Wild herbivorous mammals (genus *Neotoma*) host a diverse but transient assemblage of fungi

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ABSTRACT: Fungi are often overlooked in microbiome research and, as a result, little is known about the mammalian mycobiome. Although frequently detected in vertebrate guts and known to contribute to digestion in some herbivores, whether these eukaryotes are a persistent part of the mammalian gut microbiome remains contentious. To address this question, we sampled fungi from wild woodrats (*Neotoma* spp.) collected from 25 populations across the southwestern United States. For each animal, we collected a fecal sample in the wild, and then re-sampled the same individual after a month in captivity on a controlled diet. We characterized and quantified fungi using three techniques: ITS metabarcoding, shotgun metagenomics and qPCR. Wild individuals contained diverse fungal assemblages dominated by plant pathogens, widespread molds, and coprophilous taxa primarily in Ascomycota and Mucoromycota. Fungal abundance, diversity and composition differed between individuals, and was primarily influenced by animal geographic origin. Fungal abundance and diversity significantly declined in captivity, indicating that most fungi in wild hosts came from diet and

environmental exposure. While this suggests that these mammals lack a persistent gut mycobiome, natural fungal exposure may still impact fungal dispersal and animal health.

KEYWORDS: Mycobiome, mammal, microbiome, fungi, phylosymbiosis, diet

INTRODUCTION

All animals harbor a complex community of microorganisms that are critical for digestion, development, and immunity. Research on these microbiomes has primarily focused on bacteria, while other microbial groups such as fungi remain poorly characterized (Forbes et al. 2019). Studies on host-associated fungi have typically examined disease states or disease causing organisms (e.g., Cui et al. 2013, Fisher et al. 2012, Iliev and Leonardi 2017), with fungal communities in healthy animals receiving less attention (Peay et al. 2016). While fungi are detected in most human gastrointestinal tracts (Nash et al. 2017), and fungal exposure facilitates immune system development (Yeung et al. 2020), it is unclear whether fungi are persistent members of mammalian gut microbiomes (Fiers et al. 2019, Suhr and Hallen-Adams 2015). To address this question, we sampled the gut mycobiota from rodents in nature and after a month in captivity, quantifying how host and environmental factors influence fungal assemblages and whether fungi persist in the absence of natural sources.

Although the extent to which fungi colonize the human gastrointestinal tract remains controversial (Auchtung et al. 2018, Fiers et al. 2019), there is substantial evidence that herbivorous mammals host fungal symbionts (Hespell et al. 1997). While many fungi in herbivore guts derive from dietary sources (e.g. Lund 1980), some are mutualists that grow at mammalian body temperatures, require anaerobic conditions, and contribute to host digestion. For example, anaerobic fungi in the Phylum Neocallimastigomycota are obligate symbionts that aid in fiber degradation in both fore- and hindgut fermenting mammals (Gruninger et al. 2014, Solomon et al. 2016, Teunissen et al. 1991, Wang et al. 2019). In these herbivores,

Neocallimastigomycota composition and abundance is influenced by gut morphology and diet, as well as host taxonomy (Bauchop 1979, Boots et al. 2013, Liggenstoffer et al. 2010). This tendency for closely related hosts to harbor more similar communities (termed "phylosymbiosis") is also often seen for host-associated bacteria (Kohl 2020) and parasites (e.g., Braga et al. 2015, Cooper et al. 2012), suggesting that phylosymbiosis may be a common feature of symbiotic interactions.

Animal-associated fungal assemblages might exhibit phylosymbiosis (Harrison et al. 2021); however, these patterns could also be due to factors such as phylogenetically conserved diet, gut morphology, or geographic range (Kohl 2020). While gut mycobiota from free-ranging mammals remain poorly characterized, surveys of bats and non-human primates show that habitat, diet, and captivity all impact fungal diversity and abundance (Barelli et al. 2020, Li et al. 2018, Sun et al. 2018, Sun et al. 2021). Fungal communities are better characterized in laboratory mice and humans, and in humans, assemblages vary between individuals (Nash et al. 2017), with evidence that host genetics, immune function, lifestyle, and diet contribute to community composition (Cui et al. 2013, David et al. 2014, Hoffmann et al. 2013). Although laboratory mice typically host depauperate fungal communities, mice born to wild dams or housed in semi-natural environments harbor more fungi (Rosshart et al. 2019, Yeung et al. 2020), demonstrating that vertical transmission and environmental exposure both contribute to the maintenance and structure of vertebrate gut mycobiomes. Together, these studies suggest that host and environmental factors contribute to inter- and intraspecific differences in gut mycobiota; however, it is unclear which factors are most important, and whether patterns are driven by transient or symbiotic taxa.

Rodents in the genus *Neotoma* ("woodrats") are an ideal natural system for studying interactions between hosts and their gut mycobiota. These herbivorous small mammals are abundant in a variety of habitats across North America, with multiple species often occurring in sympatry (Reid 2006). Furthermore, their natural diets are well characterized and vary among species within the same habitat and among populations within a species (e.g., Dial 1988, Skopec et al. 2008). This ecological diversity creates an opportunity to quantify how diet, host evolutionary history, and geography contribute to microbial community structure (Kohl and Dearing 2016, Weinstein et al. 2021). Additionally, because woodrats readily acclimate to captivity (Martínez-Mota et al. 2020), wild caught individuals can be maintained in a controlled environment to facilitate the identification of core symbionts.

Here, we use wild and captive woodrats to test for a persistent gut mycobiome and examine the factors structuring this overlooked microbial community. If woodrats harbor symbiotic fungi, we predict that these fungal taxa will be adapted to an anaerobic gut environment, display evidence of phylosymbiosis, and be retained when animals are removed from natural environments. Alternatively, if fungi are transient and primarily derived from external sources such as food and nests, we expect animal diet or geographic origin to most strongly influence fungal composition, and that fungi will disappear in the absence of natural environmental sources.

METHODS

Sampling

To characterize fungal communities in woodrat gastrointestinal tracts, we sampled 120 wild individuals from 25 populations, representing 7 species across 18 sites in the southwestern United States (Supplementary Table S1). Sites were typically visited once; however, multiple trips were made to some sites (n = 3) to collect sufficient samples. We identified woodrats to species based on morphology, with the exception of *N. bryanti* and *N. lepida*, which were differentiated using microsatellite markers (Dearing et al. 2022). We captured animals using live traps (H.B. Sherman Traps Inc, Tallahassee, FL), collecting fresh feces at the time of capture (Weinstein et al. 2021). Captured woodrats were transported to the University of Utah School of Biological Sciences Animal Facility where they were housed in individual cages (48 × 27 × 20 cm) and fed an alfalfa-based, commercial chow (Teklad Global High Fiber Rabbit Diet 2031; Envigo, Indianapolis, IN). To examine mycobiome stability, we collected a second set of fecal samples after animals (n = 107) were in captivity for approximately one month. We also collected cage samples by swabbing empty cages that were left in the facility for three days after being prepared with standard bedding and enrichment items. Animal use was approved by the University of Utah IACUC (16-02011) and conducted under permits from CA (SC-8123), UT (1COLL5194-1,2), NV (333663), and AZ (SP773078).

DNA extraction

We extracted DNA from feces (n = 227), chow (n = 3), cage controls (n = 3), and kit controls (n = 12) using QIAamp PowerFecal DNA kits (Qiagen, Germantown, MD) following the manufacturer's protocol. We quantified DNA concentrations using a NanoDrop (Thermo

Scientific, Waltham, MA), and then for metabarcoding and fungal quantification, standardized DNA concentrations to 8.7 ng/µl.

Fungal 18S rRNA gene copy quantification

To quantify total fungal load, we used the FungiQuant assay, a probe-based qPCR assay that targets the fungal 18S rRNA gene and uses a plasmid standard containing a *Candida albicans* 18S rRNA gene clone (Liu et al. 2012). Amplifications were performed in triplicate as detailed in the Supplemental Methods. The mean of triplicate reactions with coefficient of variation (CV) < 0.10 was calculated, and then divided by input ng of template DNA per sample to calculate the per sample 18S copy number.

Fungal ITS2 amplicon metabarcoding and sequencing

To characterize fungal assemblages, we used previously described approaches to amplify, barcode and add Illumina adapters to ITS2 sequences (Yeung et al. 2020, see Supplemental Methods), using primers with ITS2 targeting sequences from Taylor et al. (2016) and sample-specific barcodes from Kozich et al. (2013). We included the ZymoBIOMICS Microbial Community Standard as a positive control, as it includes DNA from *Saccharomyces cerevisiae* and *Cryptococcus neoformans* fungi (ZymoResearch, Irvine, CA; #D6305). Sequencing (2 × 300bp) was performed on Illumina MiSeq at the University of Utah High-Throughput Genomics Core.

Shotgun metagenomics

For 66 wild woodrats, we also sent extracted DNA to the DNA Service Facility at the University of Illinois-Chicago for shotgun metagenomic sequencing. At this facility, input DNA was normalized to 15 ng prior to making an equal-volume pool of all samples. The pool was quantified using a Qubit DNA High Sensitivity kit (Life Technologies, Carlsbad, CA) and size distribution was assessed using an Agilent 4200 TapeStation (Agilent Technologies, Santa Clara, CA). Libraries were prepared using a Swift 2S Turbo DNA Library Kit and, for quality control and library balancing purposes, libraries were pooled and first run on an Illumina MiniSeq. Based on these results, a new pool was made, quantified as described above and sequenced on an Illumina NovaSeq 6000 with 2 × 150bp sequencing and a 1% phiX spike-in.

Bioinformatics

For shotgun metagenomic samples, we first performed quality control on reads using fastp v0.20.1 (Chen et al. 2018) and then removed host sequences by aligning to the *N. lepida* genome using bowtie2 v2.4.2 (Greenhalgh et al. 2022, Langmead and Salzberg 2012). Samples retained an average of 5,012,449 ± 943,195 reads, which we classified using Kraken2 v2.1.1 and the PlusPFP reference database (Jan 27, 2021 release --Wood et al. 2019). As this database contains a limited 38 fungal genera and only classified 12.1 ± 1.2% metagenomic reads even with the most lenient confidence thresholds, we also classified reads using a custom-built Kraken database with over 6000 fungal genera, in addition to bacteria, animals, plants, and other groups (Dentinger 2022). We report outputs from this larger database in the main text, and summarize the effects of database and confidence thresholds in the supplementary material (Table S3).

We demultiplexed and processed ITS Amplicon Sequence Variants (ASVs) using QIIME2 v 2020.2 (Bolyen et al. 2019). In brief, demultiplexed paired-end raw sequence files were read into a QIIME2 artifact and then trimmed and denoised using the ITSxpress and DADA2 denoise-paired plugins (Callahan et al. 2016, Rivers et al. 2018, see Supplementary Methods). We retained only overlapping sequences; however, we also include outputs from just forward and reverse reads in the supplemental material (Table S4). Although single-end datasets contained more reads and more ASVs, the overall patterns were similar; therefore, we present the more conservative paired-end results in the main text. We assigned taxonomy using a classifier trained in QIIME2 with Scikit-learn and the UNITE database (v8.2, QIIME release with dynamic clustering thresholds -- Abarenkov et al. 2020, Pedregosa et al. 2011). Additional processing and analyses were performed in R v4.1.0, using phyloseq v1.30.0 (McMurdie and Holmes 2013, R Core Team 2020). We first removed three ASVs that matched the *Saccharomyces cerevisiae* in the microbial community positive control. We then examined negative controls, and for each sample (n = 241), removed ASVs with < 25 read counts. This filtered dataset contained 2,545,625 reads (88.5% of original) assigned to 1,672 taxa, with 99% of reads assigned to phylum, class, order, family, and genus, and 96.7% to species. To examine patterns in fungal prevalence, we merged ASVs at the species level, retaining unknown species as separate taxa. We examined the distribution of the resulting 551 taxa and calculated the percent detected in more than 50% of wild hosts, as this is often used as a minimum threshold for defining core microbiota (Neu et al. 2021).

We next tabulated four types of ecological data for the ~950 fungal taxa with > 10 ASV counts per sample, using ecological data from literature (Supplementary Table S4). For each

taxon, we assigned a primary niche based on associations with fungi, insects, lichen, plants, rock, soil/debris/dung, and vertebrates, including two additional categories for ubiquitous taxa and taxa with poorly characterized ecology. We then classified species with edible fruiting bodies as potential diet items. However, as animals could be ingesting spores or DNA from the environment, potential diet items were only considered food if > 100 read counts were present in an individual woodrat. Recognizing that a variety of factors influence ASV read counts, this conservative minimum threshold was selected based on fungal cells averaging approximately 100 copies of ribosomal DNA (Lofgren et al. 2019). Next, we assigned a trophic mode (i.e., saprotroph, pathotroph, symbiotroph) and finally noted whether fungal taxa were known mammal colonists. Acknowledging that many fungi are poorly studied and that some belong in multiple categories, we used these data to characterize the ecology of fungi in woodrats, rarefying data to 1000 ITS read counts per host when calculating the proportion of counts assigned to each ecological niche or trophic mode.

Statistical analyses

We used sequencing and qPCR outputs to examine how host and environmental factors influenced fungal quantity, diversity, and composition in wild and captive woodrats. Using wild samples, we first tested whether fungal amounts from metagenomic, qPCR, and metabarcoding approaches were correlated, using Kendall's tau to measure correlations between 18S rRNA gene copies per nanogram (c/ng), total ASV counts, and the percent of shotgun reads assigned to fungi.

We next examined how host and environmental factors influenced total fungal quantity in wild woodrats. To test whether more diverse diets exposed animals to more fungi, we characterized natural diets in 115 wild individuals via plant metabarcoding, using observed plant families as a proxy for diet diversity (Weinstein et al. 2021). To test whether fungal amounts increased at wetter sites, we downloaded annual precipitation normals from the weather station closest to each site using the package rnoaa v1.3.4 (Chamberlain 2021, Supplementary Table S1). We then used linear models to test whether host species, site, diet diversity and precipitation predicted total fungal quantity. We $\log_{10}$ transformed fungal quantity, removed one outlier (see results), visually assessed model fit and identified the best models using backward selection. As precipitation was measured at the site level, we analyzed site and precipitation in separate models.

Using the ITS metabarcoding data, we tested whether the same factors predicted fungal diversity in wild hosts. We first examined impacts on observed ASVs, and then rarefied samples to an even depth of 1000 read counts (removing 14 samples with counts below this threshold), and repeated analyses using observed ASVs and the Shannon index from these subsampled communities. As we expected the effects of diet diversity to be strongest on plant-associated fungi, we also ran the same models using observed diversity of plant-associated ASVs as the response variable. We analyzed models with the MASS v7.3-51.5 package (Venables and Ripley 2002), using a Gaussian error distribution for the Shannon index and a negative binomial error distribution for observed ASVs, assessing models as previously described.

We predicted that mycobiomes would be more similar in wild animals that were closely related, closely located or feeding on similar diets. To compare the composition of fungal assemblages, we calculated mycobiome dissimilarity from rarefied community data using Jaccard and Bray-Curtis indices. We first tested whether communities differed between species and sites using the adonis function, testing for homogeneity of dispersion using the betadisper and permutest functions in vegan v2.5-6 (Oksanen et al. 2019). Next, following methods described in Weinstein et al. (2021), we converted host phylogeny (using branch lengths from Matocq et al. 2007), sampling location, and individual diet composition data into distance matrices using phangorn v2.5.5 and geosphere 1.5-10 (Hijmans 2019, Schliep 2011). For the 102 animals with complete phylogeny, location and diet data, we then used ecodist v1.5.0 to perform multiple regression on distance matrices (Goslee and Urban 2007), before calculating the relative importance of each factor using variance partitioning. To further test whether diet effects were primarily driven by plant-associated fungi, we also ran these analyses with fungal communities divided into plant and non-plant associates.

Finally, we examined how fungal assemblages changed in captivity. We first confirmed that extracted samples from wild and captive animals contained similar DNA concentrations using a Welch's t-test. Using the same test, we then tested whether captivity reduced total fungal quantities, removing the previously mentioned outlier from the wild dataset and $\log_{10}$ transforming quantities. We also tested for a correlation between total fungal quantities in wild and captive animals using linear regression, also $\log_{10}$ transforming fungal quantities. Using the metabarcoding data, we next tested whether total ASV counts and observed ASVs differed between wild and captive animals using paired Wilcoxon signed rank tests. We then tested for a

correlation between ASVs observed in wild and captive animals using a linear model. Due to low total ASV counts in captive rats, we did not rarefy data for these comparisons.

RESULTS

Most wild woodrat feces contained fungal DNA; however, amounts varied among individuals (Fig. 1). Fungal reads were present at low abundance in all metagenomic samples (Fig. 1A); however, relative amounts varied depending on the reference database and classification parameters (Supplemental Table S2). Using the larger reference database (and a 0.05 confidence score -- Ye et al. 2019), 2.5 ± 1.8% of all shotgun metagenomic reads were classified, with fungi comprising 3.8 ± 10.6 % of classified reads per host. The probe-based qPCR assay detected the fungal 18S rRNA gene in 118 of 120 wild woodrats. Although one sample had over 730,000 c/ng, other samples with detectable fungi averaged 1,740 ± 2,453 c/ng (Fig. 1B). ITS metabarcoding detected fungal reads in 116 of 120 wild individuals. These samples averaged 15,970 ± 25,056 total ASV counts, excluding the previously identified outlier, which had > 441,000 total ASV counts (Fig. 1C). Total ITS ASV counts and 18S rRNA gene c/ng were highly correlated even when excluding the outlier (Kendall's tau = 0.40, $p < 0.0001$). Fungal relative abundance in shotgun metagenomic samples also correlated with 18S rRNA gene c/ng and ITS ASV counts, but only when the outlier was included (18S: metagenomics tau = 0.25, p = 0.01; ITS: metagenomics tau = 0.42, p = < 0.0001). Notably, as we were unable to assign taxonomy to most shotgun metagenomic reads, subsequent characterization of fungal assemblages and quantities is based on ITS amplicon and 18S qPCR data, respectively.

Wild woodrats contained a diverse and highly variable assemblage of fungi. Most ITS reads were assigned to Ascomycota (50% of reads, 76% of ASVs), Mucoromycota (32% reads, 5% ASVs), and Basidiomycota (18% reads, 15% ASVs), with small amounts of Mortierellomycota (0.17% reads, 0.9% ASVs), Basidiobolomycota (0.06% reads, 0.2% ASVs), and Chytridiomycota (0.02% reads, 0.7% ASVs) also recovered. In total, wild woodrats contained 1,509 ASVs assigned to 147 families, 289 genera, and 315 fungal species. Nearly half (47%) of these taxa occurred in just one wild host, 13% occurred in at least 10% of animals, and only 11 (2%) were found in in > 50% of animals (Table 1, Fig. 2).

While the most prevalent taxa in wild woodrats were coprophilous (Table 1), nearly half of all ASVs (41%) detected in wild individuals were plant associates (Fig. 2). Approximately 30% of taxa were associated with soil, debris and dung, 16% were from mixed/unknown habitats, and 10% were classified as ubiquitous environmental fungi (e.g., *Cladosporium*, *Mucor*, *Alternaria* spp.). We also detected rock-inhabiting, lichen-forming or lichen associates, and insect, fungi or vertebrate associates; however, each comprised less than 1.5% of ASVs and total ASV counts. Fungal taxa typically associated with vertebrates were rare -- only *Arthroderma* and *Kazachstania* spp. were detected, with each found in only two wild animals. While vertebrate symbionts were rare, opportunistic mammal colonists were common, particularly *Alternaria*, *Aureobasidium,* and *Aspergillus* spp. which occurred in 64%, 56%, and 32% of wild hosts. *Candida* spp. were only detected in two wild woodrats, with *C. arabinofermentans* and *C. membranifaciens* each in one individual.

Approximately 16% (19/120) of wild samples contained fungi potentially consumed as food. *Phallus* and *Agaricus* spp. each occurred in five animals, while *Geastrum*, *Rhizopogon*, *Gautieria*, *Itajahya*, *Phellorinia*, *Calvatia*, *Coprinopsis*, *Coprinus*, and *Tubaria* spp. were each detected in one animal. Although a small percentage (4.9%) of total ASV counts across all individuals, these diet components represented 30 ± 31% of counts in these 19 woodrats. This potential mycophagy occurred in five *Neotoma* species collected at nine sites, with no clear taxonomic or geographic clustering.

For wild woodrats, total fungal quantity differed between sites, but was not influenced by host species identity, diet diversity or local precipitation (Table 2). Fungal diversity varied among animals, with wild samples containing an average of 16.0 ± 11.1 identified families, 19.1 ± 15.5 genera, 18.5 ± 16.2 species, and 38.3 ± 35.8 observed ASVs. Fungal diversity differed between sites for all diversity metrics (i.e., Shannon index from rarified data and observed ASVs from both rarefied and non-rarefied data), but only differed between host species when measured using observed ASVs from rarefied data (Fig. 3A, Table 2). As for fungal quantity, precipitation and diet diversity had no effect on fungal diversity, even when analyses were restricted to only plant-associated fungal taxa (Table 2).

Fungal composition also differed between sites and species. Site explained substantially more variance than species identity (PERMANOVA, Site: $R^2 = 0.27$, $p = 0.001$; species: $R^2 = 0.05$, $p = 0.001$); however, significant differences in these models could be due to differences in dispersion (both site and species $p < 0.005$). Animals with more similar evolutionary history, geographic origin, and diet had more similar fungal communities (Fig. 3B). Site, diet, and

phylogeny all significantly predicted natural mycobiome structure (multiple regression on distance matrices, all $p < 0.002$, Table 3), and together explain approximately 10% of observed variation. Individually, site was the most important factor (explaining 8.4% of variance). Diet and host phylogeny explained only 2.8 and 1.9% of the variance, respectively, and much of this variance was also explained by site (as seen in overlapping regions of Fig. 3B). Plant associates contributed to observed diet effects (Table 3) and when excluded from the fungal community, the variance uniquely explained by diet decreased to < 0.5%.

Captivity substantially altered fungal assemblages (Fig. 4). Fecal samples from wild and captive rats contained similar total DNA concentrations (wild 75.5 ± 46.0, captive 67.2 ± 25.9 ng/µl, Welch's two-samples t-test; $t(166.96) = -1.6$, $p = 0.10$); however, qPCR and high-throughput sequencing analyses showed substantial reductions in fungal quantity and diversity. Total fungal quantities significantly decreased in captivity (Fig. 4C, wild (excluding outlier) 1,739 ± 2,453 c/ng, captive 528 ± 1,210 c/ng; $t(216.69) = -7.7685$, $p < 0.0001$), with no correlation between amounts in wild and captive individuals ($F_{1,100} = 1.415$, $p = 0.24$). The impacts of captivity were even more pronounced in the ITS metabarcoding data. Of 107 captive woodrats, only 35 contained any ITS reads. The only captive animal with > 1000 total ASV counts had more than >10,000 counts from *Kazachstania* (Ascomycota, Saccharomycetaceae), and was the same animal that, in the wild, contained > 400,000 ASV counts assigned primarily to *Mucor* and *Thelebolus* species. Alongside the significant reduction in total ASV counts (Fig. 4A, wild: 20,932 ± 48,584, captive: 142 ± 1004; paired Wilcoxon signed rank test: $p < 2.2e\text{-}16$), observed ASVs also decreased (Fig. 4B, wild: 39.1 ± 36.6, captive: 0.76 ± 1.50, Welch's t-test: $t(106) = -10.82$, p

< 2.2e-16), with no correlation between the number of ASVs seen in wild and captive conditions ($F_{1,105}$ = 0.006, p = 0.94).

In total, we detected only 32 ASVs in captive woodrats. Captive individuals with fungal reads contained an average of 2.3 ± 1.8 ASVs, 1.1 ± 1.3 species, 1.5 ± 1.3 identified genera, and 2.1 ± 1.4 families. Most ASVs (18/32) in captive woodrats were also seen in at least one wild individual, but with little evidence that wild animals were retaining these taxa in captivity (Fig. 5A, Figure S1). Of these 32 ASVs, only those from the genera *Cladosporium* (in 17 of 107 captive individuals) and *Alternaria* (9 of 107) were seen in more than two captive animals. Eight ASVs (in the genera *Malassezia*, *Saccharomyces*, *Candida*, *Saccharomycopsis*, *Wallemia*, and *Lichtheimia*) were detected only in captive rats, but each occurred in only one animal. Approximately 40% of the ASVs in captive rats were also seen in chow, with these chow-associated ASVs representing 76.9 ± 35% of the reads per captive animal (Fig. 5B). Chow was mostly comprised of Ascomycota (90.1% of reads) associated with plants, soil, and debris and, compared to animal samples, had substantially higher fungal DNA quantities, read counts and richness (Fig. 4). In contrast, cage controls contained almost no detectable fungi.

DISCUSSION

Longitudinal sampling of woodrats suggests that these wild mammals harbor a diverse but transient assemblage of fungi derived almost exclusively from environmental sources. Consistent with the high heterogeneity and geographical clustering seen in other fungal communities (Peay et al. 2016), fungi in wild woodrats were highly variable and primarily

structured by sampling site. Most fungal taxa occurred in few hosts, with shared exposure to dung and decaying plants likely producing the small number of apparently core taxa. The majority of fungi, including these core species, were no longer detected when animals were removed from natural environments. Combined, these results suggest that these mammals experience high fungal exposure, but do not host a persistent gut mycobiome.

Fungi comprised a small fraction of the microbial material in woodrat guts. The relative abundance of fungi in shotgun metagenomic data suggests that wild woodrats might harbor more fungal material than humans (Nash et al. 2017, Qin et al. 2010) and lab mice (Dollive et al. 2013). However, fungal abundance estimates from metagenomic data should be interpreted cautiously as genomic databases have poor fungal coverage, confidence thresholds substantially influence outputs, and read abundance does not necessarily equate to population size (Nilsson et al. 2019). Nevertheless, similar to other surveyed mammals (Dollive et al. 2013, Qin et al. 2010), woodrat guts appear to contain substantially more bacterial than fungal diversity.

Woodrats did not appear to host Neocallimastigomycota, the fiber-degrading fungal symbionts found in many other herbivores. These anaerobic gut fungi can be difficult to detect without taxon-specific methods (Edwards et al. 2017); however, they have been detected using similar extraction protocols (Zhang et al. 2017) and primers (Cox et al. 2021). Furthermore, the majority of reference sequences in the order Neocallimastigales are predicted to be detected by these primers (Taylor et al. 2016). Although one rodent, the 8kg Patagonian mara (*Dolichotis patagonum*), hosts Neocallimastigomycota in its hindgut (Teunissen et al. 1991), smaller

mammals likely do not have the gut capacity or residence times required to support the metabolism of these symbionts (Gruninger et al. 2014).

In wild woodrats, most fungi were saprophytes associated with soil, dung, debris or vegetation. Prevalent taxa included widespread species like *Alternaria*, *Cladosporium*, and *Aspergillus* spp., which are also common in human (Nash et al. 2017, Suhr and Hallen-Adams 2015), wild animal (Li et al. 2018, Sun et al. 2021) and environmental samples (Dietzel et al. 2019). Woodrats also contained a variety of plant pathogens and endophytes, likely acquired from their herbivorous diet. In nature, woodrats consume fresh plants, as well as feces and vegetation stored in their large, multichambered middens (Vaughan 1990). In these middens, high humidity, constant temperatures, and abundant nutrient resources enhance fungal growth (Whitford and Steinberger 2010, Zak and Whitford 1988). Frequent coprophagy likely explains the abundance of coprophilous fungi, while feeding on stored, decaying vegetation could increase exposure to saprophytes. These fungal saprophytes might compete with woodrats for resources. Alternatively, woodrats might benefit from fungal degradation of plant fiber and toxic secondary metabolites. For other small mammals (e.g. pika -- Dearing 1997), caching plants enriches nitrogen and improves nutritional quality, likely via fungal activity. Multiple invertebrates have harnessed fungal degradation for their own benefit (Mueller et al. 2005, Silliman and Newell 2003), and as middens create ideal conditions for fungal domestication (Branstetter et al. 2017), further study of mammal-fungi interactions in these microhabitats may prove interesting.

Fungal assemblages in wild woodrats were influenced by sampling site, animal diet, and host species identity. Although moisture is often an important determinant of fungal diversity (Tedersoo et al. 2014), we found no evidence that animals from wetter habitats harbored more fungi, or more diverse fungal communities. As no site received more than 70 cm of annual precipitation, moisture differences may have been too small to impact fungal diversity. Alternatively, most fungal exposure might occur in middens, which create mesic microhabitats even in dry environments (Desjardin et al. 1992). Although host phylogeny strongly predicts bacterial communities in woodrats (Weinstein et al. 2021), this factor explained relatively little variation observed in fungal assemblages. Diet was also a small, but significant predictor of fungal composition, perhaps due to host-specific plant endophytes and pathogens, like the cactus pathogen, *Tintelnotia opuntiae*, found only in cactus-feeding woodrats (Ahmed et al. 2017). Consistent with the dispersal limitations and high regional endemism seen in other fungal communities (Higgins et al. 2014, Peay et al. 2016), sampling site was the strongest predictor of fungal composition. Although the strongest predictor, geographic proximity still explained less than 10% of observed variation. More variation might be explained by differences in season, host age, or immune status; however, if the majority of host-associated fungi are haphazardly acquired from heterogenous natural environments, these assemblages may remain largely unpredictable.

Most fungi disappeared in captive woodrats, similar to reductions seen in captive primates (Sun et al. 2021) and humans consuming controlled diets (Auchtung et al. 2018). Captive woodrats contained *Malassezia* and *Candida* species, both common human commensals potentially transferred during animal care. Most other detected fungi were

ubiquitous species (e.g., *Alternaria*, *Cladosporium*, and *Wallemia* species) that are common in both natural and indoor environments. Captive animals also hosted *Kazachstania heterogenica*, a pathogen known to cause disease in laboratory mice (Kurtzman et al. 2005) and to be more abundant in captive compared to wild primates (Sawaswong et al. 2020). These studies suggest that captivity facilitates *K. heterogenica* growth. However, as *K. heterogenica* was abundant in only one captive woodrat, the same individual with exceptionally high wild fungal loads, susceptibility to this pathogen is likely also influenced by host physiology, condition, or exposure history.

Of the limited fungal material detected in captive rats, most came from the chow that animals consumed. Digestion degrades DNA (Deagle et al. 2006), and as fungal DNA in processed chow was likely already fragmented, further digestion might have degraded most DNA to the extent where it was no longer detected via metabarcoding. As the fungal quantification assay typically relies on a shorter amplicon (350 v 250-500 bp), this assay might have detected more degraded DNA (Liu et al. 2012, Taylor et al. 2016). This could explain why the FungiQuant assay detected some fungi in captive rats with no ITS reads. Alternatively, the 18S targeting FungiQuant assay might have detected taxa missed by the ITS2 targeting primers (Tedersoo and Lindahl 2016), or be cross-amplifying some host or plant DNA. While this FungiQuant assay has been widely used with human and mouse samples (e.g., Boutin et al. 2021, Tirelle et al. 2020), more validation may be needed for non-model systems.

Most fungi in woodrats appear to be transient; however, exposure to these taxa could still impact animal health and development. Many fungi produce secondary metabolites that

are toxic to mammals and microbes (Keller et al. 2005). For example, aflatoxin produced by *Aspergillus* spp. is both acutely toxic and carcinogenic in vertebrates (Keller et al. 2005), while the epicorazines and flavipin produced by *Epicoccum nigrum* inhibit bacterial and fungal growth (Baute et al. 1978, Brown et al. 1987). Many of the prevalent fungi in wild woodrats, including *Mucor*, *Aspergillus*, and *Alternaria* spp. are also opportunistic pathogens that can cause disease, particularly in animals that are stressed, immuno-compromised or experiencing bacterial dysbiosis (Dollive et al. 2013, Seyedmousavi et al. 2018). Although fungal infections can alter bacterial communities and exacerbate disease states (van Tilburg Bernardes et al. 2020), fungi are also critical for immune system development. For example, fungal exposure increases circulating granulocytes in rewilded laboratory mice (Yeung et al. 2020) and in gnotobiotic mice, commensal *C. albicans* induces Th17 cells that protect against pathogenic fungi (Bacher et al. 2019). Even in highly controlled model systems, fungi have complex and context-dependent impacts, suggesting that these interactions will be even more nuanced in wild animals with more extensive and prolonged fungal exposure.

Mammal-fungal interactions may also impact fungal populations. Many of the fungi consumed by woodrats rely on mammals for dispersal (Bradshaw et al. 2022, Johnson 1996). Although fungi are a small component of woodrat diets, where woodrats are abundant, they may substantially contribute to the dispersal of these ectomycorrhizal species (e.g. Stephens and Rowe 2020). Beyond what is consumed as food, woodrats likely disperse other fungi, including coprophilic and pathogenic taxa. Within an animal's natural range, this may be an important mechanism for facilitating sexual reproduction and increasing genetic diversity in fungal populations. However, when animals are moved, the same processes can also spread

fungi to novel habitats. Notably, the diversity of transient plant and mammal pathogens in wild mammalian feces underscore the importance of animal quarantines prior to relocation.

In conclusion, our results suggest that these wild mammals contain a diverse, but transient assemblage of fungi in their guts. In wild woodrats, most fungi came from diet and the local environment, resulting in fungal assemblages that were less structured and less predictable than bacterial communities in the same hosts (Weinstein et al. 2021). We found no evidence of a symbiotic mycobiome; however, our non-invasive molecular approaches may have missed commensal fungi that were highly localized, or at very low abundance. Confirming the absence of commensal fungi, or detecting their presence, will require integrated sequencing, *in situ* visualization and culture-based approaches to target metabolically active and potentially site-specific fungi. Whether transient or symbiotic, mammalian-fungal interactions are expected to substantially impact both animal and fungal fitness, particularly in natural systems where animals are continuously exposed to diverse fungal communities.

Acknowledgments We thank Rodolfo Martínez-Mota, Tess E. Stapleton, Dylan M. Klure, Teri J. Orr, Kaylene Yamada, James Patton, James Malcolm, Madeline Nelson, and Margaret Doolin for assistance with sample collection and animal husbandry, and Bryn Dentinger and Alexander Bradshaw for discussions on fungal ecology and access to reference databases.

Funding Support was provided by NSF Dimensions DEB 1342615, NSF IOS 1656497, and Ruth L. Kirschstein National Research Service Award NIH T32AI055434. ITS amplicon sequencing was

performed at the University of Utah's High-Throughput Genomics facility which is supported by NIH award number P30CA042014.

Data and code availability Sequencing data can be found on the SRA under BioProjects PRJNA824056 and PRJNA722312. Code is available on GitHub at https://github.com/SBWeinstein/Neotoma_fungi.

DECLARATIONS

Ethics approval Animal use was approved by the University of Utah IACUC (16-02011) and conducted under permits from CA (SC-8123), UT (1COLL5194-1,2), NV (333663), and AZ (SP773078).

Conflicts of interest The authors declare that they have no conflict of interest.

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Figure Legends

Fig. 1 Wild woodrats varied in (A) percent of classified metagenomic reads assigned to fungi, (B) 18S rRNA gene copies per ng DNA, and (C) total ASV counts. Along the x axis, samples are ordered by increasing total ASV count. In A, B, and C, grey points respectively represent samples that were not analyzed, inconclusive qPCR results, and animals with no read counts after filtering. Plot A contains data from 66 animals (black points), while B and C have 120 (black and grey points). One sample consistently had more fungal material (primarily assigned to *Mucor* and *Thelebolus* spp.) and this outlier is shown as an open circle in all plots

Fig. 2 Most fungal taxa were rare, with only 11 species found in more than 50% of wild woodrats (**A**). (**B**) Of the 950 fungal taxa with > 10 ASV counts per sample, most were assigned to taxa associated with soil/debris/dung or plants (outer ring), and of the 74% of taxa with an assigned trophic mode, saprotroph (Sapro), was most common, followed by pathotroph (Path) and symbiotroph (Sym; inner Euler diagram). (**C**) On average, within an individual rat, most ASV counts came from fungi associated with soil/debris/dung or ubiquitous environmental taxa. For

B and C, niches assigned to < 1.5% of ASVs or total ASV counts are unlabeled, and correspond to rock−inhabiting fungi (0.3% of ASVs, 1.3% of total ASV counts); lichen forming/lichen associates (<0.1, 0.9), vertebrate associates (0.5, 0.2), fungal associates (<0.1, 0.2), and insect associates (<0.1, 0.2)

Fig. 3 Fungal diversity and composition differed among sites and species for wild woodrats. (**A**) The number of observed ASV differed among sites. Pairwise comparisons of estimated marginal means were calculated with emmeans v1.5.0 (Lenth 2020), and sites that do not significantly differ are displayed using letters a, b and c (**B**) Animals with more similar diets, evolutionary history (phylogeny) and geographic origins (site) had more similar mycobiomes, with site explaining substantially more variance than other factors

Fig. 4 In captivity, woodrats had fewer fungi in their feces, with significant decreases seen in (**A**) fungal quantity (**B**) total ASV counts and (**C**) ASV diversity. Notably, the alfalfa-based commercial chow fed to captive woodrats contained over 100× and 450× more fungal DNA (dark grey dashed line) than did wild and captive animal samples. One animal with substantially higher ASV counts and fungal quantities is shown as an open circle in A and B

Fig. 5 (**A**) Only 32 ASVs were detected in captive rats. Of these, 25% occurred only in captive animals, 56% were also seen in wild animals, and 40% occurred in chow. (**B**) Sequences from chow-associated fungi comprised >76% of total ASV counts in captive rats

Table Legends

Table 1. The most prevalent fungi in wild woodrats, their prevalence and typical ecology (see Supplementary Table S4 for references).

Table 2. Analysis of deviance table for best fit models. We present the full model, followed by the factors (in bold) retained in the best fit model, degrees of freedom (Df), deviance, residual degrees of freedom (Deviance Resid. Df), residual deviance (Resid. Dev), and p-value (Pr(>Chi)). Models for observed ASVs had a negative binomial error distribution and were assessed using a likelihood ratio test. Models for Shannon index and total fungal quantity had a normal error distribution and were compared with F tests. For these latter two models, values under columns labeled Deviance, Resid Df, Resid Dev and Pr(> Chi) refer instead to the sum of squares, Mean squares, F value, and Pr(>F), respectively.

Table 3. Outputs from multiple regression models for wild woodrats, using two metrics of community dissimilarity (Bray-Curtis (BC) and Jaccard (J)), applied to either the full fungal dataset (Wild), or that dataset separated into plant associates (Wild-P) and fungi with other non-plant-associated ecologies (Wild-NP). For each metric, we list variance explained by a full

model with Diet, Phylogeny, and Site (DPSr), then variance explained by models with just Site (Sr), Diet (Dr), and Phylogeny (Pr). The next four columns provide p-values for each model, followed by the variance uniquely attributed to Diet (Du), Phylogeny (Pu), and Site (Su).

Figure 1

Click here to access/download;Figure;Figure1.jpg

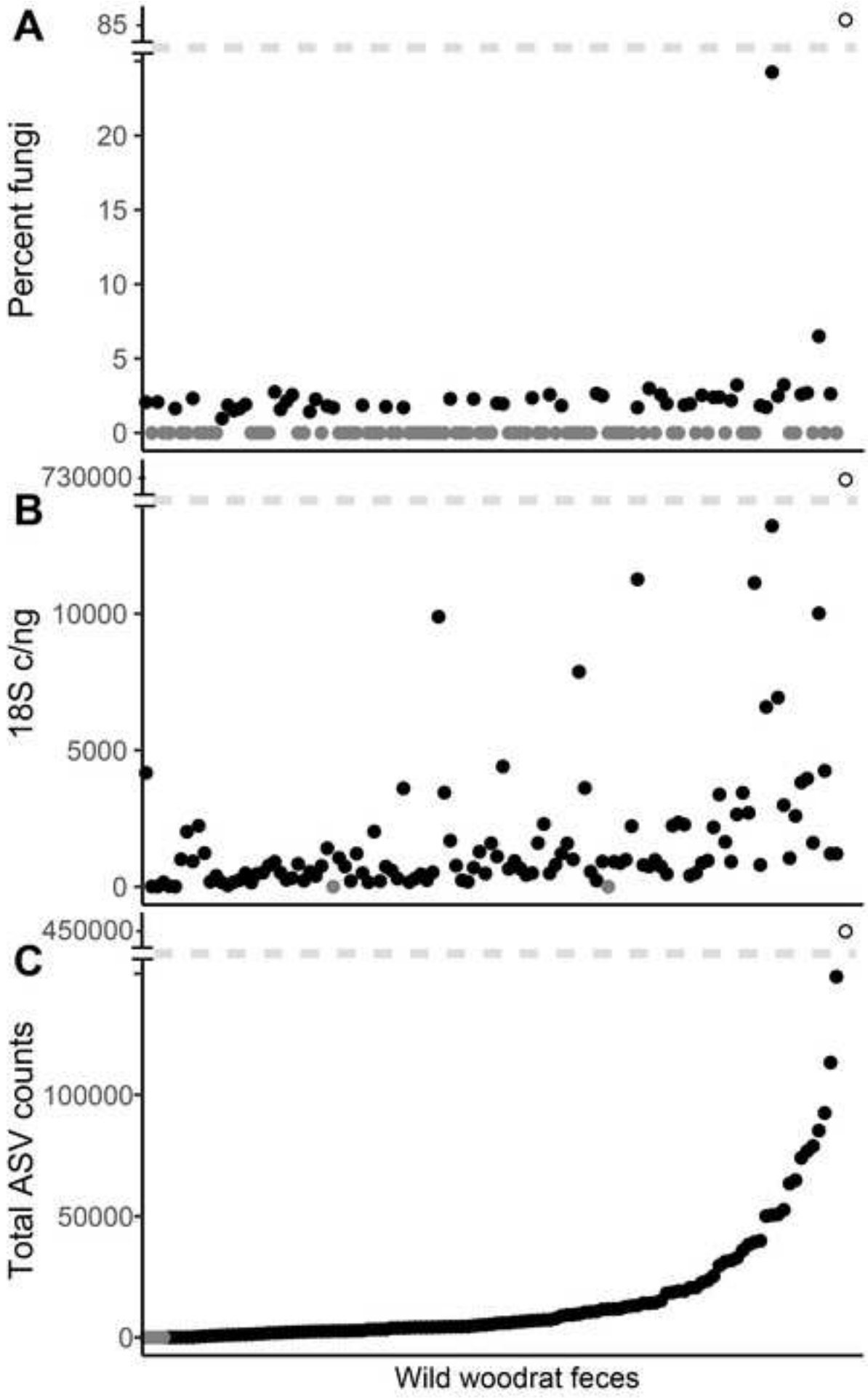

Figure 2

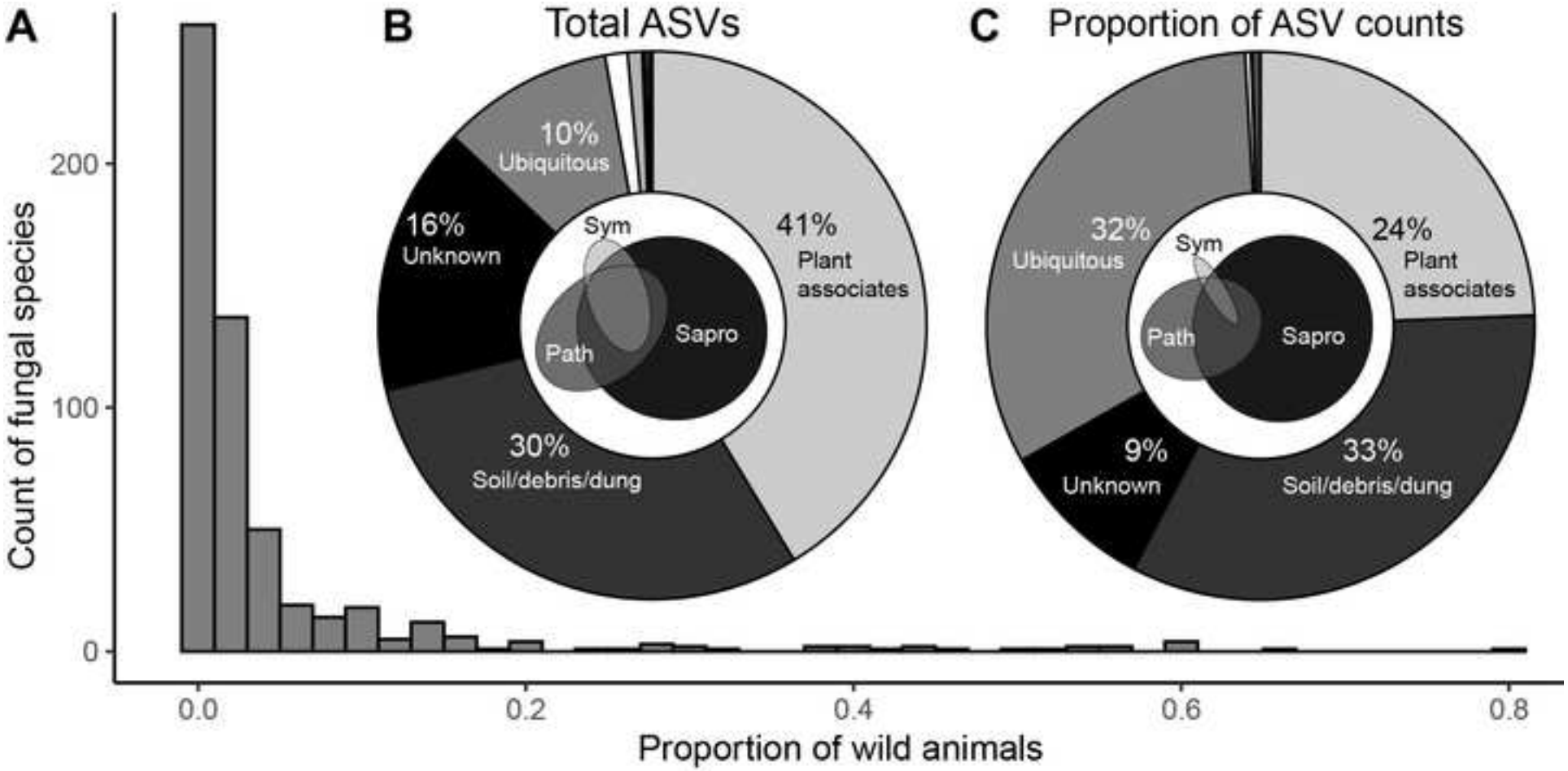

Figure 3

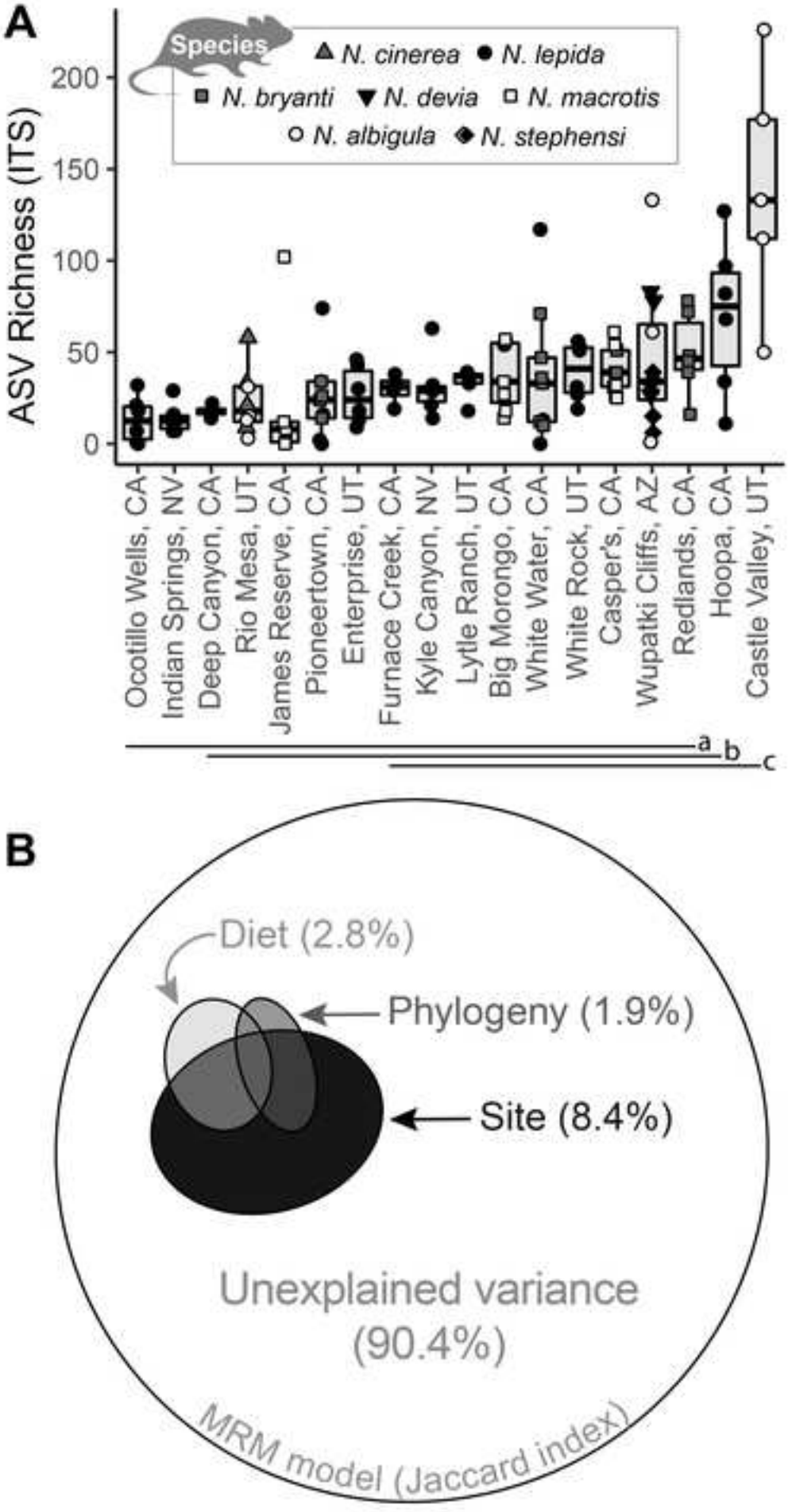

Figure 4

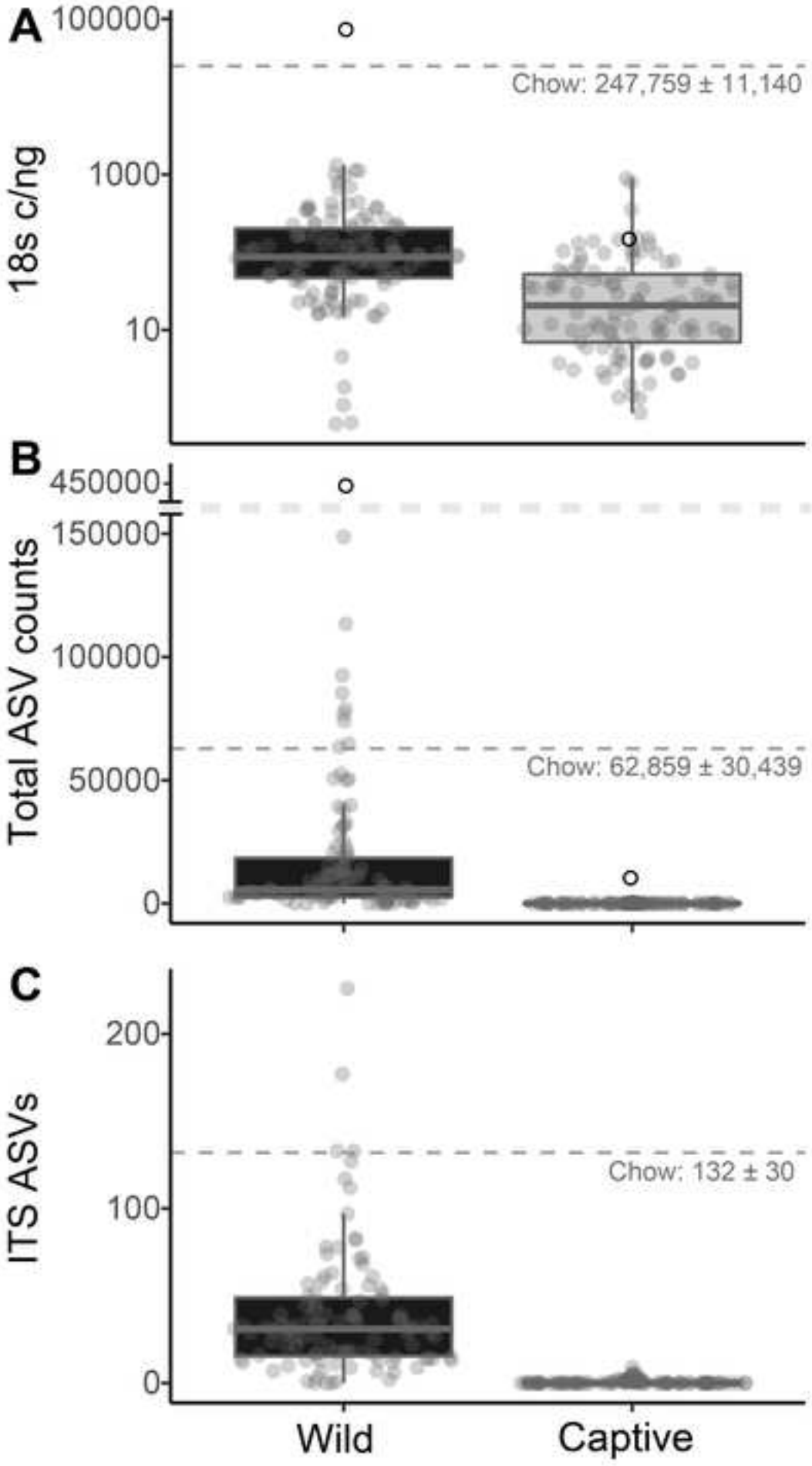

Figure 5

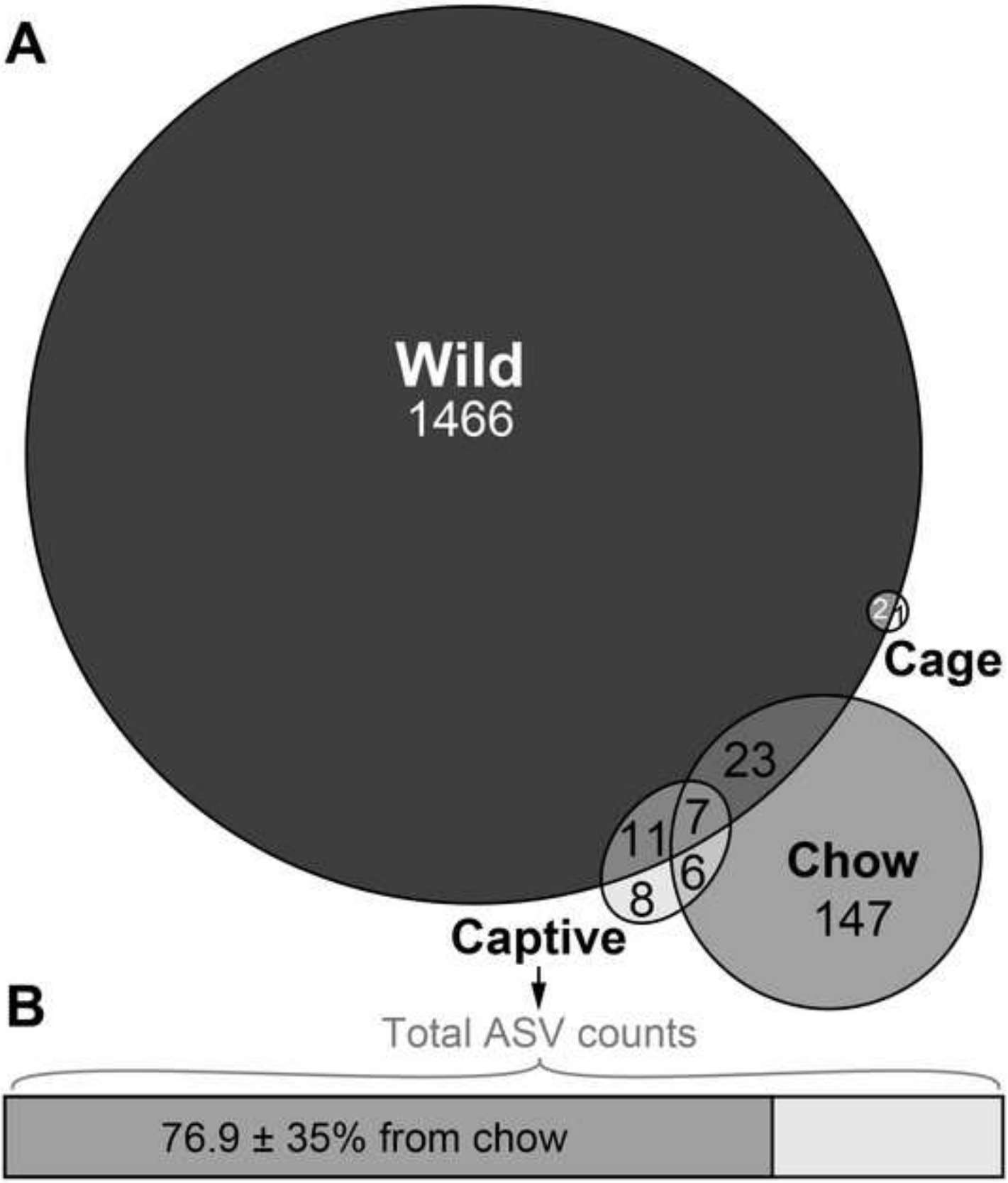

Table 1.

Fungal Species	**Prevalence**	**Ecology**
Botryotrichum spirotrichum	81%	Coprophilous, associated with herbivore dung
Sporormiella intermedia	67%	Coprophilous
Alternaria alternata	61%	Cosmopolitan saprobe and plant pathogen
Ulocladium chartarum	59%	Saprobe associated with soil and decaying plants
Didymellaceae sp.	59%	Family of predominately plant pathogens
Chaetomiaceae sp.	59%	Family isolated from soil, dung, dust, leaf litter, air
Mucor racemosus	57%	Ubiquitous in soil and decaying plant material
Thelebolus globosus	56%	Coprophilous
Mucor circinelloides	54%	Ubiquitous in soil and decaying plant material
Cladosporium sp.	54%	Hyperabundant plant pathogens and saprobes

Table 2

Model	Factor	Df	Deviance	Resid. Df	Resid. Dev	Pr(>Chi)
Observed ASVs ~ **Site** + Species + Precipitation + Diet diversity						
	Site	17	63.0	102	139.2	<0.0001
Observed ASVs (rarified) ~ **Site** + **Species** + Precipitation + Diet diversity						
	Site	17	92.6	88	120.8	<0.0001
	Species	5	12.3	83	108.6	0.03
Observed Plant associated ASVs ~ **Site** + **Species** + Precipitation + Diet diversity						
	Site	17	87.8	102	148.5	<0.0001
	Species	5	11.9	97	136.6	0.04
Shannon Index (rarefied) ~ **Site** + Species + Precipitation + Diet diversity						
	Site	17	16.2	0.95	2.9	0.0007
Log_{10}(Total Fungal Quantity) ~ **Site** + Species + Precipitation + Diet diversity						
	Site	16	10.1	0.63	2.0	0.02

Table 3

		Model Variance (r)				Model significance (p)				Factor Variance (u)		
Animals	**Metric**	**DPSr**	**Sr**	**Dr**	**Pr**	**DPSp**	**Sp**	**Dp**	**Pp**	**Du**	**Pu**	**Su**
Wild	BC	0.095	0.083	0.027	0.019	0.001	0.001	0.001	0.001	0.009	0.003	0.054
Wild	J	0.096	0.084	0.028	0.019	0.001	0.001	0.001	0.002	0.009	0.003	0.055
Wild-P	J	0.069	0.049	0.036	0.005	0.001	0.001	0.001	0.02	0.020	< 0.001	0.031
Wild-NP	J	0.077	0.068	0.019	0.018	0.001	0.001	0.001	0.003	0.005	0.003	0.044