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Research Article

Syntopic diet divergence inferred from metabarcoding in a pair of parulid warblers: *Setophaga virens* (Black-throated Green Warbler) and *Seiurus aurocapilla* (Ovenbird)

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ABSTRACT

The role of diet in driving ecological differences across the radiation of parulid wood warblers has been a topic of substantial debate. The inferences made in Robert MacArthur's original study of their niche differences relied heavily on observations from microhabitat partitioning. How these different foraging behaviors translated to distinct diets and, more specifically, individual prey items has been less clear. Here, we use fecal metabarcoding of 2 syntopic insectivorous warblers—*Setophaga virens* (black-throated Green Warbler) and *Seiurus aurocapilla* (Ovenbird)—to complement and expand previous work, and we address past limitations by densely sampling many birds at the same location over a short period of time. We found highly significant differences in diet composition using several multivariate measures of diversity. In an analysis of individual diet proportions of insect orders, *S. aurocapilla* consumed more beetles and flies (Coleoptera and Diptera), whereas *S. virens* consumed more "true bugs" (Hemiptera) and stoneflies (Plecoptera). At the arthropod species level, we found that both warblers readily consumed invasive spongy moths (*Lymantria dispar*), and we identified 9 other arthropod species that significantly differed between the warblers. Of those, for 3 spider taxa, we combined warbler diet information with observations from arthropod collections and showed that spiders, which were more likely to be encountered on the ground, were exclusively eaten by *S. aurocapilla* whereas those encountered in the canopy were more likely to be consumed by *S. virens*, fitting with the expected vertical foraging stratification of the warblers. We interpret these diet differences as likely due to these 2 warbler species "opportunistically" encountering different arthropod assemblages in distinct foraging strata as opposed to "preferentially" consuming different prey. Our research emphasizes the benefits of extending analyses to more distantly related taxa—beyond those considered by MacArthur—and suggests a need for similar fine-scale studies within genera to enhance our understanding of dietary dynamics.

Keywords: diet, ecological competition, metabarcoding, molecular ecology, warblers

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LAY SUMMARY

- We asked why two common forest warblers—*Setophaga virens* (Black-throated Green Warbler) and *Seiurus aurocapilla* (Ovenbird)—eat different prey: by choice, or because they encounter different insects when feeding at different heights.
- Using fecal metabarcoding, we found that these warblers eat distinct insects, though this was not entirely surprising given the species are at the ends of the phylogenetic and ecological spectrum of warblers. Ovenbirds consumed more beetles and flies, while Black-throated Green Warblers ate more true bugs and stoneflies.
- What this analysis allowed, however, was to characterize what specific diet items—at the arthropod species level—that differed between the warbler species.
- For a subset of spider species where we also had collection records, ground spiders were more often eaten by Ovenbirds; canopy spiders by Black-throated Green Warblers (i.e., an intuitive match between predator and prey life histories). These results support Robert MacArthur's idea that warbler diet differences reflect foraging location rather than strong prey preferences.

Divergencia sintópica en la dieta inferida mediante meta-codificación de barras en dos reinitas parúlidas: *Setophaga virens* y *Seiurus aurocapilla*

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RESUMEN

El rol de la dieta en la generación de diferencias ecológicas a lo largo de la radiación de las reinitas parúlidas ha sido objeto de un debate considerable. Las inferencias realizadas en el estudio original de Robert MacArthur sobre las diferencias de nicho se basaron en gran medida en observaciones de partición del micro-hábitat. Cómo estos distintos comportamientos de forrajeo se traducen en dietas diferentes y, más específicamente, en distintas presas individuales, continúa estando menos claro. Aquí utilizamos meta-codificación de barras de materia fecal de dos reinitas insectívoras sintópicas—***Setophaga virens*** y ***Seiurus aurocapilla***—para complementar y ampliar trabajos previos, y abordamos limitaciones anteriores mediante un muestreo intensivo de muchas aves en un mismo sitio durante un corto período de tiempo. Encontramos diferencias altamente significativas en la composición de la dieta utilizando varias medidas multivariadas de diversidad. En un análisis de las proporciones individuales de órdenes de insectos en la dieta, ***S. aurocapilla*** consumió más escarabajos y moscas (Coleoptera y Diptera), mientras que ***S. virens*** consumió más "chinches verdaderas" (Hemiptera) y plecópteros (Plecoptera). A nivel de especie de artrópodo, encontramos que ambas reinitas consumieron con frecuencia la polilla ***Lymantria dispar***, e identificamos otras nueve especies de artrópodos que difirieron significativamente entre las reinitas. De ellas, para tres taxones de arañas, combinamos la información de la dieta de las reinitas con observaciones de colecciones de artrópodos y demostramos que las arañas más propensas a encontrarse en el suelo fueron consumidas exclusivamente por ***S. aurocapilla***, mientras que las encontradas por encima del suelo fueron más probablemente consumidas por ***S. virens***, lo que concuerda con la estratificación vertical de forrajeo esperada para estas reinitas. Interpretamos estas diferencias en la dieta como probablemente debidas a que estas dos especies de reinitas encuentran de manera "oportunista" distintos conjuntos de artrópodos en diferentes estratos de forrajeo, más que a una "preferencia" por diferentes presas. Nuestra investigación enfatiza los beneficios de extender los análisis a taxones más distantemente relacionados—más allá de los considerados por MacArthur—y sugiere la necesidad de realizar estudios similares a escala fina dentro de los géneros para mejorar nuestra comprensión de las dinámicas de la dieta.

Palabras clave: competencia ecológica, dieta, ecología molecular, meta-codificación de barras, reinitas

INTRODUCTION

Classically studied adaptive radiations are characterized by species that show strong ecological differentiation driven by natural selection (Schluter 2000). Studies of *Geospiza* finches, in particular, have focused on morphological traits linked to fitness, like bill size and shape, that diverged in response to variation in diet items, such as seeds varying in their size and hardness (e.g., Grant and Grant 2006). By examining various morphological traits and their association with survival-linked diet items, these studies have provided valuable insights into the mechanisms underpinning the remarkable diversification of these groups.

By contrast, the role of diet differences in driving other radiations, like the wood warblers (family: Parulidae) of North, Central, and South America, has been a topic of more substantial debate (MacArthur 1958, Kaspari 2008). This is because the most conspicuous phenotypic differences across the parulid family are not ecomorphological, but in visual and vocal signals, which conventional wisdom suggests results from divergent sexual selection (Price et al. 2000). Historically, this prompted debate over the ecological "equivalence" of warblers, and in part motivated the seminal study of niche partitioning of 5 syntopic species (i.e., in the same habitat at the same time) by MacArthur (1958). His initial motivation stemmed from the notion that any distinctions in the species' foraging behaviors were "quite obscure" (MacArthur 1958), but subtle specializations translated into important diet differences. While natural historians have described consistent habitat differences among parulid warblers, how resource partitioning has been associated with their divergence has not been clear as in other avian radiations (Kent and Sherry 2020, Price et al. 2000, Rosamond et al. 2020).

MacArthur's original study of diet differences relied on observations of micro-habitat partitioning, specifically by quantifying the time individuals of each species spent in different "feeding zones" of coniferous trees (MacArthur 1958). For example, he found that *Setophaga virens* (Black-throated Green Warbler) spent more time at the outer tops of trees compared to *S. coronata* (Yellow-rumped Warbler). Combined with qualitative assessments of stomach contents from these species, such foraging differences were equated by MacArthur as reflecting diet differences and, subsequently, niche partitioning that facilitated the various warbler species' coexistence: "there is every reason to believe that the birds behave in such a way as to be exposed to different kinds of food" (MacArthur 1958).

Miller et al. (2025) recently tried to more rigorously address some of the shortcomings of MacArthur's original study, in part by directly characterizing the insect diet of co-occurring warblers using cytochrome-c oxidase subunit I (COI) metabarcoding of fecal samples. While there were quantifiable and significant differences among some of the warbler species, Miller et al. (2025) found overall high diet overlap among a set of 13 warbler species (including 3 of the 5 originally studied by MacArthur). Moreover, while morphology and foraging ecology showed evidence of competition-driven divergence over evolutionary time among the species—using phylogenetic canonical correspondence analysis—arthropod diet did not, and instead showed a weak signal of phylogenetic conservation.

That said, there were some warbler species that showed significantly more divergence in their diet than others. This includes *Seiurus aurocapilla* (Ovenbird), which has a natural history, morphology, and ecology that is highly distinct from other warblers ("Ovenbirds" get their name from their covered nest, where the dome and side entrance make it resemble a Dutch oven). *Seiurus aurocapilla* are also the most phylogenetically distinct among parulids, with previous authorities debating whether they should or should not be included within Parulidae, though modern phylogenies based on a range of genetic data support their inclusion (Lovette et al. 2010; Bennett et al. 2025).

While not a part of MacArthur's original studied species, this high level of diet and ecological divergence compared to other parulid warblers facilitates a more direct test of a central tenet of MacArthur's conclusions that could not be addressed by Miller et al. (2025): for those warbler species that do differ in their diets, is this because they (1) preferentially choose to feed on different prey items that occur in multiple potential foraging strata (the "preference" hypothesis), or is it more akin to MacArthur's original inference, that (2) different microhabitat foraging niches bring these different warbler species into contact with different prey items (an "opportunistic" hypothesis). These alternatives can only be addressed by knowing something about the ecology of the prey items themselves, which is challenging to characterize at the appropriate resolution.

Here we address this directly by contrasting the diets of 2 syntopic warblers: *S. aurocapilla* and *Setophaga virens*. These species differ strongly in their diet (Miller et al. 2025) and their foraging ecology: *S. virens* tend to spend their time higher in the canopy, foraging among the arboreal vegetation (Morse and Poole 2020; MacArthur 1958), whereas nearly 90% of foraging for *Seiurus aurocapilla* is spent lower in the canopy and often directly on the ground (Porneluzi et al. 2020). We first confirm the diet differences of these 2 warbler species at the level of arthropod order. Second, facilitated by a large sample of birds in a very restricted temporal window and geographic scope, we ask what *specific* diet items—to the arthropod species level—differ between the warblers. This allowed us, for a subset of arthropod taxa for which we have some knowledge of their natural history and which have little ecological differences across life stages, to use collection records to characterize the vertical stratum (i.e., above-ground or at ground level) where they are more likely to be found. Combined with the distinct foraging behaviors of the warblers, this allowed us to directly distinguish between preference versus opportunism.

Finally, given the expansion of invasive insects throughout North America—and the observation of Miller et al. (2025) of a high proportion of an invasive weevil as a top diet item across all warblers—there is the potential for such introduced prey items to have indirect effects on altering the complex food webs and/or influencing potential competitive interactions. We therefore opportunistically leverage these data to quantify the prevalence of invasive arthropods in their diet, establishing a baseline for future studies on outbreaks of these species—like *Lymantria dispar* (spongy moths) and *Lycorma delicatula* (spotted lanternfly)—and how they may (or may not) alter the dynamics of this system.

METHODS

Study Site and Fecal Sampling

To control for phenological variation in insect abundance, as well as environmental variation among sites, we intensively studied diet in warblers over one month in a small geographic region (Figure 1). Our study focused on a region—roughly 260 km^2—in central Pennsylvania, within Rothrock State Forest (Figure 1). To catch birds, we traveled the matrix of forestry roads listening for territorial male warblers singing and used audio lures and mist nets to catch, band, and release them after taking a fecal sample. Sampling occurred in 2021 between May 17 and June 20, catching approximately 5 to 8 birds per day when conditions were suitable. In many cases we attempted to catch both species in the same net. Given *Setophaga virens* are the less common of the 2, we would typically listen for them first, attempt to capture the territorial male and, if successful, then also lure an *Seiurus aurocapilla* that had an overlapping territory. We obtained fecal samples from *Setophaga virens* ($n = 60$) and *Seiurus aurocapilla* ($n = 59$) over this short period of the breeding season. All of the individuals were adult (i.e., "after hatch year") and all but one bird was identified as male by plumage (in the case of *Setophaga virens*), the extent of cloacal protuberance, and/or behavior (i.e., strongly responding to playback).

Fecal DNA Extraction and Library Preparation

To obtain fecal samples, immediately after capture and extraction of the birds from the mist net we placed them into a large brown paper bag, placed this bag into a cloth holding bag, and left the individual in a quiet location for at least 10 min. In our experience, >98% of individuals produced a fecal sample within this 10-min period. We then placed the sample into a 0.5% SDS lysis buffer and shook the tube vigorously. When returned to the lab the samples were placed into −80°C until DNA extraction. To extract DNA from avian fecal samples, we followed the protocol described by Vo and Jedlicka (2014) with minor adjustments (Baiz et al. 2023). Before DNA extraction, fecal samples were thawed at room temperature and then centrifuged to concentrate fecal material at tube bottoms. Using bleached laboratory spatulas and/or pipettes, we transferred fecal material into 2-mL screw-cap microcentrifuge tubes each containing 0.25 g of 0.1-mm and 0.25 g of 0.5-mm zirconia-silica beads. Our target was 0.05 g of solid fecal material, but this was not possible for many samples, so we supplemented with a suitable volume of storage buffer as necessary. We immediately added 818 µL of warmed (65°C) lysis buffer (Vo and Jedlicka 2014) and homogenized samples using a Precellys 24 Tissue Homogenizer (Bertin Instruments) set to 3 cycles of 6,800 rpm for 30 s with a 30 s pause between cycles. After transferring the supernatant to clean microfuge tubes, we incubated samples with Qiagen Solution C3 (Qiagen DNeasy PowerSoil 12888-100-3) to remove polymerase chain reaction (PCR) inhibitors. Next, we removed DNA from the supernatant using homemade solid phase reversible immobilization (SPRI) magnetic beads ("Serapure" beads). Serapure beads were added at 1.9x supernatant volume and, after cleaning with 80% ethanol, were eluted in 10 mM Tris-HCL. Extracted DNA was stored at −20°C before proceeding with library preparation.

From extracted fecal DNA, we generated multiplexed dual-index cytochrome oxidase C subunit 1 (COI) amplicon libraries for Illumina MiSeq sequencing. Prior to PCR, we randomized the plate order of samples to avoid within-plate batch effects during amplification. We used the "ANML" general arthropod primer pair (LCO1-1490/CO1-CFMRa) described in Jusino et al (2019) modified with overhanging Illumina adapter sequences:

LCO1-1490-overhang (5′-**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**GGTCAACAAATCATAAAGATATTGG-3′) and CO1-CFMRa-overhang (5′-**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**GGWACTAATCAATTTCCAAATCC-3′).

The product was 180 base pairs (bp) (excluding primer sequence). We performed initial PCR amplification in 30 µL reactions comprising 0.24 µL Platinum II *Taq* Hot Start DNA Polymerase (Invitrogen 14966005), 6 µL 5X Buffer (Invitrogen 14966005), 1.5 µL of each primer (10 uM working solution), 16 µL molecular grade water, and 0.6 µL 10 mM dNTP mix (Promega U151A) and 4 µL of fecal DNA. DNA concentrations were not normalized prior to amplification. Reaction conditions were: 94°C for 2 m, followed by 5 cycles of 94°C for 15 s, 45°C for 15 s, 68°C for 15 s, followed by 35 cycles of 98°C for 5 s, 68°C for 15 s, followed by a final extension at 68°C for 5 m, and hold at 12°C. We cleaned PCR products by incubating with a 1x volume of Serapure beads and eluting the bound DNA in 10 mM Tris-HCL. We evaluated amplification success by visualizing cleaned product on a 1.5% agarose gel.

Next, we appended dual P5 and P7 Illumina indexes to each library via PCR. Reactions were 30 µL and contained 15 µL KAPA HiFi HotStart ReadyMix (Roche 7958935001), 3 µL of each primer (10 µM working solutions), and 9 µL DNA (cleaned

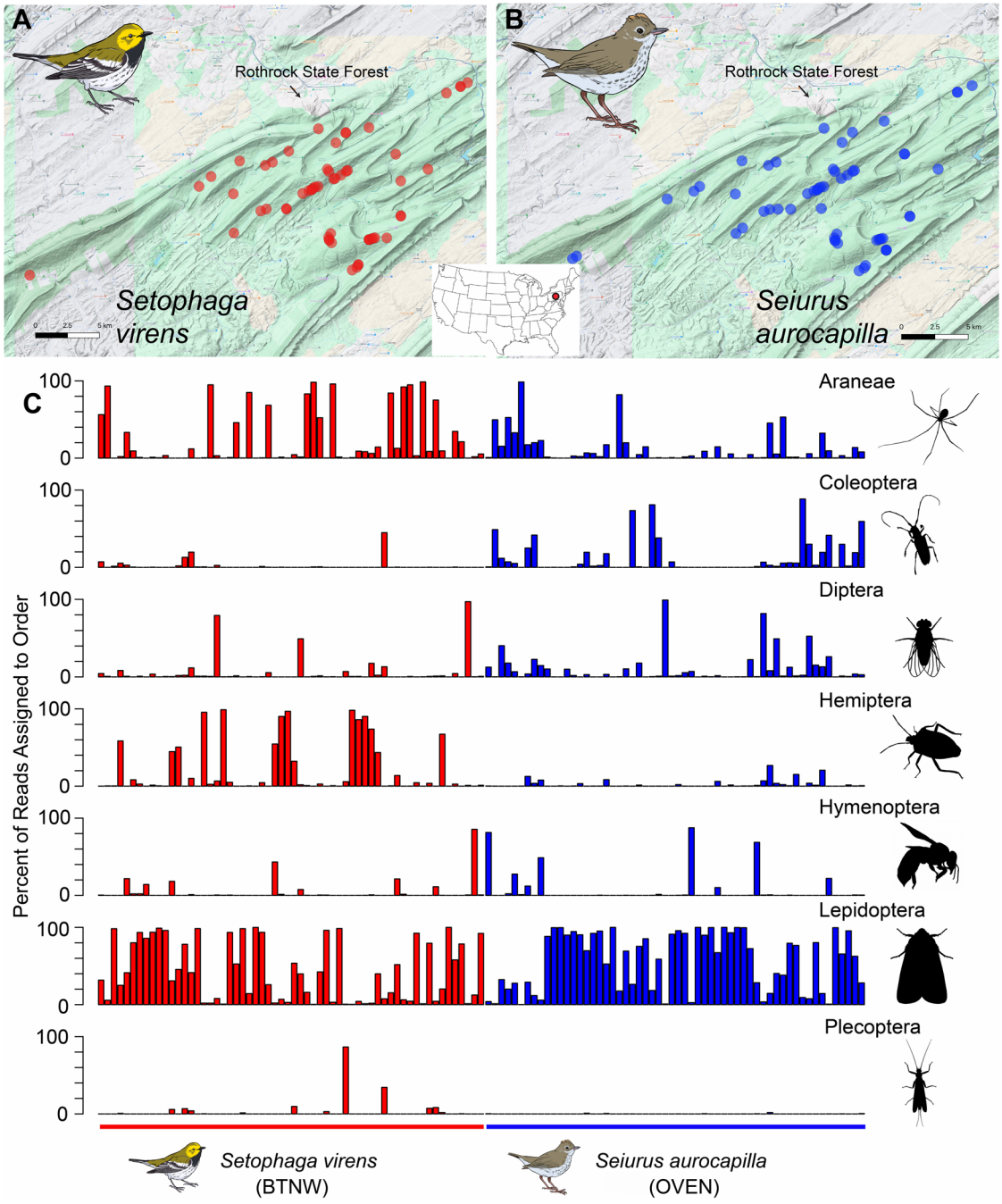

FIGURE 1. Map of sampling locations where individual *Setophaga virens* (red) (**A**) and *Seiurus aurocapilla* (**B**) were captured in Rothrock State Forest in Central Pennsylvania. Scale bars indicate 5 km. (**C**) Individual diet proportions of different insect orders. Each bar is an individual warbler of either *S. virens* (left; red) or *S. aurocapilla* (right; blue). Warbler illustrations by Rush Dhillon.

initial PCR product). Reaction conditions were 98°C for 45 s, followed by 7 cycles of 98°C for 15 s, 60°C for 15 s, 72°C for 15 s, followed by a final extension at 72°C for 1 m, and hold at 12°C.

We cleaned the indexed PCR product using a double-sided Serapure bead procedure. We first removed potential high-molecular weight contamination by incubating PCR product with a 0.75x volume of Serapure beads. After placing the samples on the magnet, we transferred the supernatant to fresh tubes and incubated it with a 1x volume of Serapure beads to remove potential low-molecular weight contamination. DNA was eluted in 10 mM Tris-HCL, and we evaluated amplification success as for the initial PCR. We quantified resulting DNA with a Qubit 4.0 Fluorometer (Invitrogen) using Broad Range reagents. We then normalized library concentrations and pooled libraries to a targeted 20 nM final pool concentration. We submitted the final pool to the Penn State Genomics Core Facility, where final quality assessment was performed on a Bioanalyzer Tape Station to confirm pool concentration with qPCR. Resulting libraries were sequenced on a single run of Illumina MiSeq using the 600-cycle kit run as 2 × 250 paired-end sequencing.

Bioinformatic Analysis

We used the QIIME2 (version 2022.2) analysis pipeline for our bioinformatic analyses (Bolyen et al. 2019). We first imported the de-multiplexed raw data. Using the *demux summarize* function we visually inspected the sequencing quality for the run. Following the QIIME2 recommendations, we denoised with *dada2* and trimmed after base pairs where the 25th percentile dropped below a PHRED quality score of 30 (for the forward read this was "p-trunc-len-f 247" and reverse read "p-trunc-len-r 219"). We also set the criterion for expected errors for both forward and reverse reads (p-max-ee) to 5 and trimmed 25 and 23 bp from the forward and reverse reads, respectively, to remove primer sequences.

We then used *vsearch* to cluster reads using an open reference based on Barcode of Life Data (BOLD) repository. These sequences were acquired from BOLD 78 between July 1, 2020 and August 8, 2020 by Devon O'Rourke and trimmed to the region covered by the ANML primers (Robeson et al. 2021, O'Rourke et al. 2020). As recommended, we generated clusters based on a 97% identity ("p-perc-identity 0.97"). To link COI sequences to their taxonomic assignment, we used the Naïve Bayes classifier *bold_anml_classifier*—originally trained by O'Rourke et al. (2020)—using *feature-classifier classify-sklearn*. Because of strict version control for this function, this step (and only this step) was run in an older version of QIIME2 (version 2019.10; Bolyen et al. 2019).

To estimate the rarefaction threshold for the phylogenetic analyses, we used the *diversity alpha-rarefaction* function and visually inspected the output to identify the diversity plateau, which we determined to be a sampling depth of 8,000 reads. This process retained 97 samples above this threshold for the phylogenetically informed diversity measures (i.e., the Unifrac analyses, below). We characterized diet composition for individual birds by identifying prey items at a range of taxonomic levels, but for visualization we focus on the order level. To do this, we used the *qiime tools extract* function.

Statistical Analyses

To compare individual-level diet proportions of arthropod orders, we filtered our data to include only those samples where we had paired *Setophaga virens* and *Seiurus aurocapilla* caught at the same time in the same mist-net ($n = 44$ pairs). In cases where there was more than one bird from either species captured (e.g., 3 birds, usually 1 *Setophaga virens* and 2 *Seiurus aurocapilla*) caught at the same time and place, we randomly assigned pairs among the individuals—using the *sample* function in R—such there was always only one *Setophaga virens* paired with a *Seiurus aurocapilla* for the analysis (<10 pairs). For each individual, we then calculated a standardized read proportion: the number of reads assigned to each arthropod order, divided by the total number of reads for that sample. While all methods to estimate diet have some bias (Hoenig et al. 2022), using a proportion per sample allowed us to control read abundance variation among samples, recognizing that we are losing some information on the variation among samples in the total amount of prey they are consuming.

To analyze the proportion of arthropod orders in the diets of *Setophaga virens* and *Seiurus aurocapilla*, we used zero-inflated beta regression models implemented in the *pscl* package in R (R Core Team 2024) to account for an excess of zero proportions (instances where a given insect taxon was entirely absent; Zeileis et al. 2008). Because our response values are proportions, we adopted the beta family distribution. For each model, warbler species (*Setophaga virens* vs. *Seiurus aurocapilla*) was included as a fixed effect (with *Seiurus aurocapilla* used as the reference), and net ID was included as a random effect to account for paired individuals captured at the same net. We extracted coefficients and tested species differences using a Wald z-test. For Lepidoptera, only the conditional model was run, as no individuals lacked prey items from this order in their diet.

We also calculated several diversity metrics at the operational taxonomic unit (OTU) level. To compare differences in arthropod diversity at this scale, we used the "diversity core-metrics-phylogenetic" command in QIIME2. Some of these metrics are phylogenetically informed; thus, we created a phylogeny from the representative sequences from the previous clustering step using the *phylogeny align-to-tree-mafft-fasttree* command and used the rooted tree output. These metrics also require a read rarefaction threshold, for which we used 8,000 reads. We calculated alpha diversity using the *diversity alpha-group-significance* command in QIIME2. For beta diversity measures, we used the four standard measures of distance calculated by QIIME2: Jaccard, Bray-Curtis, Weighted-Unifrac, and Unweighted-Unifrac. The Bray-Curtis dissimilarity is calculated using taxonomic occurrence data and abundance information. By contrast, Jaccard distance is based solely on presence/absence data and does not take into account the abundance of OTUs. UniFrac distances consider both the occurrence table (abundance) and the phylogenetic diversity (sequence distance) between samples. UniFrac distances are either weighted or unweighted, depending on whether they take into account relative abundance or only focus on presence/absence information, respectively. For each, to determine whether there were significant differences between the 2 species in beta distances we used the *diversity beta-group-significance* command, which conducts a PERMANOVA with 999 permutations. We conducted post-hoc Welch 2 sample t-test for each axis in R, with species identity as the grouping factor.

Arthropod Species-Level Analysis

We also tested whether any particular arthropod species/OTUs were more abundant in the diet of either warbler. To do this, we used a multivariable association analysis using linear models in MaAsLin2 (Mallick et al. 2021). We used OTU relative abundances as input in the model by dividing the OTU count by the sum of reads in each sample. Warbler species was the fixed effect variable, with *Setophaga virens* as the reference, and we otherwise used default settings including Benjamini–Hochberg correction for multiple comparisons and a q-value threshold of 0.25 for significance. We then focused on only those OTUs that were unambiguously identified to the species level.

For 3 species of spider that the MaAsLin2 analysis found differed significantly between *Setophaga virens* and *Seiurus aurocapilla* (see Results), we contextualized these findings by comparing them to capture data obtained from "Symbiota Collections of Arthropods Network" (i.e., SCAN), which is a portal of arthropod species occurrences collated from museum records from across the globe. We focused on spiders because, unlike the insects identified by MaAsLin2, they undergo direct development and do not exhibit distinct metamorphic life

stages with drastically differing ecologies. Metabarcoding cannot distinguish between such life stages, making it difficult to infer ecological context for insects like Lepidoptera, which include caterpillars, pupae, and adult moths. In contrast, although spiders do show some phenological variation, we can make more reliable inferences about their life stage at the time of consumption (Foelix 2010). Moreover, spider habitat preferences are relatively consistent across life stages, making it easier to associate them with specific microhabitats than for lepidopterans, whose different stages occupy ecologically distinct niches.

To obtain a sufficient number of records, we focused those observations from the same spider family with a *samplingProtocol* field filled. We then binned each protocol as "ground" (e.g., pan traps, pitfall traps, etc.), "off ground" (e.g., Malaise traps, beat sheets, Lindgren funnels, etc.), or "unclear," for instances where either was ambiguous (e.g., collectors identified "hand collected" or "looked down"). We then calculated the proportion of each to compare across the spider species.

Given the differing ecologies of *Setophaga virens* and *Seiurus aurocapilla*, we expected *Seiurus aurocapilla* to obtain nearly all of their food items from close to the ground, whereas *Setophaga virens* would have a broader vertical stratification. If these warblers are *preferentially* obtaining diet items that occur across a broad vertical spectrum, then we would not expect differences in the collecting methods across these spider species. By contrast, if they are *opportunistically* obtaining diet items that are more likely to occur in these different spaces, then we would expect to observe different proportions of the "ground" versus "off ground" methods.

RESULTS

Sequencing resulted in 4.6 million reads prior to filtering. Following quality filtering, denoising, and clustering, this resulted in a mean of 38,686 reads per individual. At the level of arthropod order, from the conditional and zero-inflated beta regression models, the largest differences between *Setophaga virens* and *Seiurus aurocapilla* were in the proportion of Coleoptera and Lepidoptera, which were more common in *Seiurus aurocapilla*, as well as Hemiptera and Plecoptera, which were more common in *Setophaga virens* (Table 1; Figure 2). Alpha diversity between the 2 warblers was not significantly different (Shannon's $H = 0.102$, $P = 0.75$). By contrast, all the multivariate analyses of beta diversity were highly significant between the 2 species (*Setophaga virens*, $n = 54$; *Seiurus aurocapilla*, $n = 43$; PERMANOVA: Bray-Curtis $P = 0.001$, Jaccard $P = 0.001$, Unweighted UniFrac $P = 0.001$, Weighted UniFrac $P = 0.001$), with Jaccard and Unweighted UniFrac showing the strongest separation of the species along the first 2 axes (Figure 3). The post hoc *t*-test results also showed significant differences between the diet composition of the species, with varying levels of statistical significance along the first 4 axes (Table 2).

At the level of arthropod taxa identified to species level, based on either relative read abundance, or using a proportion of all samples with a minimum threshold of 20 reads (a conservative threshold), 3 species were much more abundant compared to all others in the diet of both bird species: *Heterocampa guttivitta* (order: Lepidoptera, common name: saddled prominent moth), *Speranza pustularia (*order*:* Lepidoptera, common name: lesser maple spanworm moth*)*, and *Philodromus rufus* (order: Araneae, white-striped running crab spider). These were observed in 30 (25%), 35 (29%), and 39 (32%) warbler samples (over a 20 read threshold), respectively. The 2 moth species are both very common in the region; however, the spider is comparatively less common based on observations obtained from the Global Biodiversity Information Facility (GBIF.org 2024a, 2024b, 2024c).

We identified 16 OTUs with different relative abundances between species (using the default threshold for maaslin2 significance of $Q < 0.25$), 9 of which could be assigned to species-level taxonomy (Table 3). Five diet items were more abundant in *Setophaga virens*, including 2 Aranae, 2 Hemipterans, and 1 Lepidopteran. Four diet items were more abundant in *Seiurus aurocapilla*, including 1 Araneae, 1 dipteran, and 2 lepidopterans. For the 3 spiders, SCAN records from collections from the same families of the 2 taxa (*Philodromus rufus* and *Anyphaena pectorosa*) that were most common in the diet of *Setophaga virens* (Philodromidae and Anyphaena, respectively), showed much higher proportion of collection protocols that were from off the ground (Figure 4A and B). By contrast, the family-level collection records from *Pirata praedo*, found exclusively in the diet of *Seiurus aurocapilla* (family: Lycosidae), were found at a much higher proportion using ground-based collection techniques (Figure 4C).

DISCUSSION

We focused on the arthropod diet of 2 syntopic warblers: *Setophaga virens* and *Seiurus aurocapilla*. Our goal was to focus, in sharp detail, on diet differences between the 2 species and to attempt to avoid the confounding variables that have been present in previous work. By using data collected from a very fine geographic scale—in most cases with birds caught in the same mist-net (e.g., Figure 2)—and sampled at the same time of year and sequenced on the same lane, we were able to

TABLE 1. Output from linear models comparing the diet proportions of paired Setophaga virens and Seiurus aurocapilla for conditional (first 4 columns) and zero-inflated (final 4 columns, with "–0") models.

Order	Estimate	SE	*Z*	*P*	Estimate-0	SE-0	*Z*-0	*P*-0
Aranae	–0.29	0.28	–1.06	0.29	0.16	0.57	0.28	0.78
Coleoptera	0.42	0.33	1.29	0.20	**–1.23**	**0.45**	**–2.73**	**0.00625****
Diptera	0.22	0.26	0.85	0.40	–0.15	0.54	–0.27	0.79
Hemiptera	**–0.96**	**0.32**	**–2.98**	**0.00288****	0.19	0.44	0.44	0.66
Hymenoptera	0.11	0.36	0.31	0.76	0.46	0.43	1.07	0.28
Lepidoptera	**0.64**	**0.28**	**2.28**	**0.0226***	NA	NA	NA	NA
Plecoptera	**–1.05**	**0.42**	**–2.51**	**0.012***	–0.11	0.47	–0.24	0.81

Significant values are indicated by bold text (*P < 0.05, **P < 0.01).

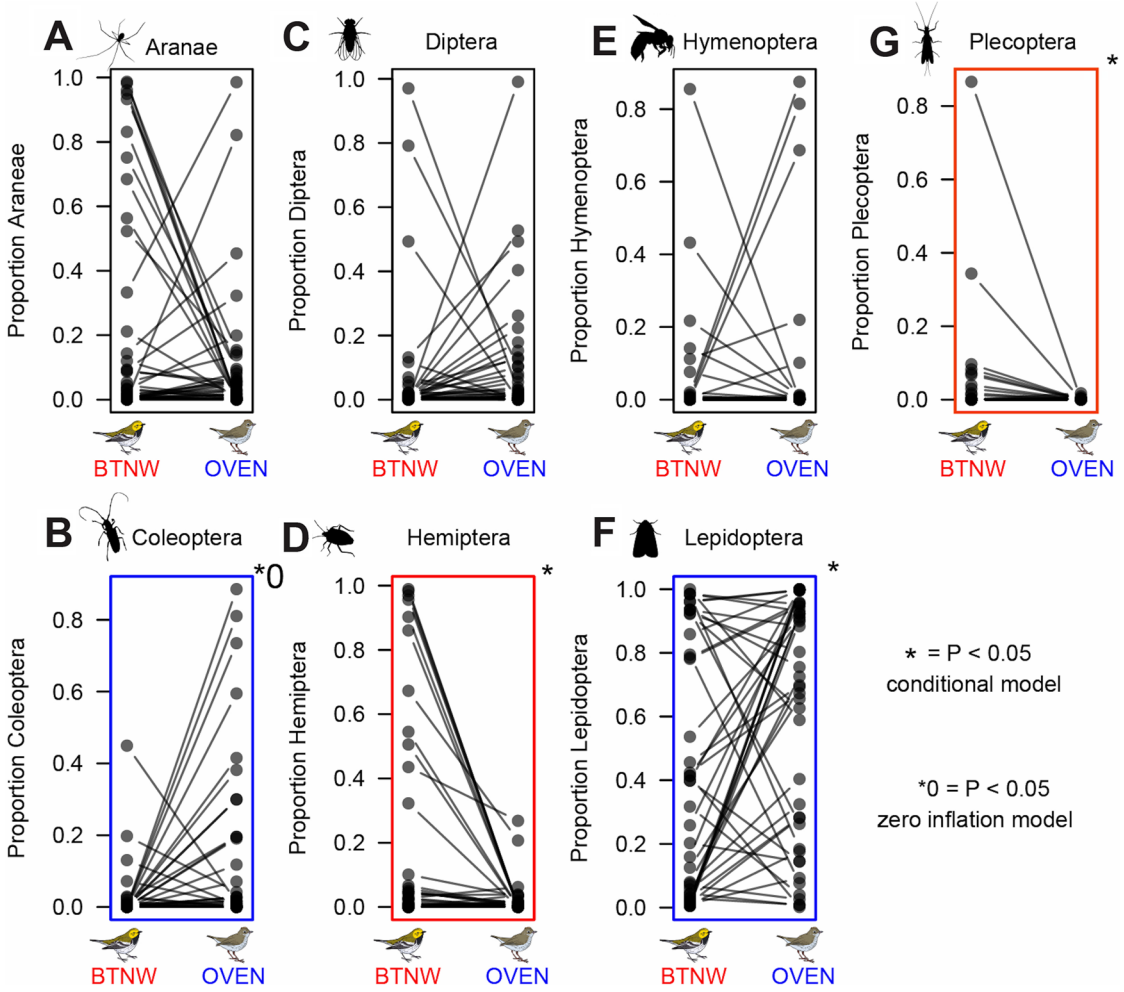

FIGURE 2. Paired diet proportions for those birds that were caught in the same net ($n = 44$ pairs). Colored plot frames show the results of the conditional and zero-inflated beta regression models when $P < 0.05$ and the colors indicate which species (*Setophaga virens*, BTNW, red, left, or *Seiurus aurocapilla*, OVEN, blue, right) had higher diet proportions of a given arthropod taxon (Table 1). Warbler illustrations by Rush Dhillon.

characterize each species' diet at a fine resolution using fecal metabarcoding. Not only did we find significant differences in diet composition between the 2 species—as has been demonstrated by previous work (Miller et al. 2025)—but we were able to document *how* their diets differ, down to the level of arthropod species in some cases, and how the ecologies of the prey taxa aligned with known foraging strategies of each warbler species.

Fine-Scale Diet Partitioning

At the broadest scale of diet composition (i.e., individual diet proportions at the arthropod order level), in the conditional models, *Seiurus aurocapilla* appears to prefer butterflies and moths (or their caterpillars, Lepidoptera; Table 2). By contrast, *Setophaga virens* prefer "true bugs" (Hemiptera) and stoneflies (Plecoptera). The zero inflation models show a significant effect for *Seiurus aurocapilla* for beetles (Coleoptera), suggesting *Setophaga virens* is significantly more likely to have *no* beetles in their diet than *Seiurus aurocapilla* (i.e., *S. aurocapilla* prefers coleopterans). A study of stratification of tropical insects by de Souza Amorim et al. (2022) found beetles and flies were significantly more abundant below 8 m, whereas peak abundance for hemipterans was above 24 m, consistent with the predicted vertical stratification of these warblers. While the beta-diversity statistics are not helpful in identifying what specific diet items differ between the warblers, the first 2 axes of the multivariate analysis of diet composition, Jaccard and unweighted UniFrac, appear to separate the species most strongly (Figure 3), though all the measures strongly differentiate the species (Table 1). The different diet compositions of *Setophaga virens* from *Seiurus aurocapilla* could be due to two-dimensional spatial niche partitioning (i.e., *Setophaga virens* and *Seiurus aurocapilla* occurring in nonoverlapping portions of the forest and eating different diet items). However, we found no obvious geographic differences in the birds or their insect prey. Moreover, in this paired analysis, we only considered birds captured in the same net at the same time, suggesting any differences between these species are maintained in extreme syntopy.

Analysis of the specific diet items—identified to arthropod species levels by MaAsLin2—provides the most novel aspect of our findings (Table 3). First, it is notable that some of the specific species that differentiate the warblers are not necessarily in the orders that differ most strongly between the taxa (e.g., Araneae and Diptera). Moreover, even for those orders, like

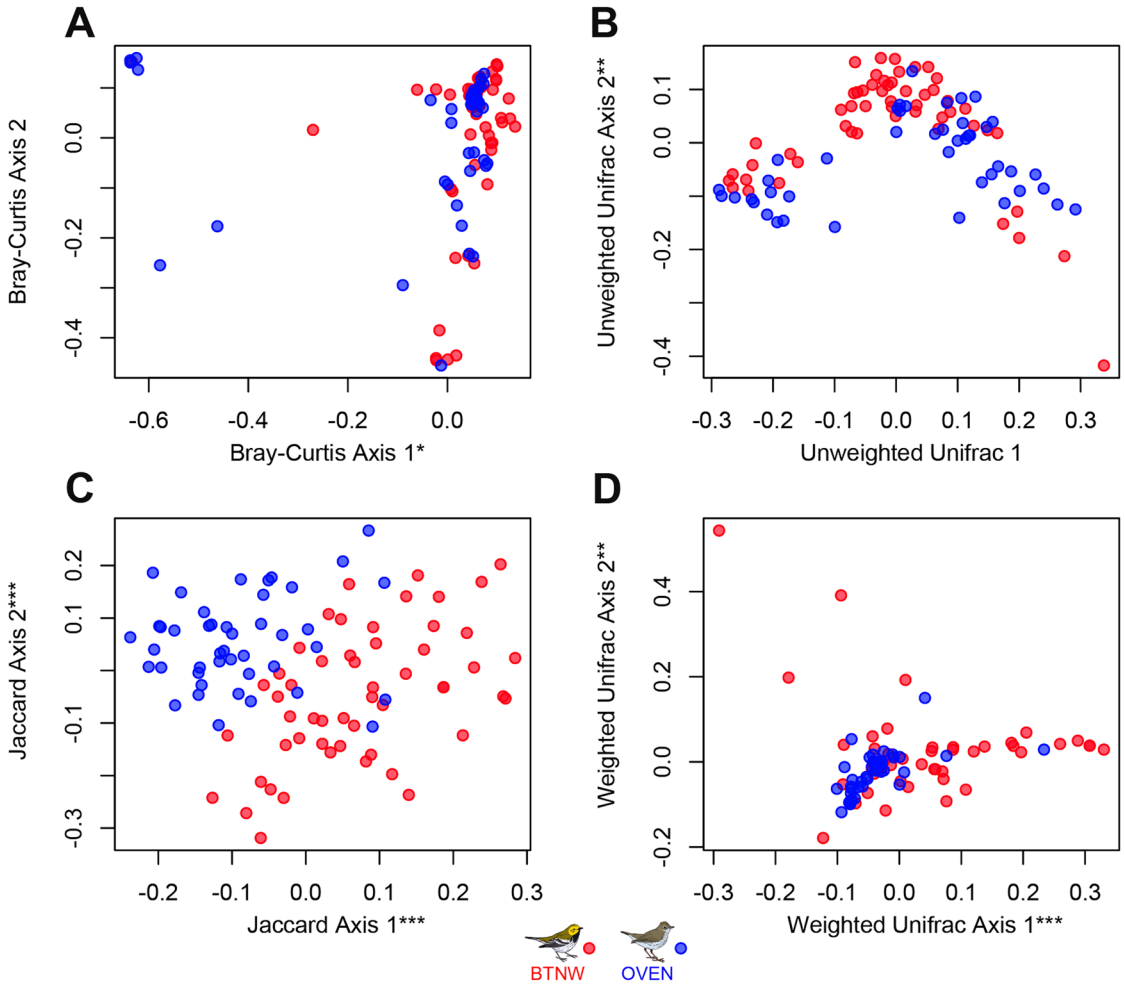

FIGURE 3. Multivariate beta-diversity of rarefied diet contrasts between *Setophaga virens* (blue; BTNW) and *Seiurus aurocapilla* (red; OVEN). Asterisks represent significance values for the first and second axes as described in Table 2 (bold; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$). Jaccard distance, which is based solely on presence/absence data and does not take into account the abundance of OTUs, shows the strongest separation between the species along the first 2 axes.

TABLE 2. Post hoc t-test of multivariate axes versus species differences.

Test	Axis 1	Axis 2	Axis 3	Axis 4
Bray-Curtis	*	$P>0.05$	**	**
Jaccard	***	***	$P>0.05$	$P>0.05$
Unweighted Unifrac	$P>0.05$	**	***	***
Weighted Unifrac	***	**	***	$P>0.05$

$^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

Lepidoptera, that differ strongly between *Setophaga virens* and *Seiurus aurocapilla*, the directionality of the association is not always clear (e.g., *Setophaga virens* has a higher diet composition of *Chionodes pereyra*, the Twirler moth, whereas *Seiurus aurocapilla*'s diet includes significantly more *Gypatopa simlicella*, a scavenger moth). This suggests that order-level analyses are helpful to describing high-level diet differences, but fine-scale analyses such as this can provide more nuanced information about specific diet differences.

We contrasted the foraging ecology of the warblers based on 2 hypotheses. First, a foraging "preference" of the warblers, would imply a consistent, selective behavior where they actively choose certain food types that occur across foraging strata occupied by both *Setophaga virens* and *Seiurus aurocapilla*. In contrast, "opportunistic" foraging involves taking advantage of food sources that are encountered in the different foraging strata (primarily above the ground for *Setophaga virens*, and on the ground for *Seiurus aurocapilla*). While previous work on this system (e.g., MacArthur 1968, Kent and Sherry 2020, Miller et al. 2025) indirectly supported the "opportunistic" hypothesis, here we explicitly test this by focusing on the spiders that differed between the warbler species. We focused on spiders because, unlike insects that can have drastically different life stages (with concomitant different life histories; e.g., caterpillars versus butterflies), with directly developing spiders we can be more confident about their natural history. We used observations from the SCAN network of arthropod observations to bin the spider observations in "on ground," "off ground," and "unclear." While there are potential differences related to different microhabitat use between juvenile and adult spiders, we assumed that the vast majority of the SCAN observations and foraged spiders by the warblers were adults.

The 2 spiders more common in the diet of *Setophaga virens* (*Philodromus* and *Anyphaena*) are from spider families that

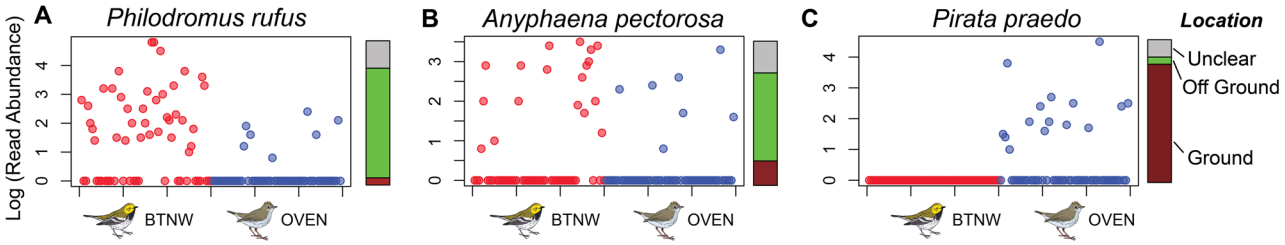

FIGURE 4. Log of the relative read abundance for species-level differences for 3 spider taxa that MaAsLin2 (Table 3) found significantly different between *Setophaga virens* (red; BTNW) and *Seiurus aurocapilla* (blue; OVEN). The bar chart to the right illustrates the proportion of arthropod collections that were classified as either "on the ground" (brown), "off the ground" (green), or "unclear" (grey).

TABLE 3. Arthropod OTUs exhibiting significant differences in relative abundance between warbler species. Association indicates the warbler species exhibiting elevated OTU abundance.

OTU ID	Order	Family	Species	Common name	Association	Coef.	*P*	FDR (*Q* value)
9421964	Aranae	Philodromidae	*Philodromus rufus*	White-striped Running Crab Spider	*Setophaga virens*	–3.27	<0.001	<0.001
9507813	Aranae	Anyphaenidae	*Anyphaena pectorosa*	Ghost spider	*S. virens*	–1.41	0.02	0.05
7687722	Hemiptera	Cicadellidae	*Oncopsis coloradensis*	Leafhopper	*S. virens*	–1.31	0.01	0.03
3210602	Hemiptera	Aphrophoridae	*Aphrophora cribrata*	Pine spittlebug	*S. virens*	–1.00	0.06	0.13
3376548	Lepidoptera	Gelechiidae	*Chionodes pereyra*	Twirler moth	*S. virens*	–0.99	0.07	0.13
3338681	Lepidoptera	Blastobasidae	*Hypatopa simplicella*	Scavenger moth	*Seiurus aurocapilla*	1.07	0.04	0.10
7092434	Lepidoptera	Notodontidae	*Heterocampa guttivitta*	Saddled prominent moth	*S. aurocapilla*	1.17	0.14	0.21
3483215	Diptera	Tipulidae	*Tipula submaculata*	Crane fly	*S. aurocapilla*	1.66	<0.001	<0.001
3406410	Aranae	Lycosidae	*Pirata praedo*	Pirate wolf spider	*S. aurocapilla*	1.67	<0.001	<0.01

Coef = MaAsLin2 model coefficient; FDR = false discovery rate (i.e., *Q* value), where the default threshold for significance in maaslin2 is $Q < 0.25$.

are much more likely to be observed off the ground. The finding that some *Seiurus aurocapilla* also foraged on these spiders is consistent with either these species spending some of their time on the ground, or *Seiurus aurocapilla* foraging at a slightly higher vertical stratum. By contrast, the results for *Pirata praedo* showed a very strong difference: this species was found in the diet of 15 *Seiurus aurocapilla* but no *Setophaga virens*. This wolf spider is a ground-dwelling spider, and like many in the genus *Pirata*, it tends spend its time in low vegetation or on the ground, often near water sources like ponds, streams, or wetlands, and it would be extremely unlikely to observe it high in the canopy (Nørgaard 1951, Foelix 2010). This is highly consistent with the SCAN results: while the other 2 spiders were much more likely to be observed above the ground, 82% of the observations for Lycosidae were from the ground and only 5% were above ground.

Taken together, as the prey taxa that differed significantly in diet composition among warbler species also varied in their availability across foraging microhabitats (e.g., canopy vs. ground), these patterns are unlikely to reflect true diet preference (i.e., choice under equal availability) or selective foraging (i.e., disproportionate consumption relative to availability). Instead, they suggest opportunistic consumption of prey encountered within the species' primary foraging habitats. While this result is in line with MacArthur's (1968) original interpretation of ecological differentiation among parulid warblers—those differences in foraging behaviors brought warblers into contact with different suites of prey—as Miller et al. (2025) recently showed, in reality this does not translate into extremely strong differences in diet among most species. It could also be that those arthropod taxa that are differentially preferred between the warbler species are not as strongly vertically stratified as the spiders investigated in detail here. Moreover, the 2 species chosen here—*Seiurus aurocapilla* and *Setophaga virens*—are at the ends of the ecological and phylogenetic spectrum, thus it is not altogether surprising to confirm overall diet differences. While being able to identify individual prey items, at the species level, that differ between warblers was not a part of previous work, future research should approach similar questions with more closely related taxa. For instance, among MacArthur's original 5 taxa, *Setophaga virens* and *S. fusca*, are 2 closely related *Setophaga* warblers that MacArthur suggested both foraged high in the canopy, with *S. virens* showing a slight propensity for time spent lower and towards the trunk of the tree (MacArthur 1968). Do these more subtle foraging differences lead to measurable diet variation at finer taxonomic resolutions? Answering such questions would provide deeper insights into the ecological mechanisms driving niche partitioning among closely related warbler species.

Invasive Species

We were also interested in opportunistically documenting whether invasive arthropod taxa were present in the warblers' diets. Metabarcoding and environmental DNA approaches have been used to detect the presence and range shifts of these species in other taxa (e.g., Comtet et al. 2015, Borrell et al. 2017). Miller et al. (2025) documented that one of the most prevalent prey items among 13 species of warbler in upstate New York was in fact an invasive leaf weevil. Here, we did not find evidence of spotted lanternfly (*Lycorma delicatula*), which has become a significant invasive pest in southeastern Pennsylvania (Urban 2020). This is not entirely unexpected, as *Lycorma* was only recently documented in Centre and Huntingdon County—where our work took place—and were initially at very low numbers at the invasion front (when our study took place; though recently much more abundant). They are also

toxic, depending on whether they fed on tree of heaven (*Ailanthus altissima;* Johnson et al. 2025), and this could have been learned by the birds. We did, however, find a large number of warblers that had eaten spongy moths (OTU "*Lymantria* sp. AN-2017"), an invasive Lepidopteran of great concern due to their impact on deciduous forests (Wu et al. 2020, Nunez-Mir et al. 2022). Using a conservative minimum threshold of 20 sequencing reads, we found evidence of *Lymantria* in 22 samples: 10 *Setophaga virens*, and 12 *Seiurus aurocapilla*. Spongy moths have expanded southwards substantially, from New York state into northern and central Pennsylvania, and there has been an outbreak between 2021 (when these samples were taken) and 2023. This has resulted in significant spraying of *Bacillus thuringiensis* (BT; a bacterium that non-discriminately kills lepidoptera), across state forests and parklands in the state, which is particularly relevant for bird species, like those studied here, that frequently feed on moth caterpillars. Much effort goes into surveying for spongy moth, and given our results, fecal metabarcoding could be a useful alternative or complementary approach to objectively quantify potential outbreaks in given regions. One of the key differences in a metabarcoding approach like ours and stomach contents analysis is that the latter can provide insights into the developmental stage of prey, whereas metabarcoding cannot. For instance, it is possible that most of the Lepidoptera were caterpillars or pupae and not adult moths. Future work could add a temporal component of sampling, to quantify whether changes in diets reflect local abundances of caterpillars versus adult moths at appropriate points throughout the breeding season.

In addition, determining to what extent invasive insects such as these alter the competitive interactions within this avian community will be useful to quantify in the long term, with our work here a useful baseline for these 2 warbler species. These kinds of anthropogenetic influences on foraging have been quantified in a range of species, including most notably the loss of bill dimorphism in populations of *Geospiza* finches on the Galápagos where human foods have been introduced (Hendry et al. 2006). That said, as outlined above, diet differences per se do not seem to have had as large a role in driving overall species differences in warblers—as compared to the Galápagos finches—though unpredictable responses to introduced and invasive taxa are clearly possible.

Conclusions

Given the phylogenetic distance between *Setophaga virens* and *Seiurus aurocapilla*, it may not be overly surprising to have documented significant diet differences. *Setophaga* represent some of the youngest species in the radiation (Oliveros et al. 2019), whereas *Seiurus aurocapilla* is the only member of its genus and, for a long time, was debated whether it was even a member of the parulid family (Lovette et al. 2010). That said, the role of ecological differences among parulids has been a subject of substantial debate (e.g., MacArthur 1958, Price et al. 2000), and had not been considered explicitly within a phylogenetic context until Miller et al. (2025). Therefore, these new diet data are useful in providing additional substance to these discussions, which have traditionally focused on very indirect proxies of ecological overlap (e.g., Price et al. 2000). In particular, being able to identify *how* these warblers differ in their diet—down to species-level arthropod taxa in some cases—allows us to connect these patterns with knowledge of the natural history of the prey themselves, and better infer the evolutionary and ecological processes at play. This scale of analysis lays the foundation for studies to identify the underlying traits that might actually facilitate the niche differentiation, such as differences in vision that make certain species better adapted in exploiting foraging territories within a certain light environment versus others. More generally, these new data provide useful insights into the natural histories and ecologies of these common North American songbirds, which have been traditionally difficult to study in this way.

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Ethics statement

Permits for work were support by the USGS (Master banding permit #24043), the Pennsylvania Game Commission (#46121), and the PA Dept. of Conservation and Natural Resources. All samples in this study were collected under a protocol approved by IACUC at Penn State University (protocol no. 201900879).

Conflict of interest statement

The authors declare no conflict of interest.

Author contributions

D.P.L.T., L-N.P., S.J.S., and A.W.W. we involved with the design of the project. M.D.B., A.B.C, A.R.D, L-N.P., S.J.S., and A.W.W. were involved with the field sampling. A.W.W. conducted the molecular methods. A.R.D. collated arthropod specimen data. D.P.L.T. and M.D.B. performed the bioinformatic analyses. D.P.L.T. wrote the original draft of the manuscript. All authors reviewed and edited the final manuscript.

Data availability

Code and sample information files are available at Zenodo (Toews et al. 2025; 10.5281/zenodo.16573644). Metabarcoding data are available at NCBI SRA: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA889412/.

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