bayesian mixing models (R package *MixSIAR*; Stock et al., 2018), first using measured AA_{ESS} $\delta^{13}C$ values and then using LDA coordinates of sources and consumers (Protocol S4; sensu Manlick & Newsome, 2022). For both approaches, we assumed direct routing of AA_{ESS} and applied TDFs of zero (Manlick & Newsome, 2022). Additionally, to quantify alternative pathways for the assimilation of microbially synthesized AA_{ESS} via insectivory, we estimated proportional contributions of each AA_{ESS} source to grasshoppers using an identical analytical approach with grasshoppers grouped by general foraging strategy (C_3, C_4 or mixed C_3-C_4) according to bulk tissue (whole body) $\delta^{13}C$ values (Table S11). Grasshoppers and other insects are merely a conduit for AA_{ESS}, which are ultimately sourced from the organisms that synthesized them de novo (e.g. C_3 plants, C_4 plants and gut microbes), and therefore were not included as sources in any of the small mammal models.

As a preliminary exploration of seasonal shifts in diet and associated changes in gut microbiome AA_{ESS} provisioning, we analysed the AA $\delta^{13}C$ and $\delta^{15}N$ values of RBCs collected from one *D. merriami* individual (DIME-2417), two *D. ordii* individuals (DIOR-2329 and DIOR-2316) and three *D. spectabilis* individuals (DISP-2497, DISP-2165 and DISP-2288) re-captured in two or three different months in 2017 (Table S10).

RESULTS

16S and 18S rRNA gene sequencing revealed distinct microbial communities, particularly for bacteria, among *D. ordii*, *D. merriami* and *D. spectabilis* faeces (Figures S3–S11; Tables S2 and S3). Overall, we identified 2284 amplicon sequence variants (ASVs) from our 16S reads and 414 ASVs from our 18S reads. 16S rRNA gene sequencing indicated the most abundant bacterial phyla in faeces across species were Firmicutes (42.1%–71.7%) and Bacteroidetes (18.9%–55.5%; Figure S5), and the two most abundant bacterial families in faeces across species were Muribaculaceae (18.6%–54.9%; Bacteroidetes) and Lachnospiraceae (9.5%–50.7%; Firmicutes; Figure S6). 18S rRNA gene sequencing revealed Basidiomycota (0.1%–85.8%) and Ascomycota (0.0%–21.6%) comprised

the most abundant fungi in faeces across species (Figure S10). Eukaryotes from the phylum Preaxostyla, which contains known flagellated animal gut endosymbionts, were also abundant (0.0%–98.6%; Figure S10). Given these findings, Firmicutes, Bacteroidetes, Basidiomycota and Ascomycota likely contributed the most to gut microbial AA_{ESS} $\delta^{13}C$ fingerprints.

AA $\delta^{15}N$ values varied among small mammal taxa and potential AA_{ESS} sources (Tables S4 and S5; Figure S12). Offsets between the $\delta^{15}N$ values of trophic and source AAs ($\Delta^{15}N_{TrophicAA\text{-}SourceAA}=\delta^{15}N_{TrophicAA} - \delta^{15}N_{SourceAA}$) varied significantly among taxa (Figure S13). Using Val and Lys $\delta^{15}N$ values to estimate mean (±SD) TP (see Statistical analysis in Materials and Methods), *D. spectabilis* had the lowest TP (2.0±0.7), followed by *D. ordii* (2.2±0.7), *D. merriami* (2.3±0.7), *Peromyscus* spp. (2.4±0.7) and finally *O. arenicola* (3.3±0.8; Figure 1).

LDA on the $\delta^{13}C$ values of five AA_{ESS} (Ile, Leu, Phe, Thr and Val) (Tables S6 & S7) yielded perfect separation among potential AA_{ESS} sources as indicated by a 100% successful overall reclassification rate for C_3 plants, C_4 plants and gut microbes (Figure 2). Leu $\delta^{13}C$ values drove the first linear discriminant axis (LD1, β=0.69), while Ile $\delta^{13}C$ values drove the second linear discriminant axis (LD2, β=−0.62; Table S8). Using this LDA model, 8/10 of *D. merriami* samples, 7/10 of *D. ordii* samples, 6/10 of *D. spectabilis* samples, 5/10 of *Peromyscus* spp. samples and 4/10 of *Onychomys arenicola* samples classified with gut microbes (Table S9). Only two samples (one *D. spectabilis* and one *Peromyscus* spp.) classified with C_3 plants, and the remaining samples (n=18) classified with C_4 plants (Table S9). We found substantial seasonal variation in AA_{ESS} sources for the six *Dipodomys* individuals captured across multiple months, with individuals switching between predominant reliance on C_3 or C_4 plants and gut microbes (Figure S14; Table S10). Using the same LDA model, 11/13 of grasshopper samples classified with C_3 or C_4 plants and only 2/13 classified with gut microbes (Figure S15; Table S11).

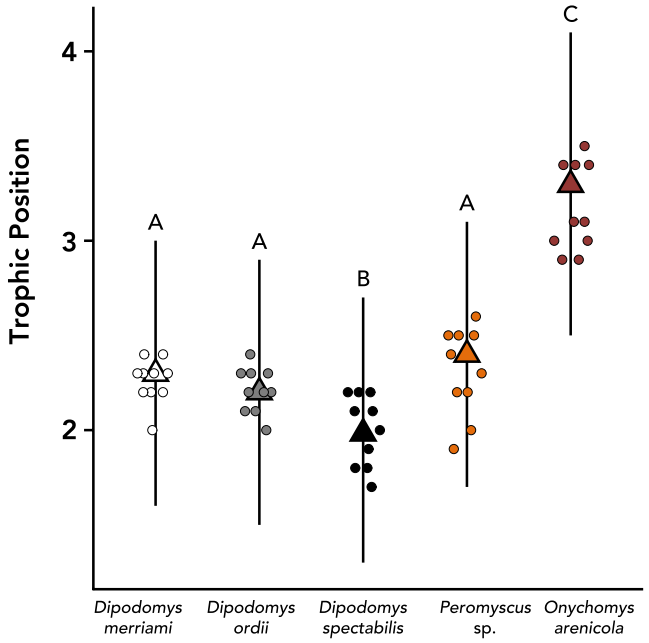

FIGURE 1 Trophic position (TP) estimates of five small mammal taxa. TP estimates were calculated using the 'trophic' amino acid valine and the 'source' amino acid lysine. The trophic discrimination factor (mean±standard deviation=3.3±0.6‰) was estimated by defining *Dipodomys spectabilis* as a primary consumer (TP=2). A β-value of 1.1±2.1‰ for C_3 and C_4 plants was taken from Besser et al. (2022). TPs are displayed as means (triangles) with error (black lines) determined using second-order Taylor Expansion with the *propagate* package in R. Circles represent TP estimates for individuals using the mean trophic discrimination factor and mean β-value with no error propagation.

MixSIAR models using measured AA_{ESS} $\delta^{13}C$ values or LDA coordinates provided nearly identical results (Figure S16), however, models using measured AA_{ESS} $\delta^{13}C$ values showed substantially higher posterior correlations, particularly between C_3 plant and gut microbes, so we report only models using LDA coordinates here. The top model using LDA coordinates carried 72.4% of the weight and included taxon as a fixed effect (Table S12). This model indicated *D. merriami*, *D. ordii* and *D. spectabilis* received nearly equal proportions of AA_{ESS} from C_4 plant (medians of 39%, 42% and 45% respectively) and gut microbial (medians of 43%, 50% and 40% respectively) sources, whereas *Peromyscus* spp. and *O. arenicola* received greater contributions of AA_{ESS} from C_4 plants (median=58% for both taxa) than from gut microbial sources (medians of 16% and 25% respectively; Figure 3 and Figure S17). C_3 plants were the least important source of AA_{ESS} across taxa (medians of 18% for *D. merriami*, 7% for *D. ordii*, 16% for *D. spectabilis*, 26% for *Peromyscus* spp. and 18% for *O. arenicola*; Figure 3 and Figure S17). The MixSIAR model for grasshoppers indicated negligible contributions of AA_{ESS} from gut microbial sources (medians of 3%–8%) regardless of whether they consumed C_3 plants, C_4 plants or a mixture of the two (Figure S18).

DISCUSSION

The functional role of the gut microbiome is an emerging research focus in the field of ecophysiology, and our data provide the first direct evidence that AA_{ESS} synthesized by gut microbiota are assimilated into the tissues of wild mammals. Specifically, we show that ~44% of the AA_{ESS} in the RBCs of predominantly granivorous mammals are synthesized by their gut microbiome, while omnivorous and insectivorous mammals obtain most of their AA_{ESS} from food chains supported by C_4 primary production (Figures 1 and 3). This finding was consistent with a weak, but notable, correlation between TP and gut microbial subsidization of AA_{ESS} to host tissue, where granivores receive a greater proportional contribution of AA_{ESS} synthesized by gut microbes than omnivores and insectivores (Figure 3 and Figure S19).

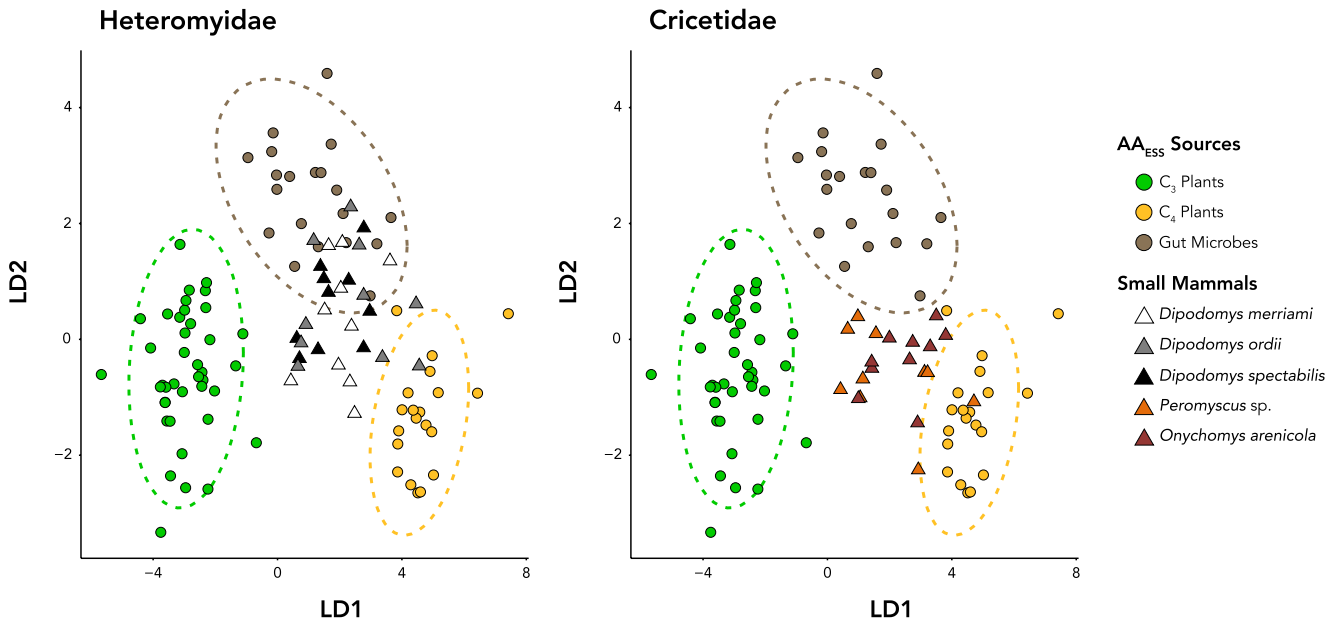

FIGURE 2 AA$_{ESS}$ δ^{13}C fingerprints of five small mammal taxa and their potential AA$_{ESS}$ sources. Linear discriminant analysis was performed using the δ^{13}C values of five AA$_{ESS}$ (isoleucine, leucine, phenylalanine, threonine and valine). Dotted lines represent 95% confidence intervals. Linear Discriminant 1 (LD1) explains 82.7% of the variation among AA$_{ESS}$ sources and LD2 explains the remaining 17.3%. The overall successful reclassification rate for AA$_{ESS}$ sources is 100%.

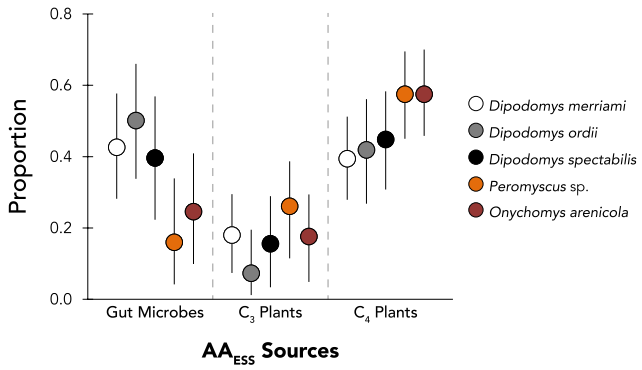

FIGURE 3 Proportional contributions of three AA$_{ESS}$ sources to five small mammal taxa. Proportions were estimated using MixSIAR models on the linear discriminant analysis coordinates from Figure 2. The circles represent medians and the black bars represent 95% confidence intervals.

Our three granivorous species assimilated nearly equal proportions of AA$_{ESS}$ synthesized by symbiotic gut microbes and C$_4$ plants (Figure 3), indicating their C$_4$ forage alone is deficient in the protein needed to maintain nitrogen balance. *D. spectabilis* and *D. merriami* received slightly lower proportions of AA$_{ESS}$ synthesized by gut microbiota (40% and 43% respectively) than *D. ordii* (50%; Figure 3). This pattern may be the result of low sample sizes or may be mediated by varied foraging strategies and behaviour. Notably, *D. merriami* and *D. spectabilis* received greater proportional contributions of AA$_{ESS}$ synthesized by C$_3$ plants (18% and 16% respectively) than *D. ordii* (7%); C$_3$ plants typically contain less carbohydrates and more protein than C$_4$ plants and are thus considered to be of higher nutritional quality (Barbehenn et al., 2004; Barbehenn & Bernays, 1992). Behaviour may also play a minor role, as *D. spectabilis* is the largest of the three sympatric *Dipodomys* species and has been shown to agonistically outcompete *D. merriami* and *D. ordii* for habitat and higher quality seed forage (Brown & Munger, 1985; Frye, 1983). *D. merriami* and *D. ordii* could then be forced to forage on lower quality plants and opportunistically prey on insects to supplement their protein intake, thereby increasing their TPs slightly above those of a primary consumer (2.3±0.7 and 2.2±0.7 respectively; Figure 1). Consumption of insects could supply AA$_{ESS}$ derived from the C$_4$ plants (grasses) that dominate net primary productivity at our study site (Noble et al., 2019), as our data demonstrate for grasshoppers (Figures S15 and S18; Table S11). Stomach content analyses of small mammals at our field site showed that arthropods seasonally accounted for up to 20% (on average) of stomach volume in *D. merriami* and *D. ordii*, while only 3% of *D. spectabilis* stomachs included arthropod remains (Hope & Parmenter, 2007). Given that proportional contributions of AA$_{ESS}$ from gut microbiota were highest for *D. ordii* (50%) and lowest for grasshoppers (3%–8%), gut microbial contributions are most likely mediated by seed forage quality and do not originate from gut microbiota in prey.

Another possible explanation for the trophic patterns observed in *Dipodomys* species is that assimilating AA$_{ESS}$ synthesized by gut microbiota elevates the TPs of *D. merriami* and *D. ordii* by effectively adding an extra trophic step between primary production and primary

consumption, where the microbial processing of recalcitrant organic matter within the gut represents an *intrinsic* 'brown' (i.e. microbial) energy channel. The microbial processing of organic matter in terrestrial food webs and the subsequent importance of brown energy channels has gained traction in recent years (Allison, 2006; Hagen et al., 2012; Manlick et al., 2023; Steffan & Dharampal, 2019). This philosophy has been further supported by recent isotopic work (Pollierer et al., 2020; Potapov et al., 2019; Steffan et al., 2017), including a controlled feeding experiment that suggested heterotrophic bacteria are trophic analogues of animals, at least regarding isotopic discrimination associated with nitrogen metabolism (Steffan et al., 2015). However, most work to date has focused on extrinsic processes for liberating brown energy, such as microbial decomposition of organic matter in soils, rather than intrinsic processes like gut microbial activity. Here, we suggest gut microbial processing of recalcitrant dietary carbohydrates acts as an intrinsic brown energy channel to maintain protein balance. Given the broad functional diversity of the gut microbiome (Alberdi et al., 2016; Holman et al., 2022) and the low-quality diets consumed by many herbivores and omnivores (Dearing & Kohl, 2017), this intrinsic brown energy channel may be more widespread among wild animals than currently appreciated.

Accordingly, we found that omnivorous *Peromyscus* spp. and insectivorous *O. arenicola* obtained a significant proportion (~20%) of their AA_{ESS} from gut microbes (Figure 3). This finding is supported by controlled feeding experiments on omnivorous house mice (*Mus musculus*) in which gut microbes supplied between ~2% and 60% of the AA_{ESS} used to synthesize skeletal muscle in their host, even in mice fed diets containing ample quantities of protein (up to 40%) (Newsome et al., 2020). Importantly, Newsome et al. (2020) found that AA_{ESS} provisioning by gut microbiota to mouse muscle varied by AA_{ESS} and dietary protein content. Gut microbial contributions of Lys were low (<5%) relative to Val (35%–40%) and Ile (~30%) in micefed diets containing high amounts (~20%–40%) of protein, while contributions of Val and Ile nearly doubled to 60% and 50%, respectively, when mice were fed diets containing only 9% protein. Although we were unable to examine contributions for each AA_{ESS} individually, the LD1 and LD2 coefficients in our analysis indicate Leu and Ile $\delta^{13}C$ values were the most important for separating sources using LDA (Table S8). Small mammal AA_{ESS} $\delta^{13}C$ fingerprints were plotted within this same multivariate framework, suggesting these two branch-chained AA_{ESS} may be supplied by gut microbiota at higher rates than other AA_{ESS}. For omnivores (*Peromyscus* spp.) and insectivores (*O. arenicola*) that consume protein-rich diets, excess dietary AAs can be catabolized to intermediaries in glycolysis (e.g. pyruvate) and the TCA cycle (e.g. oxaloacetate) to generate energy via gluconeogenesis (Wester et al., 2015). The catabolic pathways of Ile and Leu are among the most efficient in converting energy to ATP (59.1% and 57.6% respectively) relative to other AAs (Wu, 2009). *Peromyscus* spp. and *O. arenicola* may catabolize some dietary AA_{ESS} for energy and rely on their gut microbiota for a small, but measurable, proportion of the AA_{ESS} needed for tissue synthesis. Our TP estimates confirm *O. arenicola* likely consumes a more protein-rich diet than *Peromyscus* spp. (Figure 1), yet *O. arenicola* receives a greater proportion of AA_{ESS} synthesized by gut microbiota (25%) than *Peromyscus* spp. (16%; Figure 3). In addition to catabolizing dietary AA_{ESS} for energy, *O. arenicola* may feed in brown energy channels or consume prey whose AA_{ESS} budget is subsidized by their gut microbiota. However, the AA_{ESS} $\delta^{13}C$ fingerprints of the grasshoppers we analysed indicate they route most of their AA_{ESS} from the plants they consume (Figures S15 and S18), suggesting both of these pathways are unlikely to be significant sources of AA_{ESS} for small mammals.

Although the enzymatic pathways utilized by microbes to synthesize the carbon skeletons of AA_{ESS} de novo are relatively well-characterized, our understanding of how symbiotic gut microbes metabolize and process the nitrogen needed to aminate these carbon skeletons is lacking. The nitrogen gut microbes use to synthesize AAs de novo is ultimately sourced from plants, and extensive transamination of AAs typically increases residual AA $\delta^{15}N$ values (O'Connell, 2017). Generally, the AA $\delta^{15}N$ values of our gut microbe samples more closely matched those of small mammals than those of C_3 and C_4 plants (Figure S12; Table S4), suggesting gut microbes and their hosts likely process nitrogen from the same central nitrogen pool. Interestingly, the only AA that displayed significantly different $\delta^{15}N$ values between gut microbes and plants was the trophic AA Val, potentially confirming de novo synthesis by gut microbes or indicating extensive transamination of Val directly assimilated from diet (plants) by the host (Table S5). However, interpretations of our AA $\delta^{15}N$ data are limited by small sample sizes and a lack of paired host tissues with associated gut microbes, thus numerous questions remain. Three commonly employed source AAs—Phe, Lys and Met—are all AA_{ESS} and are presumably directly routed from diet into consumer tissues. If gut microbiota provision their host with these compounds, the pool of source AA_{ESS} used by the host to build tissues may have different $\delta^{15}N$ values than in primary producers at the base of the food web. In our study, Phe $\delta^{15}N$ values do not statistically differ among plants, gut microbes and small mammals (Figure S12; Tables S4 and S5). This pattern likely indicates direct routing of dietary Phe by small mammals and suggests direct routing by gut microbes as well, however, the wide range of Phe $\delta^{15}N$ values observed in plants makes this difficult to parse out. In contrast, Lys $\delta^{15}N$ values are significantly lower in gut microbes than in *D. merriami* and *D. ordii*, which

may result from gut microbial de novo synthesis of AAs with ^{15}N-depleted nitrogen available in the gastrointestinal tract (Figure S12; Tables S4 and S5). Future work should aim to better characterize relationships between the nitrogen metabolisms of hosts and their associated gut microbiota and the subsequent impacts on AA δ^{15}N values to help improve our understanding of gut microbiota as facilitators of intrinsic brown energy channels.

Connecting gut microbial communities to specific metabolic pathways that may have beneficial impacts on nutrient acquisition for their host is crucial to understanding how animals deal with seasonal shifts in the quantity and quality of resources. Further exploration of these links is needed to contextualize emerging evidence that suggests gut microbiota played crucial roles in enabling dietary specialization within mammalian herbivores (Moeller & Sanders, 2020). We found clear distinctions in faecal microbial communities, particularly for bacteria, among *Dipodomys* species (Figures S3–S11; Tables S2 and S3). Recent work has correlated changes in gut microbial diversity and activity to seasonal dietary shifts in wild black howler monkeys (*Alouatta pigra*; Amato et al., 2015) and plateau pika (*Ochotona curzoniae*; Wang et al., 2020), and advances in genomic sequencing and metabolite profiling now allow for direct comparisons between gut microbial community composition, gut microbial activity and host digestive environment (Lu et al., 2018). Our study demonstrates the utility of AA δ^{13}C and δ^{15}N analysis in connecting host nutrient acquisition to gut microbial activity and provides preliminary evidence of seasonal variation in the proportional contributions of gut microbiota as AA_{ESS} sources in *Dipodomys* (Figure S14; Table S10). In the small mammal community we studied, seasonal variation in AA_{ESS} supplementation by gut microbiota is likely influenced by phenological shifts in plant communities and/or depletion of preferred cached food stores. For example, at our field site in the northern Chihuahuan Desert, precipitation during the relatively cold winter and spring fuel the production of C_3 plants, while warm monsoon rains favour C_4 plant production (Collins et al., 2010). Given the vastly different digestibility and nutritional qualities of C_3 and C_4 plants (Barbehenn et al., 2004; Barbehenn & Bernays, 1992), we expect greater AA_{ESS} provisioning by gut microbiota during the summer monsoon when lower quality C_4 plants dominate the landscape. Our small data set including six individuals re-captured in two or three different months demonstrated that two *Dipodomys* individuals (DIME-2417 and DIOR-2316) obtained most of their AA_{ESS} from gut microbes during the summer (August–October), and several individuals obtained most of their AA_{ESS} from gut microbes during the early spring (March–May) when preferred cached food stores were likely depleted (Table S10). By extension, individual dietary specialization is common in *Dipodomys* (Manlick et al., 2021) and may also influence the gut microbiome's role in host nitrogen balance, such that individuals specializing on lower quality forage may rely more heavily on their gut microbiota for AA_{ESS} than conspecifics specializing on higher quality resources. Future work should aim to better characterize seasonal shifts in small mammal diet, gut microbiome composition and gut microbial contributions of AA_{ESS} through the analysis of paired host and faecal samples in both controlled feeding experiments and from repeated measures of wild individuals. Additionally, given the distinct gut microbial communities we observed across *Dipodomys* species, more studies characterizing the gut microbiomes of species across the trophic spectrum are needed to clarify the ecological and taxonomic controls on mammalian gut microbiome composition and function.

CONCLUSIONS AND FUTURE DIRECTIONS

Our study provides a comprehensive framework for applying AA_{ESS} δ^{13}C fingerprinting to quantify gut microbial AA_{ESS} provisioning across consumer groups and our data clearly demonstrate substantial proportional contributions of AA_{ESS} synthesized by gut microbiota to rodent hosts. These contributions generally decreased with increasing TP, though deviations from this trend suggest that the functional role of gut microbes may also be influenced by dietary protein quality, the microbial processing of nitrogen within the gut and the catabolism of excess dietary AAs for energy. TP estimation based on AA δ^{15}N analysis has exploded in popularity in recent decades, but the assumptions behind this method have largely ignored the potential influence of gut microbial activity on source and trophic AA δ^{15}N values. Here, we provide evidence that these assumptions require careful consideration and advocate for further investigation into relationships between gut microbiome and host nitrogen cycling. Improving our understanding of the interplay between the AA metabolism of mammalian hosts and their symbiotic gut microbiota is critical for interpreting the nutritional plasticity of wild populations that experience seasonal variation in food quality and abundance. As plant communities in desert ecosystems change in response to hotter and drier conditions, the gut microbiota of mammalian species living in these environments may be integral to their ability to acclimate to shifting resource landscapes.

AUTHOR CONTRIBUTIONS

ACB, CMB and SDN designed the study; CDTV and SDN provided reagents and laboratory equipment; ACB and CMB collected the data; ACB and PJM

analysed the data; ACB, PJM, CDTV and SDN interpreted the data; ACB wrote the article; PJM, CDTV and SDN provided substantial contributions to editing the article.

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DATA AVAILABILITY STATEMENT

Amino acid $\delta^{13}C$ and $\delta^{15}N$ data presented in this article are archived in the Dryad Digital Repository and can be accessed at https://doi.org/10.5061/dryad.tdz08kq49 (Besser 2023a). 16S and 18S rRNA gene sequence data presented in this article are archived in NCBI's Sequence Read Archive (SRA) and can be accessed at https://www.ncbi.nlm.nih.gov/bioproject/970345 (Besser 2023b).

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ORCID

Alexi C. Besser https://orcid.org/0000-0003-3384-6793
Philip J. Manlick https://orcid.org/0000-0001-9143-9446
Seth D. Newsome https://orcid.org/0000-0002-4534-1242

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SUPPORTING INFORMATION

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