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SPECIAL FEATURE: Advances In Avian Diet Methods and Applications

DNA metabarcoding reveals broad woodpecker diets in fire-maintained forests

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ABSTRACT

Ecological disturbance is a key agent shaping the spatial and temporal landscape of food availability. In forests of western North America, disturbance from fire can lead to resource pulses of deadwood-associated arthropods that provide important prey for woodpeckers. Although the foraging strategies among woodpecker species often demonstrate pronounced differences, little is known about the ways in which woodpeckers exploit and partition prey in disturbed areas. In this study, we employed DNA metabarcoding to characterize and compare the arthropod diets of 4 woodpecker species in Washington and California, USA—Black-backed Woodpecker (*Picoides arcticus*), Hairy Woodpecker (*Dryobates villosus*), Northern Flicker (*Colaptes auratus*), and White-headed Woodpecker (*Dryobates albolarvatus*)—primarily using nestling fecal samples from burned forests 1–13 years postfire. Successful sequencing from 78 samples revealed the presence of over 600 operational taxonomic units (OTUs) spanning 32 arthropod orders. The nestling diets of two species in particular—Northern Flicker and Black-backed Woodpecker—proved to be much broader than previous observational studies suggest. Northern Flicker nestlings demonstrated significantly higher diet diversity compared to other focal species, all of which displayed considerable overlap in diversity. Wood-boring beetles, which colonize dead and dying trees after fire, were particularly important diet items for Black-backed, Hairy, and White-headed woodpeckers. Diet composition differed among species, and diets showed limited differences between newer (≤5 yr) and older (>5 yr) postfire forests. Our results show mixed evidence for dietary resource partitioning, with three of the four focal species exhibiting relatively high diet overlap, perhaps due to the pulsed subsidy of deadwood-associated arthropods in burned forests. Woodpeckers are frequently used as management indicator species for forest health, and our study provides one of the first applications of DNA metabarcoding to build a more complete picture of woodpecker diets.

Keywords: Black-backed Woodpecker, Hairy Woodpecker, molecular scatology, next-generation sequencing, niche partitioning, Northern Flicker, White-headed Woodpecker, wildfire

LAY SUMMARY

- Forest fire creates a "resource pulse" of arthropod prey for woodpeckers, yet little is known about the composition of woodpecker diets in postfire areas.
- We used DNA metabarcoding to characterize and compare the diets of four woodpecker species, focusing on nestling fecal samples from burned forests in the western USA.
- Woodpecker diets were broader than previous studies have reported. Northern Flicker samples showed especially high nestling diet diversity compared to other focal species.
- Black-backed, White-headed, and Hairy Woodpecker nestlings showed a high prevalence of beetles in their diets.
- Woodpeckers are frequently used as indicators to evaluate forest management activities, and detailed characterization of woodpecker diets represents an important step in understanding their resource requirements.

La meta codificación de barras de ADN revela dietas amplias de pájaros carpinteros en bosques mantenidos por incendios

RESUMEN

El disturbio ecológico es un agente clave que modela el paisaje espacial y temporal de la disponibilidad de alimentos. En los bosques del oeste de América del Norte, el disturbio causado por el fuego puede generar pulsos de recursos de artrópodos asociados con la madera muerta, que representan presas importantes para los pájaros carpinteros. Aunque las estrategias de forrajeo entre las especies de pájaros carpinteros a menudo demuestran diferencias pronunciadas, se sabe poco sobre las formas en que los pájaros carpinteros explotan y se reparten las presas en áreas disturbadas. En este estudio, empleamos meta codificación de barras de ADN para caracterizar y comparar las dietas de artrópodos de cuatro especies de pájaros carpinteros en Washington y California, EEUU—*Picoides arcticus*, *Dryobates villosus*, *Colaptes auratus* y *D. albolarvatus*—principalmente utilizando muestras fecales de polluelos de bosques quemados de 1–13 años después del incendio. La secuenciación exitosa de 78 muestras reveló la presencia de más de 600 unidades taxonómicas operativas que abarcan 32 órdenes de artrópodos. Las dietas de los polluelos de dos especies en particular, *C. auratus* y *P. arcticus*, demostraron ser mucho más amplias de lo que sugieren los estudios de observación anteriores. Los polluelos de *C. auratus* demostraron una diversidad de dieta significativamente mayor en comparación con las otras especies focales, las cuales mostraron una superposición considerable en la diversidad. Los escarabajos perforadores de madera, que colonizan árboles muertos y moribundos después de un incendio, fueron elementos de la dieta particularmente importantes para *P. arcticus*, *D. villosus* y *D. albolarvatus*. La composición de la dieta difirió entre las especies, y las dietas mostraron pocas diferencias entre los bosques post-incendios más nuevos (≤5 años) y más viejos (>5 años). Nuestros resultados muestran evidencia mixta sobre la partición de los recursos de la dieta, con tres de las cuatro especies focales exhibiendo una superposición de dieta relativamente alta, tal vez debido al pulso de artrópodos asociados con la madera muerta en los bosques quemados. Los pájaros carpinteros se utilizan con frecuencia como especies indicadoras de la salud de los bosques, y nuestro estudio proporciona una de las primeras aplicaciones de la meta codificación de barras de ADN para construir una imagen más completa de las dietas de los pájaros carpinteros.

Palabras clave: Colaptes auratus, Dryobates albolarvatus, Dryobates villosus, *escatología molecular*, *incendio forestal*, *partición de nicho*, Picoides arcticus, *secuenciación de próxima generación*

INTRODUCTION

Dietary niche partitioning is an important mechanism for the coexistence of ecologically similar consumers through alleviation of resource competition (Schoener 1974, Kent and Sherry 2020). Partitioning among coexisting species that share morphological traits or behaviors is often expected for landscapes with a relatively consistent and predictable resource base (Tilman 1994, Kartzinel et al. 2015). In many landscapes, however, resource availability varies widely over space and time due to a variety of natural and anthropogenic factors. In some cases, resources may occur in a shifting mosaic where localized patches experience periods of elevated resource availability (Wimberly 2006). Under these circumstances, characterizing predator-prey interactions and comparing diets between consumers can provide useful insights into the origin and maintenance of biodiversity in ecosystems with patchy and unpredictable resources.

Ecological disturbance is a common agent behind the changing landscape of resource availability. In forested areas, disturbances such as fire, insect outbreaks, or drought can fundamentally alter communities of consumers and their prey. After forest fire, many arthropods take advantage of regenerating vegetative growth (He et al. 2019), and deadwood-associated insects quickly colonize patches of standing, dead trees (Ray et al. 2019). As forest succession advances in the years following fire, arthropod populations change along with shifts in vegetation and deadwood availability (He et al. 2019). In temperate and boreal forests, woodpecker species arrive quickly after disturbance events to take advantage of abundant prey and nesting opportunities in dead trees (Virkkala 2006, Stillman et al. 2019a). One species, in particular, the Black-backed Woodpecker (*Picoides arcticus*), is closely associated with recently burned forests in the western USA (Hutto 2008, Tingley et al. 2020), and observational studies suggest that they forage primarily on the larvae of wood-boring beetles found commonly in burned areas (Murphy and Lehnhausen 1998). In some cases, local populations in recently burned areas show population trajectories that mirror the rise and fall of food availability as time since fire increases (Saab et al. 2007, Nappi et al. 2010, Tingley et al. 2018). While the Black-backed Woodpecker's association with fire has been well-documented, many studies have noted that other woodpecker species are also commonly found in burned areas. For example, generalist species like the Hairy Woodpecker (*Dryobates villosus*) and Northern Flicker (*Colaptes auratus*) frequently nest in postfire forests (Saab et al. 2007, Tarbill et al. 2015). The at-risk White-headed Woodpecker (*Dryobates albolarvatus*) is also found in burned areas (Lorenz et al. 2015), although this habitat association is enigmatic because the species is elsewhere associated with live trees. It is not known whether these woodpecker species use burned forests solely because of an abundance of snags for nesting (e.g.,

Lorenz et al. 2015), or if they also benefit from the pulse of arthropod prey.

Because woodpecker populations respond to forest structure, woodpeckers are frequently used as indicator species to guide forest management and biodiversity conservation (Walters 1991, Virkkala 2006, Drever et al. 2008). Woodpeckers play a keystone role as primary cavity excavators in forest systems, providing an important resource for other wildlife (Martin and Eadie 1999, Tarbill et al. 2015). The specific habitat requirements of many woodpecker species make them sensitive to forest management practices that remove trees, and woodpecker populations in North America are frequently monitored to assess the ecological impact of management actions (Walters 1991, Saab et al. 2009, Tingley et al. 2020). Some species, such as Black-backed and White-headed woodpeckers, are also considered species of conservation concern. The disturbance-prone forests of western North America host a relatively high diversity of woodpecker species, yet little is known about the arthropod diets of these species or how their diets may overlap or change during resource pulses following disturbance.

Studies of woodpecker diets are conventionally conducted by carefully observing foraging or provisioning individuals (Beal 1911, Murphy and Lehnhausen 1998, Kozma and Kroll 2013). While these methods can be effective for understanding general trends in prey consumption, they are biased towards larger prey and may not provide reliable data on prey taxonomic identity. Dissecting and identifying prey items from fecal samples can provide added taxonomic resolution, but these time-consuming methods often overlook soft-bodied prey items like larval insects (Pompanon et al. 2012, Hoenig et al. 2021). Advances in molecular approaches using DNA metabarcoding provide powerful tools to address these shortcomings and enhance our ability to identify taxonomic groups present in fecal material (Pompanon et al. 2012, Jedlicka et al. 2013, Jusino et al. 2019). General primers can be used to amplify the DNA of many prey species simultaneously, allowing fine-scale characterization of predator-prey interactions and subsequent analysis of dietary niche partitioning between sympatric consumers.

In this study, we used DNA metabarcoding to characterize and compare the diets of four woodpecker species living in disturbance-prone forests of Washington and California, USA: Black-backed Woodpecker, Hairy Woodpecker, Northern Flicker, and White-headed Woodpecker. Our approach used the "ANML" primer pair (Jusino et al. 2019) to amplify arthropod DNA from fecal samples and cloacal swabs primarily collected from nestlings in recently burned forests. Our objectives were to (1) describe the arthropod diet of nestlings of each species within a landscape of pulsed arthropod resources; (2) test for resource partitioning among provisioning woodpeckers by comparing nestling diets among the four related species; and (3) examine whether nestling diet composition differs in newer (≤5 yr postfire) vs. older (>5 yr postfire) burned areas, which likely witness successional changes in arthropod communities. We predicted that woodpecker diets would reflect species-specific differences in behavioral foraging strategies such as excavation (Black-backed Woodpecker, Hairy Woodpecker), bark and foliage gleaning (White-headed Woodpecker), and ground foraging (Northern Flicker). In addition to providing one of the first applications of DNA metabarcoding to study the diets of woodpeckers, our study also provides new insights into the food resources provisioned by two at-risk species (Black-backed Woodpecker, White-headed Woodpecker), which can be used to inform conservation measures.

MATERIALS AND METHODS

Study Species and Sample Collection

Field observations and examinations of stomach contents have indicated that all four woodpecker species in our study primarily feed on arthropods (Beal 1911). Consistent with their specialized habitat associations, previous work suggests that Black-backed Woodpeckers likely have a narrow diet that consists almost entirely of the larvae of wood-boring beetles (e.g., Cerambycidae and Buprestidae), which are abundant in dead and dying conifers, particularly after fire (Villard and Beninger 1993, Murphy and Lehnhausen 1998). In our study region, Hairy and White-headed woodpeckers forage on both dead and live trees, where they likely take advantage of a variety of bark-living insects (Kozma and Kroll 2013, Lorenz et al. 2016). In addition, White-headed Woodpeckers are also known to forage extensively on cut stumps and live foliage in our study region (Lorenz et al. 2016). Past studies have documented that common prey items for these two species include beetle larvae, adult beetles, ants, and caterpillars (Kozma and Kroll 2013, Lorenz et al. 2016). In contrast to the other three woodpeckers, Northern Flickers are thought to forage predominantly on the ground, where ants (Formicidae) and ground beetles (Carabidae) make up important diet items (Beal 1911, Gow et al. 2013).

We collected fecal samples provided by woodpeckers from April to July 2017–2018, in coniferous forests with active fire regimes in central Washington (Yakima county) and Northern California (Plumas, Lassen, and Shasta counties), USA. In Washington, sampling sites were located in mixed ponderosa pine (*Pinus ponderosa*) and Douglas-fir (*Pseudotsuga menziesii*) forests of the Cascade Range. Sampling sites in California were predominantly Sierran mixed conifer forest dominated by *P. ponderosa*, *Pinus jeffreyi*, *Abies concolor*, *Abies magnifica*, *Pseudotsuga menziesii*, and *Calocedrus decurrens*, with some sampling sites located in eastside pine forest composed of *P. ponderosa*, *P. jeffreyi*, *C. decurrens*, and *Juniperus occidentalis*.

The vast majority (96%) of samples came from areas that had been burned, either by prescribed fire (53% of total) or wildfire (43% of total), within the last 13 years (mean = 5 years since fire).

We located woodpecker nests by conducting nest searches in burned areas or by tracking radio-tagged adults to nesting areas, and we monitored nests on a regular basis to predict fledging dates. Between 1 and 5 days before fledging, we accessed nests using the hole-saw method (Ibarzabal and Tremblay 2006), extracted nestlings for standard in-hand processing (e.g., morphometric measurements), and immediately replaced nestlings into the nest cavity when processing was complete. Fresh fecal samples were collected opportunistically if a nestling defecated during the processing time or while being held in a cloth bag. In some cases, nestlings were placed in a shoebox lined with fresh white paper for up to 15 min to increase the chances of obtaining a sample. While our sampling focused on nestling woodpeckers, we also collected fecal samples opportunistically from adult or juvenile woodpeckers during behavioral observation bouts that we conducted as part of a series of separate studies (Stillman et al. 2019b, 2021). These opportunistic samples showed low DNA recovery rates, likely due to a longer time period between sample collection and freezing. In all cases, fecal samples were lifted off the substrate using a sterile cotton-tipped applicator, placed in a DNA-free 1.5-mL microcentrifuge tube, and stored out of direct sunlight (e.g., in a portable cooler with ice packs) until they could be transported out of the field and stored in a −18°C freezer.

In addition, we obtained 15 cloacal swab samples from nestling and adult Black-backed Woodpeckers in Washington to complement diet analysis and, if sequencing success allowed, provide information on the utility of cloacal swab samples for assessing diet. We rubbed sterile cotton-tipped applicators around the cloacal opening, placed the swabs in DNA-free 1.5-mL microcentrifuge tubes containing sterile cell lysis solution (CLS; Lindner and Banik 2009), and otherwise treated them like fecal samples. Both fecal and cloacal swab samples were stored until Spring of 2020, when they were processed at the University of Florida.

DNA Extraction and Sequencing

Each fecal sample was thawed at room temperature for 10 min and homogenized before DNA extraction. Fecal DNA was extracted using the Qiagen Power Soil DNA kit according to the manufacturer's protocol (Qiagen, Germantown, Maryland) for fecal samples. For cloacal swabs preserved in CLS, we extracted DNA according to the modified CLS/ glass milk extraction protocol described in Jusino et al. (2016). We included negative extraction controls for both extraction methods. We then used high-throughput amplicon sequencing to identify the dietary components. We amplified the mitochondrial gene cytochrome oxidase C subunit 1 (COI) using the ANML pair primers (Jusino et al. 2019). Forward and reverse ANML primers were modified for metabarcoding by adding Illumina Nextera v2 adapters (Illumina, San Diego, California) to each primer.

PCR reactions were performed in 15 μL reactions using the following reagents: 7.88 μL of DNA-free molecular grade water, 3 μL of 5X Green GoTaq buffer (Promega, Madison, Wisconsin) for a final concentration of 1x, 3 μL of DNA, 0.3 μL of 10 mM dNTPs (Promega, Madison, Wisconsin) for a final concentration of 0.2 mM for each dNTP, 0.12 μL of 20 mg mL^{-1} BSA (New England BioLabs, Ipswich, Massachusetts), 0.3 μL of 10 mM of each primer for a final concentration of 0.2 μM of each primer, and 0.1 μL of 5uM μL^{-1} GoTaq® DNA Polymerase (Promega, Madison, Wisconsin). This step included our negative extraction controls in addition to traditional PCR negative controls. Thermocycler conditions were set according to Herbert et al. (2003). PCR products were stained with SYBR Green I (Lonza Bioscience, Rockland, Maine) and run in a 2% agarose gel. Successfully amplified samples were then dual-barcoded using an 8 cycle PCR reaction using the Illumina Nextera v2 Kit (Illumina, San Diego, California).

Indexed products were visualized in a 2% agarose gel and combined by 3 based on band intensity. Combined samples were then cleaned and size-selected at ≥200 bp with Zymo Select-A-Size Clean-up kits (Zymo Research, Irvine, California) according to the manufacturer's instructions. Cleaned and size-selected products were quantified using an Invitrogen Qubit 4.0 Fluorometer and Qubit 1X dsDNA HS Assay kit. Then, samples were equilibrated at 3,000 pM and combined. Libraries were sequenced using v3 2 × 300 bp sequencing chemistry on the Illumina MiSeq at the University of California Riverside Institute for Integrative Genome Biology.

Bioinformatics

We bioinformatically processed our high-throughput amplicon sequencing data using AMPtk version 1.4.1 (Palmer et al. 2018; http://amptk.readthedocs.io). We preprocessed our merged, individually barcoded reads using *USEARCH* (version 9.2.64) and *vsearch* (version 2.11.1) and then removed the forward and reverse ANML primers. We discarded any reads shorter than 170 bp. Reads longer than 181 bp were trimmed to 181 bp. Sequence reads were then quality-filtered with expected errors less than 1.0, de-replicated, and denoised using UNOISE3 (Edgar and Flyvbjerg 2015). The resulting amplicon sequence variants were then validated and clustered at 97% to generate operational taxonomic units (OTUs), and sequence reads were mapped back to the resulting OTUs. The OTUs were assigned taxonomy using the hybrid taxonomy algorithm

in AMPtk combined with the built-in COI database (Jusino et al. 2019). Following taxonomy assignment, all non-arthropod OTUs were removed. We manually curated the taxonomies for all OTUs that occurred at least 5 times in our database, as well as the most frequently occurring OTUs for each woodpecker group, by comparing sequences to reference sequences in the Barcode of Life Database (BOLD) and Basic Alignment Search Tool (BLAST). OTU identification criteria for database searches followed appendix B in Trevelline et al. (2016).

We used a single-copy equimolar arthropod mock community (Jusino et al. 2019) to parameterize our bioinformatics approach and to account for observed rates of barcode crossover using the filter module in AMPtk. This spike-in mock community was amplified, indexed, and sequenced alongside our genomic DNA products and serves as a positive community control.

In addition, we repeated these methods for a question-specific mock community that we created for this study consisting of DNA extracted directly from insects following the insect extraction protocol in Jusino et al. (2019). The mock community included 4 beetle species that we expected to find in our diet samples: golden buprestid (*Cypriacis aurulenta*), mountain pine beetle (*Dendroctonus ponderosae*), white-spotted sawyer (*Monochamus scutellatus*), and ribbed pine borer (*Rhagium inquisitor*).

Statistical Analysis

We characterized and compared woodpecker diets using OTU presence–absence data, which reduces biases associated with using sequence read numbers as proxies for prey abundance in fecal samples (Jusino et al. 2019). Prior to analysis, we tested for differences between fecal samples and cloacal samples using permutational multivariate analysis of variance (PERMANOVA) and Wilcoxon tests for per-sample taxonomic richness. Because both sampling methods provided a similar diet snapshot and used the same bioinformatics pipeline, we then pooled data from nestling fecal samples (n = 66) and nestling cloacal swabs (n = 6) for additional analysis. For instances where an individual had successful sequencing for both sample types, we combined presence-absence data to yield one measurement per individual. Sample size constraints prevented formal analysis of the overall utility of cloacal swab samples as a complement to fecal samples, which we recommend as a topic for future study.

To compare nestling diet richness and diversity between species, we produced rarefaction curves using the R package *iNEXT* (Hsieh et al. 2020). We generated interpolated estimates of diet richness, Shannon diversity (exponentiated Shannon entropy), and Simpson diversity (inverse Simpson concentration), and we used 95% confidence bands around estimates to test for differences between diets (Chao et al. 2014). Taxonomic richness estimates for each species group were rarefied to n = 10 to facilitate comparisons. We also compared the taxonomic richness of nestling diet items per sample at the order and OTU levels using 2 approaches. First, we tested for differences between species using generalized linear models with Poisson error in R version 4.0.3 (function glm(); R Core Team 2020). We omitted Hairy Woodpeckers due to low sample size and split Black-backed Woodpeckers between Washington and California, yielding 4 species groups in total (all other focal species were only sampled in Washington). These models used taxonomic richness (i.e., either the number of OTUs or the number of orders) as the response variable and species group as the independent variable. To generate pairwise comparisons for each combination of species, we ran the models four times and assigned a different species to the intercept each time. This approach allowed us to statistically compare each species to the remaining species using estimated contrasts at a significance level of $\alpha = 0.05$. Second, we tested for differences in diet taxonomic richness between recently burned areas (≤5 yr postfire) and older burned areas (>5 yr postfire) using a Wilcoxon test for each species. Although relatively little is known about the temporal dynamics of prey species in our study region, postfire snag attrition peaks around 5 years after fire and likely leads to shifts in prey availability (Grayson 2019). For example, we expected that woodboring beetle abundance would increase immediately following fire but decrease 5–10 years postfire as snags decay. For Black-backed Woodpeckers and Northern Flicker nestlings, we pooled data into a binary index representing whether the sample came from an area ≤5 yr or >5 yr postfire to allow enough sampling variation for within-species comparisons. We omitted White-headed and Hairy woodpeckers from this analysis due to an inadequate sample size.

Although the true, total diets of nestmates likely have some measure of dependency due to shared parents, our samples represent single diet snapshots with substantial variation between nestmates. Therefore, we elected not to pool samples from the same nest when comparing rarefied estimates between species. Moreover, pooling nestmate samples in our study runs the risk of introducing bias when comparing richness and diversity due to species-specific differences in clutch size. In our dataset, Northern Flickers tended to have 3 times as many samples per nest compared to other species (mean samples per Northern Flicker nest = 4.0, all other species = 1.3–1.8). To explicitly test for potential non-independence among nestmates, we calculated the order-level dissimilarity matrix for all sample pairs within each species, decomposed to represent two dissimilarity vectors: beta diversity between nestmates and beta diversity between non-nestmates. We then compared these diversity measures using Wilcox rank-sum tests.

We summarized the diet composition for each woodpecker group using two descriptive metrics: frequency of

occurrence and the weighted proportion of occurrence, following equations from Deagle et al. (2019). The weighted proportion of occurrence represents a relative expression of a diet item's contribution to the whole, where each item is weighted in proportion to the number of OTUs in a sample (e.g., less weight to OTUs in a mixed meal). To test for resource partitioning in diet composition, we compared the diets of nestling woodpeckers using PERMANOVA at the OTU level (Anderson 2001), implemented using the *adonis* function from the *vegan* package (Oksanen et al. 2019) version 2.5-7 in R version 4.0.3 (R Core Team 2020). We pooled samples by species, state, and sampling site (i.e., burned area) and included a categorical effect for each species-state combination, a binary variable for time since fire using a threshold of ≤5 yr postfire, and the interaction between both terms. In addition, we used the R package *pairwiseAdonis* to test for differences between each pair of species-state groupings. We investigated interspecific differences in multivariate dispersion between species groups, a metric of diet variability, using the *betadisper* function in the *vegan* package (Anderson 2006, Oksanen et al. 2019). Last, we visualized dietary niche space using non-metric multidimensional scaling (NMDS). All comparisons used the modified Raup-Crick dissimilarity index (method "raup" in *vegan*; Chase et al. 2011). We repeated this analysis using read abundances and the Bray-Curtis dissimilarity index (method "jaccard") and confirmed that results did not change (Supplementary Material Table S1).

RESULTS

We successfully recovered arthropod DNA from 66 out of 91 nestling fecal samples and 2 out of 15 adult fecal samples. We also successfully sequenced DNA from 10 out of 15 cloacal swab samples, representing 6 nestling and 4 adult Blacked-backed Woodpeckers. Illumina sequencing generated 4.2 million COI sequences, which reduced to 4 million reads after quality filtering and clustered into 668 unique OTUs assigned to the phylum Arthropoda. Of these, 662 OTUs were assigned to a taxonomic order, 197 OTUs were assigned to a family, and 131 OTUs were identified at the species level. Of the 6 adult woodpecker samples with successful sequencing, 4 cloacal swabs came from Black-backed Woodpeckers in Washington, and 1 fecal sample came each from a Hairy Woodpecker (Washington) and Black-backed Woodpecker (California). To avoid biased conclusions based on only a single sample, we only included adult Black-backed Woodpeckers from Washington in our subsequent analysis. Due to low sequencing success for nestling Hairy Woodpecker samples, we omitted this species from statistical comparisons and present descriptive information only.

Per-sample taxonomic richness values for nestling Black-backed Woodpeckers from Washington were not statistically different between swab samples (n = 6) and fecal samples (n = 10) at the OTU level (difference in medians = 0.5, Wilcox test: W = 30.0, P > 0.99) or order level (difference in medians = 1.5, Wilcox test: W = 43.5, P = 0.15). PERMANOVA results indicated slight differences in community composition based on sampling method, which explained 27–30% of variation at both taxonomic levels (order-level F = 5.3, P = 0.05; OTU-level F = 6.0, P = 0.02). Two Washington Black-backed Woodpecker nestlings yielded both a fecal and cloacal sample with successful sequencing, and we combined results from these samples to aid diet characterization. Pairwise comparisons between nestmates (n = 64 comparisons) and non-nestmates (n = 507 comparisons) showed similar amounts of dissimilarity in Hairy Woodpeckers, White-headed Woodpeckers, and California Black-backed Woodpeckers (Wilcox rank-sum tests: W = 4.0–227.5, P = 0.14 – 0.91), with somewhat greater similarity between nestmates in Northern Flickers (difference in mean dissimilarity = 0.02) although this difference was not statistically significant (Wilcox rank-sum tests: W = 4408.5, P = 0.08). In general, nestmates had limited diet overlap (e.g., dissimilarity > 0.2). Dissimilarity values for Washington Black-backed Woodpeckers were lower in nestmates vs. non-nestmates (Wilcox rank-sum test: W = 181.0, P = 0.005), largely due to a single nest with four samples that all contained DNA from the order Siphonaptera (e.g., fleas), despite the absence of this diet item from all but one other sample.

For our spike-in controls, we were able to recover and identify all members of the single-copy equimolar arthropod mock community, which clustered into 32 OTUs with 268–7145 reads per OTU (mean = 1652). This mock community included two members with known sequence variants that clustered with the originating sequence (Jusino et al. 2019). Our question-specific mock community clustered into 21 OTUs and successfully recovered the four beetle species that we included. There was variation in read counts among these four taxa of interest, likely reflecting PCR bias in read abundance: *C. aurulenta* (19,203), *D. ponderosae* (8,577), *M. scutellatus* (1,212 reads), and *R. inquisitor* (14,649). This mock community also generated 1 additional OTU identified as *R. inquisitor* with only 62 reads.

Taxonomic Richness and Diversity

Dietary richness estimates were substantially higher for nestling Northern Flickers compared to other focal species—raw OTU richness was over three times greater than other woodpecker species in our study (Table 1). Nestling Black-backed Woodpeckers had significantly lower richness estimates in Washington compared to

TABLE 1. Number of sequenced samples, raw diet richness, and rarefied diet richness estimates for four woodpecker species in Washington and/or California, USA. Taxonomic richness estimates for each species group were rarefied to $n = 10$ to facilitate comparisons. Rarefied numbers give the mean estimate along with 95% confidence intervals in parentheses

		OTU level		Order level	
Species group	Samples	Raw richness	Interpolated ($n = 10$)	Raw richness	Interpolated ($n = 10$)
Nestlings					
Black-backed Woodpecker (California)	18	126	81.6 (72.6, 90.6)	16	13.5 (11.2, 15.8)
Black-backed Woodpecker (Washington)	14	74	57.7 (48.8, 66.5)	10	9.1 (7.1, 11.0)
Hairy Woodpecker (Washington)	4	43	–	10	–
Northern Flicker (Washington)	24	396	207.4 (194.5, 220.3)	29	20.3 (16.5, 24.1)
White-headed Woodpecker (Washington)	10	82	82 (71.1, 92.9)	12	12.0 (8.8, 15.2)
Adults					
Black-backed Woodpecker (Washington)	4	41	–	9	–

California at both taxonomic levels, and Washington Black-backed Woodpecker samples consistently had lower richness estimates than the other three species (Table 1, Figure 1). Rarefied richness estimates were higher for adult Black-backed Woodpeckers compared to nestlings in Washington, but adults were similar to nestlings in California (Table 1).

Shannon and Simpson diversity values showed a similar pattern to dietary richness when compared between nestlings from different species groups. Diversity was significantly higher for Northern Flickers compared to Black-backed and White-headed woodpeckers. Diversity was lowest for Black-backed Woodpeckers from Washington and confidence bands did not overlap with rarefied estimates for California nestlings at the OTU level. Samples from Black-backed Woodpecker adults often had higher diversity compared to nestlings in Washington, although differences were not significant (Supplementary Material Tables S2 and S3).

Individual samples contained a median of 12 OTUs (range: 1–90) and a median of 5 orders (range: 1–17). Species identity was a strong predictor of per-sample taxonomic richness at both levels and significantly reduced model deviance compared to a null model (analysis of deviance: $P < 0.001$). Nestling Black-backed Woodpecker samples from Washington contained fewer OTUs on average compared to those from California or other species (generalized linear model: coefficient estimates ± standard deviation [SD] for species contrasts ranged from $\beta = 0.32 \pm 0.14$ to $\beta = 1.28 \pm 0.10$, $P < 0.02$), and Northern Flicker samples contained significantly more OTUs per sample than those from other species groups (generalized linear model: coefficient estimates ± SD for species contrasts ranged from $\beta = -0.96 \pm 0.10$ to $\beta = -1.28 \pm 0.10$, $P < 0.001$; Figure 2). There were no meaningful differences between per-sample order richness for Black-backed and White-headed woodpeckers, but Northern Flicker samples contained more orders than other woodpeckers (generalized linear model: coefficient estimates ± SD for species contrasts ranged from $\beta = -0.64 \pm 0.14$ to $\beta = -0.82 \pm 0.16$, $P < 0.001$; Figure 2). Black-backed Woodpecker samples from California had

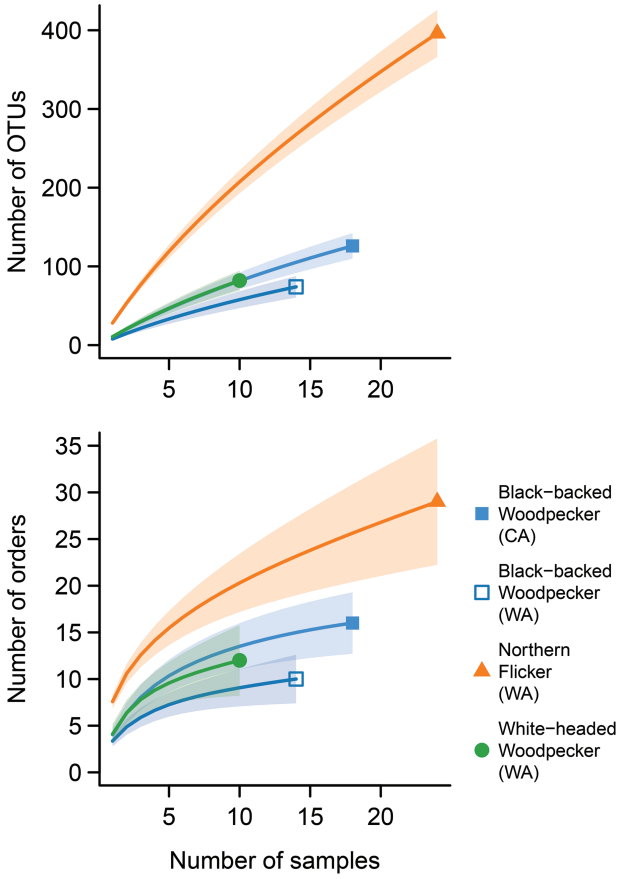

FIGURE 1. Diets of nestling woodpeckers showed high taxonomic richness, particularly for Northern Flickers. Points indicate observed diet richness at the true sample size for each species, and shaded regions show 95% confidence intervals. Samples were analyzed separately, where applicable, depending on study region (California vs. Washington).

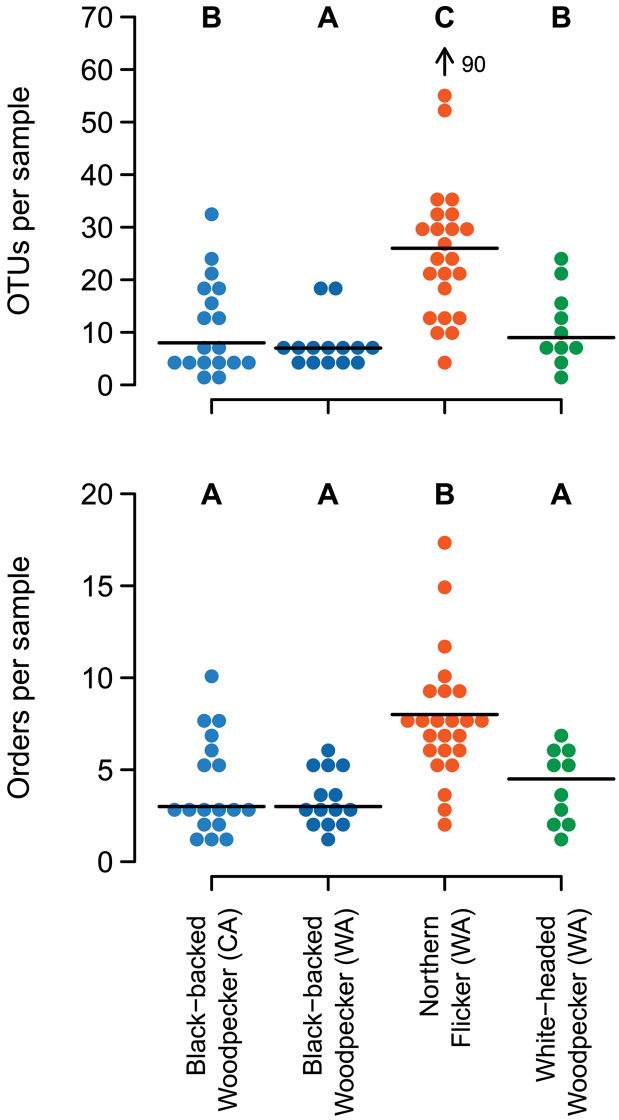

FIGURE 2. Northern Flicker nestlings had greater taxonomic richness per sample compared to Black-backed and White-headed woodpeckers. Each point represents a nestling, black lines show the median value, and the arrow (top) gives the value of an outlier. Letters represent groupings that are significantly different from each other ($P < 0.05$) based on a generalized linear model. Samples were analyzed separately, where applicable, depending on study region (California vs. Washington).

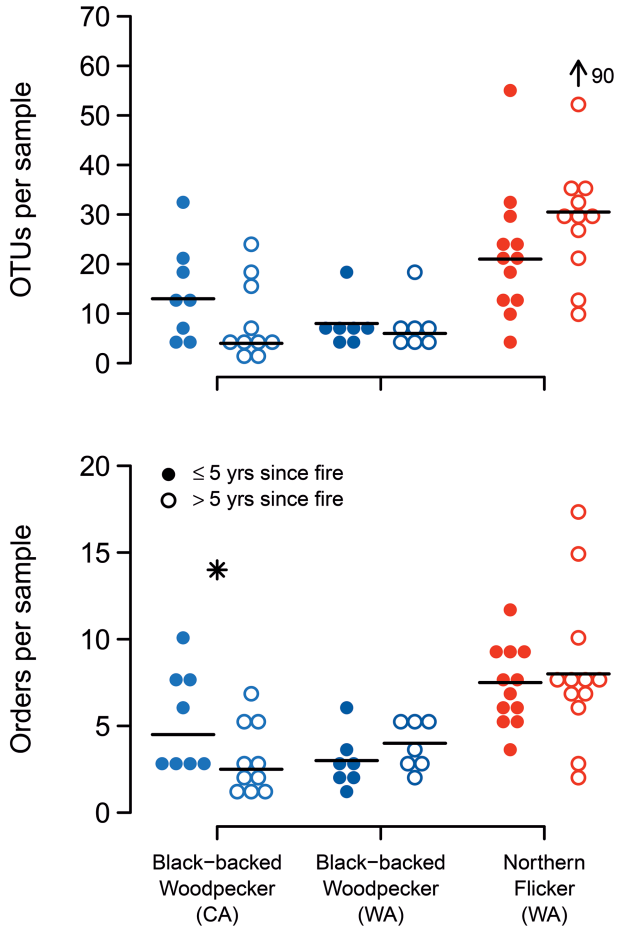

FIGURE 3. Nestling diets showed varied responses to time since fire at the nest site. Each point represents the per-sample taxonomic richness for an individual, black lines show the median value, and the arrow (top) gives the value of an outlier. Pairs that are significantly different from each other ($P < 0.05$) are marked with an asterisk.

significantly greater order richness in burned areas ≤ 5 yr postfire compared to areas > 5 yr after fire (Figure 3; Supplementary Material Table S4). Pairwise comparisons for nestling Northern Flickers and Washington Black-backed Woodpeckers did not detect meaningful differences in per-sample richness based on time since fire (Figure 3; Supplementary Material Table S4).

Diet Composition

Overall, 32 orders and 68 families were detected across woodpecker samples. Coleoptera was the most frequently detected order for Black-backed, White-headed, and Hairy woodpeckers, occurring in 80–100% of samples (Table 2) and demonstrating the highest weighted percent occurrence for those species (Supplementary Material Table S5). The most frequently detected OTUs for nestlings of these species were identified to the family Buprestidae, and in California Black-backed Woodpeckers, taxonomy of the top two OTUs was assigned to *Buprestis lyrate* and *Buprestis laeviventris* (Supplementary Material Table S6). For the subset of samples with OTUs matched at the family level, the Coleoptera families with the highest incidence for Black-backed Woodpeckers (n = 36) were Cerambycidae (69.4%), Buprestidae (52.8%), and Elateridae (22.2%). The most common Coleoptera families for White-headed Woodpeckers (n = 9) were Elateridae (55.6%) and Buprestidae (44.4%), and all 3 Hairy Woodpecker samples with family-level taxonomy contained Buprestidae. We detected DNA from bark

TABLE 2. Frequency of occurrence (%) of prey orders identified in the diets of 4 focal woodpecker species, presented separately by study region (California vs. Washington) and/or age (nestling vs. adult). Data are shown for the 15 orders with highest overall frequency

	Nestling					Adult
	Black-backed Woodpecker		Hairy Woodpecker	Northern Flicker	White-headed Woodpecker	Black-backed Woodpecker
	(CA)	(WA)	(WA)	(WA)	(WA)	(WA)
Order	(*n* = 18)	(*n* = 14)	(*n* = 4)	(*n* = 24)	(*n* = 10)	(*n* = 4)
Coleoptera	94.4	92.9	100	62.5	80	100
Diptera	38.9	78.6	50	95.8	50	75
Araneae	38.9	35.7	75	83.3	40	100
Hymenoptera	50	21.4	25	95.8	50	50
Lepidoptera	44.4	35.7	50	83.3	30	50
Hemiptera	11.1	0	25	66.7	70	25
Entomobryomorpha	16.7	7.1	0	29.2	10	25
Sarcoptiformes	0	0	0	50	0	0
Trichoptera	22.2	7.1	0	20.8	20	0
Diplostraca	11.1	0	0	29.2	0	0
Isopoda	16.7	14.3	0	4.2	30	0
Ephemeroptera	0	0	0	25	0	25
Trombidiformes	5.6	7.1	0	20.8	0	0
Blattodea	27.8	0	0	4.2	0	0
Siphonaptera	0	35.7	25	0	0	0

beetles (*Dendroctonus ponderosae* and *Dendroctonus valens*) in only 2 samples: 1 Hairy Woodpecker and 1 nestling Black-backed Woodpecker in Washington. However, wood-boring beetles—here represented by Cerambycidae, Buprestidae, and Zopheridae (*Phellopsis porcata*)—occurred in the majority of samples for Black-backed, Hairy, and White-headed woodpeckers. The frequency of wood-boring beetle DNA in Black-backed Woodpecker nestling samples decreased slightly from 95% in areas ≤ 5 yr after fire to 82.4% in areas > 5 yr postfire. This trend was largely driven by changes in the frequency of Cerambycidae, which decreased from 90.0% to 47.1%, and Buprestidae, which increased from 35.0% to 76.5% in newer vs. older burned forests. Supplementary Material Table S6 summarizes the most frequent OTUs for each woodpecker species.

Prey items in the Order Diptera were also frequently consumed by nestlings, particularly Northern Flickers (Table 2). Northern Flicker samples showed high family-level diversity within this order—4 families occurred in 2 out of 23 samples (Bibionidae, Ceratopogonidae, Muscidae, Tabanidae), while the rest occurred in only 1 sample each. The Order Hymenoptera had the highest weighted proportion of occurrence for Northern Flickers (Supplementary Material Table S5), and 82.6% of Northern Flicker samples contained DNA from ants in the family Formicidae. Compared to other species, White-headed Woodpeckers provisioned a relatively high proportion of Hemipterans, dominated by aphids in the family Aphidae (55.6%).

The taxonomic composition of OTU-level nestling diets (pooled by species and burn site) varied significantly between species groupings (*pseudo-F* = 5.0, R^2 = 0.47, *P* = 0.001). We did not detect differences in multivariate dispersion between species groups (*F* = 0.34, *P* = 0.795). Pairwise comparisons between species-state groupings revealed that this variation was partially driven by differences in the diet composition of Northern Flicker nestlings compared to the other study species. California Black-backed Woodpeckers also differed from all other species groups, including Black-backed Woodpecker nestlings in Washington (*pseudo-F* = 3.2, R^2 = 0.30, *P* = 0.041). Accounting for differences between species, we did not find an effect of time since fire on diet composition (*pseudo-F* = 1.2, R^2 = 0.04, *P*= 0.380). NMDS ordination generated a stable 2-dimensional solution (stress = 0.149) and demonstrated divergence in diet composition between nestling Northern Flickers and other species groups (Figure 4).

DISCUSSION

Woodpeckers are frequently used as indicator species in forest ecosystems, and our study provides one of the first applications of DNA metabarcoding to build a more complete picture of woodpecker diets. While our results show the importance of several deadwood-associated insect taxa (e.g., woodboring beetles) as prey, our findings also highlight the considerable diversity of diet items in 4 focal species. This information provides a framework for

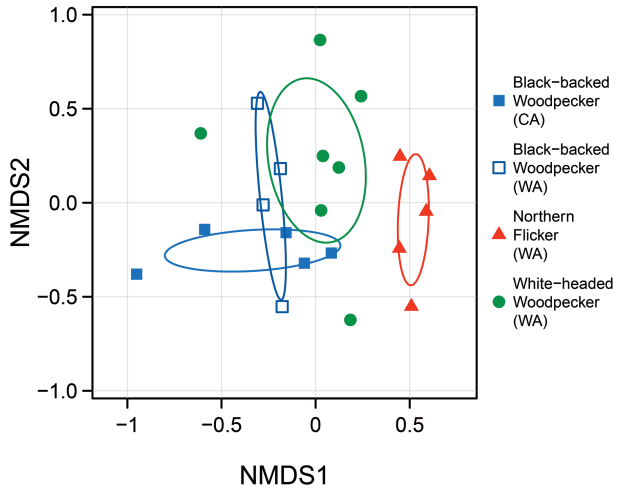

FIGURE 4. NMDS ordination of nestling diet composition shows limited overlap between Northern Flickers and the remaining three focal groups (stress = 0.149). Points represent diet composition based on OTUs, pooled for each species and sampling site (i.e., burned area) combination. Ovals indicate 95% confidence intervals around the spatial median for each species and state. Samples from Black-backed Woodpeckers were split between two study regions.

interpreting the habitat relationships, foraging behaviors, and resource requirements for woodpeckers in disturbed forests of the western U.S. For example, differences in Black-backed Woodpecker occupancy rates between burned vs. beetle-killed forests may reflect access to preferred prey during the breeding season (Tingley et al. 2020).

Our DNA metabarcoding approach revealed the presence of over 600 OTUs representing 32 arthropod orders in the diets of 4 woodpecker species. Nestling woodpecker diets had greater taxonomic richness and diversity than previous studies (which have relied on observational methods) would suggest. For example, Northern Flickers are sometimes considered ant specialists, and previous diet analysis using fecal sample dissections suggests that over 99% of Northern Flicker diets are made up of ants (Gow et al. 2013). While we found a similarly high frequency of occurrence for the order Hymenoptera (Table 2), our results demonstrate significantly higher diet richness and diversity in nestling Northern Flickers compared to other focal species. Along with ants, fecal samples from nestling Northern Flickers at our study sites frequently contained arthropods in the orders Diptera (flies), Araneae (spiders), Lepidoptera (butterflies and moths), and Coleoptera (beetles). Diet characterization using weighted proportion of occurrence suggested that, rather than simply supplementing an ant-based diet, these prey items make up a large component of Northern Flicker provisioning to nestlings (Supplementary Material Table S5). Moreover, we found higher diet diversity in Black-backed Woodpeckers than expected based on previous studies which have emphasized the importance of wood-boring beetle larvae in the diet of this species (Villard and Beninger 1993, Murphy and Lehnhausen 1998). While our study confirms this strong association with wood-boring beetles, we were surprised by the relatively high occurrence of additional prey items in samples from nestlings (Table 2). Although Black-backed Woodpeckers primarily forage by excavating into dead wood, our findings provide evidence that this species likely deviates from its typical foraging strategy to glean prey from bark, catch prey on the ground, or catch flies on the wing. Indeed, our diet diversity estimates among all four focal species suggest that woodpecker foraging behaviors are marked by a degree of foraging plasticity and opportunism (Lorenz et al. 2016).

Although sample size constraints prevented a formal analysis comparing molecular results from cloacal swab and fecal samples, our limited data show considerable overlap in diet characterization results from the two sampling techniques. In the field, cloacal swabs are often quicker to obtain compared to fecal samples, allowing a higher sampling success rate under permitting constraints that limit handling time for birds. Despite potential benefits in the field, however, the cloacal swabs in our study likely contained lower DNA concentrations than fresh fecal samples, which could make them more prone to contamination. Moreover, our fecal samples were stored frozen while cloacal swabs were stored in buffer, leading to potential differences in DNA preservation between the two techniques. These observations suggest that further research comparing field sampling techniques is warranted and timely given the need to understand avian resource requirements in the face of climate- and human-induced changes in arthropod prey populations (Montgomery et al. 2020).

The period of the breeding season when birds are actively provisioning altricial young is marked by high energetic demands with critical impacts on nestling survival and juvenile recruitment (Bortolotti et al. 2011, Santos and Nakagawa 2012). In woodpeckers, nestling diets may show responses to habitat type (e.g., burned vs. unburned forest; Tremblay et al. 2016), tree species composition, and even seasonal weather fluctuations (Lorenz et al. 2020). However, nestling diets could also be somewhat buffered from species-specific environmental effects due to prey selection by provisioning parents. For instance, Black-backed Woodpecker provisioning rates and the size of prey deliveries tend to increase as nestlings grow older (Loverin et al. 2021). Western Bluebirds (*Sialia mexicana*) show high overlap

in diet items between nestlings and adults (Jedlicka et al. 2017), but diet studies from marine birds demonstrate differences between prey selected for provisioning vs. prey captured for self-feeding (Ydenberg 1994, Davoren and Burger 1999, Alonso et al. 2012). In cases where adult birds exhibit strong prey selection when provisioning young, this raises the possibility that diets may show greater interspecific convergence among nestlings compared to adults. In burned forests, for example, different woodpecker species may take advantage of pulsed arthropod food sources in similar ways when selecting prey for nestlings but exhibit greater separation between species during the nonbreeding season, particularly if prey scarcity in the winter leads to more interspecific competition. Although sample size constraints affected our ability to test for age-specific diets, future work using DNA metabarcoding may provide insights into the potential similarities and differences between adult and nestling diets in terrestrial birds.

The advent of DNA metabarcoding has greatly enhanced our ability to investigate avian diets by reducing bias toward more conspicuous or less degradable prey (Pompanon et al. 2012, Hoenig et al. 2021). In our study, one outcome of this increased resolution was the unexpected prevalence of relatively small arthropods in nestling diets. For example, we frequently detected Diptera DNA in samples from all 4 woodpecker species, including over 95% of Northern Flicker samples. Although flycatching behavior seems to be uncommon in woodpeckers (Raphael and White 1984), our results suggest that these species may supplement their diets with aerial insects—or their larvae—relatively frequently. Another example is the high prevalence of aphids in the diet of White-headed Woodpeckers, which are known to forage on conifer cones or clusters of needles (Lorenz et al. 2016). Aphids identified to the genus *Cinara* (giant conifer aphids) occurred in 30% of White-headed Woodpecker samples and included one of the most common OTUs for this woodpecker species (Supplementary Material Table S6). Moreover, the importance of small diet items in nestling diets raises intriguing questions about provisioning behavior in the context of central-place foraging (Houston 1985). While adults often carry larger prey items (e.g., wood-boring beetle larvae) individually to nestlings (Loverin et al. 2021), adult woodpeckers foraging on smaller prey likely need to gather multiple items in a single foraging trip. The energetic balance of these provisioning behaviors could exert considerable influence over foraging patch selection and food delivery rates (Houston 1985).

Diet richness and composition remained similar between newer burns ≤5 yr and older burns >5 yr postfire. Fire-killed trees gradually decay and fall as time since fire increases (Grayson et al. 2019), likely leading to declines in deadwood-associated arthropods that are important diet items for Hairy, White-headed, and Black-backed woodpeckers (Nappi et al. 2010). Despite potential changes in prey availability over time, these three woodpecker species still displayed similar nestling diets in newer and older burns. Rather than switching to alternative prey items, bark- and trunk-foraging woodpeckers may instead demonstrate numerical responses to time since fire that track resource availability. For example, postfire population trajectories tracking the pulse and decline of postfire resources have already been documented in Hairy and Black-backed woodpeckers (Saab et al. 2007, Tingley et al. 2018). In comparison to these species, Northern Flicker nestlings in our study had high diet diversity, and samples showed a low prevalence of deadwood-associated beetles (Table 2). It is possible that high diet diversity, combined with a ground-based foraging strategy in open areas, may allow Northern Flickers to maintain stable or increasing populations in the decade following forest fire (Wiebe 2014). In western Idaho, for example, Saab et al. (2007) found that Northern Flicker nest densities increased between 1 and 10 years postfire.

While our results support the hypothesis that Northern Flickers occupy a distinct dietary niche in postfire landscapes compared to other focal species, we observed more overlap in diet richness and diversity than expected among Hairy, Black-backed, and White-headed woodpeckers. Under the resource partitioning hypothesis, we predicted that these three sympatric species would demonstrate divergence in diet characteristics in response to interspecific competition for food resources (Schoener 1974). However, the diets of species with different foraging strategies have been shown to converge in times of high food supply, such as abundant ants for overwintering migrants (Kent and Sherry 2020), or seasonally emergent aquatic insects in riparian habitats (Trevelline et al. 2018). Similarly, 3 sympatric hummingbird species in California demonstrate broadly overlapping diets with limited evidence for dietary niche partitioning (Spence et al. 2021). Our samples primarily came from burned forests in Washington and California, where abundant fire-killed trees produce a resource pulse of deadwood-associated insects (Costello et al. 2011, Ray et al. 2019). Thus, our observed diet similarities between Black-backed, White-headed, and Hairy woodpeckers (which usually exhibit different foraging strategies) may represent a case of diet convergence during opportunistic foraging in the presence of a pulsed food source. If this is the case, sympatric woodpecker species should show greater diet divergence in unburned forest compared to burned forest, although this prediction has not been tested. Ecological disturbances such as forest fire create complex landscapes of resource availability with cascading effects on the community structure of

sympatric consumers (He et al. 2019). Our results highlight the utility of molecular tools for characterizing avian diets and testing predictions about resource partitioning among insectivores living in this dynamic landscape.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Ornithology* online.

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Author contributions: T.J.L., M.A.J., and A.N.S conceived the ideas and designed the methodology; A.N.S. and T.J.L. collected the data; M.V.C. and M.A.J. completed molecular analysis; A.N.S and M.W.T analyzed the data and led manuscript preparation. All the authors contributed critically to the drafts and gave final approval for publication.

Data availability: Statistical analyses reported in this article can be reproduced using the datasets provided by Stillman et al. (2022). All metabarcoding data associated with this work are available at the NCBI Sequence Read Archive (accession number PRJNA735755).

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