**DNA metabarcoding reveals within-season variation in the diet of the insectivorous black-throated blue warbler (*Setophaga caerulescens*)**

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**Abstract**

Changes in leaf phenology from warming spring and autumn temperatures have lengthened the green season and potential breeding window for migratory birds in North America. However, the potential fitness benefit of an extended breeding season for multiple brooded species will depend on whether late season food supplies are sufficient to support rearing additional broods, and whether species exhibit the flexibility in their diets to respond positively to changes in early and late season prey availability. We used DNA metabarcoding to characterize spatial and temporal variation and diversity in the diet compositions of the insectivorous, migratory black-throated blue warbler (*Setophaga caerulescens*) at the Hubbard Brook Experimental Forest, New Hampshire. We examined how their diet varied along a 600-m elevation gradient encompassing a two-week difference in green season length, and over the duration of the breeding season. Successful sequencing of 98 samples identified 395 taxa spanning 17 arthropod orders, 257 of which were identified to species-level. Most taxa were rare; 48.6% of them were detected in only one sample. Our results demonstrated differences in diet composition across survey periods, and by age. Our results also showed differences in diet diversity between survey periods.. This study demonstrates that black-throated blue warblers have flexibility in their diet and are able to respond to temporal pulses in prey availability, which will be an important asset in the face of climate change and a lengthening green season.

**Introduction**

The growing ‘green’ season, and thus the potential breeding window for migratory bird populations, has been lengthening in North America with earlier leaf emergence and delayed leaf senescence due to climate change (Richardson et al. 2006, Zhu et al. 2012, Buitenwerf et al. 2015). Deciduous tree leaf phenology is mostly governed by temperature; leaf emergence is dictated by warming spring temperatures while cooling autumn temperatures activates the onset of leaf senescence (Richardson et al. 2006). Spring leaf phenology affects early seasonal events in the annual cycle of migratory birds, including the timing of spring arrival on the breeding grounds and timing of breeding (Gordo 2007, Vega et al. 2021), behaviors that have become progressively earlier over the last 50 years (Knudsen et al. 2011). For multiple brooding species, early breeding birds may have a fitness advantage over later breeding birds by raising additional broods after successfully rearing a first brood (Townsend et al. 2013a). However, this potential fitness benefit of an extended breeding season will depend on whether late-season food supplies are sufficient to support rearing additional broods (Nagy and Holmes 2005a), and if species exhibit the flexibility in their diets to respond positively to potential changes in late season prey availability.

Insectivorous birds are particularly sensitive to the effects of changing seasonality on insect availability (Visser et al. 2006). Changes in phenology from warming spring and autumn temperatures are associated with the earlier emergence and spring migration of insects, additional insect generations late in the growing season, and delayed autumn migration and insect diapause (Gallinat et al. 2015). These changes in early and late season insect availability suggest that insectivorous birds might be able to take advantage of the fitness gains from multiple brooding and extend their breeding season. Understanding how the diet composition and diet diversity of migratory birds varies over the green season will help predict their response to potential changes in insect availability over a lengthening green season.

Advances in fecal DNA-based metabarcoding methods have revitalized studies of avian diets and trophic dynamics that could be affected by changing seasonality (Fayet et al. 2021, Bumelis et al. 2022, Hoenig et al. 2022, Spence et al. 2022). DNA metabarcoding techniques use genetic markers to characterize the species composition and diversity of prey from a complex mixture of fragmented DNA found in fecal samples (Silva et al. 2019). These methods can have high taxonomic detectability and specificity relative to other methods for studying avian diets (Hoenig et al. 2022) and may enable the identification of rare prey species and soft-bodied prey items, such as caterpillars, which are often overlooked using traditional methods that identify prey directly from foraging observations or from feces or stomach contents (Hoenig et al. 2022). DNA metabarcoding is also a much less invasive technique than other methods such as regurgitation studies, which can harm birds (Carlisle and Holberton 2006). While DNA-based methods do have important limitations, including a limited ability to quantify prey species abundance or biomass (Deagle et al. 2019), or to differentiate between larval and adult life stages of insect prey (Hoenig et al. 2022), these methods can be used to characterize fine-scale differences in the composition and diversity of diets and are therefore suitable for investigating dietary variation between early and late breeding birds experiencing changing seasonality on their breeding grounds.

In this study, I use DNA metabarcoding to characterize within-season spatial and temporal variation in the diet of the insectivorous, migratory black-throated blue warbler (*Setophaga caerulescens*) at the Hubbard Brook Experimental Forest (Hubbard Brook), New Hampshire. Since the 1980s when research on this population began, leaf emergence dates have advanced 2.1 days per decade (8.4 days in total) in response to increasing spring temperatures (Richardson et al. 2006, Rustad et al. 2012, Campbell et al. 2021) and autumn leaf senescence has occurred 2.8 days later per decade (11.2 days total) (Melaas et al. 2016), resulting in a 19.2 day extension of the green season. Black-throated blue warblers have exhibited flexibility in their timing of arrival and breeding in response to earlier spring leaf emergence (Townsend et al. 2013a, Lany et al. 2016) and an increased propensity to double brood later in the breeding season (Germain et al. 2021), suggesting an extension of their breeding window both early and late in the season. However, prey availability limits the duration of the breeding season by affecting the probability that females will initiate second broods (Sillett 2000, Nagy and Holmes 2005a, Kaiser et al. 2015). At Hubbard Brook, the seasonal pattern of insect availability for breeding birds (i.e. caterpillars and flying insects) is highly variable throughout the breeding season with a shifting pool of arthropods with diverse life histories and larval feeding times (Stange et al. 2011, Lany et al. 2016). Thus, to understand the capacity of black-throated blue warblers to respond to potential changes in insect availability with a lengthening green season, I examine variability in their diets corresponding to spatial and temporal pulses in insect availability. My objectives were to (1) describe the arthropod diets of black-throated blue warblers in a temperate mixed-hardwood forest experiencing changing seasonality; (2) compare how their diet composition and diversity varies over the duration of the green season and across an elevation gradient encompassing a two-week difference in green season length; (3) compare the diet composition and diversity among age classes and sexes; and (4) examine spatial and temporal associations between diet and insect availability.

**Methods**

***Study Population***

I studied the diet of the insectivorous, migratory black-throated blue warbler as part of a larger demographic study of this species at the 3,160 ha Hubbard Brook Experimental Forest (Hubbard Brook), New Hampshire (43.56ºN, 71.45ºW). The study area is a northern hardwood forest spanning a 600-m elevation gradient with an overstory dominated by sugar maple (*Acer saccharum*), American beech (*Fagus grandifolia*), and yellow birch (*Betula alleghaniensis*), with red spruce (*Picea rubens*), balsam fir (*Abies balsamea*), and white birch (*B. papyrifera*) increasing in abundance at higher elevations (Schwarz et al. 2003, van Doorn et al. 2011). The shrub layer is dominated by hobblebush (*Viburnum lantanoides*), the preferred nest substrate for the warblers (Holmes et al. 2020a), along with saplings of striped maple (*Acer pensylvanicum*) and the major canopy species (Schwarz et al. 2003, van Doorn et al. 2011). Black-throated blue warblers primarily forage in the green food web by gleaning prey from foliage in the shrub and lower forest canopy (Robinson and Holmes 1982). Foraging observations and earlier studies of their stomach contents at Hubbard Brook indicate that they consume Lepidoptera larvae and adults, adult Diptera, Coleoptera, spiders and other arthropods including Homoptera and Hymenoptera, and small snails (Robinson and Holmes 1982, Holmes et al. 1986).

The breeding ecology of the black-throated blue warbler has been studied extensively at Hubbard Brook (Holmes 2007, 2011, Holmes and Likens, 2016, Holmes et al. 2020a). Males and females arrive in early May, establish territories (1–4-ha), pair, and initiate breeding in mid- to late-May, depending on spring leaf expansion (Lany et al. 2016, Holmes et al. 2020a). Females build open cup nests <1 m from the ground in the shrub layer, lay one egg per day (mean clutch size = 3.6, range = 2–5 eggs), and incubate for 12 days. Both females and males will feed nestlings for 9 days until fledging (Holmes et al. 2020a). Small nestlings are most commonly fed small flying insects (Diptera and Hymenoptera), spiders, and small, smooth-skinned caterpillars (Harding 1931, Holmes et al. 2020a), while larger nestlings are fed crane flies, moths, and both small and large caterpillars (Rodenhouse and Holmes 1992, Holmes et al. 2020a). At Hubbard Brook, 31% of black-throated blue warbler pairs attempt second broods (i.e. double brood) when food resources are favorable (Nagy and Holmes 2005b, Townsend et al. 2013b, Kaiser et al. 2015).

***Field Methods***

I collected data on a 333 ha study plot at three elevation zones (low: 382–499 m [85 ha], mid: 500–599 m [123 ha], high: 600–740 m [125 ha]) during three 4-wk survey periods from May–Aug 2021 (Table 1). The field crew mapped the birds’ territories, captured and marked adults, collected fecal samples from birds, and found and monitored nesting attempts. The boundaries of male territories were mapped throughout the breeding season relative to a 50 x 50 m virtual grid by recording the locations of singing males and agonistic encounters between neighboring males. All individuals were captured within their territories between 0600–1400 and marked with a unique combination of three colored leg bands and one U.S. Geological Survey leg band. Nests were found by following females during nest building, following adults carrying food, and searching vegetation. Nests were monitored every other day throughout all nest stages, with daily checks near clutch completion and anticipated hatch and fledge dates. Males and females were aged as hatch-year (HY), second-year (SY) or after-second-year (ASY) breeders based on plumage characteristics (Holmes et al. 2020b). Males were lured into 6-m mist nets with song playback and a male decoy. Females were captured by flushing them off their nests into a 6-m net placed in front of their nest. At capture, adults were placed in a new paper bag with an index card at the bottom for a maximum of 10 min or until defecation. Fecal samples were collected off the paper bag or index card into sterile 2 mL tubes containing 1 ml DNA/RNA Shield (Zymo Research, Irvine, CA) and stored at room temperature until DNA extraction in Sept–Oct 2021. I captured 44 birds that did not defecate after being held for 10 min that were released without obtaining a sample. All banders sterilized their hands with alcohol wipes after handling each bird to minimize the risk of cross-contamination. I collected field blanks at each elevation and during each survey period to monitor for background contamination from the bags and index cards used to collect samples.

I examined spatial and temporal variation in insect availability across the three elevation zones and over the breeding season from 15 May–15 Aug. Visual caterpillar surveys were conducted in the shrub layer across four transects at each of the three elevation zones during six 2-wk survey periods (Holmes et al. 1979, Holmes and Schultz 1988). Caterpillars and spiders were identified to family, counted, and measured on 100 leaves each of striped maple, American beech, and hobblebush at 10 points along each transect. We converted caterpillar and spider measurements to wet biomass (mg) using length-mass regressions (Rogers et al. 1977). I summed biomass per 3000 leaves sampled on the leaves of all three tree species on each of the four transects within a given 2-wk survey period. I calculated mean daily sampling rate for three caterpillar families (Geometridae, Noctuidae, Notodontidae) and spiders (Araneae). I combined two 2-wk survey periods and calculated the mean (±SE) sampling rate for each taxonomic group within each elevation zone and during each of three 4-wk survey periods (i.e. early, mid, and late).

ADD SOMEWHERE IN PARAGRAPH ABOVE: To facilitate comparisons between arthropod survey results and fecal samples, we grouped survey data into three survey periods matching the three temporal windows of fecal sampling.Two 24-hr samples of flying insects were collected each week from two Malaise traps at each of the three elevation zones. Malaise samples were sorted and identified to family (Rodenhouse and Holmes 1992). I measured mean daily sampling rate as the 24-hr sampling rate per Malaise trap and included only individuals greater than 4 mm in length. I calculated sampling rates for four insect families (Tachinidae, Rhagionidae, Tipulidae, Ichneumonidae), and five arthropod orders (Diptera, Hymenoptera, Lepidoptera, Coleoptera, Araneae), and calculated the mean (±SE) sampling rates for each taxonomic group within each elevation zone and during each of three survey periods.

ADD SOMEWHERE IN PARAGRAPH ABOVE: Same survey period grouping as above. In addition, it’s worth noting that we carefully filtered the Malaise trap dataset to remove taxa with known or potential bias in capture rates. The resulting dataset included 14 taxonomic groups with high confidence in the Malaise trap results: Hemiptera.Pentatomidae, Hemiptera.Other, Plecoptera, Coleoptera, Lepidoptera, Diptera.Tipulidae, Diptera.Rhagionidae, Diptera.Other, Hymenoptera.Ichneumonids, Hymenoptera.Not.Ichneumonids, Homoptera, Homoptera:Leafhopper, Trichoptera, Panorpidae.

(you might consider giving these more intuitive names... hymenoptera-not-ichneumonids is a little clunky).

***DNA Extraction and Sequencing***

I used DNA metabarcoding to characterize diet composition with taxonomic specificity (Garfinkel et al. 2020). I randomly placed samples into groups spanning elevation zones and survey periods for DNA extractions to avoid batch effects. Samples were homogenized through bead beating on a benchtop vortex for 20 min prior to extraction. I extracted fecal DNA in a clean lab with UV decontamination using a Quick-DNA Fecal/Soil Microbe MiniPrep Kit following the manufacturer’s protocol (Zymo Research, Irvine, CA), with an elution volume of 75 µl. For each group of extractions, I included one negative extraction control and one positive insect control (i.e. mock prey community DNA). The positive insect control was created by selecting five insect families collected from Malaise traps that represent known prey items of black-throated blue warblers at Hubbard Brook (Cantharidae, Cerambicidae, Curculionidae, Ichneumonidae, Rhagionidae). I extracted DNA from the legs and heads of insects using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany), with an elution volume of 100 µl.

I used a two-step PCR protocol, using the ANML primers (Jusino et al. 2019) to amplify a 200 base pair sequence of the mitochondrial gene, cytochrome oxidase C subunit 1 (COI), a mitochondrial marker frequently used in avian diet analyses to identify dietary components (Hoenig et al. 2022). The ANML primer pair has been shown to be more effective than other primers such as ZBJ because it has better taxonomic coverage (Jusino et al. 2019, Forsman et al. 2022). Forward and reverse ANML primers were modified for metabarcoding with the addition of TruSeq tails for use in the second PCR. I ran PCRs on 96-well plates including fecal samples, field blanks, negative extraction controls, one positive insect control, and two no-template negative controls. PCR reactions were performed in 25 µl reactions with 12.5 µl AmpliTaq Gold 360 Master Mix (Applied Biosystems, Waltham, MA), 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM) and 10.5 µl template, and 10.5 µl water for the no-template negative control. The positive insect control contained 9.5 µl water and 1 µl mock prey community DNA, which was made by mixing 10 µl of each of the 5 insect families chosen. I ran PCRs in duplicate to minimize error from false negatives or poor amplification (Taberlet 1996). Thermocycling conditions were: an initial denaturation step at 95ºC for 10 min followed by 35 cycles of 95ºC for 30 s, 50ºC for 30 s, 72ºC for 30 s, and a final extension of 72ºC for 7 min. PCR products were visualized using gel electrophoresis and duplicate PCR runs were combined and diluted prior to sequencing at the Hubbard Center for Genome Studies (Durham, New Hampshire). Prior to sequencing, a second-step PCR was performed at the sequencing facility to add unique dual-barcoded indexes to each sample, while sequencing was performed using 5% of a NovaSeq lane.

***Bioinformatics***

I processed the sequences using Qiime2 v2021.4 (Bolyen et al. 2019). I first checked the read quality and number of reads per plate, before trimming primers from both the 3’ and the 5’ ends of the reads using the *cutadapt* plugin (Martin 2011). Next, I filtered out low-quality reads and denoised the reads using the *DADA2* plugin (Callahan et al. 2016), truncating the length of sequences to 130 bp and specifying a minimum overlap of 50 bp between the forward and reverse reads. After denoising, I merged the sequence reads from each plates of samples and assigned taxonomy to the sequences using a pre-trained COI classifier. This naïve Bayes classifier was trained on COI sequences downloaded and curated from the Barcode of Life Database (BOLD) using the *RESCRIPt* plugin (Robeson et al. 2021) and trimmed to the ANML region by O’Rourke et al. (2020). Following taxonomy assignment, I removed non-arthropod reads (0.7% of reads) and calculated alpha-rarefaction curves to determine rarefaction depth. The non-arthropod reads were identified as either unassigned to any taxon, undetermined Animalia, Fungi, Nematoda, Tardigrada, Heterokontophyta (Protozoa), Mollusca, or Rotifera, and I omitted them because the ANML primers are primarily suitable for detecting arthropod COI sequences. We used the mock community to confirm correct taxonomic assignment of major known prey families. Prey species were identified against prey known to occur at Hubbard Brook. For any species that had not been observed at Hubbard Brook, we used BLASTn (NCBI) to determine certainty percentage and query cover. If either metric was below 95%, we adjusted the taxonomic identification so that it was above the 95% threshold.

***Statistical Analyses***

We characterized black-throated blue warbler diets using presence-absence data to reduce biases associated with sequence read abundances (Deagle et al. 2019, Jusino et al. 2019). To test our hypotheses about factors affecting diet composition, we performed PERMANOVA at the taxonomic level of prey families using the *adonis2* function from the *vegan* package in R version 4.2.2 (Oksanen et al. 2019, R Core Team 2022). The right-hand side of the formula included independent variables for survey period, elevation, age, and the interaction between survey period and elevation. We controlled for potential differences between sexes using a blocked design where permutations are constrained within each sex. We investigated differences in multivariate dispersion between groupings of samples using the *betadisper* function in *vegan* (Anderson et al. 2006), followed by Tukey tests to conduct pairwise comparisons of mean dispersions. This approach tests for heterogenous variances between groups using the average distance of group members to a group-specific centroid in multidimensional space. Next, we visualized dietary niche space using non-metric multidimensional scaling (NMDS). All comparisons used the modified Raup-Crick dissimilarity index for presence-absence data (Chase et al. 2011). We summarized the frequency of occurrence for each prey family separately for the early, mid, and late survey periods.

We compared diet richness and diversity between survey periods using rarefaction curves to interpolate to the lowest common sample size (n = 24; R package iNEXT; Hsieh et al. 2020). All comparisons used species-level prey taxonomies to generate estimates of diet richness, Shannon diversity (exponentiated Shannon entropy), and Simpson diversity (inverse Simpson concentration). We used 95% confidence bands around estimates to interpret differences between groups (Chao et al. 2014). In addition, we tested for differences in per-sample prey species richness for the early, mid, and late survey periods using a Kruskal-Wallice test.

In our study, warbler fecal sampling accompanied two field efforts to sample arthropod communities along a gradient of seasons and elevations: visual caterpillar surveys and Malaise trap surveys. We used these survey data to test the hypothesis that seasonal changes in warbler diets reflect changes in the underlying availability of arthropod prey. First, we used the caterpillar survey results to calculate the proportional biomass of three Lepidopteran families in each survey period (proportional biomass = family-specific biomass / total biomass \* 100). Plotting the changes in fecal frequency of occurrence and proportional biomass between survey periods highlights areas where diet tracks availability or, conversely, where foraging birds may select certain prey relative to availability. Second, we used a linear modeling approach to compare Malaise survey results to fecal samples. We grouped fecal samples into the same 14 taxonomic groups employed by the Malaise survey and calculated a taxon-specific frequency of occurrence for each survey period. Next, we calculated the Malaise capture frequency for each taxon by dividing the number of captures for each taxon by the total captures. Here, values represent the percent of the total captures in each survey period. Using each taxon and survey period combination as a sampling unit (n = 42), we modeled the linear relationship between fecal frequency of occurrence and Malaise capture frequency. A positive slope in this model indicates that prey in the diet generally track availability, and residual outliers show evidence for selection or avoidance of specific taxa.

**Results**

From May–Aug in 2021, I collected fecal samples from 99 adult black-throated blue warblers and successfully recovered arthropod DNA from 98 of the samples (Table 1). Illumina sequencing generated 34 million COI sequences with 25 million of these reads passing quality filters, from which we identified 395 taxa in the phylum Arthropoda. These represented 17 arthropod orders, 121 families, and 249 were identified to the species level. Most taxa were rare; 192 (48.6%) of the taxa were detected in only one sample. All orders in the mock community were correctly identified.

***Diet Composition***

The family-level composition of black-throated blue warbler diets showed significant variation between the three sampling periods (Table 1). NMDS ordination generated a 3-dimensional solution (stress = 0.23) which highlighted differences in diet composition for the late survey period compared to early and mid (Figure 1a). Diet composition also showed strong differences between age groups and weak differences between elevations (Figure S1). There was no interaction effect between period and elevation. Variation within diets differed based on survey period but not elevation or age (Table 1). Birds had increased diet variability as the season progressed, and pairwise comparisons between periods demonstrated that mean multivariate dispersion was greater in the late survey period compared to the early (p = 0.042) and mid (p < 0.001) periods (Figure 1b).

LINDSEY, TEXT TO ADD: Next, it’s time to add 1-2 paragraphs with the important descriptive results – similar to what you’ve already done for your honors thesis. The goal here is to highlight any important patterns in FOO results and diet composition.

* Reference the FOO table and highlight the families with especially high occurrence. Let me know if you need total FOO calculations (i.e., not split up by period).
* Bring up any special cases worth noting. E.g., is it worth reporting the invasive taxa you found in the samples? Anything especially surprising or unexpected?
* Many taxa showed patterns between seasons. Reference Figure 2.

If you want to include the full family-level FOO table or a species-level FOO table in the supplement, reference it here.

Lepidoptera was the most frequently detected order, occurring in 100% of samples, and Notodontidae was the most frequently detected family, occurring in 90.8% samples. At the species level, the species with the highest frequency of occurrence in black-throated blue warblers diets were: 1*)* *Heterocampa guttivitta* (saddled prominent; FOO = 83.3%) and *Philodromus rufus* (a running crab spider; FOO = 75.0%) during the early survey period, 2) *Heterocampa guttivitta* (90.3%) and *Orthofidonia exornata* (a geometrid moth; FOO = 62.5%) during the mid survey period, and 3) *Heterocampa guttivitta* (FOO = 96.9%) and *Theridion frondeum* (Eastern long-legged cobweaver; FOO = 90.6%) during the late survey period. At each elevation zone, the species with the highest frequency of occurrence were: 1) *Heterocampa guttivitta* (FOO = 90.0%) and *Theridion frondeum* (FOO = 56.7%) at low elevation, 2) *Heterocampa guttivitta* (FOO = 89.7%) and *Philodromus rufus* (FOO = 79.3%) at mid elevation, and 3) *Heterocampa guttivitta* (FOO = 92.9%) and *Theridion frondeum* (FOO = 92.9%) at high elevation. The species with the highest frequency of occurrence in each age class were: 1) *Heterocampa guttivitta* (FOO = 96.0%) and *Theridion frondeum* (FOO = 68.0%) for HY, 2) *Heterocampa guttivitta* (FOO = 80.0%) and *Orthofidonia exornata* (FOO = 80.0%) for SY, and 3) *Heterocampa guttivitta* (FOO = 84.4%) and *Theridion frondeum* (FOO = 56.3%) for ASY.

The frequency of occurrence of several prey species differed across the three survey periods and elevation zones (Fig. 3a-b), potentially driving the observed differences in dietary composition. *Philodromus rufus* (a running crab spider) increased in frequency in diets from the early to late survey periods. *Rhagio mystaceus* (the common snipe fly) were most frequent in diets during the late survey period with lower frequencies during the early and mid survey periods. *Helophora insignis* (a sheetweb spider) and *Deraeocoris grandis* (a plant bug) were more frequent during the early survey period with lower frequencies during the mid and late survey periods. Comparing elevation zones, *Eutrapela clemataria* (a curved-toothed geometer moth) and *Tetragnatha shoshone* (an American orbweaver spider) were rare at the high elevation zone but more frequent at the low and mid elevation zones. *Pandemis lamprasona* (the woodgrain leafroller moth)*, Rhagio mystaceus,* and *Philodromus rufus* were more frequent at the mid elevation zone compared to low and high elevation zones. Lastly, *Helophora insignis* and *Deraeocoris grandis* were both most frequent at the high elevation zone.

***Diet Richness and Diversity***

Individual samples contained a median of five orders (range = 2–9, *n* = 17), 12 families (range = 4–26, *n* = 121), and 12 species (range = 3–25, *n* = 249). The number of prey species present in each sample showed weak differences between survey periods (χ2 = 5.25, df = 2, p = 0.072) with the highest per-sample richness in samples from early-season surveys (Figure S2). For all samples combined, rarefied prey richness estimates were substantially higher in the early survey period (203 species, n = 24) and lower in the mid (164 species, interpolated n = 24) and late (165 species, interpolated n = 24) survey periods (Table S1). Similarly, Shannon and Simpson diversity of prey items was highest in the early survey period, although Simpson diversity values showed considerable overlap in confidence intervals between groups (Table S1).

***Comparisons to arthropod surveys***

LINDSEY TO ADD: Might be good to start each section off with a descriptive summary of the Malaise or lep survey data (totals, most common captures, etc.)? It’s up to you whether we break this into 2 paragraphs or include it as a single paragraph. I included the relevant statistical results text below.

[ADD: 1-2 sentences to summarize the Lep survey]

The proportional biomass of Geometridae and Notodontidae caterpillars increased throughout the season, yet the high occurrence of these taxa in warbler diets remained notably consistent (Figure 3). This pattern represents potential selection for these prey items early in the breeding season when availability is relatively low. Caterpillars from the family Noctuidae showed similar temporal patterns between survey periods, although the occurrence of Noctuids in fecal samples was much lower than expected giving the high availability of this prey item (Figure 3).

[ADD: 1-2 sentences to summarize the Malaise survey]

Among the 14 sampled taxa, the frequency of occurrence in warbler diets showed a strong, positive relationship with survey-based capture frequencies (Figure S3, slope = 0.23, 95% CI = 0.15, 0.31). Here, a positive slope supports a general pattern of diet composition tracking the availability of prey. The order Hemiptera demonstrated notable deviations from this pattern; prey items from this order occurred in fecal samples at much higher rates than expected from Malaise surveys (linear model residuals < -11). Insects from the orders Diptera and Hymenoptera tended to occur in fecal samples less than expected from Malaise surveys. These results provide evidence that black-throated blue warbler diets respond to seasonal changes in the availability of prey, yet several taxa may be consistently selected or avoided throughout the breeding season.

**Discussion**

Insectivorous, migratory birds are expected to take advantage of a lengthening green season by extending their breeding season depending on whether insect availability is sufficient to support raising late season broods and the flexibility in their diets to respond to effects of changing seasonality on insect availability. I examined how the diet composition and diversity of multiple-brooded, black-throated blue warblers varied over the green season (early, mid, and late survey periods) and across an elevation gradient that encompasses a two-week difference in green season length (low, mid, and high elevation zones), as well as differences based on age. I then examined associations between spatial and temporal variation in diet and insect availability. My results showed that both survey period and age had significant effects on black-throated blue warbler diet composition, and there was a significant difference in overall diversity between the early and mid periods. Differences in species richness between survey periods were weak but present. . These patterns provide a framework for understanding the capacity of black-throated blue warblers to respond to potential changes in insect availability over a lengthening green season.

The lower diet diversity that I found in the late survey period is especially relevant for multi-brooded birds that could raise second broods depending on food resources late in the breeding season (Nagy and Holmes 2005a). Field-based food supplementation studies, studies of “natural” food supplementation (i.e. insect outbreaks), and studies measuring per-territory food availability have shown that higher food availability increases the likelihood of double brooding in insectivorous birds (Nagy and Holmes 2005a, Norris and Martin 2014, Seward et al. 2014, Kaiser et al. 2015). Lower diet diversity during the late survey period could present a barrier to multi-brooded species for extending their breeding and increasing their reproductive fitness. While it does not appear that prey diversity or prey abundance is lower in the late survey period according to insect data from Malaise traps and caterpillar surveys, the lower diversity in the diets suggests that black-throated blue warblers are relying more heavily on fewer species later in the breeding season. Thus, although food abundance does not appear to be lower late in the breeding season under current environmental conditions, the lower diet diversity could become prevent black-throated blue warblers from raising second broods if the prey species they rely on experience declines due to climate change. An alternative explanation for the low diet diversity in the late survey period is that adults were primarily feeding on particular prey. Specifically, insect sampling showed that the peak availability of Geometridae and Notodontidae occurred in the late survey period. At Hubbard Brook, caterpillar availability is highly variable among years with no predictable seasonal peak (Lany et al. 2016) because of a high diversity of caterpillars that use different environmental cues to transition between different stages of their life cycles (Stange et al. 2011). Incorporating fecal diet analyses into the long-term demographic study of the black-throated blue warbler will be important to document changes in diet and insect diversity late in the breeding season.

Diet composition differed significantly among survey periods, suggesting that black-throated blue warblers are able to be flexible in their diets based on pulses in insect availability that vary over time. Studies have shown that even in systems that do have strong pulses in caterpillar abundance, birds are able to utilize diet flexibility to avoid the effects of phenological mismatch with their prey (Mallord et al. 2017), a phenomenon which is exacerbated by climate change. Given the incredible arthropod diversity at Hubbard Brook (Stange et al. 2011) and the lack of seasonal peaks in caterpillar abundance (Lany et al. 2016), black-throated blue warblers might benefit even more from this dietary flexibility. It is important to note that in frequency of occurrence data, the importance of certain rare prey items can be overstated as FOO data is based on presence-absence data and not abundance. The relative importance of different prey in each individual diet is not determined because a rare prey item is treated equally to a common prey item detected in any given fecal sample. This issue becomes more pronounced in cases where an organism is consistently eating small amounts of a rare prey item such as a grazing herbivore (Deagle et al. 2019). For more selective insectivorous birds, the issue would not be as pronounced, so frequency of occurrence data still gives meaningful insight into how important certain taxa are across samples, and is therefore very useful in determining diet composition.

Diet diversity and composition did not differ significantly across elevation zones. Although the study plot encompasses a 2-wk difference in green season length across an elevation gradient, the differences in elevation (low: 382–499 m, mid: 500–599 m, high: 600–740 m) were potentially not broad enough to detect significant differences in arthropod diversity or composition in diets. For example, a fecal DNA metabarcoding study of blue tits (*Cyanistes caeruleus*) in Scotland with a similar elevation gradient as in this study showed that dietary richness (i.e. number of species present) did not vary with elevation (Shutt et al. 2020). Few studies have examined the diets of insectivorous birds over an elevation gradient to compare patterns, and even fewer over an elevation gradient larger than in this study such as in high mountain elevations (Sam et al. 2017). Therefore, it is unclear whether diet diversity would differ over a larger elevation gradient. Similar to dietary richness, the elevation gradient may not be significant enough to alter the composition of the arthropod community at this scale. Few studies have examined how diet composition differs over elevation gradients to compare to this study. Studies examining how diet diversity and composition varies across elevation gradients are needed, as one of the primary responses of migratory songbirds to climate change is to shift breeding distribution up in elevation (Rodenhouse et al. 2008, Tatenhove et al. 2019).

The spatial and temporal variation in the diets of black-throated blue warblers that I found – particularly in their primary prey, Lepidoptera – somewhat reflect the availability of insects sampled across elevation zones and over the breeding season. For example, the frequency of occurrence of Geometrids in diets was lower both during the mid survey period and at the mid elevation zone, which mirrored the same pattern in Geometrid availability documented on caterpillar surveys across elevation zones. The frequency of occurrence of Noctuidae in diets was lower relative to Geometridae and Notodontidae, which reflected the comparatively low sampling of Noctuids on caterpillar surveys. Black-throated blue warblers are known to feed primarily on Lepidoptera larvae (Robinson and Holmes 1982, Holmes et al. 1986). It follows that the frequency of occurrence of different families of Lepidoptera in their diets would match trends in Lepidoptera families sampled on caterpillar surveys. The similar trends in the diet and caterpillar availability also suggest that adults exhibit flexibility in their caterpillar consumption based on what is available at a given elevation zone or at a given time.

Comparing arthropods sampled in Malaise traps, most arthropods had mean daily sampling rates that were comparatively lower than their frequency of occurrence in the diets, with the exception of Diptera. The four taxa in the Malaise samples with the highest frequency of occurrence in the diets were: Araneae, Tipuloidea, Lepidoptera, and Diptera. Araneae (spiders) had an especially low mean daily sampling rate in comparison to its frequency of occurrence in the diet. However, Malaise traps are designed to capture flying insects which explains why they were largely underrepresented. Lepidoptera also had a low mean daily sampling rate in comparison to the frequency of occurrence in the diet, which is likely due to the fact that a large portion of the Lepidoptera consumed by black-throated blue warblers are larval, which would also not be present in Malaise traps. Interestingly, Tipuloidea (crane flies) had a very high frequency of occurrence in the diets but a relatively low mean daily sampling rate, suggesting that adults are selecting for crane flies even at lower abundance. It is also possible that Malaise traps are simply inadequate for sampling crane flies, however this is unlikely given that Malaise traps are commonly used in studies of crane flies (Autio et al. 2013), and because Malaise traps have been shown to be one of the most effective ways to survey Diptera, the order which includes crane flies (Montgomery et al. 2021). In general, the trends in frequency of occurrence in diets did not clearly correspond with trends in mean daily sampling rate in the Malaise traps across elevation zones and survey periods.

We did find significant differences in diet composition between the three age classes. Althoughthe sample size of HY birds was small (*n* = 5) compared to SY (*n* = 49) and ASY (*n* = 31) and all HY samples were collected in the late survey period, the results of the PERMANOVA were consistent even when removing HY birds from the analysis. We lack avian studies on how diet varies by age in adult birds. However, we would only expect SY and ASY black-throated blue warblers to have different diets if they were foraging in territories that differed in insect diversity and composition and if younger birds were less specialized on particular prey because of lower foraging success and experience.

Contrary to our predictions, diet diversity and composition did not differ by breeding stage. I had expected birds to be more selective on Lepidoptera larvae while feeding young. Studies have shown that caterpillars comprise 60-87% of prey biomass brought to black-throated blue warblers nestlings (Goodbred and Holmes 1996), thus I expected their diets to be less diverse during the parental stage and be comprised of mostly Lepidoptera. However, my results suggest that adults may select the same prey items to feed their young that they eat themselves throughout all breeding stages. Need to say that it’s unlikely that they would expend energy foraging for different prey than what they feed their young, but not sure what to cite.

Fecal DNA metabarcoding allowed us to examine the diets of black-throated blue warblers at a specificity that is not possible to replicate in observational studies. We were able to identify 249 species in 98 fecal samples. While it is possible to classify prey items at the species-level by examining fecal samples or stomach contents directly, it is challenging and time consuming even for expert taxonomists, and certain arthropod parts may be degraded (Hoenig et al. 2022). In addition, fecal DNA metabarcoding is minimally invasive in comparison to other methods such as emetics studies, which can be harmful to birds (Carlisle and Holberton 2006). Although DNA metabarcoding does not provide information on prey abundance, we can utilize frequency of occurrence data to understand the relative importance of different prey items based on the percentage of diets in which each taxon occurs (Deagle et al. 2019). These data can help us to better understand how declines in specific prey might affect black-throated blue warbler.

To better understand how the community may respond to a lengthening green season, it would be informative to examine the diets of other insectivorous songbirds with differing life histories. Ovenbirds (*Seiurus aurocapilla*) and wood thrushes (*Hylocichla* mustelina) utilize the brown, forest floor food web, whereas black-throated blue warblers utilize the green, foliar food web. Ovenbirds are typically single-brooded (Porneluzi et al. 2020), whereas double-brooding is common in black-throated blue warblers at Hubbard Brook (30% of females; Holmes et al. 2020a), as well as in wood thrushes (INSERT FRIESEN ET AL CITATION HERE). As these species differ in their propensity to double-brood and have differing reliance on the green and brown food webs, they may respond differently to a longer green season. Understanding their diets and how they differ would help us make predictions about their resilience to climate-induced changes in green season length.

**Conclusions**

Black-throated blue warblers demonstrated differences in diet diversity between the mid and late survey periods, as well as differences in diet composition across survey periods and elevation zones. Trends in the frequency of occurrence of Lepidoptera families in the fecal samples reflected their availability in our insect sampling, particularly that of Geometridae and Noctuidae, demonstrating flexibility in prey choice based on availability. DNA metabarcoding is a powerful tool to assess avian diets at levels of specificity that are not achievable by other diet analysis methods. It will be important to continue to examine within-season variation in the diets of black-throated blue warblers as well as assess between-year variation to examine the effects of a lengthening green season on diet. Insectivorous birds are being affected by insect population declines worldwide (Tallamy and Shriver 2021), and this study shows black-throated blue warblers’ ability to be flexible in their diet in response to spatial and temporal pulses in food availability, which may help shield them from some of the effects of these insect declines.

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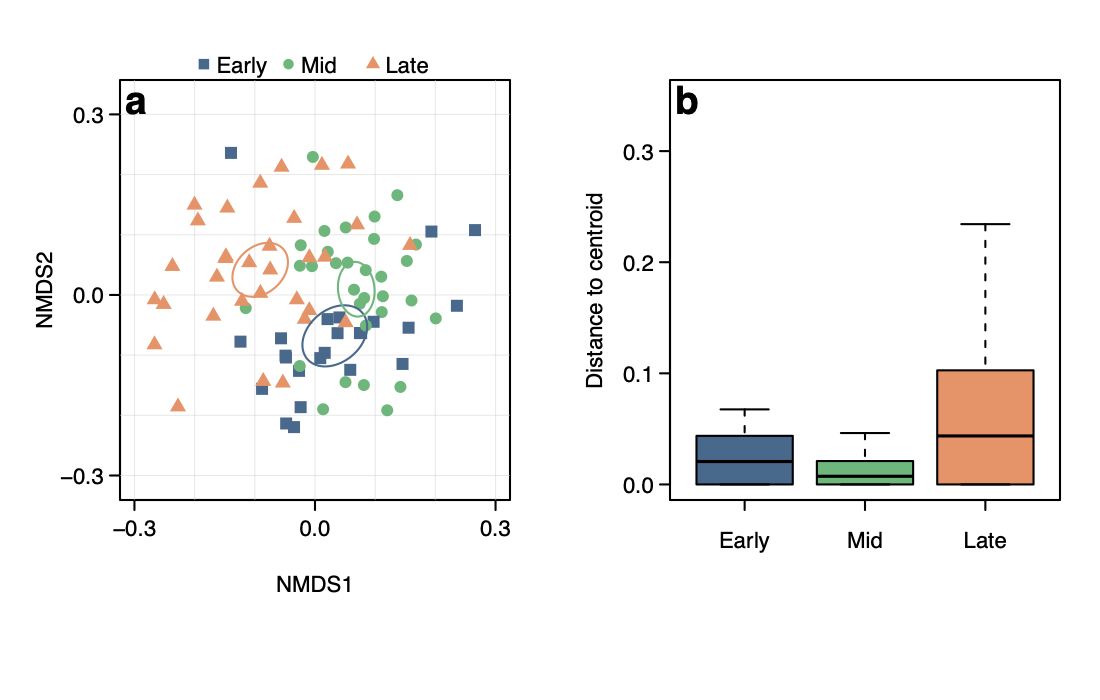
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| **Table 1.** Sample sizes of black-throated blue warbler fecal samples by elevation zone, survey period, age class, sex, and breeding stage at the Hubbard Brook Experimental Forest, New Hampshire, USA. | | |
| **Variable** | **Group** | ***N*** |
| Elevation zone | Low | 33 |
|  | Mid | 32 |
|  | High | 34 |
|  |  |  |
| Survey period | Early | 29 |
|  | Mid | 34 |
|  | Late | 36 |
|  |  |  |
| Age class | HY | 5 |
|  | SY | 57 |
|  | ASY | 37 |
|  |  |  |
| Sex | Male | 66 |
|  | Female | 33 |
|  |  |  |
| Breeding stage | Fertile | 8 |
|  | Incubation | 45 |
|  | Parental | 21 |
|  | Unknown | 25 |

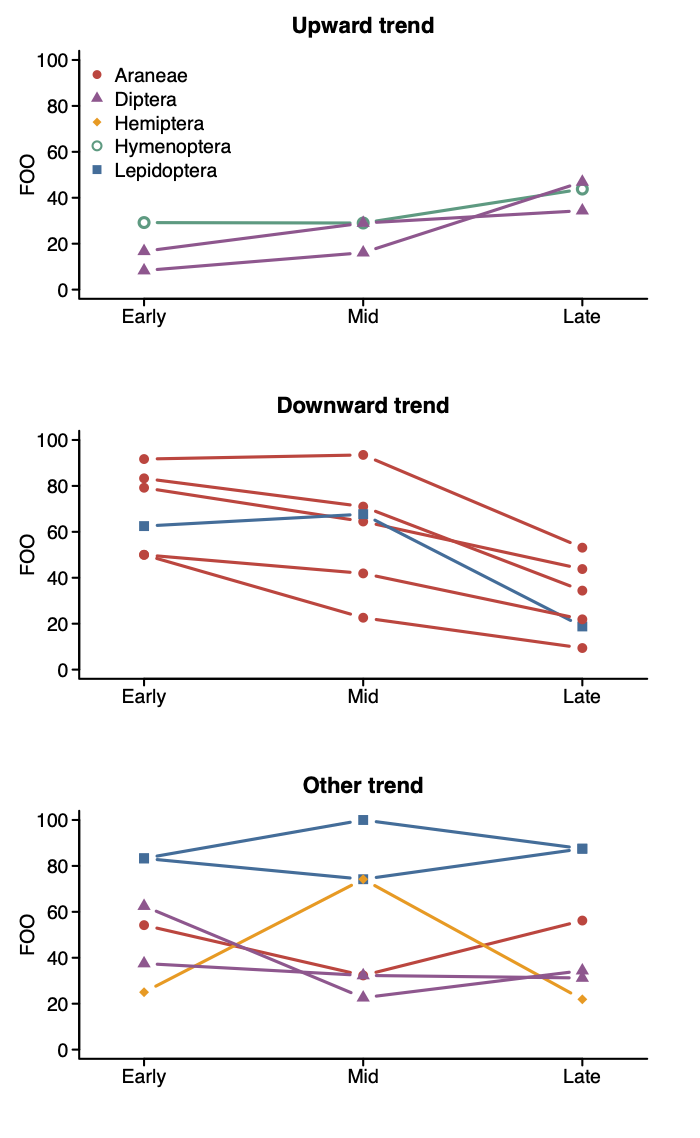
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| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Table 2.** Frequency of occurrence (%) of 21 prey species identified in the diets of black-throated blue warblers by survey period at the Hubbard Brook Experimental Forest, New Hampshire, USA. Each line represents a different species, identified to the highest taxonomic rank possible from the Barcode of Life Database (BOLD). | | | | | | | | | |
|  |  |  |  |  |  |  | **Survey Period** | | |
|  |  |  |  |  |  | **Total** | **Early** | **Mid** | **Late** |
| **Phylum** | **Class** | **Order** | **Family** | **Species** | **Common Name** | ***n* = 99** | ***n* = 29** | ***n* = 34** | ***n* = 36** |
| Arthropoda | Insecta | Lepidoptera | Notodontidae | *Heterocampa guttivitta* | Saddled prominent moth | 79 | 83.3 | 100 | 87.5 |
| Arthropoda | Arachnida | Araneae | Theridiidae | *Theridion frondeum* | Eastern Long-legged cobweaver | 53 | 70.8 | 93.5 | 21.9 |
| Arthropoda | Arachnida | Araneae | Philodromidae | *Philodromus rufus* | Running Crab spider | 47 | 75.0 | 64.5 | 28.1 |
| Arthropoda | Insecta | - | - | *-* | Insect | 43 | 62.5 | 41.9 | 46.9 |
| Arthropoda | Insecta | Lepidoptera | - | - | Butterfly/Moth | 33 | 54.2 | 51.6 | 12.5 |
| Arthropoda | Arachnida | Araneae | Araneidae | *Cyclosa conica* | Conical trashline orbweaver | 33 | 70.8 | 32.3 | 18.8 |
| Arthropoda | Arachnida | Araneae | Dictynidae | *Emblyna maxima* | Cribellate araneomorph spider | 30 | 45.8 | 41.9 | 18.8 |
| Arthropoda | Insecta | Lepidoptera | Geometridae | *Orthofidonia exornata* | Geometrid moth | 28 | 4.2 | 25.8 | 59.4 |
| Arthropoda | - | - | - | - | Arthropod | 28 | 37.5 | 6.5 | 53.1 |
| Arthropoda | Insecta | Lepidoptera | Tortricidae | *Pandemis lamprosona* | Woodgrain leafroller moth | 27 | 33.3 | 51.6 | 9.4 |
| Arthropoda | Insecta | Lepidoptera | Geometridae | - | Geometrid moth | 26 | 4.2 | 25.8 | 53.1 |
| Arthropoda | Insecta | Diptera | Cecidomyiidae | - | Gall gnat | 25 | 37.5 | 29 | 21.9 |
| Arthropoda | Arachnida | Araneae | Tetragnathidae | *Tetragnatha shoshone* | Spider | 22 | 50.0 | 22.6 | 9.4 |
| Arthropoda | Insecta | Hemiptera | Miridae | *Deraecoris grandis* | Plant bug | 20 | 16.7 | 48.4 | 3.1 |
| Arthropoda | Arachnida | - | - | - | Spider | 20 | 12.5 | 12.9 | 40.6 |
| Arthropoda | Arachnida | Araneae | Linyphiidae | *Helophora insignis* | Sheetweb spider | 20 | 4.2 | 12.9 | 46.9 |
| Arthropoda | Arachnida | Araneae | Philodromidae | *Philodromus praelustris* | Running crab spider | 20 | 37.5 | 19.4 | 15.6 |
| Arthropoda | Arachnida | Araneae | Araneidae | *Araneus saevus* | Fierce orbweaver | 20 | 33.3 | 9.7 | 28.1 |
| Arthropoda | Arachnida | Araneae | Araneidae | *Araneus guttulatus* | Red-backed orbweaver | 20 | 12.5 | 35.5 | 18.8 |
| Arthropoda | Arachnida | - | - | - | Spider | 19 | 12.5 | 19.4 | 31.2 |
| Arthropoda | Arachnida | Araneae | Linyphiidae | *Pityohyphantes costatus* | Hammock spider | 18 | 37.5 | 16.1 | 12.5 |

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Table 3.** Frequency of occurrence (%) of 15 prey families identified in the diets of black-throated blue warblers by survey period at the Hubbard Brook Experimental Forest, New Hampshire, USA. | | | | | | | |
|  |  |  |  |  | **Survey Period** | | |
|  |  |  |  | **Total** | **Early** | **Mid** | **Late** |
| **Class** | **Order** | **Family** | **Common Name** | ***n* = 99** | ***n* = 29** | ***n* = 34** | ***n* = 36** |
| Insecta | Lepidoptera | Notodontidae | Prominent Moths | 79.0 | 83.3 | 100 | 87.5 |
| Insecta | Lepidoptera | Geometridae | Geometer Moths | 71.0 | 83.3 | 74.2 | 87.5 |
| Arachnida | Araneae | Theridiidae | Cobweb Spiders | 68.0 | 91.7 | 93.5 | 53.1 |
| Arachnida | Araneae | Araneidae | Orbweavers | 53.0 | 79.2 | 64.5 | 43.8 |
| Arachnida | Araneae | Philodromidae | Running Crab Spiders | 53.0 | 83.3 | 71.0 | 34.4 |
| Insecta | Lepidoptera | Tortricidae | Tortricid Leafroller Moths | 42.0 | 62.5 | 67.7 | 18.8 |
| Arachnida | Araneae | Linyphiidae | Sheetweb and Dwarf Weavers | 41.0 | 54.2 | 32.3 | 56.2 |
| Insecta | Hemiptera | Miridae | Plant Bugs | 36.0 | 25.0 | 74.2 | 21.9 |
| Insecta | Diptera | Rhagionidae | Snipe Flies | 33.0 | 62.5 | 22.6 | 34.4 |
| Arachnida | Araneae | Dictynidae | Meshweavers | 32.0 | 50.0 | 41.9 | 21.9 |
| Insecta | Hymenoptera | Ichneumonidae | Ichneumonid wasps | 30.0 | 29.2 | 29.0 | 43.8 |
| Insecta | Diptera | Cecidomyiidae | Gall and Forest Midges | 29.0 | 37.5 | 32.3 | 31.2 |
| Insecta | Diptera | Tachinidae | Bristle Flies | 24.0 | 16.7 | 29.0 | 34.4 |
| Insecta | Diptera | Mycetophilidae | Fungus Gnats | 22.0 | 8.3 | 16.1 | 46.9 |
| Arachnida | Araneae | Tetragnathidae | Long-jawed Orbweavers | 22.0 | 50.0 | 22.6 | 9.4 |

**Figure 2.** NMDS plot of diet composition shows limited overlap between the diets of black-throated blue warblers between survey periods at the Hubbard Brook Experimental Forest, New Hampshire, USA. Points represent diet composition of individuals based on species. Ovals represent standard error (???).



**Figure 3.** Frequency of occurrence (FOO) of top 15 prey families (families that occur in >20 samples) in the diets of black-throated blue warblers across survey periods at the Hubbard Brook Experimental Forest, New Hampshire, USA.



**Figure 4.** Comparison of the frequency of occurrence of prey items in the diets of black-throated blue warblers with proportional biomass of target Lepidoptera families caterpillar surveys across survey periods at the Hubbard Brook Experimental Forest, New Hampshire, USA.

