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Successes and limitations of quantitative diet metabarcoding in a small, herbivorous mammal

Running title: Accuracy of Metabarcoding to Determine Diet

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Abstract

DNA metabarcoding is widely used to determine wild animal diets, however whether this technique provides accurate, quantitative measurements is still under debate. To test our ability to accurately estimate abundance of dietary items using metabarcoding, we fed wild caught desert woodrats (*Neotoma lepida*) diets comprised of constant amounts of juniper (*Juniperus osteosperma*, 15%) and varying amounts of creosote (*Larrea tridentata*, 1-60%), or cactus (*Opuntia* sp., 0-100%), and commercial chow (0-85%). Using metabarcoding, we compared the representation of items in the original diet samples to that in the fecal samples to test the sensitivity and accuracy of diet metabarcoding, the performance of different bioinformatic pipelines, and our ability to correct sequence counts. Metabarcoding, using standard *trnL* primers, detected creosote, juniper, and chow. Different pipelines for assigning taxonomy performed similarly. While creosote was detectable at dietary proportions as low as 1%, we failed to detect cactus in most samples, likely due to a primer mismatch. Creosote read counts increased as its proportion in the diet increased, and we could differentiate when creosote was a minor and major component of the diet. However, we found that estimates of juniper and creosote varied. Using

previously suggested methods to correct these errors did not improve accuracy estimates of creosote, but did reduce error for juniper and chow. Our results indicate that metabarcoding can provide quantitative information on dietary composition, but may be limited. We suggest that researchers use caution in quantitatively interpreting diet metabarcoding results unless they first experimentally determine the extent of possible biases.

Key words: DNA metabarcoding, diet analysis, herbivory, mammals

Introduction

All animals eat, but quantifying what an animal eats remains a challenge. Diet is an essential part of animal biology and knowledge of an animal's diet is critical for determining niches, managing populations, and predicting responses to environmental change. Popular methods of quantifying diet such as direct observations (Collopy, 1983; Henley, Smith, & Raats, 2001; Pisani, Distel, & Bontti, 2000), microhistology (Alipayo, Valdez, Holochek, & Cardenas, 1992; Bergstrom, 2013; Wydeven & Dahlgren, 1982), and stable isotopes can provide valuable information (Ben-David, Flynn, & Schell, 1997; Burns, Trumble, & Castellini, 1998; Inger et al., 2006). However, these techniques all have limitations. Small, nocturnal, fossorial, or otherwise elusive species are difficult or impossible to directly observe (Lafage et al., 2020; Soininen et al., 2013). Not all food leaves detectable fragments (e.g., fluids such as nectar and sap), and when present in feces, fragments may be digested beyond recognition, and difficult to resolve to species

(Holechek, Vavra, & Pieper, 1982; Moreby, 1988; Peterson & Fry, 1987). Stable isotopes can detect seasonal diet shifts, but isotopic signatures are often not different enough to distinguish food items (Traugott, Pázmándi, Kaufmann, & Juen, 2007). Finally, many of these techniques are labor intensive, impeding large scale diet surveys.

Advances in sequencing technology have led to increased use of DNA-based diet determination, particularly DNA metabarcoding (Taberlet, Coissac, Pompanon, Brochmann, & Willerslev, 2012). Metabarcoding involves the extraction of total DNA from a sample, such as feces or stomach contents, using a suitable marker gene to amplify a broad range of possible diet components via high-throughput sequencing, and assigning taxonomy by comparing sequences to a reference database. This technique can provide rapid and non-invasive diet characterization for large numbers of samples and, with increasing use, has resulted in ever-growing reference databases for improved taxonomic resolution (Taberlet et al., 2012). In addition, because metabarcoding produces sequence counts, this technique also generates data with the potential to provide relative abundance estimates of diet components.

Diet metabarcoding has become increasingly common, with recent studies applying this technique on samples from omnivorous, herbivorous, and carnivorous species in both terrestrial and aquatic environments. Metabarcoding has revealed the broad range of diets and niche partitioning exhibited by mammalian, insect, and avian insectivores (Arrizabalaga-Escudero et al., 2018; Kaunisto, Roslin, Sääksjärvi, & Vesterinen, 2017; McClenaghan, Nol, & Kerr, 2019). Diet metabarcoding has also demonstrated that human influence increases dietary niche overlap in mesocarnivores (J. A. Smith, Thomas, Levi, Wang, & Wilmers, 2018) and that large, African herbivores use novel food items after predators are extirpated (Atkins et al., 2019). It is even possible, using multiple

primer sets, to gain insight into the diet of omnivores (De Barba et al., 2014), although detection of incidentally consumed taxa presents a challenge (Tercel, Symondson, & Cuff, 2021). As sequencing technology continues to improve and become more affordable, the use of diet metabarcoding will likely increase.

Despite recent advances, there are many limitations to diet metabarcoding. Metabarcoding can be used to quantify diet components, but due to both technical and biological biases such as primer bias or differential degradation of DNA, sequence proportions may not accurately reflect the biomass of what was consumed (Alberdi, Aizpurua, Gilbert, & Bohmann, 2018; Deagle, Thomas, Shaffer, Trites, & Jarman, 2013; Murray et al., 2011; Pompanon et al., 2012). Every step of the workflow has the potential to introduce bias. Biological bias can be introduced before sample collection through the amount of biomass consumed, density of DNA in the consumed tissues, individual variation in digestion of consumed tissues, and differential degradation of DNA (Deagle, Chiaradia, McInnes, & Jarman, 2010; Thomas, Jarman, Haman, Trites, & Deagle, 2014). Technical bias can be introduced through the DNA extraction methods (Datukishvili, Gabriadze, Kutateladze, Karseladze, & Vishnepolsky, 2010; Djurhuus et al., 2017; Henderson et al., 2013), primer mismatch, and unequal primer binding; all of which can affect the amplification of different diet components (Alberdi et al., 2018; Clarke, Soubrier, Weyrich, & Cooper, 2014; Deagle, Eveson, & Jarman, 2006). In addition, increasing numbers of primer mismatches, as well as their position, can exacerbate these amplification biases (Piñol, Senar, & Symondson, 2019a). In considering these biases, many studies conservatively interpret sequence counts as frequency of occurrence (presence/absence) data, reporting lists of taxa found within samples and frequency of recovered taxa in populations (Biffi et al., 2017; Elbrecht & Leese, 2015; Kartzinel & Pringle, 2015). Although this technique can provide some quantitative information about dietary items, it also has

drawbacks. For example, it gives the same weight to all dietary components, potentially overestimating the contribution of rare taxa while underestimating the contribution of major components (Deagle et al., 2019).

Many studies spanning multiple species make quantitative measurements of diet based on relative read abundance (RRA; Buglione et al., 2018; Kartzinel & Pringle, 2015; McInnes et al., 2017) and several studies have evaluated the accuracy of this approach. However, these evaluations have been done primarily on species with carnivorous and insectivorous diets, with few focusing on herbivores. One of the early empirical studies, in which Little Penguins (*Eudyptula minor*) were fed a fish-based diet to determine the accuracy of diet metabarcoding, cautioned against using metabarcoding as a quantitative measure because the raw relative abundance data did not reflect the proportions in the diet (Deagle et al., 2010). However, subsequent work provided corrections for PCR amplification and digestion disparities to improve the accuracy of quantifying diet components (Thomas, Deagle, Eveson, Harsch, & Trites, 2016). Experiments to assess accuracy have also been conducted in insectivores (Clarke et al., 2014; Mata et al., 2019; Tournayre et al., 2020), focusing primarily on the effects of bias introduced via PCR.

Very few studies have tested the quantitative accuracy of metabarcoding in herbivores, despite herbivory being one of the most common terrestrial feeding strategies (Choat & Clements, 1998). It is important to assess the accuracy and precision of this technique in a range of species because key physiological differences exist between organisms with different dietary strategies. Herbivores harbor a complex digestive system that differs from carnivores and insectivores in ways that may impact our ability to recover DNA from diet items. Herbivores masticate their food to a greater extent and have a highly segmented gastrointestinal tract with increased

surface area, longer gut transit times, and pouches, such as the cecum (Karasov, William & Rio, 2020; Karasov & Douglas, 2013). These adaptations may affect the degradation of plant DNA. The few studies that examine the accuracy of metabarcoding for herbivores focused primarily on domesticated and wild ruminants (Nakahara et al., 2016; Scasta et al., 2019; Willerslev et al., 2014), which also have a unique digestive strategy that differs from non-ruminants (Holechek et al., 1982). To the best of our knowledge, diet metabarcoding has not been evaluated in a wild, non-ruminant herbivore.

To determine whether diet metabarcoding accurately describes the diets of non-ruminant herbivores, we conducted diet trials using the desert woodrat (*Neotoma lepida*). Woodrats are an excellent system for diet studies because they feed on numerous plant species across their range (Cameron & Rainey, 1972; Skopec, Kohl, Schramm, Halpert, & Dearing, 2015; F. A. Smith, Murray, Harding, Lease, & Martin, 2014), and rapidly acclimate to novel, plant-based diets in a laboratory setting. Furthermore, woodrats are non-ruminants that may provide insight into the accuracy of metabarcoding for other wild herbivores with similar gut morphologies.

We investigated the accuracy of metabarcoding with respect to woodrat diets. First, we compared the use of different bioinformatics pipelines in evaluating metabarcoding results to determine whether there was any possible bias in the pipelines. Next, we experimentally evaluated the ability of metabarcoding to detect different dietary components, particularly, rare components, those with varying abundances, and a component with primer mismatches. Third, we assessed the accuracy of RRA to that of known dietary composition. Then, we investigated our ability to correct RRA estimates in samples of known content using relative correction factors. Finally, we compared the accuracy of RRA to weighted occurrence, another commonly used method for interpreting diet data.

Methods

Animal Collection

We used two populations of *N. lepida* (hereafter "woodrats") for this study (Table S1). Animals in diet trial 1 and the cactus diet trial were born in captivity to mothers from Furnace Creek, California (36.46°N, 116.86°W). These captive-born animals (n = 5) were housed individually after weaning and fed commercial high fiber rabbit chow ("chow"; Envigo Teklad formula 2031). We conducted trials after animals reached adult size (> 90g) with animals weighing an average of 117.9g ± 22.9 at the time of trials. Diet trial 2 was conducted using wild caught woodrats from the Mojave National Preserve, California (35.12°N, 115.43°W). We collected adult woodrats using Sherman live traps baited with oats. Woodrats were housed individually and transported to the University of Utah where they were fed chow and acclimated to captivity for two weeks before starting the diet trial. These animals (n = 5) weighed an average of 124.72g ± 17.46. Animal use and procedures were approved by the University of Utah IACUC (16-02011). Animals were collected under California Department of Fish and Wildlife permit SC-2105 issued to Jim Patton.

Diet Preparation

For feeding trials, we prepared diets using powdered chow and three plants naturally consumed by woodrats. Experimental diets for diet trial 1 ("low creosote") and 2 ("high creosote") contained creosote bush (*Larrea tridentata*) and juniper (*Juniperus osteosperma*). Diets for the cactus diet trial contained cactus pads of a single, hybrid *Opuntia* species (*Opuntia macrorhiza* × *O. polyacantha* and juniper. We chose cactus because it is a common dietary item for wild woodrats, however cacti in the genus *Opuntia* have two mismatches in the *trnL h* primer at the third and third from last primer position. These mismatches likely impair primer

binding, and thus may not enable detection of cactus at low concentrations. Because creosote, juniper, and cactus contain toxins that limit food intake (Mangione, Dearing, & Karasov, 2004; Skopec et al., 2015), we mixed these natural diet components with the alfalfa and soy-based chow to ensure that animals could consume sufficient food to maintain body mass. Chow was sterilized by the manufacturer via heat processing at 48°C. We collected creosote from the Mojave Preserve, California in October 2018, juniper from Castle Valley, Utah in February 2017, and cactus pads from Castle Valley, Utah in February 2019. All plant material was transported on ice and then stored at −20°C until use.

Diets were made fresh daily to prevent desiccation and DNA degradation. For both juniper and creosote, diets were made only from plant leaves. The natural components of the diet (juniper, chow, and cactus) were not sterilized prior to being fed to animals. To prevent food caching by woodrats, we homogenized diets by first freezing diet components in liquid nitrogen and then grinding them in a commercial blender until able to pass through a 1mm sieve. For cactus, we removed the spines, including the areole, before blending. We blended cactus with chow at room temperature and then evaporated it using a Büchner funnel for 12 hours to remove excess moisture. Plant proportions were measured by dry weight and all diets were thoroughly homogenized in a commercial blender. All diets, except the 100% cactus and creosote diets, contained 15% juniper and 25-85% chow. For the low creosote diet trial, we prepared diets with 0%, 1%, 5%, and 20% creosote. For the high creosote diet trial, we prepared diets with 0%, 20%, 60% and 100% creosote (Figure 1A). For the cactus diet trial, we prepared diets with 0%, 20%, 60%, and 100% cactus (Figure S2). Portions of each homogenized diet prior to digestion by an animal ("diet samples", n = 11) were stored at −80°C and analyzed alongside fecal samples (n = 65).

Feeding Trials

Animals were housed in metabolic cages (Lab Products Inc.) during feeding trials to prevent food caching and contamination of fecal samples. Each day, animals were given 15g of freshly prepared diet and their food intake was recorded (Tables S2−S4). Animals were weighed daily and removed from the trial if they lost more than 10% of their initial mass. Animals were given each diet for two days to ensure passage through the gut (Kohl, Weiss, Cox, Dale, & Dearing, 2014). After two days on the diet, the most recent fecal samples were collected and stored at −80°C until DNA extraction. All animals lost more than 10% of their initial mass after one day on the 100% creosote diet. As this indicates that animals did not consume this diet, 100% creosote samples were excluded from all analyses.

DNA Extraction and Sequencing

Total DNA was extracted from feces, diet samples, and blanks (n = 4) using QIAamp® PowerFecal® DNA kits following the manufacturer's protocol. In order to determine the accuracy of sample pooling, we also extracted pooled fecal samples, made by homogenizing equal portions of feces from each animal on a given diet.

To determine if cactus would amplify using the *trnL g* and *h* primers, we amplified and visualized extracted DNA from samples containing various amounts of cactus. These primers are widely used in plant metabarcoding and amplify the P6 loop of the chloroplast *trnL* (UAA) intron (Taberlet et al., 2007). We tested the amplification of cactus alone and mixed with spinach (*Spinacia*

oleracea), in mixtures containing 90% cactus, 70% cactus, and 50% cactus DNA. To determine if digestion impacted amplification of cactus, we also amplified DNA extracted from the feces of animals that had been fed 100%, 60%, and 20% cactus in the cactus diet trial. Each 25 µL PCR reaction contained 12.5 µL Taq 2X Master Mix (New England BioLabs), 0.5 µL 10µM of each primer, 10.5 µL of water and 1 µL of DNA. Amplification began with an activation step of 10 min at 95° C, followed by: 35 cycles of 30 s at 95°C, 30 s at 55°C and 30 s at 72°C, and a final extension step of 2 min at 72°C. Products were visualized using gel electrophoresis with a 4% agarose gel.

For NGS sequencing using the same *trnL g* and *h* primers, extracted DNA was sent to the DNA Service Facility at the University of Illinois-Chicago for amplification, library preparation, and sequencing on an Illumina MiniSeq platform (2 × 153 bp paired-end reads). Initial PCR amplifications were performed in 10 µL reactions under the following conditions: 95°C for 5 minutes, followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. A second PCR amplification was performed in 10 µL reactions in 96-well plates where each well received a separate primer pair with a unique barcode from an Access Array Barcode Library for Illumina. Conditions for the second PCR were as follows: 95°C for 5 minutes, followed by 8 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. Libraries were pooled in equal volume and purified using an AMPure XP cleanup (0.8X, vol/vol; Agencourt, Beckman-Coulter) to remove fragments larger than 300 bp. Pooled libraries were loaded onto an Illumina MiniSeq mid-output flow cell and were re-pooled based on the distribution of reads per barcode. The re-pooled library was purified as previously described and then loaded onto a Miniseq flow cell and sequenced. Sequencing resulted in an average of 50,720 (SD ± 30,965) reads per sample.

Constructing the trnL *Reference Database*

To construct a reference database for the diet components, we downloaded chloroplast nucleotide sequences and their accompanying taxonomies in the XML format from the NCBI on June 3, 2020 using version 11.0 of the Entrez Direct utilities. We downloaded sequences for the following families: Asteraceae, Brassicaceae, Chenopodiaceae, Fabaceae, Plantaginaceae, Poaceae, Cupressaceae and Zygophyllaceae. Cactaceae and Bromeliaceae sequences and taxonomies (downloaded September 29, 2021 and March 17, 2021, respectively) were later added to supplement this information. The database contains 15,048 species with a total of 3,706 unique sequences. Sequences that could not be assigned to at least the genus level, or those flagged as "environmental samples", were not included in the reference database. All other sequences were converted into the FASTA format with a custom Python script (xmls_to_fasta.py) employing functions from the 1.76 release of Biopython. Sequences were subsequently trimmed using Cutadapt 2.10 and the *trnL g* and *h* primers, and any sequence containing indels or more than two mismatches in either primer region was discarded. A second script (build_db.py) was used to construct a DADA2-compatible taxonomy database from the Cutadapt-trimmed FASTA. To ensure only high-quality sequences were included in the database, the length of the target region had to be between 8 and 175 bp and sequences could not have ambiguous nucleotides anywhere in the primer or target regions. All shell commands, the Python scripts, and the resulting DADA2 FASTA are available at https://github.com/tess-stapleton/neotoma-trnl-metabarcoding.

Sequence Processing and Bioinformatics

In order to compare sequence processing pipelines, sequences were merged, aligned, clustered, and assigned to taxonomy using two popular pipelines: VSEARCH/QIIME2 and DADA2/BLAST+ (Bolyen et al., 2019; Callahan et al., 2016; Rognes, Flouri,

Nichols, Quince, & Mahé, 2016). For the VSEARCH/QIIME2 pipeline, sequence processing parameters were as follows. In order to retain high quality sequences of our species of interest, minimum sequence length after trimming was set to 20 base pairs, minimum acceptable PHRED score was increased to 20, and minimum overlap was reduced to 10 bp; all other parameters were left at default settings. The amplified *trnL* region for cactus, creosote, juniper, and alfalfa (the main component of chow) are all > 40bp, therefore this threshold should not remove relevant taxa. Sequences were assigned to operational taxonomic units (OTUs) at the 100% identity level using *de novo* clustering. Chimeric sequences were identified and removed using *de novo* chimera checking in VSEARCH. Based on the contents of extraction blanks sequenced alongside samples, we removed OTUs represented by less than 20 reads per sample, and OTUs that appeared in less than 5 total samples (average of 0.5% of reads per sample, SD ± 0.6). Taxonomy was assigned to OTUs using the Scikit-learn classifier in QIIME2 version 2019.4, trained on our custom reference database. Sequenced chow-only diet samples contained OTUs belonging to Asteraceae, Brassicaceae, Chenopodiaceae, Fabaceae, Plantaginaceae, and Poaceae. Reads assigned to those families in plant and fecal samples were classified as chow. Sequences assigned to taxa not identified as belonging to chow and that were not resolved to at least the family level were considered unclassified (0.32% of total sequences).

Taxonomy Assignment with BLAST+

To assign taxonomy with BLAST+, we first performed read pair joining, quality control, and filtering in DADA2, using parameters that matched those used in VSEARCH. After assigning sequences to amplicon sequence variants (ASVs) with DADA2, we assigned taxonomy using BLAST+ 2.9.0+; this step was taken as many of the *trnL* sequences amplified in plants are shorter than the minimum length required for taxonomic assignment by DADA2. ASV tables were converted into the FASTA format using

Biopython 1.76 functions (csv_to_fasta.py), and were aligned against the taxonomy sequence database using blastn with the following parameters: -task "blastn-short" -evalue 1 -word_size 4 -perc_identity 90. The database sequence(s) with the lowest E-value, highest percent identity, and the closest length to the ASV sequence were used to assign taxonomy. No taxonomy was assigned if there were no database sequence hits with an E-value less than 1, a percent identity greater than or equal to 90, or a length within 10% of that of the ASV sequence. When multiple taxonomic best hits were available for an ASV sequence, taxonomy was assigned at each successive rank (kingdom, phylum, etc.) so long as at least 50% of the best hits shared that rank. The 0.42% of sequences not identified as chow or not resolved to the family level were removed, as described previously. DADA2 was implemented in R version 3.6.1 (R core team, 2019). The shell commands and script used to assign sequences (assign_taxonomy.py) are available at https://github.com/tess-stapleton/neotoma-trnl-metabarcoding.

Correcting Read Counts Using Relative Correction Factors and Assessing the Accuracy of Weighted Occurrence Data

Correction factors calculated from known diets can be used to correct biases in RRA metabarcoding data (Thomas et al., 2016, 2014), but have not been tested on a plant-based diet. We used two approaches to calculate correction factors for our diet components. First, we used diet samples containing only juniper and chow from the high creosote trial to calculate respective relative correction factors for those taxa ("juniper-chow correction factors"). Next, we created "control correction factors" using 50/50 mixtures of target components (chow, creosote, and juniper) and a control species as done in Thomas et al., 2016. For the control species, we used the leaves from the crown of pineapple (*Ananas comosus*). We chose this species because pineapple crown contains similar fiber levels to

our species of interest (Brito et al., 2020; Skopec et al., 2015) and pineapple belongs to a plant family (Bromeliaceae) that is unlikely to appear in natural woodrat diets (Givnish et al., 2011; Patton, Huckaby, Álvarez-Castañeda, & Ticul, 2014). We ground and mixed the species of interest (chow, creosote, and juniper) and the pineapple leaves as described for other diet trials. DNA was extracted from these diet samples, samples were sequenced, and sequences were analyzed and classified as previously described using the VSEARCH/QIIME2 pipeline. Following Thomas et al., 2016, we calculated both juniper-chow and control correction factors using the formula $(M_t/M_c) \times (S_c/S_t)$, where t is the species of interest, c is the control species, M_t and M_c are the true mass percentages of the test and control species, and S_c and S_t are the observed percentages of the control and test species, respectively. We then applied these correction factors to raw sequence counts from fecal samples by multiplying the sequence counts for each sample by the appropriate correction factor. Finally, we calculated the RRA of each dietary component using the adjusted read counts.

Weighted percent of occurrence has also been proposed as an alternative method to measure diet while minimizing the biases of RRA (Deagle et al., 2019). Therefore, we tested the accuracy of this approach as an alternative to uncorrected RRA for our system. Weighted occurrence scales each dietary component to an equal proportional weight (i.e., our samples contain 3 dietary items, therefore the weighted occurrence of each dietary item is 1/3 or 33%).

Statistical Analysis

To determine the accuracy of creosote, juniper, and chow relative abundances in fecal and diet samples, we calculated the percent error of each component as $(|\text{Actual abundance} - \text{Observed abundance}|) / (\text{Actual abundance}) \times 100$. We then used generalized linear mixed models (GLMMs) to test how different pipelines influenced percent errors of estimated diet components,

running separate models for each plant species of interest. In addition, we used GLMMs to test how the percent error of each dietary component was influenced by our different diets (i.e., changing proportions of creosote). For creosote and juniper, samples that did not contain the dietary component (i.e., 0% diets) were not included in these analyses. We ran models in the package glmmTMB (Brooks et al., 2017), inspected model residuals to confirm goodness of fit, and selected the best models using Akaike information criterion (AIC) (Akaike, 1974). Models were fit with a gamma error distribution to account for proportional data, and included individuals as a random effect, but did not include diet trial as it resulted in overfitting. We conducted post-hoc comparisons using Tukey-adjusted estimated marginal means in the package emmeans (Lenth, 2021). To account for the multivariate nature of the data, we also tested the same hypotheses using generalized linear models (GLMs) in the package mvabund (Wang, Naumann, Wright, & Warton, 2012).

We next examined the relationship between consumed plant proportions and plant proportions detected in the feces. To account for the proportional nature of the data as well as meaningful zero counts, we used zero-inflated beta-distributed GLMMs for these analyses. As both the high and low creosote trials included a 20% creosote diet, we first tested whether estimated creosote differed between the trials. Next, we tested whether recovered relative abundance of creosote and recovered relative abundance of juniper differed by the actual amount of creosote in the diet. For all models, we included individual as a random effect. To test the quantitative accuracy of metabarcoding, we assessed the relationship between the actual creosote in the diet and the relative abundance of recovered creosote using zero-inflated beta-distributed GLMMs, noting that in these models, we did not include individual as a random effect as it resulted in overfitting. We calculated quasiR^2 values as 1 − (deviance/null deviance) and conducted post-hoc comparisons using emmeans, as previously described. We again tested these same hypotheses using multivariate models in

mvabund. However, because mvabund cannot account for the proportions and meaningful zeros, we applied these models to counts of sequences rather than relative abundances. To account for uneven sequencing depth, sequences were first rarefied to 9,000 sequences per sample (the lowest read count of any one sample). Finally, as standard curves are typically calculated as simple linear models (Larionov, Krause, & Miller, 2005; Moosavi & Ghassabian, 2018), to determine whether we could create a standard curve for the proportion of creosote in the diet, we used linear models to evaluate the relationship between the relative abundance of creosote recovered from feces and the relative abundance of creosote in the diet. All statistical analyses were conducted in R version 3.6.1.

Results

Dietary Component Detection

The VSEARCH/QIIME2 and DADA2/BLAST+ pipelines performed similarly (Table 1). After filtering, the VSEARCH/QIIME2 pipeline produced 1,010,382 reads with an average of 28,868 ± 13,512 reads per sample and the DADA2/BLAST+ pipeline produced 1,950,528 reads with an average of 48,763 ± 24,599 reads per sample. Both pipelines assigned the majority of creosote and juniper reads to the family level and the DADA2/BLAST+ pipeline was able to assign some juniper reads to the correct genus level (Table 1). Using our reference database, neither pipeline could assign juniper reads to the species level due to the fact that multiple species from several genera within Cupressaceae have the same *trnL* sequence as *J. osteosperma*. Therefore, subsequent analyses treat all Cupressaceae reads as juniper; diets contained no other species from this family.

Extracted cactus DNA amplified only when cactus was the sole component of the mixture. We were able to visualize PCR products for both the 100% cactus plant sample and 100% cactus fecal sample, but the addition of another plant to the mixture resulted in reduced or no amplification of cactus (Figure S1). After sequencing, the VSEARCH/QIIME2 pipeline identified no reads in any sample as belonging to the cactus family (Cactaceae), even when animals consumed a diet of 100% cactus (Figure S2). The DADA2/BLAST+ pipeline identified cactus reads in three samples, but only recovered a maximum of ~50 reads identified as cactus. All other fecal samples from the cactus trial, no matter the amount of cactus in the diet, also consisted only of juniper and chow. Samples from animals feeding on the 100% cactus diet had very low sequence counts with an average of 4,221 SD ± 4,336 reads per sample compared to an average of 97,199 SD± 47,322 in other fecal samples from this feeding trial. For animals consuming 100% cactus, 84.0% (SD ± 26.0) of reads were retained after filtering; however, all of these reads were identified as juniper or chow. These NGS results, combined with poor amplification in PCR trials using the same primers, suggests that cactus DNA failed to amplify prior to sequencing. As cactus was not appreciably amplified using the *trnL* primers, subsequent analyses focus only on the creosote diets. Because the VSEARCH/QIIME2 pipeline produced fewer unclassified reads, all following analyses were conducted using data from this pipeline.

Creosote was detectable in feces even at very low diet amounts (Fig. 1A–C). After OTU filtering, creosote reads were found in only one 0% sample. This sample, from diet trial 2, contained only 40 creosote reads (0.13% of total sequences) that likely represent contamination. Creosote was successfully detected in all 1% and 5% samples (Fig. 1C). Estimated percentages of creosote varied between individuals and diets. Because there was no significant difference between relative abundance of creosote in fecal samples

from woodrats fed the 20% creosote diet between the two feeding trials (GLMM, F-Ratio = 1.41, $p = 0.280$), we combined data from both trials for subsequent analyses. The relative abundance of creosote in fecal samples significantly differed with differing amounts of creosote in the diet (GLMM, F-Ratio = 19.9, $p < 0.0001$; GLM, LRT = 23.1, $p < 0.001$; Table 2).

Although all animals were fed diets with 15% juniper, estimated amounts of juniper in fecal samples ranged from 10.2% to 83.7% (Fig. 1D). Estimated proportions of juniper in fecal samples significantly differed between diets (GLMM, F-Ratio = 7.0, $p < 0.0001$; GLM, LRT = 23.1, $p < 0.001$; Table 3). The relative abundance of juniper detected in fecal samples decreased as the proportion of creosote in the diet increased, although the relationship was weak (GLMM, z-value (25) = −2.42, $p = 0.02$, quasiR^2 = 0.18; GLM, LRT = 57.7, $p < 0.001$). Despite the inverse relationship between creosote and chow abundance in experimental diets, there was no relationship between the relative abundance of chow and the amount of juniper recovered in the feces (GLMM, z-value (25) = −1.3, $p = 0.19$). When using a multivariate model, there was an effect of the amount of creosote in the diet on the abundance of the other dietary items (GLM, LRT = 57.7, $p < 0.001$).

Accuracy of Relative Abundance Data

Sequencing overestimated creosote and juniper proportions, and underestimated chow proportions. There was no significant difference in percent error between pipelines for recovered creosote (GLMM, F-Ratio = 0.09, $p = 0.77$), juniper (GLMM, F-Ratio = 1.37, $p = 0.25$), or chow (GLMM, F-Ratio = 0.77, $p = 0.38$). When using a multivariate model, pipeline did not have a significant effect on percent error for any diet item (GLM, LRT = 0.9, $p = 0.9$). Relative abundance of creosote from feces of animals had a range of 1.0–17.2% on the 1% diet, 8.4–27% on the 5% diet, 22.6–81.2% on the 20% diet, and 14.4%–77.6% on the 60% diet. The

estimated relative abundance of creosote in fecal samples had an average percent error of 142.2% (SD ± 275.3) and percent error significantly differed between the different diets (GLMM, F Ratio = 7.8, $p = 0.002$; GLM, LRT = 21.7, $p < 0.001$); Table 4). On all diets, the relative abundance of juniper ranged from 10.4–74.6%. The average percent error of juniper sequences was 147.5% (SD ± 115.9) and also differed between diets (GLMM, F-Ratio = 3.73, $p = 0.02$; GLM, LRT = 21.7, $p < 0.001$). The 1% diet had a significantly higher percent error (average 312.7% SD ± 68.5) for juniper sequence counts than the 60% diet (62.7% SD ± 52.7). Chow amplified less than expected in each diet, never reaching more than 65.7% of recovered reads despite an expected value of 85% in the 0% creosote diet (Fig 1B). Chow had an average percent error of 53.9% SD ± 35. There was no significant difference in the percent error of samples pooled before sequencing to those averaged after sequencing (GLMM, F-Ratio = 0.08, $p = 0.79$; GLM, LRT = 0.07, $p = 0.74$). In general, fecal samples had significantly higher percent error than diet samples (GLMM, F-Ratio = 8.6, $p = 0.005$; GLM, LRT = 5.6, $p = 0.04$).

The proportion of estimated creosote in feces was significantly correlated with the actual amount of creosote in the diet (Spearman's correlation, $R^2 = 0.91$, $p < 0.001$). Although more creosote was detected in animals consuming diets with higher concentrations of creosote, the relationship between the amount of creosote in diets and that detected in feces was not linear. While we found a significant, positive relationship, the quasi-R^2 value was relatively low (GLMM, z-value (29) = −2.63, quasiR^2 = 0.28, $p = 0.008$; GLM, LRT = 23.18, $p < 0.001$). In addition, we evaluated linear models for use as a standard curve to predict the amount of creosote recovered from either fecal or diet samples from the known amount of creosote in the diet. Although standard curves derived from diet samples had a high R^2 value (0.97) and explained a large proportion of the variance ($F(1,4) = 168$, $p < 0.001$; Fig. 2A), the

same model constructed using fecal samples had a much lower R^2 value ($F(1,33) = 66.4$, $p < 0.001$, $R^2 = 0.66$) and even larger prediction intervals (Fig. 2B).

Adjusting Dietary Estimates Using Correction Factors and Weighted Occurrence

We tested whether uncorrected RRA, corrected RRA, or samples weighted by the number of dietary components (weighted occurrence) most accurately measured dietary components in fecal samples. Correction factors by diet are given in Table S5. The amount of error in estimating a diet component varied across dietary components (Table 5; Figure S3). First, we applied correction factors to our sequence counts to attempt to control for bias in RRA data. Compared to the uncorrected samples, the juniper-chow correction factor improved the accuracy of estimation in 82% of samples for both juniper and chow. Applying the control (pineapple-based) correction factor improved the accuracy of chow estimates in 82% of samples, creosote estimates in 4% of samples, and never improved juniper estimates. Measuring diet using weighted occurrence rather than RRA improved the accuracy of estimated creosote in 28% of samples and in 55% of samples for both juniper and chow when compared to uncorrected samples. However, weighted occurrence data was never the most accurate method of estimation for any of our dietary components. For creosote, uncorrected RRA yielded the lowest average percent error for creosote (Table 5). In contrast, for chow, the control correction factor-corrected RRA yielded the lowest average percent error (Table 5), and for juniper, juniper-chow correction factor-corrected RRA produced the lowest average percent error (Table 5).

Discussion

Metabarcoding is an increasingly popular method for characterizing wild animal diets; however, the quantitative accuracy of this technique has not been evaluated for non-ruminant herbivores. We conducted a series of feeding trials in which herbivorous woodrats were fed diets of known proportions to evaluate the accuracy of the resulting sequence counts and analysis methods. Our results revealed the successes and limitations of this technique in a non-ruminant herbivore. Below we discuss these findings in more detail.

Metabarcoding successfully detected rare diet components, as long as primers could amplify these taxa. One benefit of metabarcoding, compared to more traditional dietary analysis techniques, is its ability to detect and accurately assign taxonomy to rare species (Nichols, Åkesson, & Kjellander, 2016; Soininen et al., 2009). Our ability to consistently detect creosote at proportions as low as 1% provides additional evidence that metabarcoding can detect rare taxa in animal diets. However, we often failed to detect cactus, even when it was the only component of the diet, due to primer mismatch. Although metabarcoding primers are often referred to as "universal", these primers do not match all species. Indeed, all publicly available sequences for the Cactaceae family have two mismatches in the *trnL h* primer that occur at the third primer position and third from last primer position, which likely strongly impairs binding. Similarly, previous studies found that increasing numbers of mismatches, particularly near the 3′ end, reduce amplification considerably (Piñol, Senar, & Symondson, 2019b; Stadhouders et al., 2010). Although we were able to amplify cactus DNA when it was the only component of the diet, it was lost once more than one plant was present in the mixture. As wild herbivores rarely feed on a single plant, diet metabarcoding studies in wild animals likely underestimate cactus feeding. Our results from the cactus diet trial underscores that primer mismatches can impact not only RRA, but also detection, even of abundant diet components.

Such detection and taxonomic biases can be avoided by checking for primer mismatches, creating mock community samples of available food sources, or using multiple primer pairs. However, these errors may be difficult to identify and control for when wild animal diets are unknown.

Sample pooling is commonly used to reduce processing time and sequencing costs for large scale studies (Ando et al., 2020), or when a single fecal sample does not contain enough DNA template, such as for many small rodents. Pooled RRA data could provide useful dietary ecology data for such situations. Previous work in insectivorous bats found that pooled fecal samples had higher error in frequency of occurrence estimates compared to individually sequenced fecal samples and that rare dietary items were often missing (Mata et al., 2019). In contrast, our results suggest that pooling samples can provide an equally accurate estimation of diet abundance as individually sequenced samples. This difference between our study and Mata et al., (2019) may be due to using frequency of occurrence rather than relative read abundance, differences in diet richness, or differences in amplification of insect DNA rather than plant DNA as some insect primers can amplify host DNA. Further work would be needed to distinguish among these possibilities. Additionally, pooling at alternative stages of the pipeline, such as pooling PCR products or final sequences, may have different effects on the resulting diet diversity. Future studies could explore differences in the accuracy of the resulting relative abundance of dietary items when pooling at various stages.

A general relationship seems to be emerging in that the amount of a dietary item present in the feces increases with the amount in the diet. The results from our trial with creosote concur with that from diet trials in sheep (Willerslev et al., 2014). In addition, sequence counts produced from diet mixtures were more accurate than those from fecal samples, demonstrating digestion introduces

substantial variability and increases error. Notably, herbivores have relatively long gut transit times, which can vary by species (Hume, 1989; Sakaguchi, 2003) and impede large DNA fragment amplification (Juen & Traugott, 2005). An additional complicating factor is that ingested plant tissues may contain differential concentrations of DNA as well as be differentially degraded by the herbivore gut. Not only do different plants have different digestibility, but woody stems contain more indigestible material than other parts of the plant, such as leaves or buds (Shipley, Illius, Danell, Hobbs, & Spalinger, 1999). Plants or plant tissues that are more thoroughly digested may result in more thoroughly degraded DNA and therefore be underrepresented in the resulting sequence counts. Though we used only leaves in our study, wild samples may be affected by ingestion and differential digestion of different tissue types. Finally, as both our study and previous studies in sheep used relatively simple diets, future studies should test more complex diets and investigate the effects of plant digestibility on sequence counts.

We found that metabarcoding produces quantitative estimates of diet; however, these estimates have substantial error. The lower concentrations of creosote were distinguishable from all other levels, until creosote exceeded 20% of the diet. We were unable to discern between the 20% and 60% diet samples, despite the threefold difference in the actual proportion of creosote in the diet, and that at 60%, creosote comprised the majority of the diet. In addition, the constant dietary component, juniper, was not a constant proportion in recovered sequences. Although all diets contained 15% juniper, we recovered fewer juniper sequences as the proportion of creosote in the diet increased. One of the limitations of metabarcoding, and of using RRA, is that error in one component propagates across all components.

One of our dietary components, chow, always under amplified compared to its actual proportion in the diet and likely contributed to the inaccuracies in estimating other dietary components. We suspect that chow amplified poorly because it is dried and processed. Heat treatment and sterilization techniques used in processed diets can significantly degrade DNA and subsequently affect PCR amplification (Hrnčírová, Bergerová, & Siekel, 2008; Särkinen, Staats, Richardson, Cowan, & Bakker, 2012). Previous studies on processed plant material found limited or no amplification for highly degraded samples, with long DNA fragments (greater than 143 bp) producing the worst yields (Bruno et al., 2019; Särkinen et al., 2012). Our ability to recover chow sequences at all may have been because our chosen primer set amplifies a very short region (10–143 bp; Taberlet et al., 2007) and that our chow was sterilized at a relatively low temperature (48°C). Our results suggest that quantitative estimates of diet proportions for animals that consume processed foods may be more difficult than unprocessed dietary items; animals in zoos, urban environments, or wildlife parks may fall into this category. Indeed, a previous study on generalist carnivores found that sequences from human-linked, highly processed foods were rarely recovered (Forin-Wiart et al., 2018). This bias may also affect the dietary estimates of animals consuming mixtures of fresh and cached foods by underestimating the contribution of cached food to the diet. While these issues are unlikely to impact dietary estimates for animals consuming a fresh, natural diet, researchers estimating diet in urbanized animals or from highly degraded samples should aim to amplify short target regions and interpret quantitative results with caution.

How to most accurately interpret sequence data remains a question in diet metabarcoding studies. Diet data is often reported using occurrence, RRA, or a combination of both methods (Deagle et al., 2019; Kartzinel & Pringle, 2015; Quéméré et al., 2013). Notably, we found that, on average, RRA data were more accurate than weighted occurrence data. The error associated with weighted

occurrence data primarily stems from overestimating the abundance of rare items. For example, in samples where creosote was only 1% of the diet, weighted occurrence considers it in equal proportion with the other two components, inflating the proportion of creosote well beyond what was in the sample. These errors were less pronounced for juniper, as the actual percentage of juniper in the diet (15%) was much closer to the weighted occurrence estimate (33%). However, even for this more abundant component, weighted occurrence increased measurement accuracy in only 55% of samples. Using weighted occurrence is often considered a more conservative method of estimating unknown diets. However, inflating the importance of rare taxa while diminishing the importance of the most common dietary item can increase error, as demonstrated in our study. In addition, using weighted occurrence artificially makes the abundance of dietary items perfectly even, which could have major repercussions when using metabarcoding to understand an animal's niche and diet ecology. The sequencing of individual dietary components against a control component has been suggested as a means to correct for technical bias in RRA; however, the success of this technique varies across studies including ours (Deagle et al., 2010; McLaren, Willis, & Callahan, 2019; Thomas et al., 2016, 2014). We created 50/50 mixtures of each of our dietary components and a control material (pineapple). However, the correction factors for juniper and creosote amplified in the opposite direction than expected. In other words, creosote and juniper sequences had a higher-than-expected relative abundance in experimental fecal and diet samples, but a lower-than-expected relative abundance in our 50/50 mixtures. Because the correction factors did not amplify consistently with our samples, this correction method rarely improved sample estimates. The chow was the only component for which the control correction factor amplified in the same direction and subsequently, we were able to increase the accuracy of chow proportion estimates. This result could be due to the processed nature of the chow, i.e., the amplification of juniper and creosote

was skewed in our experimental samples compared to amplification against the fresh, control material. Further evidence for this is that when we used a correction factor derived from 50/50 mixtures of processed chow and juniper, we saw increases in accuracy for both of these dietary components. Correction factors derived from samples composed entirely of fresh material may be inaccurate when animals simultaneously consume other dietary items that do not amplify well, such as cached versus fresh foods. Because dietary components may amplify differently depending on the composition of the entire diet, it may not be possible to generate a correction factor that provides consistent improvement across all samples. In a previous study, this correction technique was successfully applied and increased accuracy of fish-based tissue mixtures, suggesting that there may still be some merit to creating correction factors in other systems (Thomas et al., 2016, 2014). We encourage researchers to validate correction factors experimentally before use on wild or unknown samples and/or to use caution when applying these factors. Alternatively, amplifying known quantities of synthetic DNA could mitigate these biases and provide a correction factor for individual dietary components. Critically, while correction factors can account for errors due to differential amplification, error due to differential digestion and other biological factors will be more difficult to correct.

Collectively, our results have important implications for the use of metabarcoding in herbivore diet determination. We find that RRA can provide quantitative measures of diet, particularly with respect to distinguishing between major (> 20%) and minor (< 20%) components. Researchers using metabarcoding on wild samples should carefully consider animal physiology and potential dietary items when choosing their diet estimation approach. When possible, technical biases for diet components of interest should be evaluated by sequencing control samples and quantitative data from wild animals should be interpreted cautiously. The continual

advancement of sequencing technology and reference databases for different primer sets may further improve the ability of metabarcoding to accurately assess diet composition. More studies on captive herbivores fed a known diet may also better explain sources of bias in sequence counts and refine ways to alleviate these effects.

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Data availability

Raw sequence reads and metadata are accessible through the NCBI Sequence Read Archive under BioProject PRJNA766427. All scripts associated with this manuscript are available at https://github.com/tess-stapleton/neotoma-trnl-metabarcoding.

Author Contributions

TS, SBW, and MDD designed the experiments. RG wrote scripts for the DADA2/BLAST+ pipeline and the creation of the reference databases. TS performed the research and processed sequences in the VSEARCH/QIIME2 pipeline. TS and SBW analyzed data and wrote the manuscript. MDD acquired funding. All authors helped edit the manuscript.

Table 1. Percentage of total reads taxonomically assigned by each pipeline to the family, genus, or species level as the lowest taxonomic level. Depth of taxonomic assignment varied by dietary component and pipelines performed similarly.

	Creosote			**Juniper**			**Cactus**		
Pipeline	**Family**	**Genus**	**Species**	**Family**	**Genus**	**Species**	**Family**	**Genus**	**Species**
VSEARCH/QIIME2	0.07	0.85	99.1	98.5	1.52	0	0	0	0
DADA2/BLAST+	0	0.07	99.9	59.7	40.3	0	0.01	0	0

Table 2. Results of between group comparisons testing whether the mean observed relative abundance of creosote in fecal samples differed from the proportion of creosote in the diet. Bolded text denotes comparisons with statistical significance.

Contrast	Estimate	Standard error	df	t–ratio	Tukey–adjusted p–value
0–1%	–1.29	1.07	26	–1.21	0.75
0–5%	–2.6	1.05	26	–2.48	0.13
0–20%	**–4.41**	**1.03**	**26**	**–4.29**	**0.002**
0–60%	**–4.71**	**1.04**	**26**	**–4.53**	**< 0.001**
1–5%	**–1.31**	**0.40**	**26**	**–3.24**	**0.02**
1–20%	**–3.11**	**0.42**	**26**	**–7.47**	**< 0.001**
1–60%	**–3.41**	**0.50**	**26**	**–6.89**	**< 0.001**
5–20%	**–1.81**	**0.30**	**26**	**–6.02**	**< 0.001**
5–60%	**–2.11**	**0.39**	**26**	**–5.36**	**< 0.001**
20–60%	–0.31	0.25	26	–1.22	0.74

Table 3. Results of between group comparisons testing whether the mean observed relative abundance of juniper in fecal samples differed by actual proportion of creosote in the diet. Bolded text denotes statistical significance.

Contrast	Estimate	Standard error	df	t–ratio	Tukey–adjusted p–value
0–1%	**–1.29**	**0.35**	**22**	**–3.75**	**0.009**
0–5%	–0.83	0.34	22	–2.45	0.13
0–20%	–0.15	0.3	22	–0.5	0.10
0–60%	0.33	0.35	22	0.93	0.88
1–5%	0.46	0.32	22	1.41	0.63
1–20%	**1.14**	**0.30**	**22**	**3.85**	**0.007**
1–60%	**1.62**	**0.36**	**22**	**4.54**	**< 0.001**
5–20%	0.69	0.30	22	2.35	0.17
5–60%	**1.16**	**0.35**	**22**	**3.30**	**0.02**

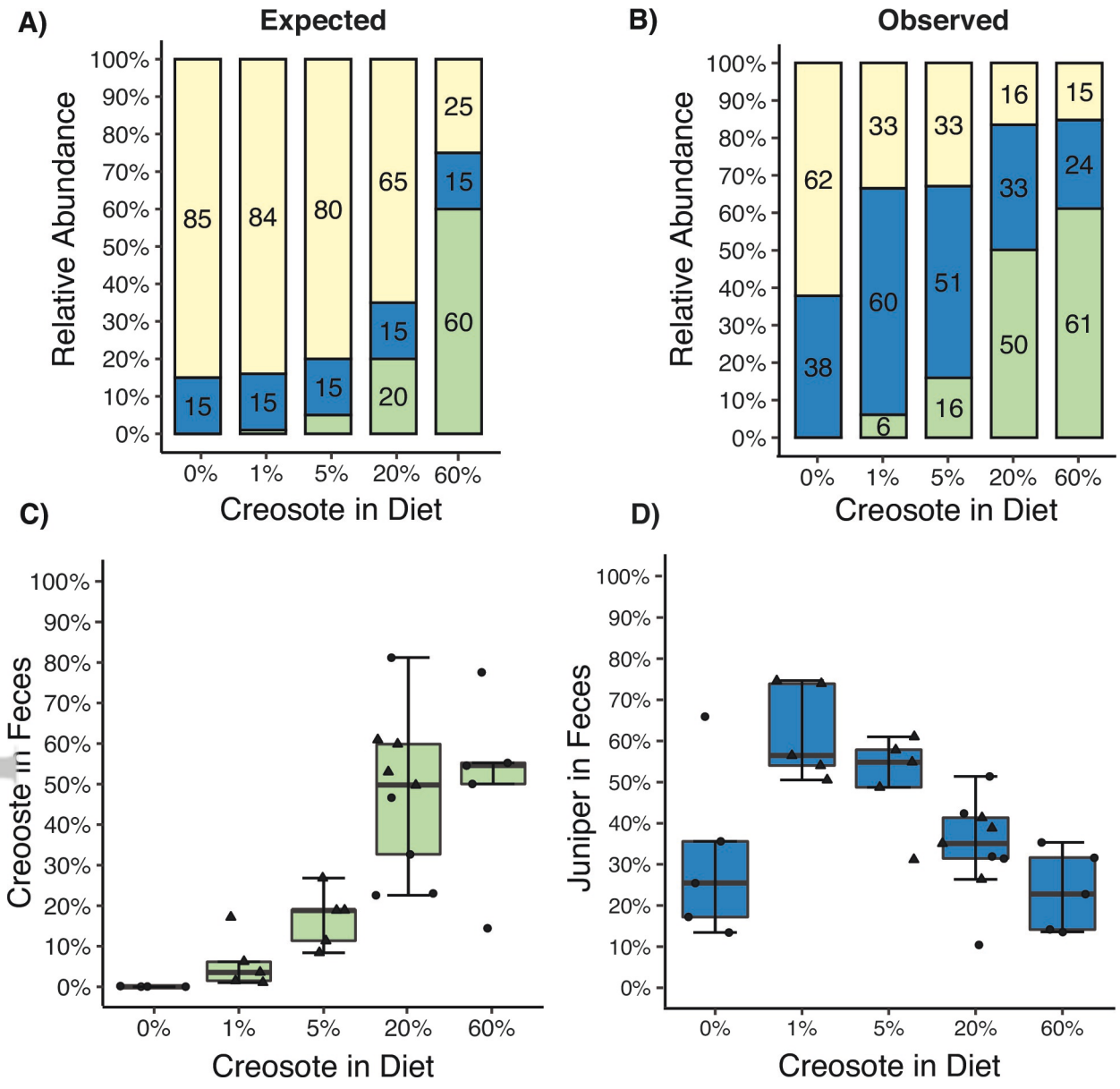
20–60%	0.48	0.31	22	1.53	0.56

Table 4. Results of between group comparisons testing whether the mean percent error of estimated creosote in fecal samples differed by actual proportion of creosote in the diet. Bolded text denotes statistical significance.

Contrast	Estimate	Standard error	df	t–ratio	Tukey–adjusted p–value
1–5%	0.72	0.62	18	1.17	0.66
1–20%	1.26	0.55	18	2.31	0.13
1–60%	**2.87**	**0.62**	**18**	**4.64**	**< 0.001**
5–20%	0.54	0.55	18	0.99	0.76
5–60%	**2.15**	**0.62**	**18**	**3.48**	**0.01**
20–60%	**1.61**	**0.55**	**18**	**2.95**	**0.04**

Table 5. Average percent error of estimated relative abundance after application of abundance weighting or correction factor for each dietary component. Bolded text indicates the lowest percent error for each dietary component.

	Creosote	Juniper	Chow
Uncorrected avg. percent error	**209%**	168%	59%
Weighted occurrence avg. percent error	817%	140%	49%
Control correction factor avg. percent error	632%	362%	**40%**
Chow correction factor avg. percent error	-	**58%**	43%



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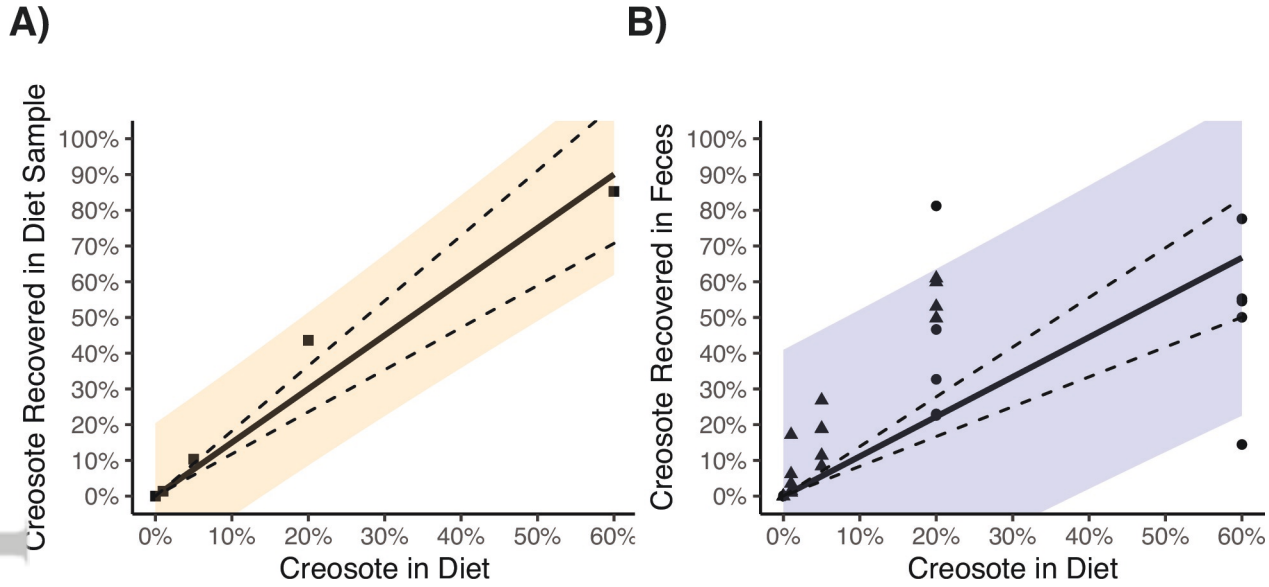

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