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RESEARCH ARTICLE

Metabarcoding reveals seasonal shifts in the Allegheny woodrat's generalist diet

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Abstract

The Allegheny woodrat (*Neotoma magister*) population has been declining for over a century, with reduced food availability being a leading hypothesis. Allegheny woodrats consume nuts, fungi, and vegetation, but no study has used a molecular tool, such as metabarcoding, to describe diet more accurately. Furthermore, few studies address seasonal diet changes in Allegheny woodrats. To address this gap, we performed a year-long DNA metabarcoding study from 2022 to 2023 by collecting fresh fecal (n = 180) and latrine (n = 240) samples from 2 populations located in Pennsylvania and New Jersey, USA. We used chloroplast trnL and internal transcribed spacer 1 (ITS1) markers to identify plants and fungi in the woodrat diet. We amplified and sequenced samples, then identified them to species using OBItools software and databases. We identified 123 families, 173 genera, and 156 species of plants and fungi in the Allegheny woodrat diet from the ITS and trnL dataset containing 19,208,635 reads. The summer season had higher diversity and richness than winter and hard mast items were not detected year-round. Fungi and invasive plant species were consumed more frequently than anticipated in each season. Fresh fecal samples detected more dietary items than latrine fecal samples, including more rare items. Findings will inform conservation plans and natural habitat enhancement actions, such as what to provide in cultivated food plots and diets for captive-bred individuals.

KEYWORDS

diet, DNA, fecal, fungi, ITS, *Neotoma magister*, New Jersey, Pennsylvania, trnL

Allegheny woodrats (*Neotoma magister*) have lived in rocky outcrops including cliff faces, talus slopes, and boulder fields across the Appalachian Mountain range for millennia (Newcombe 1930, Poole 1940, Castleberry et al. 2002, Castleberry et al. 2006, LoGiudice 2006). During the late 1900s, several states reported decreases in the species' abundance (LoGiudice 2006, Wright 2008), and Allegheny woodrats were assessed as near threatened by the International Union for Conservation of Nature (IUCN) in 2024 (Norris and Whittaker 2024). Since the late 1900s, Allegheny woodrats commonly have been categorized as endangered, vulnerable, or extirpated across their historical range (Table S1). Population reduction and range contraction are thought to be due to a number of interacting factors including habitat fragmentation, reduced connectivity and subsequent loss of genetic diversity (Balcom and Yahner 1996, Chamblin et al. 2004, Ford et al. 2006, Smyser et al. 2012, Davis et al. 2021, Muller-Girard et al. 2022), parasite mortality (McGowan 1993, Balcom and Yahner 1996, LoGiudice 2001, Smyser et al. 2011), and a decrease in food availability (e.g., loss of the American chestnut [*Castanea dentata*]) and quality (e.g., variable acorn crops) mediated by contemporary changes in the regional plant community (Hall 1987, Balcom and Yahner 1996, Castleberry et al. 2001, Smyser et al. 2011). The contribution to woodrat population declines by predators is unknown, but at least in Pennsylvania, populations of bobcat (*Lynx rufus*) and fisher (*Pekania pennanti*) have increased markedly since 2000 (Keller 2024). Evaluating the importance of decreased food availability to species persistence has been complicated by an incomplete understanding of the Allegheny woodrat diet.

Allegheny woodrats eat fungi, vegetation, berries, and nuts (Newcombe 1930, Poole 1940, Castleberry et al. 2002). Woodrats also eat insects as a minor component of their diet (Castleberry 2000, Castleberry et al. 2002, Parker 2006). Woodrats forage during summer and store food in caches for winter. These caches typically are composed of hard mast foods that store well, such as acorns and chestnuts, which are high-energy food resources consumed all year (Poole 1940, Castleberry et al. 2002, Castleberry and Castleberry 2008). Wright and Kirkland (2000) and LoGiudice (2006) argued that the American chestnut likely was a historically important food source, but this species functionally has been lost to the chestnut blight (*Cryphonectria parasitica*; Rigling and Prospero 2018). Acorns are believed to be an important food source currently (Lombardi et al. 2018), but acorns contain tannins that can inhibit digestion of nutrients (Chung et al. 1998). In addition, acorn production varies per year (Fleurot et al. 2023), and oak (*Quercus* spp.) foliage and acorn production and abundance has decreased owing to defoliation from spongy moths (*Lymantria dispar*; McManus and McIntyre 1981, Hall 1987), making it a suboptimal replacement to chestnuts. White-tailed deer (*Odocoileus virginianus*) and eastern wild turkey (*Meleagris gallopavo*) populations have increased in Allegheny woodrat habitat since the late twentieth century, and hunting of small game, such as squirrels, has decreased, thus potentially increasing competition for hard mast items (McShea and Schwede 1993, Norman and Steffen 2003, LoGiudice 2006, Boyd and Weaver 2011). These plant and animal community changes likely influence both survival and fecundity of woodrats. For example, a decline in the availability of acorns could result in increased mortality events associated with exposure and predation in winter (LoGiudice 2006; Lombardi et al. 2018, 2022) and females that survive predation and winter stress may lack suitable energetic and nutritional resources, limiting offspring in spring and summer (Mengak 2002, Manjerovic et al. 2009, Smyser et al. 2016).

Diet analyses can provide accurate and detailed information that can be used for conservation management (Iwanowicz et al. 2016, Castle et al. 2020, Goldberg et al. 2020, Lopes et al. 2020). Observations of food collected in caches (Newcombe 1930, Poole 1940), stomach content (Poole 1940, Heisler 1941), and fecal microhistological analyses (Castleberry 2000), have revealed some Allegheny woodrat dietary information (Table S2). However, much is unknown about Allegheny woodrat diets, such as how diet changes seasonally. An increased understanding of seasonal food usage can lead to better conservation management, such as what to provide in cultivated food plots, diets for captive-bred individuals, and how to improve and promote natural foraging habitats (Dierenfeld 1997, Gilbertson et al. 2018, Castle et al. 2020).

To better understand the Allegheny woodrat diet, we performed a DNA metabarcoding study of samples collected across a 1-year period from Allegheny woodrat populations in Pennsylvania and New Jersey, USA.

A high-throughput sequencing approach such as metabarcoding is a useful and non-invasive tool to accurately evaluate an organism's diet by sequencing DNA extracted from a single fecal sample to identify multiple taxa (Pompanon et al. 2012, Alberdi et al. 2019, Sousa et al. 2019). To ensure we captured a broad range of taxa, we used 2 metabarcoding markers, the trnL intron in the chloroplast genome and an internal transcribed spacer one (ITS1) locus in the nuclear ribosome genome. Our primary objectives were to examine seasonal changes in the Allegheny woodrat diet to inform our understanding of what they currently eat at these sites, determine if there is support for the food decline hypothesis, and facilitate future management recommendations. We hypothesized that if seasonality limits Allegheny woodrats' food availability, then diversity of dietary items present in fecal samples would decrease in winter relative to summer, and winter diet predominantly would be composed of food items that can be cached. Specifically, we predicted soft mast would be present predominantly during late spring and summer, whereas hard mast would be present year-round, with most abundance during fall to spring, and fungi and ferns would be present year-round. Our second objective was to compare the effect of fecal freshness on the diversity of dietary items identified to determine if less-intrusive, latrine-collected samples are adequate to study the Allegheny woodrat diet. We predicted that we would recover similar diversity and richness between fresh fecal samples and those exposed to the environment for longer periods.

STUDY AREA

We collected samples from 2 known Allegheny woodrat populations and chose sites because they were safe to access year-round. The first study site was State Game Lands 67 (40°14′32″ N 78°9′45″ W) located in Huntingdon County Pennsylvania (PA). The second site was the Palisades Interstate Park (40°59′04.8″N 73°54′21.0″W) located in Bergen County, New Jersey (NJ; Figure S1). The woodrat population at Palisades Interstate Park is the only known remaining population in the northeastern part of the historical species' range (including New York, New Jersey, and Connecticut).

States Game Land 67 is a 23-km^2 tract managed by the Pennsylvania Game Commission (PGC) with a peak elevation of 708 m (Pennsylvania Game Commission 2024). This area contains mountains and deciduous forests with the main human activities being hunting and timber extraction (Pennsylvania Game Commission 2024). Allegheny woodrats can be found in rocky outcrops, talus fields, and human-made rock piles. White-tailed deer, wild turkey, eastern grey squirrel (*Sciurus carolinensis*), and ruffed grouse (*Bonasa umbellus*) live here (Pennsylvania Game Commission 2024). Common plants are oaks, hickory (*Carya* spp.), black cherry (*Prunus serotina*), blueberry (*Vaccinium* spp.), and blackberry (*Rubus* spp.; Western Pennsylvania Conservancy 2004).

The Palisades Interstate Park is a 10-km^2 region with a peak elevation of 125 m (Palisades Interstate Park in New Jersey 2024). It is surrounded by urban and suburban development. The woodrat habitats consist of diabase cliffs, open and forested talus slopes, mesic forests, and the shoreline of the Hudson River (Amy Greene Environmental 2021). This area contains hiking trails throughout the park. The east side borders the Hudson River while the west faces the Palisades Interstate Parkway and Route 9 W. Princess tree (*Paulownia tomentosa*), black birch (*Betula lenta*), poison ivy (*Toxicodendron radicans*), and Virginia creeper (*Parthenocissus quinquefolia*) are dominant plants (Amy Greene Environmental 2021). White-tailed deer, eastern chipmunks (*Tamias striatus*), eastern grey squirrels, raccoons (*Procyon lotor*), and foxes (*Vulpes* spp.) also live here (Palisades Interstate Park in New Jersey 2024). Allegheny woodrats can be found on the east side of sheer cliffs and surrounding talus slopes.

Both study sites are in temperate forests, where minimum and maximum temperatures in PA and NJ ranged from −17°C and −14°C to 34°C and 36°C, respectively, during collection (National Weather Service 2024). Fecal collection occurred for 2 days each season, beginning 10 May (spring), 15 July (summer), 10 October (fall), and 18 January (winter, 1 day).

METHODS

Fecal collection

From 10 May 2022 to 18 January 2023, we trapped Allegheny woodrats in spring, summer, and fall at each study site to collect fresh fecal samples (Figure 1). We trapped for 2 days at each site during each season. During each trapping session, we distributed 30–60 tomahawk traps (Tomahawk Live Trap Co, Hazelhurst, WI, USA) throughout the site, as the home range of Allegheny woodrats can vary from 0.11 ha to 6.5 ha for males and 0.18 ha to 2.2 ha for females throughout the year (Castleberry et al. 2001, Mengak 2002, Castleberry 2008). In addition, woodrats are territorial, and typically 1 to 5 woodrats live per rock site or outcrop (Wood 2008), but adult females, who occupy high-quality den sites, rarely tolerate woodrats other than their young of the year within 30 m of the den site (Peles and Wright 2008). We wrapped traps halfway in plastic wrap or placed a paper bag underneath each trap, to minimize environmental contamination of the fecal samples. We baited traps with an apple slice and peanut butter, left them overnight, and inspected them the following morning. We weighed, aged, and sexed all captured Allegheny woodrats. We identified previously captured woodrats by ear tag, gave newly captured woodrats an ear tag, and then released them at the site. The trapping protocol was developed by the Pennsylvania Biological Survey,

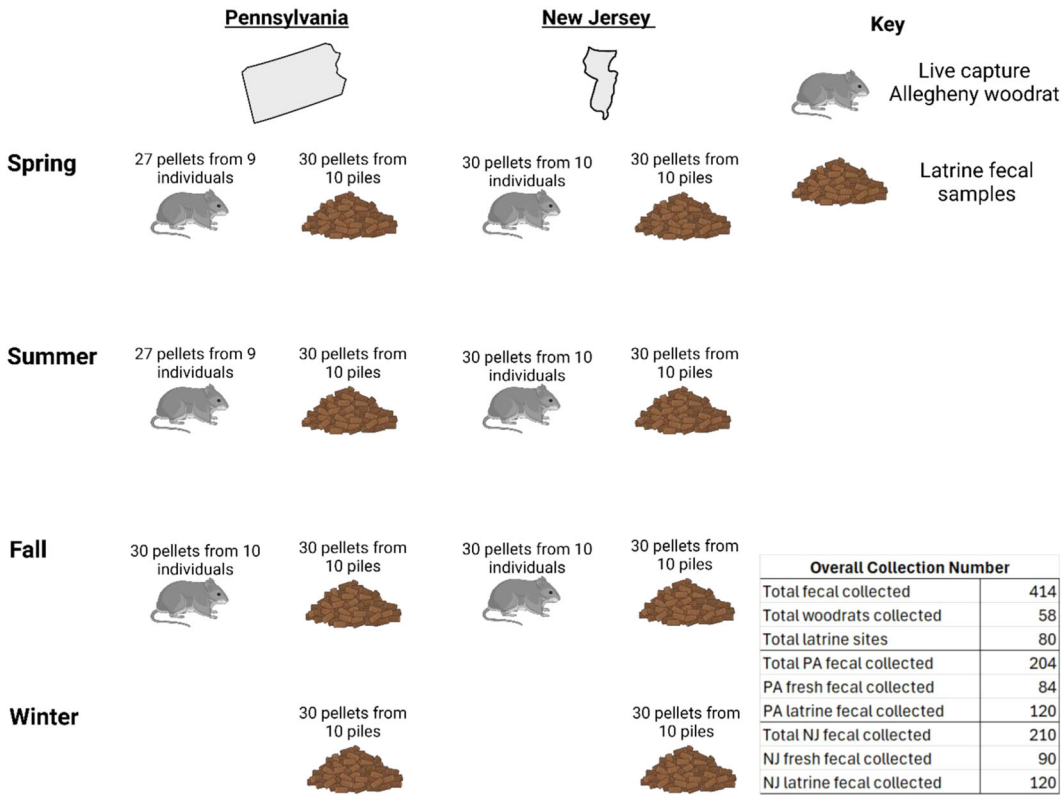


FIGURE 1 Sample collection schema used to identify seasonal diets of Allegheny woodrat from fecal pellets collected in 2022–2023 across 4 seasons in State Game Lands 67, Pennsylvania, USA (PA) and Palisades Interstate Park, New Jersey, USA (NJ). Fecal collection occurred for 2 days each season, beginning 10 May (spring), 15 July (summer), 10 October (fall), and 18 January (winter, 1 day). Figure created in Biorender (https://BioRender.com/k94q488).

Mammal Technical Committee, following Butchkoski et al. (2005). Woodrats were not trapped in winter to minimize mortality due to cold temperatures and inclement weather.

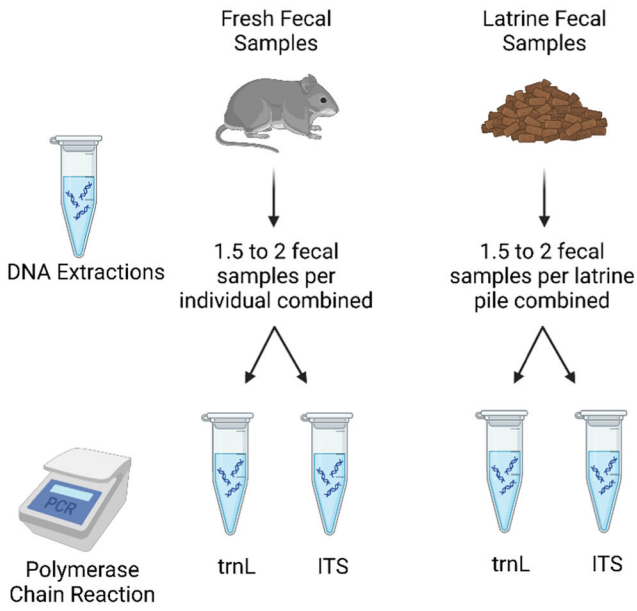
Allegheny woodrats defecate outside of their dens into piles (i.e., a latrine system; Balcom and Yahner 1996, Pennsylvania Game Commission 2021). We chose latrines for sampling if fresh scats (i.e., pellets that were still dark in color and wet) were present. We collected these samples once per season at each site and in tandem with trapping sessions. To increase the likelihood that latrine samples came from different individuals, we collected throughout the sites from 10 different latrine piles.

To evaluate the comparability of fresh and latrine fecal samples, we collected 3 fecal samples using flame-sterilized forceps from beneath each trapped woodrat and 3 samples from each latrine. We individually stored fecal samples in labeled tubes with 95% ethanol. All fecal samples were collected by members of the PGC, New Jersey Department of Environmental Protection (DEP) Fish and Wildlife Endangered and Nongame Species Program, or Towson University. We collected 414 fecal samples, including 204 from State Game Lands 67 (84 fresh, 120 latrine) and 210 from Palisades Interstate Park (90 fresh, 120 latrine), from 58 individual Allegheny woodrats and 80 latrine sites (Figure 1). For each season, we collected 27-30 fresh fecal samples (from 9-10 Allegheny woodrats) and 30 latrine fecal samples (from 10 latrine sites) per site visit (Figure 1). During winter, we collected only latrine samples.

DNA sequencing and bioinformatics analyses

We prepared all samples for DNA extraction in a bench hood sterilized with ultraviolet light and 10% bleach. We extracted genomic DNA from fecal samples using a QIAamp PowerFecal Pro DNA kit (Qiagen, Germantown, MD, USA) following manufacturer's protocol with a modified bead bash time of 30 minutes. Owing to the maximum weight of stool samples for the kit (250 mg), we combined 1.5-2 fecal samples per individual woodrat or latrine site. We extracted samples in batches of 30 samples (15 after combining) with a negative control to monitor any cross-contamination. We extracted positive controls from several species of plants separately from fecal samples. We cleaned fecal extractions with a Zymo DNA Clean and Concentrator-5 kit (Zymo Research, Irvine, CA, USA) to minimize the influence of polymerase chain reaction (PCR) inhibitors.

We performed PCR amplifications for 58 fresh fecal samples and 80 latrine samples (Figure 2). We used 2 metabarcoding markers, trnL and ITS, spanning 2 genomic regions. Locus trnL is associated with a chloroplast intron and can commonly be amplified even when using degraded DNA (Taberlet et al. 2007, Valentini et al. 2009, Mallott et al. 2018). We used universal primers g (5′GGGCAATCCTGAGCCAA3′) and h (5′CCATTGAGTCTCTGCACCT ATC3′) on the p6 loop (Taberlet et al. 2007). The other region covered was the nuclear ribosome with ITS1, which can amplify in plants and commonly is used to identify species of fungi (Nilsson et al. 2009, Bellemain et al. 2010, Schoch et al. 2012). For this region, we used primers ITS5 (5′GGAAGTAAAAGTCGTAACAAGG3′) and ITS2 (5′ GCTGCGTTCTTCATCGATGC3′; White et al. 1990). To avoid contamination, we conducted PCR procedures using separate pipettes with filtered aerosol pipette tips in a room separate from extraction and using a sterilized hood as described above. Our total PCR volume was 25 µl, 2 µl of this was DNA extract as a template. The PCR amplification mixture contained a concentration of 1X Standard Taq Reaction Buffer, 200 µM of each deoxyribose nucleotide triphosphate (dNTP), 0.2 µM of each primer, and 3 U of Taq DNA Polymerase (New England Biolabs, Ipswich, MA, USA). When using the trnL primers, we set the thermocycler program to start with an initial denaturing step of 94°C for 2 minutes, followed by 30 cycles of 94°C for 2 minutes, 63°C for 1 minute, and 68°C for 30 seconds with no final extension. For ITS, the thermocycler program started with an initial denaturing step of 95°C for 2 minutes, followed by 32 cycles of 95°C for 1 minute, 61°C for 1 minute, and 68°C for 1 minute with a final extension of 68°C for 5 minutes. We included 2 negative controls and 3 positive controls in each PCR set. All primers had an additional unique 24-nucleotide molecular tag addition to the 5′ end for Illumina sequencing (Illumina, San Diego, CA, USA). We delivered 276 PCR products (138 fecal samples per primer pair; Figure 2) to the



Sample Size After DNA Extractions	
Total fresh fecal	58
PA	28
NJ	30
Total latrine fecal	80
PA	40
NJ	40

Sample size PCR primers	
Fecal (fresh and latrine)	276
trnL total (fresh and latrine)	138
PA	68
NJ	70
ITS total (fresh and latrine)	138
PA	68
NJ	70

FIGURE 2 Description of DNA extraction and polymerase chain reaction (PCR) steps and sample sizes of fresh and latrine fecal samples collected from Allegheny woodrats in 2022-2023 in State Game Lands 67, Pennsylvania, USA (PA) and Palisades Interstate Park, New Jersey, USA (NJ). Figure created in Biorender (https://BioRender.com/w86t514).

University of Maryland Institute for Genome Sciences in Baltimore, Maryland for Illumina sequencing, where they evaluated amplicon DNA quality and performed secondary PCR for indexing, amplicon quantification, and pooling using Illumina paired-end 300-base pair sequencing and a P1 flow cell.

We received demultiplexed Illumina sequences from the University of Maryland Institute for Genome Sciences and used the OBItools pipeline (Boyer et al. 2016) to determine what plants and fungi were present in the fecal samples. Our sequence read parameters required aligned samples with an overlap score >80. We kept sequences that were present ≥10 times and did not use a sequence length requirement. From this step, 36,656 entries remained in the trnL library, and 76,281 entries were provided for the ITS library. We used the obiclean algorithm in OBItools to remove PCR and sequencing errors.

We created a global database using ecoPCR software (Ficetola et al. 2010) to simulate *in silico* PCRs on both markers with the European Molecular Biology Laboratory (EMBL) plant and fungi libraries and the National Center for Biotechnology Information (NCBI) database. For the simulated PCRs for both trnL and ITS, we allowed 3 errors, a minimum length of 10 and 50, and a maximum length of 300 and 800, respectively. We assigned taxonomic classification to the genus and family levels. We also assigned dietary items to the species level in many but not all instances, as identifying dietary items to the species level with metabarcoding can be difficult (Taberlet et al. 2007, Valentini et al. 2009, Nakahara et al. 2015, McInnes et al. 2017, Alberdi et al. 2019). We then exported 1 file per marker of the sample's identification name, scientific name, TAXID assigned by NCBI, and COUNT (number of times a sequence was present). We then used Python (version 3.8.10; Python Software Foundation, Fredericksburg, VA, USA) to search the TAXID in the NCBI database to report the taxonomic rank and the lineage of the identified scientific name organisms.

We used Excel (Microsoft Corporation, Redmond, WA, USA) to filter out the metadata. We considered sequence reads that were more abundant in negative controls than in the individual fecal samples to be contaminants and removed them. For both ITS and trnL, we reported any unclassified designation to the last identified

taxonomic classification. For example, we reassigned unclassified *Coprinellus* species to *Coprinellus* at the genus level. Also, we reassigned subgroups to their respective level. We kept only fruitbody fungi and crust fungi and removed microscopic, yeast, and mold-type fungi, as these likely represent environmental contaminants or impure food rather than food items sought out and consumed by the Allegheny woodrat (Alberdi et al. 2019, Goldberg et al. 2020).

Statistical analyses

We excluded individual woodrat fecal samples from downstream analyses if a technical error occurred and did not produce sequencing reads. We replicated analyses with 2 different data sets, one in which dietary items were assigned to genus and one in which they were assigned to family. We did not conduct analyses at the level of species, as our methodology could assign many but not all dietary items to the species level. We performed analyses in R (version 4.3.2; R Core Team 2024), primarily using tools from the vegan package (Oksanen et al. 2024), except where noted. We calculated rarefaction curves with the rarecurve function to assess how well collected fecal samples captured the full diversity of Allegheny woodrat diet within each combination of site and season.

We calculated the Shannon diversity index (Shannon 1948) for samples within each combination of sites, season, and fecal type using the diversity function in the vegan package. To ensure sample size did not influence our diversity indices (Soetaert and Heip 1990), we generated a scatter plot to determine the relationship between sample size and Shannon diversity indices, and used a linear regression to test the significance of the relationship. We used a Hutcheson *t*-test (Hutcheson 1970) to compare the significance of 2 Shannon diversity indices implemented in the ecolTest package in R (Salinas and Ramirez-Delgado 2021). We compared results between fecal types (fresh, latrine) and among seasons per site. We used Bonferroni correction to adjust the *P*-value for multiple pairwise comparisons among seasons (Bonferroni corrected *P*-value = 0.008).

We used non-metric multi-dimensional scaling (NMDS), implemented with the metaMDS function in R, to visualize similarity and dissimilarity of dietary items detected from fecal samples across sites and seasons. To reduce noise, we removed dietary items present fewer than 5 times. We calculated strength and significance of the relative contribution of each dietary item to observed differences with the envfit function in R. We evaluated differences in community composition across sites and seasons with a permutational multivariate analysis of variance (PERMANOVA) implemented with the adonis2 function in R.

We opportunistically took advantage of a recent, independently completed plant abundance survey conducted in Palisades, New Jersey (Amy Greene Environmental 2021) to consider whether woodrat diets were influenced by the relative abundance of plants on the landscape. We also used this quadrat-based survey of understory plants to compare relative abundance of taxa detected in fecal samples to relative abundance of plant taxa detected observationally at the NJ site. Forty-five species (39 genera), including saplings, vines, and herbaceous vegetation, were recorded in 1-m^2 vegetation sampling plots in the Amy Greene Environmental (2021) survey. We estimated relative abundance of taxa found in fecal samples by dividing the number of samples containing each taxon by the number of samples. We did not make any statistical comparisons for these data.

RESULTS

Of 276 samples sequenced, 250 produced sequence reads. The ITS and trnL datasets contained 8,838,398 reads that could be assigned to genus. From these reads, we identified 85 genera of plants and 88 genera of fungi in 117 Allegheny woodrat fecal samples. Fresh fecal samples detected 65 genera of plants and 74 genera of fungi, while latrine fecal samples revealed 59 genera of plants and 50 genera of fungi. Both fecal types shared 75 genera. We detected 119 genera at the PA site and 96 genera at the NJ site. Both sites shared 42 detected genera. Rarefaction

curves did not fully plateau in any group at the genus or family level, suggesting increased sampling (i.e., >10 individuals and 10 latrines) would be beneficial to detect more diet items of Allegheny woodrats (Figure S2).

Fresh vs. latrine across seasons

Depending on the season, fresh samples (when compared to latrine samples) revealed either greater or similar genera richness and Shannon diversity index values (Figure 3). For example, when considering the spring season, we detected more genera (PA: $P < 0.001$, NJ: $P < 0.001$) and generated higher Shannon diversity index values (PA: $P < 0.001$, NJ: $P < 0.001$) from fresh fecal samples than from latrine fecal samples in both PA and NJ (Figure 3). However, latrine and fresh fecal samples collected in summer and fall at the PA site were similar in diversity (PA summer: $P = 0.616$, PA fall: $P = 0.783$; Figure 3). We observed similar trends when dietary items were identified at the family taxonomic level (Figure S3). We found no correlation between sample size and Shannon diversity ($P = 0.225$, $r^2 = 0.12$).

Differences between sites and among seasons

Considering common dietary items (occurring >5 times) in both fecal types, PERMANOVA results indicated there were differences in the composition of dietary items amongst sites ($F_{1,100} = 17.5$, $P < 0.001$), seasons ($F_{3,100} = 5.46$, $P < 0.001$), and their interaction ($F_{3,100} = 4.05$, $P < 0.001$; Figure 4). Frequency of several dietary items differed

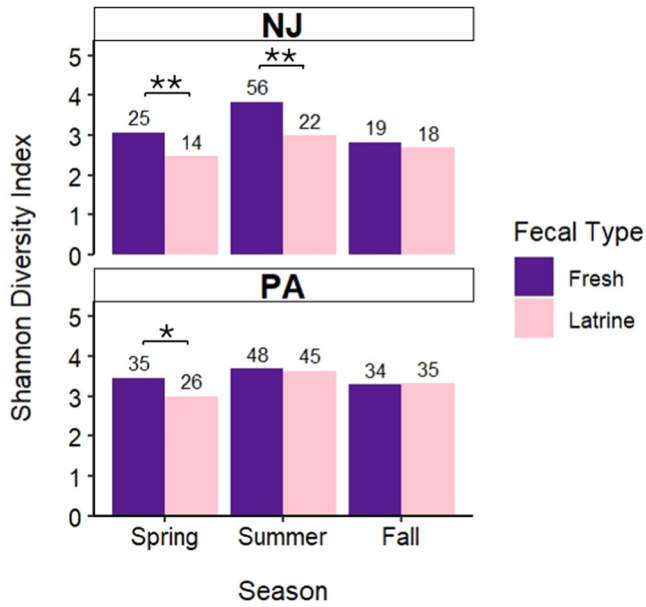


FIGURE 3 Allegheny woodrat diet Shannon diversity index identified with ITS and trnL metabarcoding markers, at the genus level. Sites are State Game Lands 67, Pennsylvania, USA (PA) and Palisades Interstate Park, New Jersey, USA (NJ). Fecal collection occurred in 2022–2023 for 2 days each season, beginning 10 May (spring), 15 July (summer), and 19 October (fall). Purple represents fresh fecal samples and pink represents latrine fecal samples. Numbers on bars indicate richness of detected items. We used a Hutcheson's *t*-test to evaluate pairwise significance of the Shannon diversity indices, shown by an asterisk if $^*P < 0.05$ or $^{**}P < 0.001$. We did not adjust *P*-values because we did not perform multiple pairwise comparisons.

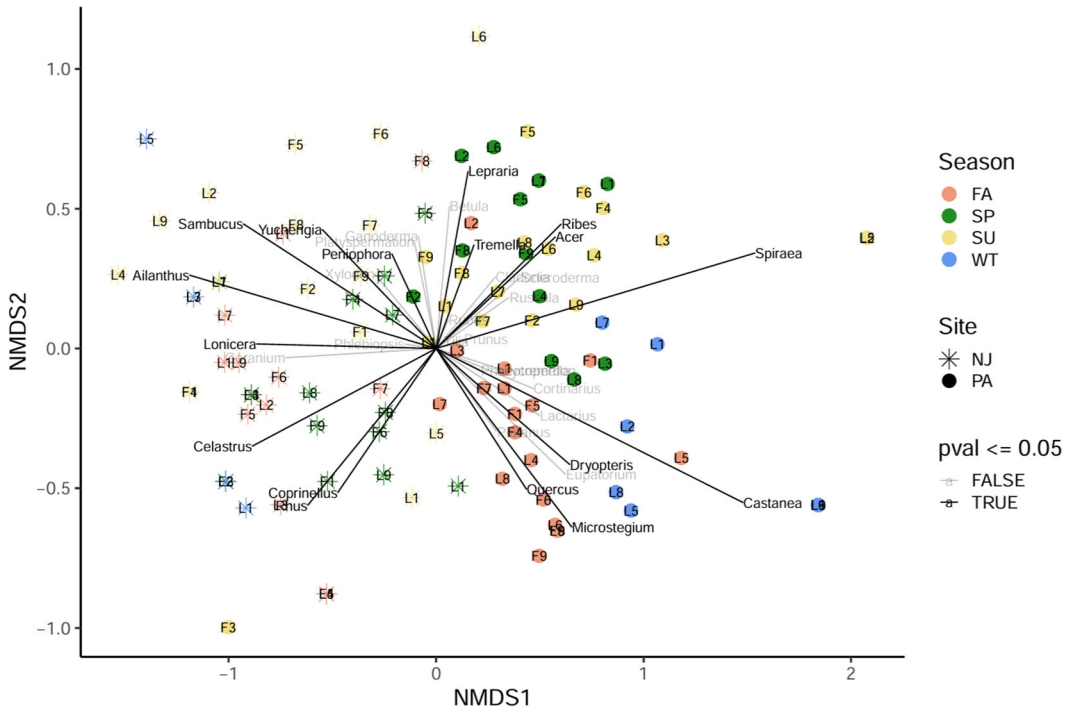


FIGURE 4 Non-metric multi-dimensional scaling (NMDS) of Allegheny woodrat diet items at the genus taxonomic level. Sites are State Game Lands 67, Pennsylvania, USA (PA) and Palisades Interstate Park, New Jersey, USA (NJ). Fecal collection occurred for 2 days each season in 2022–2023, beginning 10 May (spring, SP), 15 July (summer, SU), 10 October (fall, FA), and 18 January (winter, WT, 1 day). Points indicate individual fecal samples for which both loci (ITS and trnL) were amplified. Fresh and latrine fecal samples are indicated by an F or L, respectively, on the point. The number on the point indicates the individual fecal sample of that group (fecal type, season, site). Dietary items detected fewer than 5 times per seasonal fecal group across both sites were considered rare and removed to reduce noise. Vectors (lines) indicate direction and strength of dietary item detection along 2 primary NMDS axes. Longer vectors indicate the dietary item is more associated with a certain season or site. Dark vector lines indicate dietary items with strong evidence that they contributed to variation in diet among regions of the NMDS plot (*P* [pval] < 0.05).

among seasons and sites, contributing to distinct profiles of each sample type (Figure 4). The PA and NJ fecal samples formed distinct clusters on the NMDS plot (stress = 0.1029522), which illustrates that sites had very different detection rates of dietary items, with only 24.3% of all detected dietary items at the genus level shared between the 2 sites. For example, *Ailanthus* (tree of heaven species), *Sambucus* (elderberries), and *Celastrus* (staff vines) were detected only at the NJ site, while *Castanea* (chestnuts), *Spiraea* (meadowsweets), and *Dryopteris* (wood ferns) were detected at only the PA site. Other genera were detected at both sites, but at different frequencies. For example, *Coprinellus* was found at high frequency in NJ but low frequency in PA (Figure 4, black vectors). A few genera were found at similar frequencies at both sites (e.g., *Betula* [birches], *Ganoderma* [polypore fungi], and *Rubus* [raspberries]; Figure 4, grey vectors).

In both PA and NJ, some genera were detected every season, while others were detected a single time. For example, *Acer* (maple) and *Dryopteris* were detected during every season in PA. The most frequently detected genus during spring and summer was *Acer*, at 66.7% and 68.4%, respectively. However, *Acer* detection decreased in fall at 10.5% and winter at 12.5%. In contrast, *Dryopteris* had high detection rates during fall (47.4%) and winter (37.5%) but low detection in spring (13.3%) and summer (10.5%). The most frequently detected genus in fall at the PA site

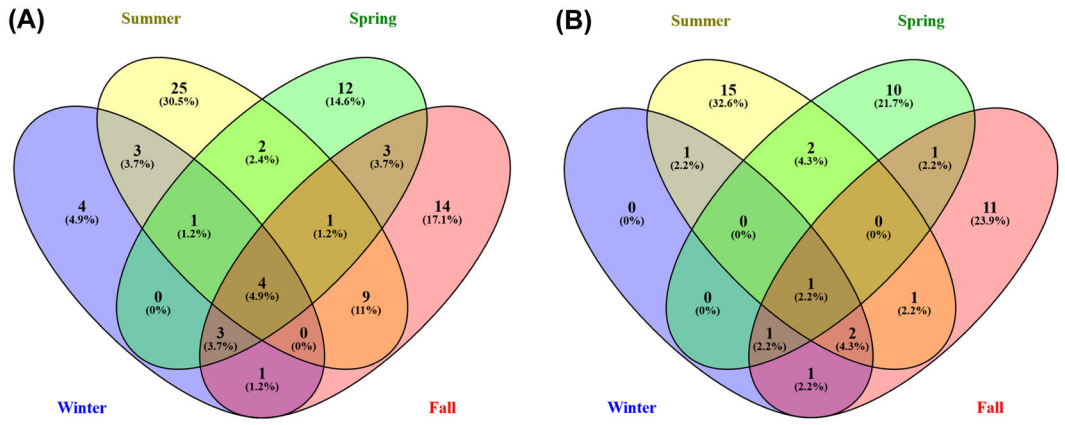


FIGURE 5 Number of dietary items of Allegheny woodrats identified to genus from (A) State Game Lands 67, Pennsylvania, USA (PA) and (B) Palisades Interstate Park, New Jersey, USA (NJ) in latrine fecal samples collected from 2022–2023. Percentages are the proportion of the entire sample represented by the number of diet items. For example, 14 diet items in fall in PA represented 17.1% of the total diet. Fecal collection occurred for 2 days each season, beginning 10 May (spring, green), 15 July (summer, yellow), 10 October (fall, red), and 18 January (winter, 1 day, blue). Venn diagrams were created using Oliveros (2007–2015) software.

was *Quercus* (oaks; 73.7% of fecal samples), which also was detected in winter and spring but at lower frequencies of 25% and 20%, respectively. At the NJ site, *Celastrus* was the most frequently detected genus in spring (64.7%), fall (41.2%), and winter (40%); it also was detected during summer at 23.5%. At the NJ site, *Ailanthus* was the most frequently detected genus in summer (52.9%); it was detected in fall (23.5%) and winter (20%) but not in spring. At the NJ site, *Sambucus* was detected each season at a similar frequency of 20–11.8%, except in the fall at 5.9%.

Several genera were detected during only 1 season but at high frequencies. For example, at the PA site, *Castanea* was detected in 100% of winter samples but was not present in spring, summer, or fall samples. In PA, *Lactarius* (milk-caps) were detected (in 42.1% of samples) only in fall. At the NJ site, *Crustodontia*, *Gelatoporia*, and *Trametes* fungi were detected only during summer in 23.5% of samples.

When considering only latrine sites (the only fecal type with sampling in all seasons), summer had the highest detection of distinct food items, while winter had the lowest in both areas (Figure 5). Each season, excluding NJ winter, exhibited a unique composition of dietary items (Figure 5). We observed similar trends at the family taxonomic level (Figure S4). Richness and diversity of genera were lower in winter compared to the other 3 seasons (PA: summer [SU]-winter [WT]: $P < 0.001$, fall [FA]-WT: $P < 0.001$, NJ: spring [SP]-WT: $P < 0.001$, SU-WT: $P < 0.001$, FA-WT: $P < 0.001$; Figure 6) except in PA in the spring (PA: SP-WT: $P = 0.009$; Figure 6). Winter diets also were lower in richness and diversity at the family taxonomic level (Figure S5). At the PA site, richness and diversity were greater in summer than during the other 3 seasons (SU-SP: $P < 0.001$, SU-FA: $P = 0.006$, SU-WT: $P < 0.001$; Figure 6). At the NJ site, richness and diversity were greater in summer when compared to spring and winter (SU-SP: $P = 0.001$, SU-WT: $P < 0.001$; Figure 6).

Relative abundance comparison

We compared relative abundance of dietary items as indicated by metabarcoding to that of an independent plant survey conducted at the NJ site (Amy Greene Environmental 2021). The comparison indicated a disproportional difference in genus preference of the woodrats (Figure 7). For example, *Parthenocissus* (creepers) had the highest relative abundance at the NJ site, but this plant was not detected in woodrat fecal samples (Figure 7). Conversely,

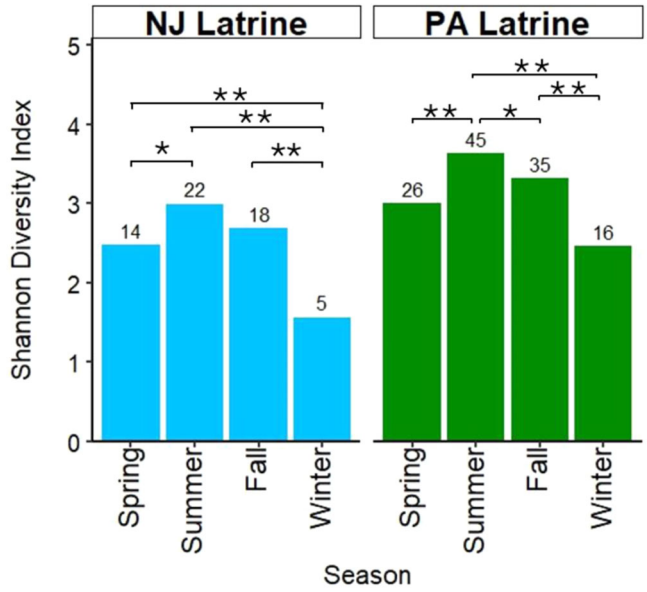


FIGURE 6 Diversity of diet items (as indicated by Shannon diversity index) of Allegheny woodrats, identified with ITS and trnL metabarcoding markers, at the genus level. Sites are State Game Lands 67, Pennsylvania, USA (PA) in green and Palisades Interstate Park, New Jersey, USA (NJ) in blue. Fecal collection occurred in 2022–2023 for 2 days each season, beginning 10 May (spring), 15 July (summer), 10 October (fall), and 18 January (winter, 1 day). Numbers on bars indicate richness of detected items. We used a Hutcheson's *t*-test to evaluate pairwise significance of the Shannon diversity indices, shown by an asterisk if $*P < 0.008$ or $**P < 0.001$. *P*-values were Bonferroni adjusted.

Celastrus frequently was found in fecal samples, despite intermediate to low abundance in the understory community.

DISCUSSION

One hypothesis for the ongoing decline of the Allegheny woodrat across its range is that populations are suffering from decreased food availability and quality, largely driven by the loss of the American chestnut and the decline in acorn production (food decline hypothesis; Hall 1987, Balcom and Yahner 1996, LoGiudice 2006). An understanding of the woodrat diet across seasons and the species' range is integral to understanding how this factor, in combination with habitat fragmentation and disease, can be combatted through management practices. Our metabarcoding study is the most detailed description of the Allegheny woodrat's diet to date, documenting over 400 dietary items. This represents more food items than past observational (e.g., caches, viscera samples) or microhistological analyses (Newcombe 1930, Poole 1940, Heisler 1941, Castleberry 2000; Table S2).

Our study indicated the use of fresh fecal samples generally resulted in higher detected diversity and richness relative to latrine fecal samples, likely because DNA in latrine fecal samples was more degraded because it had longer exposure to environmental factors than fresh fecal samples (Lindahl 1993, Oehm et al. 2011, Panasci et al. 2011, McInnes et al. 2017). If we had only used latrine samples during the spring to fall seasons, we would not have detected 38.2% of dietary items at the genus level and 24% at the family level. However, most of these diet items found only in fresh fecal samples were identified a single time when sites, markers, and spring-to-fall season data were combined, indicating these dietary items are rarer in occurrence. Overall, our results suggest both fresh

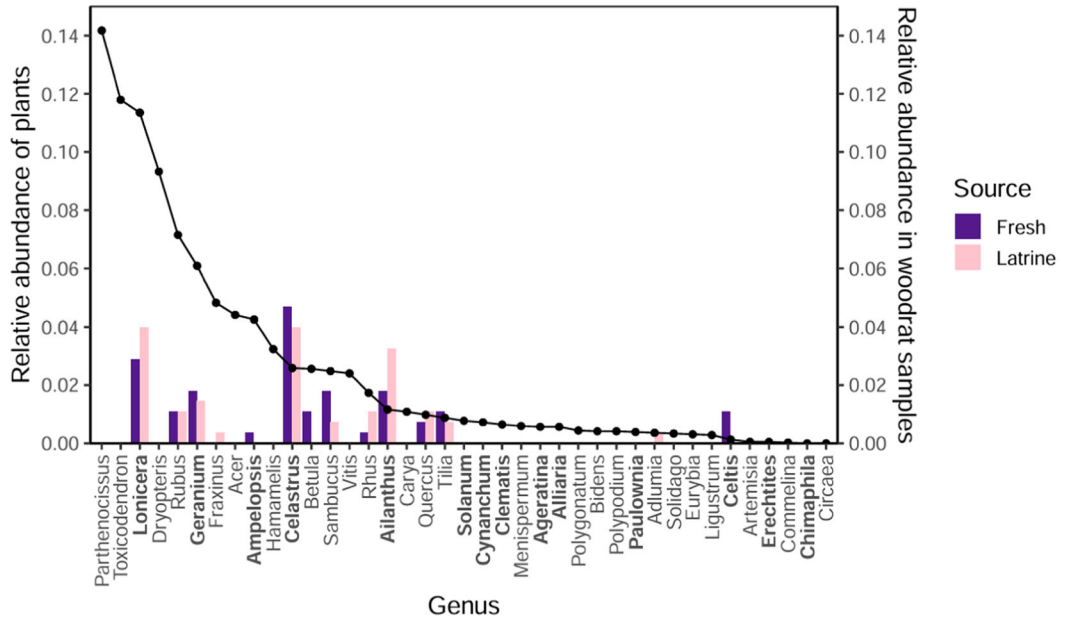


FIGURE 7 Relative abundance (calculated from percent cover data) of understory plant genera from Palisades Interstate Park, New Jersey, USA (Amy Greene Environmental 2021) and in woodrat diets as determined from fecal pellet samples. We determined relative abundance in woodrat samples by summing all occurrences of detected dietary items at the New Jersey (NJ) site and dividing by a particular taxa occurrence shown on the right *y*-axis and bars. The *x*-axis indicates the genera found at the NJ site through the Amy Greene Environmental (2021) survey. Bolded genera indicate those with invasive species. Purple represents fresh fecal samples; pink represents latrine fecal samples. Samples from 2022–2023 were combined across seasons.

and latrine fecal samples can detect common and rare items, but fresh samples will detect more rare items than latrine pellets.

Of particular interest is the contribution of mushrooms to the woodrat diet. Fungi were detected in the diet each season at both sites. Past studies have highlighted the frequency with which mushrooms were consumed by Allegheny woodrats (Castleberry et al. 2002, Parker 2006), and fungi have been reported in the diets of just 3 other woodrat species: dusky-footed (*Neotoma fuscipes*; Salmon and Gorenzel 1994), eastern (*N. floridana*; McMurry et al. 1993), and bushy-tailed (*N. cinerea*; Maser et al. 1978) woodrats. In all of these studies, fungi rarely were described with any taxonomic specificity. Furthermore, whether mushrooms are actually missing from the diets of many woodrat species (e.g., white-throated [*N. albigula*], southern plains [*N. micropus*], desert [*N. lepida*], Mexican [*N. mexicana*] woodrats) or simply underreported is unclear. Many past studies using metabarcoding of woodrat diets have used only the trnL locus (Nielsen and Matocq 2021, Stapleton et al. 2022), which cannot identify species of fungi. Studies incorporating the ITS locus or metagenomics will allow for a more accurate description of the breadth of dietary items among woodrat species.

We largely found support for our hypothesis that dietary diversity would be greatest in summer and most limited in winter. For example, as predicted, soft mast plants were more abundant in spring and summer than winter. This result is intuitive and consistent with several previous metabarcoding studies (Tiede et al. 2020, Hoenig et al. 2021, Brun et al. 2022). Growing conditions for most plants and fungi are best in summer and worst in winter in PA and NJ, influencing food availability for woodrats. Winter weather conditions also are less favorable for woodrats owing to lower temperatures and less vegetation cover to hide from predators, so foraging is less likely to occur, and reliance on caches increases (LoGiudice 2006, Castleberry and Castleberry 2008). Further, as predicted, items such as ferns and mushrooms were detected year-round.

Perhaps more importantly, we documented a decrease in dietary diversity between fall and winter, including a decrease in the presence of hard mast items. In contrast to our prediction, hard mast items were detected most frequently in the fall but decreased in frequency or were not detected in winter and spring. Woodrats cache acorns and other nuts to consume year-round (Poole 1940, Castleberry et al. 2002, Castleberry and Castleberry 2008) and the absence of these nutrient-rich and high-caloric food items has been suggested to limit reproduction and subsequent population numbers (LoGiudice 2008, Manjerovic et al. 2009). During the winter season, there was low detection of oak at the PA site and no hard mast items were detected at the NJ site. Results of this study suggest that woodrats may be unable to cache sufficient amounts of hard mast to last through winter, providing support for the food decline hypothesis. Encouragingly, our metabarcoding approach also indicates the success of a management practice meant to address food loss. The PGC deposited 227 kg of hard mast items, including European (*Castanea sativa*) and Chinese chestnuts (*C. mollissima*), throughout the PA site during the winter season of 2022–2023. Subsequently, chestnuts were found in every latrine sampled, indicating the population-wide benefit of supplemental feeding. No supplementary feeding occurred at the NJ site.

As is consistent with a generalist diet, our study and work by Castleberry et al. (2002) revealed woodrats will feed on very different items throughout their range. Only 21 dietary items were shared between our work and previous studies completed in Pennsylvania, Virginia, and West Virginia (Newcombe 1930, Poole 1940, Heisler 1941, Castleberry 2000). A wider variety of fern genera appear to be used in West Virginia than were found in either Heisler's (1941) Pennsylvania site or at our study sites (Newcombe 1930, Poole 1940). Four previous studies (Table S2) reported fungi in the diet, including several species found in West Virginia and Virginia but not in our study, such as *Boletus* species, *Clavaria* species, and *Gautieria morchelliformis* (Newcombe 1930, Poole 1940, Castleberry 2000). Plants found in Pennsylvania by Heisler (1941) but not detected at our PA site include trailing arbutus (*Epigaea repens*), partridgeberry (*Mitchella repens*), and serviceberry (*Amelanchier canadensis*). Differences in diets across woodrat range could be attributed to the plant community adjacent to den sites, latitude, and forest structure (Castleberry et al. 2002), in addition to techniques used to study diet (Nielsen et al. 2017, Massey et al. 2021). To address this unknown, future studies should compare techniques (e.g., observation, micro-histological, metabarcoding) on the same individual woodrats or caches and include more sites from a larger geographic range of this species. In addition, we found plant items that were not historically documented to be common in the diet. Particularly at the NJ site, invasive plant species such as tree of heaven (*Ailanthus altissima*), staff vine (*Celastrus*) species (possibly nonnative bittersweet [*C. orbiculatus*]), and Japanese honeysuckle (*Lonicera japonica*) were important components of the Allegheny woodrat diet. Woodrats in PA also consumed invasive species such as Japanese stiltgrass (*Microstegium vimineum*), but overall consumption of invasives was less when compared to NJ. The addition of these plants to the diet could be due to availability of food sources, increased prevalence, or detection methods. Whether these diet items represent quality foods for woodrats, or merely what is available, is uncertain. Future studies could investigate the nutritional value of these invasive plant species and the costs and benefits of removing or keeping these plants in woodrat habitats.

Despite being dietary generalists, woodrats appear to select particular food items. At the NJ site, the princess tree is one of the most common tree species present (Amy Greene Environmental 2021), but it was not detected in any woodrat fecal samples, suggesting woodrats are uninterested in this potential food source. However, Japanese honeysuckle was one of the most common ground layer species present (Amy Greene Environmental 2021) and was detected in woodrat scat each season at the NJ site. One reason woodrats might exhibit dietary preferences is that not all food items are equal in nutritional and caloric value. Mengak and Castleberry (2008) found that woodrats preferentially consume acorns when available but can persist in areas where overall hard mast is limited or non-existent. Similar to Castleberry et al. (2002) and Parker (2006), we found fungi in the diet throughout the year. Fungi are high in protein, rich in amino acids, and have antioxidant, anti-tumor, and antibacterial properties (Ayimbila and Keawsompong 2023, Li et al. 2023). However, the Amy Greene Environmental (2021) survey was not designed to compare relative abundance; thus, we could not consider all dietary items at the NJ site. Future studies of the woodrat diet would benefit from relative abundance of known dietary items.

Our conclusions should be considered in the context of the limitations of metabarcoding studies. One such limitation is being unable to tell what part of the plant is being consumed. In addition, being able to identify a diet item to family, genus, or species is dependent on robust comparison databases (Alberdi et al. 2019, Keck et al. 2022). As such, despite the substantial increase in identified dietary items by our study in comparison to past work, some food types likely go unrecognized. Finally, the use of trnL and ITS markers and potentially degraded DNA can make identifying dietary items to the species level difficult (Taberlet et al. 2007, Valentini et al. 2009, Nakahara et al. 2015, McInnes et al. 2017, Alberdi et al. 2019). Future studies might incorporate increased sampling efforts and pursue a metagenomics approach to increase the number of dietary items identified to species (Alberdi et al. 2019, Da Silva et al. 2019, Goldberg et al. 2020) and detect potentially rare food items like insects, as trnL and ITS markers are unable to detect any animal matter in the diet (Castleberry 2000, Castleberry et al. 2002, Parker 2006). Detecting differences in diet among sex, age class, and reproductive and lactating individuals would be helpful. A nutritional study could evaluate if woodrats are eating enough to be able to have sufficient offspring regularly to maintain stable populations.

MANAGEMENT IMPLICATIONS

The findings from this study will guide conservation management in several important ways. For example, the PGC cultivates food plots and uses habitat management techniques to preferentially promote desirable forage near known woodrat habitat. This work indicates which species might be planted and promoted for maximum benefit, and which items might be incorporated into diets of captive-bred woodrats. Finally, given the importance of fungi to the woodrat diet, effective conservation strategies might include leaving fallen trees down or even cultivating mushrooms. In addition to supporting the woodrats' reproductive output (LoGiudice 2008, Manjerovic et al. 2009), increasing food availability may decrease mortality from raccoon roundworm (*Baylisascaris procyonis*). Potentially, if more traditional food items were available, woodrats would not need to resort to seeds found in raccoon scat samples, leading to fewer roundworm infections (LoGiudice 2008).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

ETHICS STATEMENT

We used standard live-trapping and sample collection protocols in every collection visit. Collections followed standard mammalian sample techniques as permitted by a National Environmental Policy Act agreement between USFWS and PGC under the State Wildlife Grant agreements. All animal handling procedures used by Towson University employees were approved by the Towson University Animal Care and Use Committee (protocol #1718).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in National Library of Medicine Sequence Read Archive at https://www.ncbi.nlm.nih.gov/sra, reference number PRJNA1188638, SUB14878368.

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