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Authors: Jedlicka, Julie A., Vo, Anh-Thu E., and Almeida, Rodrigo P. P.

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RESEARCH ARTICLE

Molecular scatology and high-throughput sequencing reveal predominately herbivorous insects in the diets of adult and nestling Western Bluebirds (*Sialia mexicana*) in California vineyards

Julie A. Jedlicka,[1]* Anh-Thu E. Vo,[2] and Rodrigo P. P. Almeida[3]

[1] Department of Biology, Missouri Western State University, St. Joseph, Missouri, USA
[2] Museum of Vertebrate Zoology and Department of Integrative Biology, University of California, Berkeley, California, USA
[3] Department of Environmental Science, Policy, and Management, University of California, Berkeley, California, USA
* Corresponding Author: jjedlicka@missouriwestern.edu

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ABSTRACT

Determining the ecosystem function of high-order predators is critical for evaluation of food web interactions. Insectivorous birds are abundant predators in many ecosystems yet because they forage upon small taxa, it remains largely unknown whether birds are providing ecosystem services in the form of pest control or disservices by preying upon predaceous arthropod species. We extracted DNA from noninvasive fecal samples of adult and nestling Western Bluebirds (*Sialia mexicana*) in California vineyards. Using universal arthropod-specific primers, we sequenced prey items via massively parallel sequencing on the Illumina MiSeq platform. Bluebirds consumed a broad diet comprising 66 unique arthropod species from 6 orders and 28 families. *Aedes* sp. (mosquitoes: Culicidae), a previously unknown prey, was the most common item recovered, occurring in 49.5% of the fecal samples. Ectoparasitic bird blowfly (*Protocalliphora*) DNA was found in 7% of adult and 11% of nestling samples, presenting clear evidence of active feeding by the avian hosts on adult or larval ectoparasites. Herbivorous insects, primarily from the orders Hemiptera and Lepidoptera, represented over half (56%) of the prey items in bluebird diets. Intraguild predation (consumption of predator or parasitoid arthropods) represented only 3% of adult and nestling dietary items. Diets of adults were significantly different from nestlings as were diets from birds sampled in different vineyard blocks. Sex, date, number of young, and individual bird (based on resampled individuals) were all insignificant factors that did not explain diet variability. Nestling age was a significant factor in explaining a small amount of the variability in dietary components. In addition, our analysis of subsampling larger fecal samples and processing them independently revealed highly dissimilar results in all 10 trials and we recommend avoiding this common methodology. Molecular scatology offers powerfully informative techniques that can reveal the ecosystem function and services provided by abundant yet cryptic avian foragers.

Keywords: DNA barcoding, diet analysis, ecosystem function, fecal/faecal samples, insectivore, nest box, noninvasive, trophic ecology

La escatología molecular y la secuenciación de alto rendimiento revelan el predominio de insectos herbívoros en las dietas de adultos y polluelos de *Sialia mexicana* en los viñedos de California

RESUMEN

La determinación de la función ecosistémica de los depredadores tope es crucial para evaluar las interacciones en las redes tróficas. Las aves insectívoras son depredadores abundantes en muchos ecosistemas, pero debido a que se alimentan de taxa pequeños, se desconoce en gran medida si las aves están brindando un servicio ecosistémico como control de plagas o un perjuicio al depredar sobre especies de artrópodos depredadores. Extrajimos ADN de un modo no invasivo de muestra fecales de individuos adultos y polluelos de *Sialia mexicana* en los viñedos de California. Usando primers universales específicos para artrópodos y secuenciamos presas por medio de secuenciación masiva paralela en la plataforma Illumina MiSeq. Los individuos de *S. mexicana* consumieron una amplia dieta conformada por 66 especies únicas de artrópodos pertenecientes a 6 órdenes y 28 familias. *Aedes* sp. (mosquitos: Culicidae), una presa anteriormente desconocida, fue el ítem recuperado más común, presente en el 49.5% de las muestras fecales. El ADN de la mosca ectoparásito *Protocalliphora* fue hallado en el 7.3% de las muestras de los adultos y en el 10.7% de los polluelos, mostrando una clara evidencia de alimentación activa por parte de las aves hospederas sobre los ectoparásitos adultos o larvas. Los insectos herbívoros, principalmente de los órdenes Hemíptera y Lepidóptera, representaron más de la mitad (56%) de las presas en la dieta de *S. mexicana*. La depredación intra-gremio (consumo de artrópodos depredadores o parasitoides) representó solo en 3% de las presas de adultos y polluelos. Las dietas de los adultos fueron significativamente diferentes de la de los polluelos, como lo fueron las dietas de las aves

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muestreadas en diferentes bloques de viñedos. El sexo, la fecha y el número de jóvenes, y el ave individual (basado en el muestreo repetido de individuos) fueron todos factores insignificantes que no explicaron la variabilidad de la dieta. La edad de los polluelos fue un factor significativo que explicó una pequeña porción de la variabilidad en los componentes de la dieta. Adicionalmente, nuestros análisis de muestreos repetidos de grandes muestras fecales y los procesamientos independientes dieron resultados altamente contrastantes en los diez ensayos realizados y por ende recomendamos evitar esta metodología comúnmente empleada. La escatología molecular ofrece técnicas muy potentes e informativas que pueden revelar la función ecosistémica y los servicios brindados por las aves forrajeras, abundantes pero crípticas.

Palabras clave: análisis de la dieta, caja nido, código de barras de ADN, ecología trófica, función ecosistémica, insectívoros, muestras fecales, no invasivo

INTRODUCTION

Insectivorous birds are ubiquitous, multi–trophic-level predators, whose ecological function is poorly understood due to their high mobility and predation of relatively small organisms. Insectivorous birds reduce levels of herbivorous insects (Sekercioglu 2006, Van Bael et al. 2008, Karp and Daily 2014), preventing crop destruction (Kirk et al. 1996, Mäntylä et al. 2011, Karp et al. 2013) and forest defoliation (Eveleigh et al. 2007). Yet these same birds may function as intraguild predators, consuming intermediate invertebrate predators of the herbivores, potentially increasing plant stress (Mooney et al. 2010). Uncovering the diets of generalist insectivores, especially in agricultural landscapes, is thus vital to evaluate any ecosystem services or disservices they provide (Dobson et al. 2006, Wenny et al. 2011, Whelan et al. 2015) and to ensure avian populations have access to enough food resources to successfully reproduce in altered habitats.

Grape production in California generated over 5.2 billion dollars in 2015 from 918,000 harvested acres (USDA 2016), resulting in 88% of the United States' grapes. Increasingly over the past 20 years, wine-grape growers in northern California have placed songbird nest boxes in their vineyards to provide nesting opportunities to Western Bluebirds (*Sialia mexicana*) and other cavity-nesting bird species (Heaton et al. 2008). Western Bluebirds (hereafter simply "bluebirds") are insectivorous over the breeding season that lasts from March through July and overlaps with the grape growing season. Simultaneous breeding and provisioning of nestlings increases energetic demands and likely predatory pressure on arthropods (Holmes 1990). Bluebirds are ideal avian study systems as they are known to quickly occupy vineyard nest boxes (Jedlicka et al. 2011) and consume high volumes of insect prey. Bluebirds lay up to 2 clutches per year of 4–6 eggs each (Guinan et al. 2008). Two adults bringing food to five young at one bluebird nest are estimated to require 230 grasshoppers (at 0.54 g per grasshopper to total 124 g of arthropods) per day just to meet energetic requirements (Mock 1991). As generalist predators, bluebirds are known to consume a variety of arthropod prey items across functional guilds (including orders Orthoptera, Coleoptera, and Hemiptera; Guinan et al. 2008) although the assumed diet breadth stems mostly from stomach content analysis performed on sacrificed specimens (e.g., Beal 1915). In addition, prey selection likely varies over time and space, and adult birds may forage differently for themselves and their nestlings.

Molecular scatology opens the possibility of analyzing the arthropod prey of avian predators (Pompanon et al. 2012) and delineating their ecosystem function in newly colonized habitats. While publications analyzing bat diets have dominated the field (Symondson and Harwood 2014), significant advances in establishing effective protocols for processing minimally invasive avian samples now show it is possible to recover high quantities and qualities of DNA from avian fecal samples for downstream analysis (Vo and Jedlicka 2014). Trevelline et al. (2016) successfully applied molecular techniques to reveal that Louisiana Waterthrush (*Parkesia motacilla*) nestlings consumed larger percentages of lepidopterans and dipterans than was expected. In Australian macadamia orchards, molecular scatology uncovered that avian predators consumed 5 insect pest species, including a major pest, the green vegetable bug (*Nezara viridula*: Pentatomidae), which was found in 23% of the avian fecal samples collected (Crisol-Martínez et al. 2016). Such methods begin to make it feasible to link diet data to community ecology effects producing more robust top-down analyses of predator foraging.

In this study we apply high-throughput amplicon sequencing to address the following research question: What are the main prey items and, consequently, the ecosystem function of adult and nestling bluebirds? We also investigated the ecological, spatial, and temporal factors that could influence the foraging of these generalist insectivores. In addition, due to the high degree of heterogeneity present in avian fecal matter (where fragments of arthropod exoskeletons can be found intact) we tested whether subsampling fecal matter yielded the same or different dietary composition data. We demonstrate the power of deep sequencing to resolve trophic ecology questions in challenging study systems, revealing the hidden foraging patterns of abundant predators.

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METHODS

Study Site, Sample Collection, and Metagenomic DNA Extraction

This study utilized established bluebird nest boxes ($n = 100$) across 3 neighboring vineyards managed by the same grower in St. Helena, Napa County, California, USA (38°30′N, 122°29′W). Each vineyard grew wine grapes (*Vitis vinifera* L.) in rows grouped into blocks (Figure 1). During the avian breeding season (March–July) in 2012, fresh fecal samples were collected from adults breeding in nest boxes and their nestlings at ages zero through 20 days post-hatching. Adult bluebirds were sampled with mist nets early in the breeding season and with nest box traps once nestlings were present (Budden and Dickinson 2009). After being caught, adults were removed and placed into brown paper bags. Once a fecal sample was produced (usually a quick process as birds often defecate when handled; Burger et al. 1999), the bird was sexed, aged, banded to record repeated sampling, and released. To sample nestlings, half the young in each active nest box were placed in separate brown paper bags. Nestlings were aged and categorized as naked young (1–4 days old), partially feathered young (5–12 days old), fully feathered young (13–17 days old), or within the fledgling range (over 18 days old). Once nestlings left a fecal sample, or after 30 min, they were returned to the nest and exchanged for the remaining unsampled young. During the course of the breeding season, 37 banded birds (6 adult females, 6 adult males, and 25 nestlings) were resampled, with feces gathered at least 20 min apart and up to 11 days between samples.

Fecal samples were collected from bags within 10 min of defecation using tweezers that were sterilized between uses with hydrogen peroxide. All fecal samples were stored dry in 2 mL collection tubes, placed on ice in the field, and transferred as soon as possible to a −80°C freezer at University of California, Berkeley. Metagenomic DNA was extracted from all fecal samples ($n = 237$) using the Xpedition Soil/Fecal DNA MiniPrep Kit D6202 (Zymo Research, Irvine, California, USA) following the protocol described in Jedlicka et al. (2013) where ≤0.25 g fecal matter or less was placed in the Zymo-provided Lysis tube, combined with the Lysis/Stabilization Solution, and processed in a homogenizer (Precellys 24; Bertin Instruments, Montigny-le-Bretonneaux, France) at 6500 Hz s^{-1} for 2 cycles at 10 s each. Ten nestling fecal samples weighing over 0.25 g were subsampled by dividing them into 2–5 separate units that were processed independently to compare diet data. Elutions were stored at −20°C until use.

Arthropod COI Amplicon Generation, Library Preparation, and Sequencing

All metagenomic DNA extracts were quantified on the Qubit fluorometer using the dsDNA HS (high sensitivity)



FIGURE 1. Spatial distribution of study sites depicting vineyard blocks labeled either in capitalized letters, numbers, or lowercase letters indicating different vineyards under the same management. Each vineyard block had up to 10 bluebird nestboxes established on T-posts placed in the vines. Vegetation surrounding vineyards is oak woodland habitat.

Assay Kit (Invitrogen Q32854; Thermo Fisher Scientific, Waltham, Massachusetts, USA). Following Vo and Jedlicka (2014), DNA extracts from each sample were amplified in triplicate using degenerate, barcoded primers targeting the arthropod mitochondrial cytochrome oxidase *c* subunit I (COI) gene (ZBJ-ArtF1c and ZBJ-ArtR2c for an ∼200 base pair (bp) amplicon; Zeale et al. 2011) in 20 μL reactions containing 1X Phusion GC Buffer, 200 μM of each dNTP, 0.5 μM of each primer, 5% DMSO, 1 U Phusion high fidelity polymerase (NEB M0530S; New England Biolabs, Ipswich, Massachusetts, USA), and 0.2–1 ng DNA. Thermocycling conditions followed an initial denaturation of 98°C for 2 min; 35 cycles of 98°C for 8 s, 48.5°C for 20 s, and 72°C for 30 s; and a final extension of 72°C for 7 min. PCR set-up was performed in a UV-irradiated clean hood. PCR reactions were electrophoresed on 1.5% agarose gels and stained with SYBR-Gold (Invitrogen S-11494; Thermo Fisher Scientific) to detect amplification success.

For each sample, the triplicate PCR reactions were pooled and cleaned using Sera-Mag beads (GE Healthcare Life Sciences, Pittsburgh, Pennsylvania, USA) following Rohland and Reich (2012), except that a 1.2 volume ratio of bead solution to sample was used to discard fragments smaller than 200 bp (e.g., primer dimers). The cleaned samples were subsequently quantified using the Qubit dsDNA HS Assay Kit and pooled equimolarly to form 24 libraries of 9–10 samples and 67–153 ng of total amplicon DNA per library. The NEBNext End Repair Module, NEBNext dA-Tailing Module, and Meyer and Kircher (2010) adapter ligation protocols were used with TruSeq (Illumina, San Diego, California, USA) indexed adapters to prepare the libraries using a "with-bead" and "PCR-free" approach (Vo and Jedlicka 2014). For bead cleanups between library reactions, Fisher et al's (2011) SPRI cleanup protocol was performed with modifications

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following Vo and Jedlicka (2014) to maximize DNA recovery. The libraries were quantified via qPCR using the KAPA Library Quantification Kit (KK4824; Kapa Biosystems, Wilmington, Massachusetts, USA), pooled equimolarly, and analyzed on the Bioanalyzer (G2940CA; Agilent Technologies, Santa Clara, California, USA). The resultant pooled libraries were submitted to the University of California Davis Genome Center for a 250 bp, paired-end run on the Illumina MiSeq platform.

Data Analysis

The Illumina reads were trimmed of adapters and demultiplexed into the original libraries using CASAVA 1.8. Reads that did not pass filter were removed, and Trimmomatic was used to trim the trailing edge of each sequence to a minimum quality score of Q20 while keeping paired-end reads in sync (Bolger et al. 2014). Barcodes were error-corrected following Bystrykh (2012), and those that could not be resolved (i.e. those containing more than one error) were discarded. If one read was discarded, its paired read was also discarded. PANDAseq was used to merge paired-end reads, with a quality threshold of 0.6 (Caporaso et al. 2010). Merged reads that did not contain matching barcodes at both ends or that did not contain the correct primer sequences were also discarded.

In QIIME 1.8.0, each library was demultiplexed by barcode, and all barcode and primer sequences from the reads were trimmed before downstream analyses (Caporaso et al. 2010). With QIIME's uclust_ref protocol, sequences at least 125 bp in length were compared to a custom reference database created from all available arthropod sequences identified to species in the Barcode of Life Data Systems (BOLD) database (Ratnasingham and Hebert 2007). Operational taxonomic units (OTUs) with species identification were assigned at a 98.5% sequence similarity threshold. For OTU sequence queries that matched multiple taxa above the 98.5% threshold in the reference database, species identity was only assigned when agreed upon by all the matching reference sequences. Given the bias that PCR can introduce into amplicon libraries, we did not quantify the number of arthropod reads to report a relative abundance of each prey item within a fecal sample. Instead, arthropod sequences were analyzed as present (or absent) within fecal samples and, for each prey item, its presence among all analyzed fecal samples is reported. All identified arthropod species found in more than one fecal sample were categorized by guild (predator, herbivore, detritivore, parasitoid, other) to further summarize the composition of adult and nestling fecal samples (Triplehorn and Johnson 2005, Sasakawa 2009, Marshall 2012, Evans 2014). The "other" guild includes species such as mosquitoes (Diptera: Culicidae) exhibiting intersex dietary differences. *Chironomus* sp. (Diptera: Chironomidae) were classified as herbivores due to adult feeding ecology because larvae are aquatic (Armitage et al. 1995), and such habitats were absent from the study sites. A maximum-likelihood phylogenetic tree of all OTUs was generated using FastTree 2.1.3 as implemented within QIIME (Price et al. 2010).

Rarefaction analysis was conducted to assess the effect of sequencing depth on the number of arthropod OTUs and Shannon's diversity index observed per sample. To standardize comparisons of arthropod diversity between samples, OTU relative abundance tables were rarefied to 20 sequences per sample, the sequencing depth at which Shannon's diversity index plateaued for generally all samples. The rarefied OTU tables were then used to generate UniFrac distance matrices (Lozupone and Knight 2005) to characterize similarity among samples. UniFrac is a measure of phylogenetic distance between sets of taxa in a phylogenetic tree, and it is commonly used for the comparison of microbial communities. However, it is equally useful for the comparison of arthropod assemblages recovered from fecal samples. Given a phylogenetic tree containing all identified arthropod species, the UniFrac distance between 2 fecal samples can be understood as the fraction of the branch length of the tree that leads to arthropod species found in either one fecal sample or the other but not both.

Adonis and ANOSIM were applied (999 permutations, $\alpha = 0.05$) to examine the statistical significance of the contribution of several spatial (vineyard, vineyard block, nest box), temporal (age, sampling date), and ecological (sex, brood size) factors to variation in unweighted UniFrac among samples. Adonis partitions the UniFrac distance matrix by a factor and computes the percentage of variation explained by the factor based on squared deviations from the relevant centroids of the data. Significance tests are performed with *F*-tests based on sequential sums of squares from permutations of the raw data. ANOSIM likewise partitions the UniFrac distance matrix by a factor into 2 or more groups of samples. A significant difference among groups is determined through permutations whereby the mean rank of all distances between groups is greater than the mean rank of all distances within groups.

RESULTS

From the 237 feces collected and processed, 41 adult and 169 nestling samples produced arthropod sequences with matches in the BOLD database. The Miseq run generated 17.4 million raw paired-end reads, of which 8.2 million merged sequences passed all quality filters. A mean of 1,261 (± 3,312 SD, ranging from 2 to 22,271) merged sequences was retained per sample after quality filtering and OTU assignment against the BOLD database. Overall

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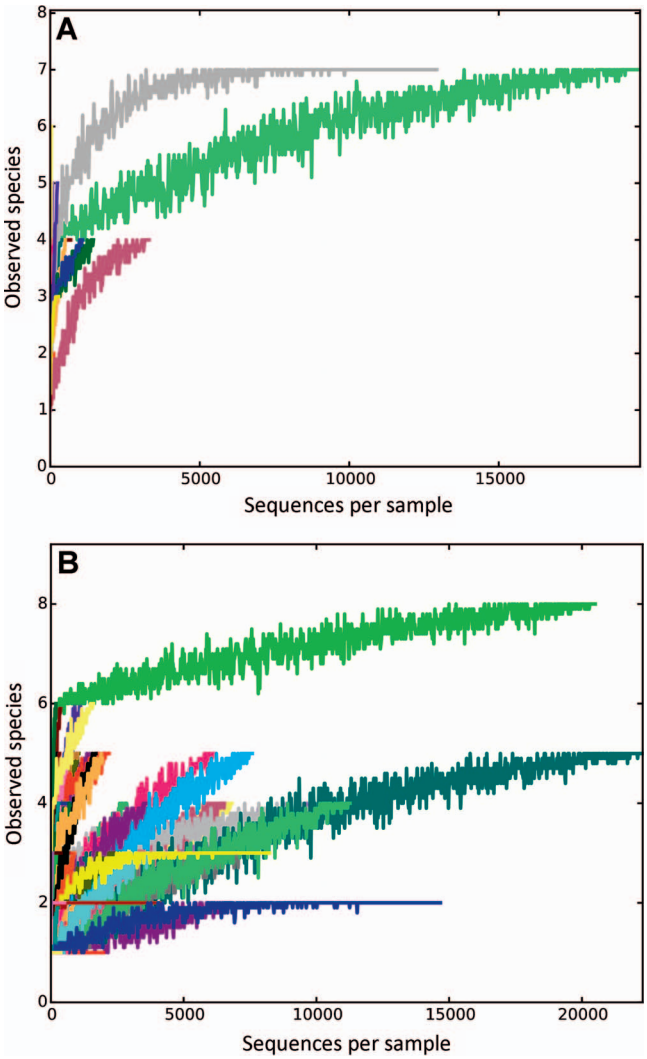


FIGURE 2. Rarefaction analysis for observed prey items in individual fecal samples from (**A**) adults ($n = 41$) and (**B**) nestlings ($n = 169$).

there was a mean of 2.6 (± 1.5 SD, ranging from 1 to 8) total prey species per sample. Rarefaction curves indicated robust characterization of the arthropod diversity given the number of sequences achieved per sample, with Shannon's diversity index observed in most samples leveling after 10 sequences (Appendix Figure 5). Of the sequences with matches to BOLD, rarefaction analysis indicated that many samples were sequenced to saturation, but additional diet items may remain undetected (Figure 2).

Dietary Components

Bluebirds consumed 66 different arthropod species representing 6 orders and 28 families (Table 1). *Aedes* mosquitoes (Culicidae) were the most commonly encountered prey item, found in 51% of the adult and 49% of the nestling fecal samples. After *Aedes* sp., herbivorous insects in the orders Diptera, Hemiptera, and Lepidoptera comprised the majority of food items, representing over 56% of OTUs identified from the fecal samples. The most commonly encountered dipteran herbivores (found in 22% of the adult and 25% of the nestling samples) were non-biting midges *Chironomus* sp. (Chironomidae) where adults consume nectar, pollen, and other sugar-rich food. In ~20% of the adult and nestling samples, herbivorous stink bugs (*Chlorochroa* sp., Pentatomidae: Hemiptera) were present. We found no vineyard insect pest species in bluebird fecal samples, although other species in common agronomic pest families were identified belonging to Lygaeidae and Cicadellidae (Hemiptera) and 2 Lepidopteran species belonging to Tortricidae. We detected bird blowfly DNA (*Protocalliphora* sp.: Calliphoridae) in 7% of adult and 11% of nestling fecal samples. Blowflies were consumed by birds associated with 6 different nest boxes located in 5 vineyard blocks at 2 vineyard sites between June 6 and July 5. At one nest, 5 young were found to repeatedly consume blowflies from 6 to 16 days old. A known ectoparasite of avian nestlings, particularly cavity-nesting species, blowflies were placed in the "other" guild category because blood-feeding larvae are trophically distinct from adults that prefer a "sugar-protein" diet (Bennett and Whitworth 1991).

Intraguild predation was seldom recorded as predaceous and parasitic arthropods comprised smaller portions of occurrence at 2.2% and 0.7% respectively. We did not detect arachnid DNA in any bluebird fecal samples. Detritivores were present in 8.5% of the fecal samples, due to a fly species from the genus *Musca* (same genus as common housefly) that was the third most common arthropod DNA sequence recovered.

Adult vs. nestling life stage ($r^2 = 0.045$, $p = 0.001$; Figure 3A) contributed to a small but significant proportion of the variation in diet composition. Coleopterans (beetles) occurred in ~20% of the adult and only 12% of the nestling samples whereas the reverse trend was found with lepidopterans (12% of nestling compared to 9% of adult). Dietary components in adult male and female bluebird samples did not differ ($r^2 = 0.03$, $p = 0.88$). Number of young within each nest was not significant when analyzed separately for adults ($r^2 = 0.409$, $p = 0.17$) and nestlings ($r^2 = 0.034$, $p = 0.70$). Neither vineyard nor nest box were significant factors, but block designation within vineyards explained a large and moderate amount of the variation in adult ($r^2 = 0.688$, $p = 0.014$) and nestling ($r^2 = 0.173$, $p = 0.048$) diets, respectively.

Nestling age was a significant factor in explaining a small amount of the variability in dietary components ($r^2 = 0.019$, $p = 0.05$; Figure 3B). Dipterans comprised 61% and 57% of the samples from naked and partially feathered young, respectively. Hemipteran taxa comprised approximately 18–24% of the nestling samples. Samples taken at

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the same time were not more similar than those taken at different times. For birds that were sampled multiple times, resamples were not significantly more similar to previous samples from the same bird relative to samples from different birds ($r^2 = 0.013$, $p = 0.50$). Fecal subsamples were not more similar in contents to each other than samples from different birds ($r^2 = 0.012$, $p = 0.92$; Figure 4). Subsamples varied in the number of taxa detected, and none contained the exact same dietary items.

DISCUSSION

Well over 50% of uncovered prey items from bluebird fecal samples were herbivorous arthropods, signaling that the predators function as second-order predators (e.g., consumers of herbivorous taxa). Predaceous and parasitic arthropods combined comprised less than 3% of the samples, indicating negligible effects on potentially beneficial taxa. Close to 50% of prey items were dipterans from numerous families, and while flies are not surprising as a prey source, it is noteworthy the extent to which both nestlings and adults relied on this prey base, feeding on similar prey items. *Aedes* mosquitoes (Culicidae) were the most commonly encountered prey item, found in 50% of all fecal samples yet are not listed as a food item for bluebirds from stomach content analyses (Guinan et al. 2008). Furthermore orthopterans (crickets and grasshoppers), widely known as common bluebird food items in their native oak woodland and savanna habitat (Beal 1915), were only found in one adult and two nestling fecal samples (*Gryllus* sp.: Gryllidae). Given that reproductive success of bluebirds in these vineyards is relatively high (Fiehler et al. 2006, Jedlicka et al. 2014), the generalist nature of bluebird foraging is evident in this dietary analysis where orthopterans were largely absent from their diets and the landscape (J. A. Jedlicka personal observation). Provisioned nest boxes in vineyard landscapes are permitting bluebirds to colonize relatively novel agricultural habitats that offer a different assemblage of arthropod taxa compared to their native oak woodlands.

We found both adults and nestlings consumed prey that were small in size, including *Aedes* mosquitoes (2–4 mm), aphids (2.5 mm), and cicadellids (3 mm). Optimal foraging theory predicts that diet choice and foraging behavior should maximize fitness by selecting the most energetically favorable (e.g., larger) prey items as these are worth the expenditure of energy adults must spend to deliver resources to the nest. Perhaps high abundances or clumped distributions of these taxa allow for more efficient foraging by birds. While individual mosquitoes and aphids are energetically less favorable than crickets (Gryllidae members often measure more than 13 mm in length; Triplehorn & Johnson 2005), future research should investigate the costs associated with dietary shifts in predator populations resulting from habitat selection.

Bluebirds were not found to regularly consume parasitoids. Three fecal samples contained a species of cluster fly (*Pollenia* sp.: Calliphoridae). These species are usually parasitoids on earthworm hosts, but some may be earthworm predators as their biology is poorly understood (Jewiss-Gaines et al. 2012). Cluster flies are widely regarded as a pest species for human habitations and received their common names for clustering on walls. There was one nestling fecal sample containing DNA from a tachinid fly parasitoid, a family well known to be beneficial for biological control. It is impossible to tell from these data whether tachinid DNA was present in avian fecal samples because of direct foraging or indirectly via consumption of lepidopteran larvae that were already parasitized by the tachinid fly.

Ectoparasitic bird blowfly (*Protocalliphora* sp.) DNA was found in 11% of nestling and 7% of adult fecal samples. Adult flies often target cavity-nesting birds and lay their eggs in nesting material. Larvae emerge and attach to nestlings to feed on blood for growth and development. Such parasitism is associated with slower development and potential death of nestlings and may induce increased feeding rates from adults (O'Brien and Dawson 2008). Because larvae usually feed from nestlings at night and retreat deep within the nesting materials during the day, it is generally thought that blowfly larvae avoid direct consumption by their avian hosts. However, our data show it is not uncommon for bluebirds to consume blowflies. Whether nestlings are foraging on the larvae directly or being fed adult or larval blowflies cannot be discerned from our data. Given the high proportion of samples that contained *Aedes* mosquito DNA, it is not improbable that nestlings are being fed adult blowflies. Regardless, we clearly detected active feeding by the hosts on their ectoparasites warranting further research.

Another surprising result from the study was the absence of spider DNA sequences in avian fecal samples. While this could be interpreted as a bias of the primers, using the same primer sets with Illumina sequencing Trevelline et al. (2016) and Crisol-Martinez et al. (2016) recovered 6 and 4 families, respectively, of arachnid taxa in avian fecal samples, and Hope et al. (2014) recovered 9 families of arachnid taxa in bat (*Myotis nattereri*) fecal samples. Arthropod sampling via pitfall traps in the field during avian fecal sampling revealed there were spiders present in the vineyards during the bluebird breeding season (J. A. Jedlicka personal observation). However, perhaps there is interannual variation in arachnid population numbers, and spiders were less abundant during the sampled season. Previous studies characterizing bluebird diets consistently list arachnids as prey items (Beal 1915), and preliminary work with 12 bluebird nestlings in

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TABLE 1. Species list and guild assignments of arthropod prey items found in Western Bluebird fecal samples, identified in the BOLD database based to 98.5% sequence similarity. Columns on the right indicate the percent frequency of occurrence in adult and nestling samples and the total number of samples where the prey was detected. Items that occurred in over 5% of the fecal samples are in bold.

Order	Family	Subfamily	Genus	Species	Guild	Frequency of occurrence in adults (%)	Frequency of occurrence in nestlings (%)	Total number of samples
Coleoptera	Cantharidae	Cantharinae	*Dichelotarsus*	*vicollis*	predator		0.6	1
	Carabidae	**Harpalinae**	***Amara***	**sp.**	**herbivore**	**7.3**	**8.9**	**18**
			Amara	*oralis*	herbivore	4.9	3.0	7
			Amara	*unis*	herbivore		0.6	1
			Anisodactylus	**sp.**	**herbivore**	**14.6**	**7.7**	**19**
			Poecilus	sp.	predator	2.4	1.2	3
	Curculionidae	Entiminae	*Sitona*	*tus*	herbivore	4.9	3.0	7
	Ptinidae	Anobiinae	*Oligomerus*	sp.	herbivore		0.6	1
	Staphylinidae	**Aleocharinae**	***Myllaena***	**sp.**	**other**	**7.3**	**11.2**	**22**
			Aleochara	*orax*	predator		0.6	1
		Staphylininae	*Quedius*	*ennis*	predator	2.4	2.4	5
Diptera	**Calliphoridae**	**Chrysomyinae**	***Protocalliphora***	**sp.**	**other**	**7.3**	**10.7**	**21**
		Calliphorinae	*Pollenia*	sp.	parasitoid	2.4	1.2	3
	Chironomidae	**Chironominae**	***Chironomus***	**sp.**	**herbivore**	**22.0**	**24.9**	**51**
			Tanytarsus	*ashii*	herbivore	2.4	1.2	3
				sp.	herbivore		1.8	3
		Orthocladiinae	*Cricotopus*	sp.	herbivore	2.4	1.2	3
			Eukiefferiella	sp.	herbivore		0.6	1
	Culicidae	**Culicinae**	***Aedes***	**sp.**	**other**	**51.2**	**49.1**	**104**
	Muscidae	**Muscinae**	***Musca***	**sp.**	**detritivore**	**19.5**	**20.7**	**43**
		Coenosiinae	*Coenosia*	sp.	predator		0.6	1
		Phaoniinae	*Phaonia*	sp.	predator		0.6	1
	Psychodidae	**Psychodinae**	***Psychoda***	**sp.**	**other**	**7.3**	**10.7**	**21**
	Sarcophagidae	Sarcophaginae	*Boettcheria*	sp.	detritivore		0.6	1
	Syrphidae	Syrphinae	*Eupeodes*	sp.	other	2.4	1.8	4
	Tachinidae	Tachininae	*Strongygaster*	sp.	parasitoid		0.6	1
Hemiptera	**Aphididae**	**Aphidinae**	***Aphis***	***civora***	**herbivore**	**9.8**	**14.2**	**28**
				aecola	herbivore	2.4	1.2	3
				riciphaga	herbivore		0.6	1
				sp.	herbivore	2.4	1.8	4
			Hyalopterus	sp.	herbivore		0.6	1
			Myzus	sp.	herbivore	2.4	1.2	3
			Rhopalosiphum	sp.	herbivore		0.6	1
		Drepanosiphinae	*Drepanosiphum*	sp.	herbivore		0.6	1
		Lachninae	*Cinara*	*ilina*	herbivore		0.6	1
	Cicadellidae	Cicadellinae	*Hordnia*	*cythura*	herbivore	2.4	1.2	3
	Lygaeidae	**Lygaeinae**	***Lygaeus***	**sp.**	**herbivore**	**7.3**	**11.8**	**23**
	Nabidae	Nabinae	*Nabis*	*icoferus*	predator		0.6	1
	Pentatomidae	**Pentatominae**	***Chlorochroa***	**sp.**	**herbivore**	**19.5**	**20.1**	**42**
			Thyanta	***ovirenssetosa***	**herbivore**	**7.3**	**5.9**	**13**
			Brochymena	*tulata*	herbivore		0.6	1
	Rhyparochromidae	Rhyparochrominae	*Megalonotus*	sp.	herbivore	2.4	1.2	3

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TABLE 1. Continued.

Order	Family	Subfamily	Genus	Species	Guild	Frequency of occurrence in adults (%)	Frequency of occurrence in nestlings (%)	Total number of samples
Hymenoptera	Formicidae	Myrmicinae	*Solenopsis*	sp.	other	2.4		1
Lepidoptera	**Erebidae**	**Arctiinae**	***Leucanopsis***	**sp.**	**herbivore**	**7.3**	**10.7**	**21**
			Amata	*olina*	herbivore	2.4	1.2	3
			Lophocampa	sp.	herbivore		0.6	1
			Termessa	sp.	herbivore	2.4	1.2	3
		Erebinae	*Euclystis*	sp.	herbivore		0.6	1
	Eutelliidae	Eutelliinae	*Paectes*	sp.	herbivore	2.4	1.8	4
	Geometridae	Larentiinae	*Euphyia*	sp.	herbivore	2.4	1.2	3
	Noctuidae	**Noctuinae**	***Leucania***	**sp.**	**herbivore**	**9.8**	**8.9**	**19**
			Dasypolia	sp.	herbivore		0.6	1
			Epipsilia	sp.	herbivore	2.4	1.2	3
			Properigea	sp.	herbivore		0.6	1
	Oecophoridae	Oecophorinae	*Heteroteucha*	sp.	herbivore		0.6	1
	Sphingidae	Macroglossinae	*Eumorpha*	sp.	herbivore		0.6	1
	Tortricidae	Tortricinae	*Olindia*	*cherana*	herbivore		0.6	1
			Tortrix	sp.	herbivore		0.6	1
Orthoptera	Gryllidae	Gryllinae	*Gryllus*	sp.	herbivore	2.4	1.2	3

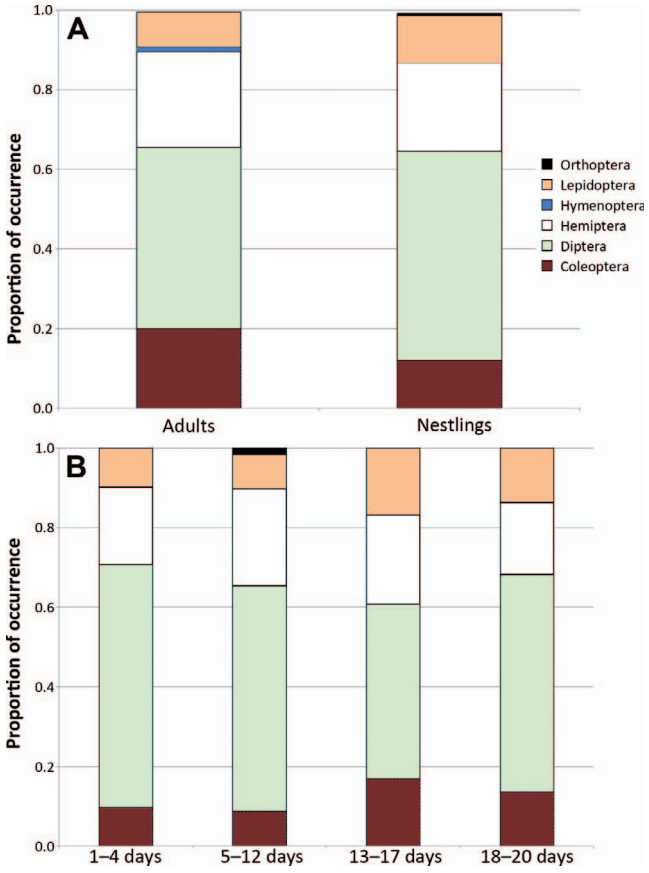


FIGURE 3. Proportion of occurrence of arthropod orders in Western Bluebird fecal samples calculated by number of fecal samples with the prey item present divided by the total number of fecal samples. (**A**) In adult ($n = 41$) diets, coleopterans comprised proportionately more than in nestling ($n = 169$) diets, which contained higher proportions of lepidopterans. (**B**) Age in days of Western Bluebird nestlings explained little of the variability in dietary components but was a significant factor ($r^2 = 0.019$, $p = 0.05$). Age categorized as naked young (1–4 days old; $n = 29$), partially feathered young (5–12 days old; $n = 80$), fully feathered young (13–17 days old; $n = 47$), or within the fledgling range (over 18 days old; $n = 13$).

vineyards found one fecal sample contained arachnid DNA (Jedlicka et al. 2013). In agricultural landscapes, there may be a lack of web-building spiders due to high frequency of machinery and people passing through crop rows.

While primer bias does not explain the spider absence, it may be the reason no isopterans were identified in bluebird fecal samples during this study. Previous research by Jedlicka et al. (2013) using different primer sets (LCO1490 and HCO2198) found isopteran DNA (*Armadillium vulgare*) in 12 out of 13 fecal samples from Western Bluebird nestlings in Napa and Sonoma County vineyards. *Armadillium vulgare* is a calcium-rich food source (Ouyang and Wright 2005) and consequently may be an important component of bluebird diets during the

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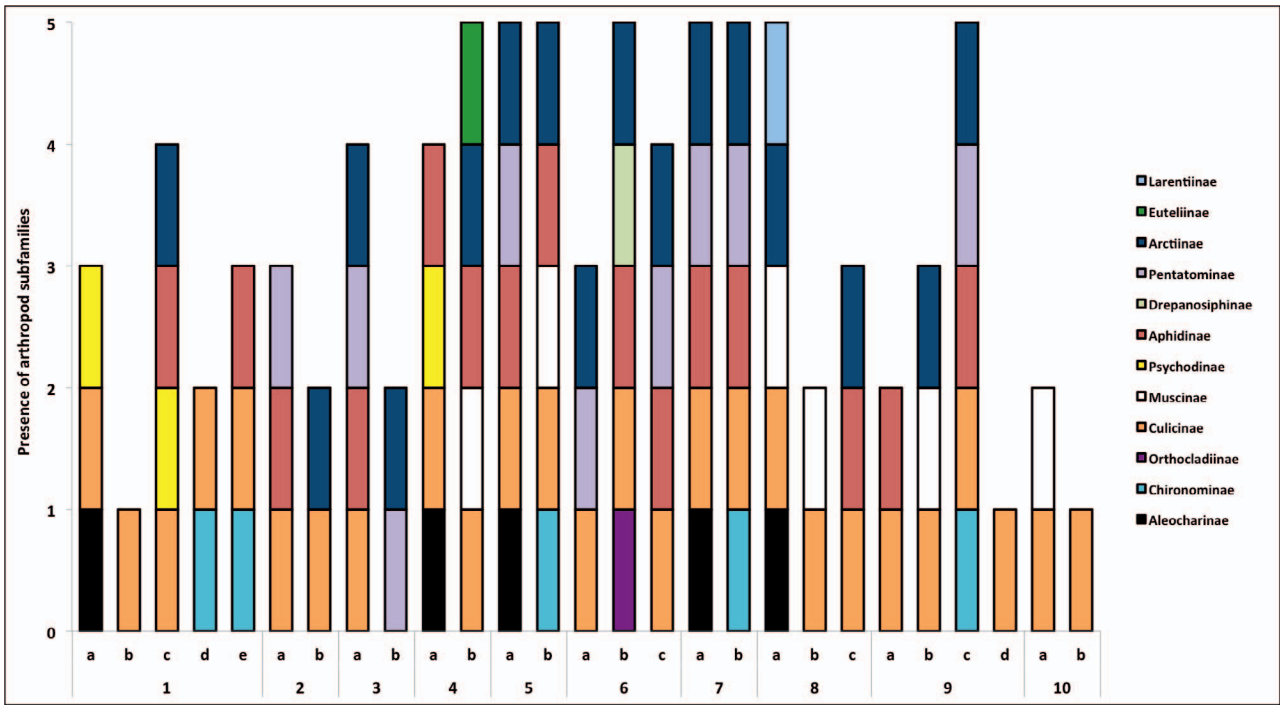


FIGURE 4. Presence of arthropod subfamilies in 10 bluebird fecal samples (labeled 1–10) that were divided into 2–5 subsamples (letters a–e) and processed independently. Subsamples from the same feces were not more similar than independent samples from different birds ($p = 0.92$).

breeding season (Tilgar et al. 1999, Dawson and Bidwell 2005). Clarke et al. (2014) show that ZBJ-ArtF1c and R2c primers used here do not provide good coverage for Isoptera, provide excellent coverage for both Diptera and Lepidoptera, and adequate coverage for Hemiptera, Orthoptera, Coleoptera, and Hymenoptera. More research on optimal primer sets or using multiple primer sets in unison is needed to continually advance applications in the molecular scatology field.

The highly generalist nature of bluebird foraging is clearly supported by the finding that nestling diets did not segregate by nest box. This indicates that intra-nest variability was high, and if any patterns between nests existed, they could not be distinguished. Likewise different vineyards were not a significant variable in our study, likely due to high arthropod variability within the vineyard. Vineyard block was the only significant spatial variable affecting diet composition of adult ($r^2 = 0.688$, $p = 0.014$) and nestling ($r^2 = 0.173$, $p = 0.048$) bluebirds. The vineyard block design (Figure 1) likely captures both the inherent patchiness of arthropod communities and the foraging territories of bluebirds during the breeding season.

Nestling age was significant in explaining only a small fraction ($r^2 = 0.019$) of the differences between dietary components in the samples. Consequently the diets of naked young ranging from 1 to 4 days old were not that dissimilar from the diets of older nestlings. In Western Bluebird nestlings, 50% of their mass is attained by 6–7 days and 90% by 10–11 days (Mock 1991). However we found the diet composition of prey in fecal samples remained fairly consistent over the nestling period.

The number of young in the nest increases foraging intensity by adults and resource competition among nestlings. Consequently nestlings in larger broods (up to 6 in this study) may be expected to suffer lower caloric intakes as they compete with their siblings for delivered prey items. Such competition may be apparent by discovering fewer OTUs or less energetically favorable prey items in fecal samples from nestlings in larger compared to smaller broods. However, brood size did not explain dietary differences among samples as there were no significant differences between the dietary composition of adults with fewer (1–3) and more (4–6) nestlings primer and the same held true for nestlings with more or fewer siblings.

In Napa vineyards, economically significant insect pests include leafhoppers and sharpshooters (Hemiptera: Cicadellidae). Some cicadellid species such as the blue-green sharpshooter (*Graphocephala atropunctata*) transmit the bacterium *Xylella fastidiosa* that causes Pierce's disease and vine mortality in grapevines. Lepidopteran larvae are the most common pest species found in grape clusters, especially taxa in the family Tortricidae (UC ANR 2013). Tortricid pests include emerging exotic species such as

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European grapevine moth (*Lobesia botrana*) discovered in Napa County, California, in 2009 and the light brown apple moth (*Epiphyas postvittana*) first found in the California North Coast in 2007. Consequently, reducing cicadellid and tortricid pests is beneficial to growers and considered a significant ecosystem service. Lepidopterans comprised 9% and 12% of adult and nestling dietary items respectively, including some tortricid moths. Although there was no direct evidence that bluebirds consume blue-green sharpshooters or lepidopteran pest species, we also did not catch any of these species in vacuum, malaise, or pitfall traps sampled weekly throughout the course of the study (J. A. Jedlicka personal observation), so their absence from bird diets is not surprising.

Some birds were resampled over the course of the breeding season, but the resampling times varied from less than an hour to more than a month apart. Our analysis of resampling diets showed that some individual birds consumed similar prey items over time whereas others contained highly differentiated prey items, and overall resampling was not a significant factor. Highly similar diets were found in the same bird sampled within 40 min and after 37 days. Nonetheless, dissimilar diets were found in a different bird sampled 50 min later and more than a month later. Consequently, while it is tempting to assume that similarity of diet among resamples may be time dependent (because prey items passing through the gut may still be detectable at later time periods), this is not always the case. Feeding trials that monitor predator consumption and track prey passage detection through fecal samples (Oehm et al. 2011) are needed on a wider breadth of predator taxa to aid in the interpretation of passage time and interpretation of molecular scatology results.

Most fecal DNA extraction kits limit the amount of fecal matter to 0.1–0.25 g at the initial stage of processing for optimal extraction performance. As a result, many researchers find it necessary to subsample from larger feces (e.g., over 1 g) down to the desired amount. Our analyses of dividing the fecal sample into 2–5 separate units and processing these independently revealed striking differences between subsamples. In 10 separate analyses we found no consistency in the subsamples processed from the same initial feces. In light of this information, we highly recommend researchers avoid simply portioning the correct mass from the sample and proceeding. Efforts to homogenize the contents need to be studied so that molecular scatology methods can be successfully applied and interpreted to larger avian fecal samples.

Our Illumina sequencing and molecular scatology approaches revealed that bluebirds predominately consumed herbivorous insects in California vineyards and identified a commonly consumed new prey item, *Aedes* mosquitoes. In addition, adult and nestling consumption of ectoparasitic blowflies was uncovered. Given the substantial amounts of food resources necessary to support avian energetic demands, both adults and nestlings appear to act as important yet largely unrecognized natural predators of herbivorous insects, including both lepidopterans and hemipterans. Potential ecosystem disservices such as avian consumption of predaceous arthropods were largely absent. Further research could pair diet analyses with pest population changes over time to clearly identify any ecosystem services these birds may provide to growers who integrate avian conservation with agricultural production.

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Author contributions: J.A.J. conceived the idea, design, and experiment, and performed the experiments. J.A.J. and A.-T.E.V. wrote the paper and analyzed the data. J.A.J., A.-T.E.V., and R.P.P.A. developed and designed methods and contributed substantial materials, resources, and funding.

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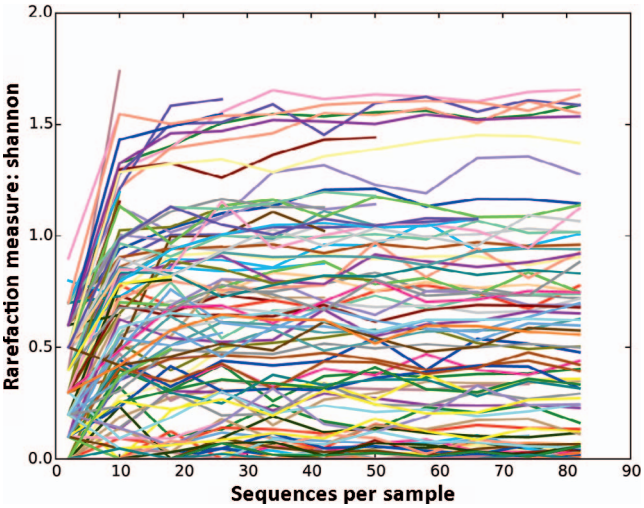
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APPENDIX FIGURE 5. Rarefaction analysis of Shannon's diversity index for arthropods in all Western Bluebird fecal samples. Each line and color depicts a different sample.

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