Appendix 1.

Stable Isotope Analysis and DNA Metabarcoding Reveals Elevational Shifts in Diet of a Montane Breeding Bird

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Supplementary material.

Supplementary Table 1. Total raw metabarcoding DNA contributions to fecal samples from 40 Swainson's Thrush (*Catharus ustulatus*) collected from the White Mountains, New Hampshire in 2019. Data includes the Order, Family, elevation (m), count, and a categorical variable, elevation bin (see dryad data depository).

Supplementary Table S2. Dissimilarity results from familial contributions of fecal samples from 40 Swainson's Thrush (*Catharus ustulatus)* collected from the White Mountains, New Hampshire in 2019. Percent values (%) describe how much each invertebrate family contributed to the difference between elevation bins. We included the top five families that contributed the most to the dissimilarity between elevations. Values were only included if they contributed to >3% of the dissimilarity between sites. For example, Scirtidae (marsh beetles) contributed to 73% of the dissimilarity between 280 m and 500 m sites.

		Sites					
Order	Family	280m vs 500m	280m vs 800m	280m vs 1,200m	500m vs 800m	500m vs 1,200m	800m vs 1,200m
Araneae	Anphaenidae	5%	--	71%†	4%	4%	58%
	Linyphiidae	--	30%	40%	52%	67%†	51%
	Philodromidae	62%†	--	60%	--	51%	--
Coleoptera	Byturidae	70%†	61%†	51%	--	--	71%†
	Cerambycidae	--	--	67%†	64%†	49%	61%
	Hydrophilidae	64%†	--	--	61%†	58%	--
	Melandryidae	67%†	--	--	72%†	66%†	--
	Scirtidae	73%†	72%†	58%	--	--	17%
	Tenebrionidae	--	--	3%	69%†	14%	7%
Diptera	Drosophilidae	--	64%†	55%	--	64%†	54%

	Tachinidae	--	69%†	48%	46%	33%	49%
Ephemeroptera	Heptageniidae	17%	8%	26%	66%†	11%	4%
Hemiptera	Cicadellidae	--	--	64%	64%†	71%†	63%†
Hymenoptera	Eulophidae	--	67%†	--	--	--	69%†
Isopoda	Philosciidae	--	--	70%†	--	69%†	65%†
Lepidoptera	Depressariidae	--	--	--	--	--	67%†
	Notodontidae	--	--	66%†	--	--	--

†Families with >70% were considered significant

Supplementary S1. Lab standards for University of New Mexico Stable Isotope Lab (2019 samples), and Cornell Stable Isotope Lab (2021 samples). Mass spectrometry from Cornell was adjusted according to previously standardized equipment from the UNM stable isotope lab.

Carbon and Nitrogen Stable Isotope Ratios

Stable isotopes were expressed in δ notation as parts per thousand (‰) derived from:

$$[(R_{sample}/R_{standard}) - 1]*1000,$$

as deviations from atmospheric nitrogen (standard for N) and Peedee Belemnite (PDM) limestone formation (standard for C). R_{sample} and $R_{standard}$ represent the proportion of heavy to light isotopes in the standard. Samples at both labs were calibrated and normalized against international reference materials provided by the International Atomic Energy Association (IAEA), and the overall standard deviation for both $\delta^{13}C$ and $\delta^{15}N$ was less than ± 0.2 ‰.

University of New Mexico Stable Isotope Lab.

Nitrogen and carbon isotope ratios were measured by Elemental Analyzer Continuous Flow Isotope Ratio Mass Spectrometry in the Center for Stable Isotopes, University of New Mexico using a Costech ECS 4010 Elemental Analyzer coupled to a ThermoFisher Scientific Delta V Advantage mass spectrometer via a CONFLO IV interface. Isotope ratios are reported using the

standard delta (d) notation relative to V-AIR and to Vienna Pee Dee Belemnite (V-PDB), respectively. Three internal, laboratory standards were run at the beginning, at intervals between samples and at the end of analytical sessions. Analytical precision calculated from the standards is ± 0.1 ‰ (1s standard deviation) for both $\delta^{15}N$ and $\delta^{13}C$. Analyses were normalized to the laboratory standards which were calibrated against IAEA N1, IAEA N2 and USGS 43 for $\delta^{15}N$ and NBS 21, NBS 22 and USGS 24 for $\delta^{13}C$. The 3 internal laboratory standards are: UNM-CSI Protein std#1, casein purchased from Sigma Aldrich with $\delta^{15}N$ and $\delta^{13}C$ values of 6.43 and -26.52; UNM-CSI Protein std#2, soy protein purchased from Sigma Aldrich with $\delta^{15}N$ and $\delta^{13}C$ values of 0.98 and -25.78; UNM-CSI protein Std#4, house made tuna protein with $\delta^{15}N$ and $\delta^{13}C$ values of 13.32 and -16.7.

Cornell University Stable Isotope Lab.

Stable isotope analyses were performed on a Thermo Delta V isotope ratio mass spectrometer (IRMS) interfaced to a NC2500 elemental analyzer. The data and quality control standards are summarized as follows: N2 and CO2 Amp (the amplitude of the sample peak in mV of the respective gas), %N and %C (the elemental percentage of these elements based on weight), 15N vs. At Air–this is the corrected isotope delta value* for 15N measured against a primary reference scale. The primary reference scale for $\delta^{15}N$ is Atmospheric Air. $\delta^{13}C$ vs. VPDB – This is the corrected isotope delta value* for 13C measured against a primary reference scale. The primary reference scale for $\delta^{13}C$ is Vienna Pee Dee Belemnite.

Cornell Isotope Laboratory in-house standards are routinely calibrated against international reference materials provided by the International Atomic Energy Association (IAEA). To ensure the accuracy and precision of the instrument an in-house standard is analyzed

after every 10 samples. For this analytical sample run the overall standard deviation for the internal animal standard ('DEER') was 0.08‰ for $\delta^{15}N$ and 0.14‰ for $\delta^{13}C$. We also quantify the ability of our instrument to accurately measure samples across a gradient of amplitude intensities using a chemical Methionine standard. Based on the results of these samples, delta values obtained between the amplitudes of 200mV and 7000mV for $\delta^{15}N$ have an error associated with linearity of 0.32‰ and between 300mV and 6000mV for $\delta^{13}C$ error is 0.18‰. Isotope corrections are performed using a two-point normalization (linear regression) of all $\delta^{15}N$ and $\delta^{13}C$ data using two additional in-house standards ('KCRN' - corn and 'CBT'- trout).

Supplementary S2. Lab methods for DNA sequencing and metabarcoding. This step by step guide to the sequencing procedures was obtained from Mayne (2022), who completed all lab work for our samples at the University of Massachusetts, Amherst. Mayne (2022) graciously combined our fecal samples with his own during lab work—therefore, the text below describes the methods he used, verbatim. Reference: Mayne, Samuel J., "Songbird-mediated Insect Pest Control in Low Intensity New England Agriculture" (2022). Masters Theses. 1164. https://doi.org/10.7275/25887248.0

Lab methods.

Genetic material was extracted from fecal samples using E.Z.N.A. Stool DNA Kit from Omega Bio-tek (Norcross, GA, USA) after a 15 second metal bead homogenization (FastPrep-24, MP Biomedicals, Illkitch, France). The arthropod cytochrome oxidase c subunit I (COI-5P) gene was amplified and indexed in a two-step PCR using ZBJ primers (Zeale et al., 2011) and rhAmpSeq index primers made by Integrated DNA Technologies (Coralville, IA, USA). First

round PCR reactions (25 μL total) included 0.75 μL DMSO, 0.25 μL Phusion High Fidelity Polymerase, 5 μL High Fidelity Buffer (all New England Biolabs, Ipswich, MA, USA), 0.5 μL of 10M dNTP mix (Promega, Madison, WI, USA), 15 μL pure water, 1.25 μL each of 10 μM ZBJ forward and reverse primer, and 1 μL of template DNA from the DNA extraction. Thermocycler conditions were 98°C for 30 sec; 35 cycles of: 98°C for 10 sec, 50°C for 30 sec, 72°C for 30 sec; 72°C for 10 min, and a final hold temperature of 12°C. The index PCR (second round) used the same reaction components, but with the template DNA and ZBJ primers replaced by 1 uL of product from the first round of PCR and 1.25 uL each of 10 μM i5 and i7 rhAmpSeq index primers. Thermocycler conditions for the second round were the same but with only 10 cycles. A bead cleanup was performed between PCR rounds to remove nontarget amplification (primer dimer), using Mag-Bind TotalPure NGS beads and protocol (Omega Biotek, Norcross, GA, USA) at a 0.8:1 bead to PCR product ratio.

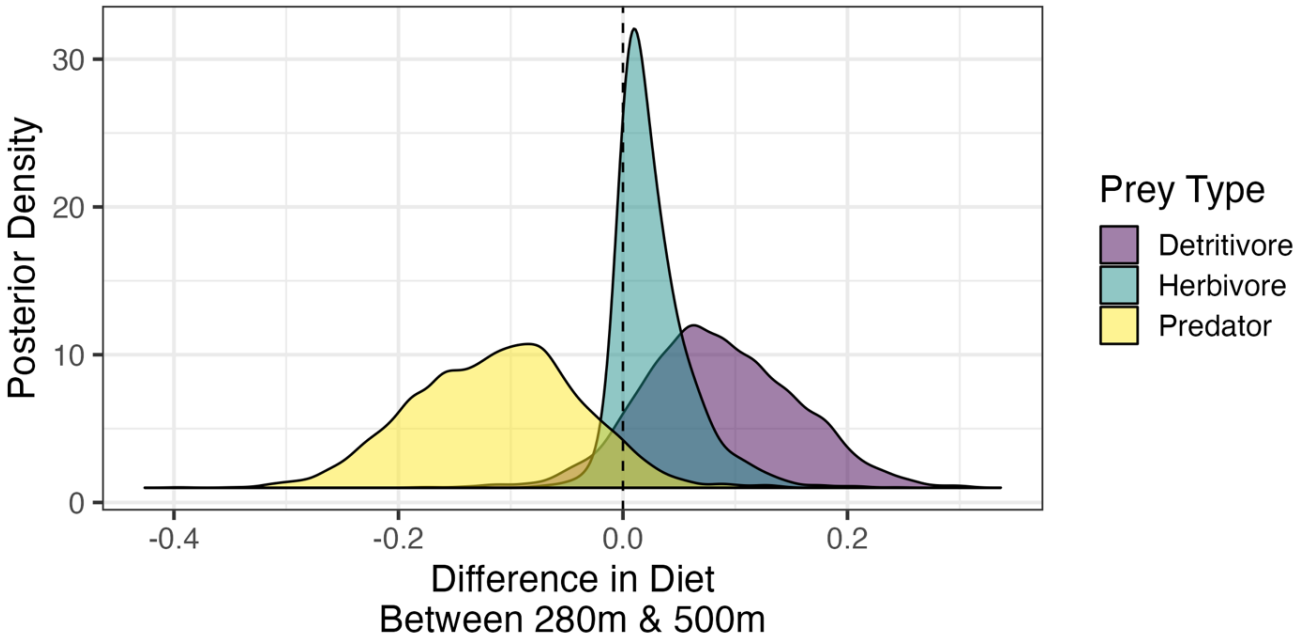
Final PCR products were combined into 4 indexed libraries and cleaned before sequencing. Two to four rounds of bead cleaning (Mag-Bind TotalPure NGS beads, Omega Biotek, Norcross, GA, USA) at a bead to PCR product ratio of 0.85:1 were used to remove nontarget amplification (primer dimer). Between each round of cleaning, 5 μL of the cleaned library was run on a 1.5% agarose gel, and if the nontarget DNA was low enough for sequencing, no more bead cleanups were performed. The four indexed libraries were sequenced by the Genomics Resource Laboratory (University of Massachusetts Amherst, MA 01003) on an Illumina MiSeq Nano v2-500 (Illumina, San Diego, CA, USA). Blank control samples run in parallel with both DNA extractions and

PCR (n = 18), and PCR only (n = 21) were sequenced alongside samples.

Genetic Database Construction.

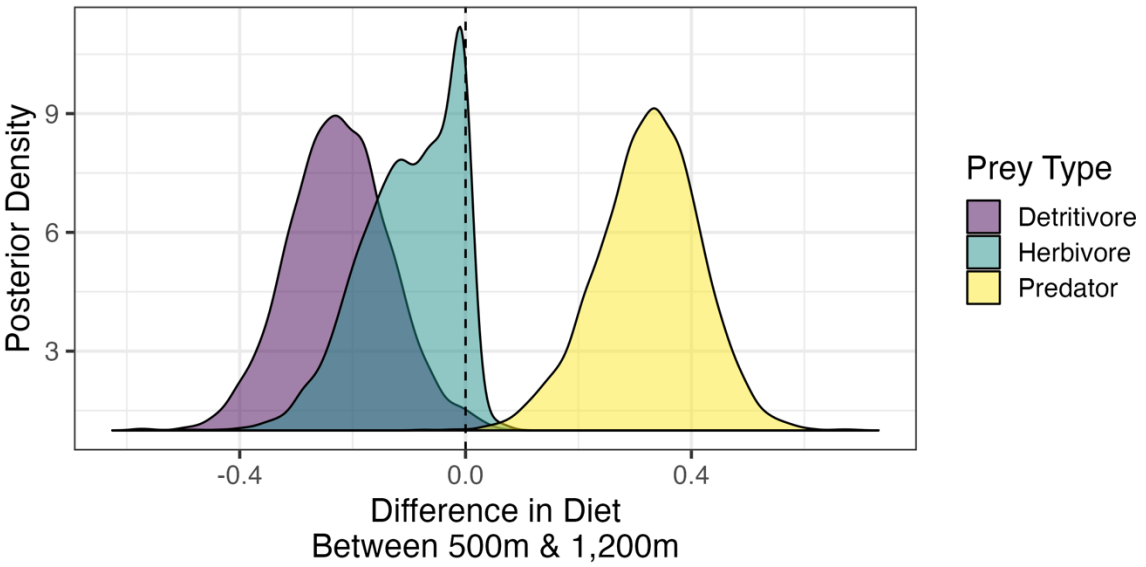
Raw sequencing reads were processed in the QIIME 2 pipeline (Bolyen et al., 2019). Sequences were demultiplexed, denoised, and assigned to amplicon sequence variants (ASVs) using DADA2 (Callahan et al., 2016). A number of quality filters were applied to remove data that were the result of contamination or PCR errors. Samples with fewer than 1000 reads before denoising were removed from analyses. ASVs present in blank control samples, identified as non-Animalia, or with bad sequence lengths (must be 144-162 bp and divisible by 3) were removed from all samples for analyses, and ASVs with a read frequency less than 5 in a given sample were removed from that sample. DNA extractions and PCR amplifications were performed in a laboratory that routinely conducts molecular work focused on the invasive winter moth, Operophtera brumata; therefore, all sequences assigned to this genus were also removed from analyses. ASVs were assigned taxonomic classifications using two naïve-Bayes (Bokulich et al., 2018) classifiers. The "tidybug" reference dataset described by O'Rourke et al. (2020), filtered to include only records from the United States and Canada, was used to train one naïve-Bayes classifier. The tidybug reference dataset includes all COI-5P records from the Barcode of Life Database (BOLD) (downloaded July 2020), filtered for quality, and trimmed to the region amplified by the ANML primers described by (Jusino et al., 2019), which includes the region amplified by the ZBJ primers used in this study. The other naïve-Bayes classifier was trained on untrimmed BOLD records from a selection of northeastern US and Canadian states and provinces, filtered for quality using a custom

Python script (Appendix B). The taxonomic classifications of our sequence library were combined using RESCRIPt (Robeson et al., 2020), maintaining identifications to the level at which both classifiers agreed where there were discrepancies, but with the more specific classification accepted when lower-level classifications agreed. Once ASVs were collapsed to taxonomic levels and converted to presence-absence, all data were exported to R (R Core Team, 2021) for statistical analysis using the vegan (Oksanen et al., 2020) package.

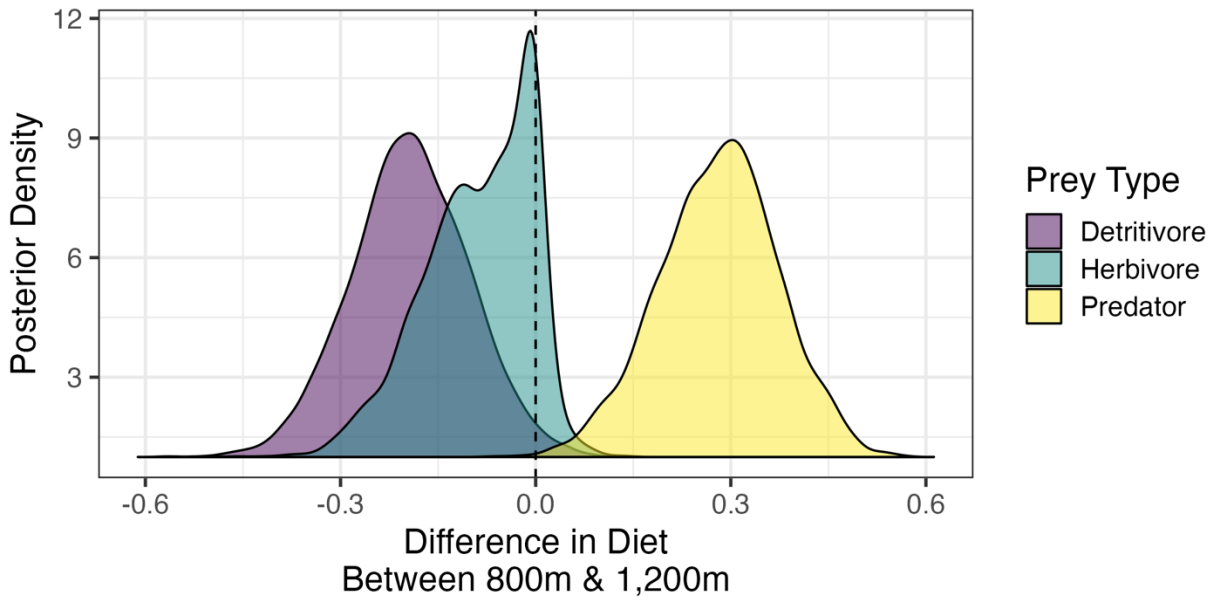


Supplementary Figure S1. Posterior densities for the difference in dietary source group prey items in Swainson's Thrush within the White Mountain National Forest. Source groups include detritivores (purple), herbivores (blue), and predators (yellow). Dietary differences are for low elevation birds (280m) versus mid elevation birds (500m). The dotted line represents zero, estimates at this threshold denote no difference in that dietary item. For example, the model

estimated a high probability that there was no dietary difference in the proportion of herbivorous invertebrate species at low versus mid elevations.



Supplementary Figure S2. Posterior densities for the difference in dietary source group prey items in Swainson's thrush within the White Mountain National Forest. Source groups include detritivores (purple), herbivores (blue), and predators (yellow). Dietary differences are for mid elevation birds (500m) versus high elevation birds (1,200m). The dotted line represents zero, estimates at this threshold denote no difference in that dietary item. For example, the model estimated a high probability that there was no dietary difference in the proportion of herbivorous invertebrate species at mid versus high elevation.



Supplementary Figure S3. Posterior densities for the difference in dietary source group prey items in Swainson's thrush within the White Mountain National Forest. Source groups include detritivores (purple), herbivores (blue), and predators (yellow). Dietary differences are for birds at 800m versus high elevation birds (1,200m). The dotted line represents zero, estimates at this threshold denote no difference in that dietary item. For example, the model estimated a high probability that there was no dietary difference in the proportion of herbivorous invertebrate species at 800m versus 1,200m.