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RESEARCH ARTICLE

Molecular analysis of nestling diet in a long-distance Neotropical migrant, the Louisiana Waterthrush (*Parkesia motacilla*)

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ABSTRACT

Elucidating the diet of Neotropical migratory birds is essential to our understanding of their ecology and to their long-term conservation. Reductions in prey availability negatively impact Neotropical migrants by affecting their survival as both nestlings and adults. Beyond broad taxonomic or morphological categories, however, the diet of Neotropical migrants is poorly documented. Using the molecular techniques of DNA barcoding and next-generation sequencing, we elucidated the diet of Louisiana Waterthrush (*Parkesia motacilla*) nestlings in Arkansas and Pennsylvania, USA. Waterthrush have been shown to respond negatively to the reduced availability of aquatic insects in the orders Ephemeroptera, Plecoptera, and Trichoptera (EPT taxa). We hypothesized that Louisiana Waterthrush nestling diet would be primarily composed of these pollution-sensitive aquatic taxa, and that changes in the riparian insect community would be reflected in their diet. Unexpectedly, the orders Lepidoptera (92%) and Diptera (70%) occurred frequently in the diet of Louisiana Waterthrush nestlings. Among EPT taxa, only the order Ephemeroptera (61%) was frequently detected whereas Plecoptera (7%) and Trichoptera (1%) were poorly represented. The frequency at which aquatic Ephemeroptera and terrestrial Lepidoptera were detected in waterthrush nestling diet differed significantly over the nesting period in Pennsylvania but not in Arkansas, suggesting that phenological shifts in the availability of non-EPT prey taxa may be an important yet undescribed factor influencing the foraging ecology of waterthrush on the breeding grounds. Furthermore, these findings suggest that terrestrial insects may be more important to waterthrush nestlings than previously thought, which enhances our understanding of this biological indicator and Neotropical migrant.

Keywords: birds, DNA barcoding, Diptera, Ephemeroptera, Lepidoptera, molecular diet analysis, next-generation sequencing

Análisis molecular de la dieta de los polluelos de *Parkesia motacilla,* un ave migrante neotropical de larga distancia

RESUMEN

Elucidar la dieta de las aves migratorias neotropicales es esencial para nuestro entendimiento de su ecología y para su conservación a largo plazo. La reducción en la disponibilidad de las presas impacta negativamente a los migrantes neotropicales al afectar la supervivencia de jóvenes y adultos. Sin embargo, más allá de categorías taxonómicas o morfológicas gruesas, la dieta de los migrantes neotropicales ha sido pobremente documentada. Usando las técnicas moleculares de códigos de barras de ADN y secuenciación de nueva generación, elucidamos la dieta de polluelos de *Parkesia motacilla* en Arkansas y Pensilvania. Se ha demostrado de *P. motacilla* responde negativamente a la disponibilidad reducida de insectos acuáticos de los órdenes Ephemeroptera, Plecoptera y Trichoptera (taxones EPT). Formulamos la hipótesis de que la dieta de los polluelos de *P. motacilla* estaría compuesta principalmente por estos taxones acuáticos sensibles a la polución y que los cambios en la comunidad de insectos ribereños se reflejarían en su dieta. Inesperadamente, los órdenes Lepidoptera (92%) y Diptera (70%) fueron frecuentes en la dieta de los polluelos de *P. motacilla*. Entre los taxones EPT sólo el orden Ephemeroptera (61%) fue detectado frecuentemente, mientras que Plecoptera (7%) y Trichoptera (1%) estuvieron pobremente representados. La frecuencia con la que los Ephemeroptera acuáticos y los Lepidoptera terrestres fueron detectados en la dieta de los polluelos de *P. motacilla* fue significativamente diferente a lo largo del periodo de anidación en Pensilvania pero no en Arkansas, lo que sugiere que los cambios fenológicos en la disponibilidad de taxones EPT podrían ser un factor importante no descrito que influye en la ecología de forrajeo de *P. motacilla* en las áreas de reproducción. Además, estos resultados sugieren que los insectos terrestres podrían ser más importantes para los polluelos de *P.*

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motacilla de lo que se pensaba previamente, lo que mejora nuestro entendimiento de este importante indicador biológico y migrante neotropical.

Palabras clave: análisis molecular de dieta, aves, código de barras de ADN, Diptera, Ephemeroptera, Lepidoptera, secuenciación de nueva generación.

INTRODUCTION

Elucidating the dietary composition and food preferences of migratory birds is essential to understanding their ecology, population dynamics, and conservation. Throughout the annual cycle, the availability of food is considered a major limiting factor for populations of birds that migrate from the Neotropics (Martin 1987, Newton 2004) and has been shown to affect migration departure and return rates (Studds and Marra 2005, Cooper et al. 2015), body condition (Marra et al. 1998, Strong and Sherry 2000, Latta and Faaborg 2002), breeding and non-breeding distributions (Burke and Nol 1998, Johnson and Sherry 2001), and rates of predation (Hoover et al. 1995). Furthermore, food availability has been shown to influence fecundity, which is considered one of the most critical factors for sustaining populations in long-distance Neotropical migrants (Sherry and Holmes 1992, Bohning-Gaese et al. 1993, Holmes et al. 1996, Sillett and Holmes 2005). Food limitations on the breeding grounds negatively affect fecundity by influencing the survival and body condition of nestlings (Rodenhouse and Holmes 1992, Sillett et al. 2000). The influence of food on fecundity is of particular conservation interest given the long-term decline of Neotropical migrants (Robbins et al. 1989, Sauer and Link 2011, Sauer et al. 2014); therefore, a detailed understanding of diet is essential to identify potential vulnerabilities and develop effective conservation strategies for these important migratory birds.

Currently, our understanding of Neotropical migrant diet is primarily derived from foraging observations and the morphological identification of insect remains from regurgitates (e.g., Robinson and Holmes 1982), gut contents (e.g., Eaton 1958), and fecal material (e.g., Deloria-Sheffield et al. 2001). These approaches are labor-intensive, expensive to analyze, require expertise in systematic entomology, and often provide an incomplete understanding of diet due to the limitations associated with identifying digested insect remains (Symondson 2002, Pompanon et al. 2012). These limitations are particularly relevant to Neotropical migrants, which commonly prey upon soft-bodied, larval Lepidoptera (e.g., Rodenhouse and Holmes 1992) that may be difficult to identify after digestion (Ralph et al. 1985, Parrish 1997).

The use of molecular techniques to describe diet from animal feces is an increasingly utilized method for studying trophic interactions. Molecular diet analyses provide ecologists with genus- or species-level taxonomic identification and can be applied to a wide range of study taxa (King et al. 2008). Fecal samples are useful for molecular diet studies because they contain residual prey DNA and can be collected with minimal disturbance to the animal (Pompanon et al. 2012). DNA barcoding coupled with next-generation sequencing technologies have enabled ecologists to investigate diet using fecal material from felids (Shehzad et al. 2012), small mammals (Brown et al. 2014), bats (Clare et al. 2014), and seabirds (Deagle et al. 2010, Bowser et al. 2013), all of which would otherwise be difficult to study. Relative to its widespread use in most major taxonomic groups, however, molecular diet analyses that utilize avian feces are underrepresented in the scientific literature. This deficiency is particularly true of perching birds (order Passeriformes), by far the largest avian order with >50% of all extant avian taxa (Raikow 1986). Notably, a recent study of Western Bluebird (*Sialia mexicana*) demonstrated the feasibility of using Illumina sequencing to elucidate diet from fecal samples (Vo and Jedlicka 2014) but has not yet resulted in widespread application. Such molecular approaches enable avian ecologists to generate a comprehensive understanding of diet, which has not been explored in such a descriptive and noninvasive manner.

The Louisiana Waterthrush (*Parkesia motacilla*) is a long-distance Neotropical migratory wood-warbler (family Parulidae). Louisiana Waterthrush are obligate riparian songbirds that occupy linear breeding territories along headwater streams throughout eastern North America (Mattsson et al. 2009; Figure 1). Louisiana Waterthrush are considered aquatic insect foraging specialists and an important biological indicator for the integrity of riparian ecosystems (Brooks et al. 1998, Prosser and Brooks 1998, Mattsson and Cooper 2006). Waterthrush that nest along degraded streams with suboptimal water quality must establish larger territories to acquire sufficient prey resources (Mulvihill et al. 2008), and they lay smaller, delayed clutches (Mulvihill et al. 2008) and rarely attempt a second brood (Mulvihill et al. 2009). These negative impacts on Louisiana Waterthrush are believed to be the result of reductions in the availability of 3 orders of pollution-sensitive aquatic insects used as biological indicators for stream quality: Ephemeroptera, Plecoptera, and Trichoptera (EPT; Mattsson and Cooper 2006, Mulvihill et al. 2008, Wood et al. 2016). Previous studies have suggested that EPT taxa are important prey for Louisiana Waterthrush (Mattsson et al. 2009) because they were found in the gut contents of 15 individuals in the only published description of waterthrush diet (Eaton 1958). Eaton (1958), however, classified nearly 60% of Louisiana Waterthrush

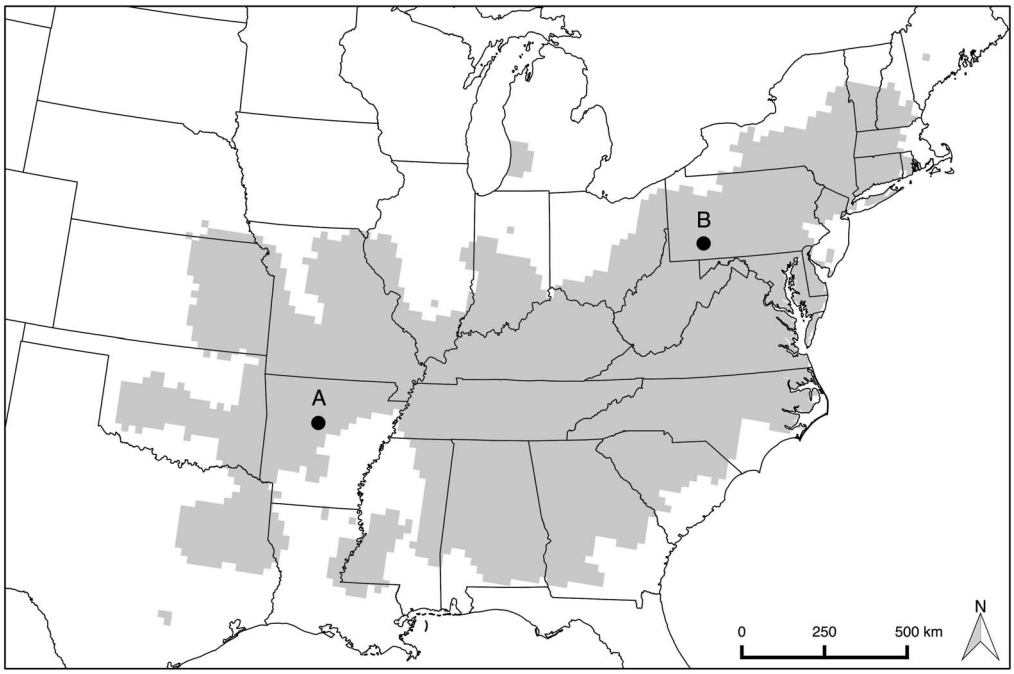

FIGURE 1. Location of study sites within the breeding range of Louisiana Waterthrush. (**A**) Study sites in Conway and Van Buren counties, Arkansas, and (**B**) Westmoreland County, Pennsylvania. Louisiana Waterthrush breeding range (shading) based on data from the North American Breeding Bird Survey (Sauer et al. 2014).

stomach contents as "undetermined fragments," which, if identified, may have revealed additional important prey items. A detailed description of Louisiana Waterthrush diet is therefore imperative to our understanding of their foraging ecology and has been identified as a priority for future research (Mattsson et al. 2009).

In this study, we utilized DNA barcoding and Illumina sequencing to describe the diet of Louisiana Waterthrush nestlings in Arkansas and Pennsylvania, USA. Based on previous diet studies and their documented response to low EPT availability, we hypothesized that Louisiana Waterthrush nestling diet would be predominantly composed of EPT taxa, and that nestling diet would differ over the course of the nesting season by reflecting changes in the riparian insect community.

METHODS

Sample Collection

Louisiana Waterthrush nests were systematically located using behavioral cues along first- and second-order streams in Van Buren and Conway counties, Arkansas (Cedar Creek, Sis Hollow, East Point Remove Creek, and Sunnyside Creek), and Westmoreland County, Pennsylvania (Camp Run, Linn Run, Loyalhanna Creek, and Powdermill Run), beginning in mid-April 2013 (Figure 1). Fecal samples were collected by placing nestlings (3–8 days post-hatching) into a clean paper bag for ~1 min. Fecal samples were immediately preserved in 20 mL of absolute ethanol and stored at room temperature for a period of ~3 months prior to DNA extraction. To investigate potential changes in diet over the course of the nesting period, fecal samples were later subdivided into three 10-day intervals (mid-May = May 12–21; late-May = May 22–31; early-June = June 1–10). Fecal samples collected outside these intervals were not included in analyses that investigated potential changes in diet over the nesting period.

Benthic macroinvertebrates were collected by Surber sampling (Barbour et al. 1999) at 10 equidistant riffles along a ~2 km segment of each stream that encompassed the foraging territories of all sampled waterthrush nests. All 10 benthic samples were combined to represent the benthic community for the entire reach and repeated every 2 weeks throughout the breeding season. A subsample of 300 (±20%) individuals (Barbour et al. 1999) was randomly selected from each benthic sample, and individuals were morphologically identified to genus by a certified aquatic entomologist (genus-level, Society for Freshwater Science). Relative abundance values were derived based on the number of individuals in an order divided by the total number of individuals in the subsample.

DNA Extraction, Amplification, and Sequencing

DNA was extracted from Louisiana Waterthrush nestling fecal samples using the QIAmp DNA Stool Mini Kit

(Qiagen) and a customized protocol for avian fecal samples adapted from Zeale et al. (2011; Appendix A). Waterthrush fecal DNA was subjected to polymerase chain reaction (PCR) using the general arthropod "mini-barcode" primers ZBJ-ArtF1c and ZBJ-ArtR2c, which amplify a 157 bp region of the cytochrome *c* oxidase I (COI) mitochondrial gene (Zeale et al. 2011). These primers were selected based on their ability to amplify degraded DNA and provide species-level taxonomic assignments from 13 arthropod orders (including EPT taxa; Zeale et al. 2011). Mini-barcode primers were modified by the addition of 5′ adapter sequences complementary to the Illumina multiplex indexing primers used in downstream sequencing protocols (Illumina 2013). PCR was conducted in 20 μL reactions with 10–100 ng of DNA template input, 4 μL of 5X high-fidelity reaction buffer (ThermoFisher Scientific), 400 μM dNTPs (ThermoFisher Scientific), 0.8 μM modified forward primer ZBJ-ArtF1c (with 5′ adapter), 0.8 μM reverse primer ZBJ-ArtR2c (with 5′ adapter), and 0.1 units of Phusion Polymerase (ThermoFisher Scientific). All reactions were prepared on ice and amplified using the following conditions: an initial denaturation phase of 2 min at 98°C, 50 cycles of 10 s at 98°C, 30 s at 45°C, 30 s at 72°C, and a final extension of 10 min at 72°C. Amplification of the COI barcode was visually confirmed by ultraviolet trans-illumination following electrophoresis through a 2% agarose-ethidium bromide gel. Amplicons were enriched through an additional PCR reaction following the standard Illumina amplicon indexing and purification protocol (Illumina 2013). Indexed amplicons were combined at equimolar concentrations into a 250 bp, paired-end Illumina MiSeq sequencing run at the Genomics Facility of the Biotechnology Resource Center, Cornell University (Ithaca, NY).

Sequence Analysis

Sequences were quality trimmed in CLC Genomics Workbench 7.0.3 and filtered using Galaxy 15.10 (Giardine et al. 2005, Blankenberg et al. 2010, Goecks et al. 2010). Once trimmed of primers and adapters, any sequences that deviated from the expected amplicon size of 157 bp were removed from the analysis. All retained sequences exhibited a mean Phred quality score $\geq$30, which translates to a base-call error rate of 1 per 1000 bases (Ewing and Green 1998, Richterich 1998).

Filtered sequences were clustered into molecular operational taxonomic units (MOTUs) based on 97% similarity (appropriate for insects as discussed in Clare et al. 2011) using the bioinformatics program QIIME 1.8.0 (Caporaso et al. 2010). After excluding MOTUs with infrequent haplotypes ($\leq$10 copies), representative sequences for each MOTU were compared to reference sequences in the Barcode of Life Database (BOLD; Ratnasingham and Hebert 2007). To ensure an accurate description of Louisiana Waterthrush diet from short fragments (157 bp) of the full-length (658 bp) COI barcode region (Hebert et al. 2003), only MOTUs that exhibited 100% similarity to a BOLD reference sequence were included in subsequent analyses (Appendix B and Supplemental Material Table S1; discussed in Clare et al. 2011).

The number of reads assigned to each successfully identified MOTU in a fecal sample was transformed into a presence or absence dataset (Supplemental Material Table S2). Louisiana Waterthrush nestling diet was summarized at the order-level based on the frequency of occurrence (number of fecal samples in which an order was detected divided by the total number of fecal samples) for each sampling region and time interval (e.g., Razgour et al. 2011, Bowser et al. 2013). This analysis approach is necessary for DNA metabarcoding studies because the proportion of sequencing reads within a sample does not necessarily reflect the relative quantities of prey consumed (Deagle et al. 2010, Pompanon et al. 2012). Tests of statistical significance across nestling diets were calculated in R using a 2-sample proportion test (function: prop.test, alternative = two.sided). Nestling diet was summarized at the order-level in the program MEGAN 5.10.6 (Huson et al. 2011) based on the number of MOTUs that matched a BOLD reference sequence at 100%. Species accumulation curves and asymptotic species richness estimates were generated in R 3.2.2 using the library vegan (functions: specaccum, method = exact; poolaccum, index = chao; Oksanen et al. 2007).

RESULTS

Field Sampling

Louisiana Waterthrush nestling fecal samples were collected from nests along all study streams in both Arkansas (16) and Pennsylvania (16; Supplemental Material Table S2). Sample collection dates were similar between Arkansas (May 14–June 19, 2013) and Pennsylvania (May 15–June 24, 2013) study regions. We collected 48 fecal samples from nestlings in Arkansas and 82 in Pennsylvania. One nest in Arkansas (3 fecal samples) and another in Pennsylvania (5 fecal samples) occurred uncharacteristically late in the breeding season (June 19 and June 24, respectively). Because these nests occurred beyond our analysis intervals, they were removed from our analysis of diet over the nesting period but remained part of our general description of Louisiana Waterthrush nestling diet (Table 1, Figures 2 and 3).

Benthic macroinvertebrates were collected in 2-week intervals from May 10 to July 7, 2013. Approximately 85% of subsampled benthic organisms were identified to the genus-level and represented 13 orders, which included EPT (Supplemental Material Table S3). The mean relative abundance of EPT taxa was similar across study streams in Arkansas (0.60 $\pm$ 0.19) and Pennsylvania (0.72 $\pm$ 0.11; Supplemental Material Table S4).

TABLE 1. Taxonomic assignment of molecular operational taxonomic units (MOTUs) detected in the diet of Louisiana Waterthrush nestlings in Arkansas and Pennsylvania. All listed taxa exhibited 100% similarity to a reference sequence in the Barcode of Life Database (BOLD). Frequency of occurrence = number of fecal samples (from a study region) in which an order was detected divided by the total number of fecal samples (from the same study region).

Class	Order	Family	Genus	Species	% Frequency of occurrence (Arkansas)	% Frequency of occurrence (Pennsylvania)
Arachnida	Araneae	Agelenidae	*Agelenopsis*	sp.	2.1	
		Anyphaenidae	*Anyphaena*	*pectorosa*		10.7
		Araneidae	*Eustala*	*anastera*	6.3	
			Larinioides	*cornutus*	4.2	
		Clubionidae	*Clubiona*	*canadensis*	4.2	4.0
		Linyphiidae	*Pityohyphantes*	*costatus*		5.3
		Salticidae	*Naphrys*	*pulex*		5.3
Insecta	Archaeognatha	Meinertellidae	*Machiloides*	*banksi*	10.4	
	Coleoptera	Carabidae	*Cyclotrachelus*	*sigillatus*		1.3
		Chrysomelidae	*Odontota*	*dorsalis*	8.3	
		Elateridae	*Athous*	*brightwelli*		16.0
				neacanthus		2.7
		Tenebrionidae	*Capnochroa*	*fuliginosa*		8.0
	Diptera	Asilidae	*Laphria*	*janus*	16.7	12.0
				prosticata		1.3
		Calliphoridae	*Calliphora*	*vomitoria*		10.7
			Phormia	*regina*		10.7
			Pollenia	*rudis*	45.8	14.7
		Empididae	*Rhamphomyia*	sp.	2.1	
		Limoniidae	*Epiphragma*	*fasciapenne*		2.7
			Eutonia	*alleni*	4.2	2.7
			Hexatoma	*spinosa*		9.3
		Pediciidae	*Tricyphona*	*inconstans*	4.2	5.3
		Scathophagidae	*Scathophaga*	*stercoraria*		1.3
		Syrphidae	*Syrphus*	*rectus*	14.6	22.7
				torvus	4.2	1.3
			Temnostoma	*alternans*		4.0
				balyras		4.0
		Tabanidae	*Chrysops*	*carbonarius*		2.7
				montanus		1.3
			Hybomitra	*lasiophthalma*		5.3
		Tipulidae	*Nephrotoma*	*virescens*	8.3	
			Tipula	*abdominalis*		1.3
				bicolor	8.3	
				fuliginosa	10.4	
				mallochi		17.3
				sp.	10.4	
	Ephemeroptera	Ameletidae	*Ameletus*	*lineatus*		13.3
		Baetidae	*Diphetor*	*hageni*		2.7
		Ephemerellidae	*Ephemerella*	*dorothea*		1.3
		Heptageniidae	*Epeorus*	*pleuralis*	41.7	48.0
			Heptagenia	sp.	43.8	45.3
			Maccaffertium	*meririvulanum*		2.7
				sp.	8.3	
				vicarium		9.3
	Hemiptera	Cicadellidae	*Gyponana*	sp.	2.1	
	Hymenoptera	Tenthredinidae	*Hemichroa*	*militaris*	4.2	
	Lepidoptera	Depressariidae	*Semioscopis*	*megamicrella*	4.2	
		Drepanidae	*Euthyatira*	*pudens*	2.1	1.3
			Habrosyne	*scripta*		4.0
		Erebidae	*Allotria*	*elonympha*	12.5	
			Catocala	*micronympha*	6.3	
				neogama	6.3	17.3
				sp.	4.2	
			Cissusa	*spadix*	10.4	

TABLE 1. Continued.

Class	Order	Family	Genus	Species	% Frequency of occurrence (Arkansas)	% Frequency of occurrence (Pennsylvania)
Insecta	Lepidoptera	Erebidae	*Idia*	*lubricalis*		1.3
			Lymantria	*dispar*	10.4	8.0
			Orgyia	*definita*	2.1	10.7
			Renia	*salusalis*	2.1	
			Zale	*minerea*	2.1	
		Geometridae	*Campaea*	*perlata*	4.2	5.3
			Epimecis	*hortaria*	14.6	17.3
			Eupithecia	*annulata*		1.3
			Heliomata	*cycladata*	2.1	
			Lomographa	*glomeraria*		26.7
			Melanolophia	*canadaria*		40.0
			Phigalia	sp.	16.7	
			Prochoerodes	*lineola*		2.7
			Tetracis	sp.	2.1	
		Lasiocampidae	*Malacosoma*	*disstria*		4.0
			Tolype	sp.	2.1	
		Noctuidae	*Achatia*	*distincta*	12.5	37.3
			Acronicta	*impleta*	4.2	
			Amphipyra	*pyramidoides*	2.1	
			Cerastis	*tenebrifera*	4.2	
			Eupsilia	*morrisoni*		9.3
				sp.	8.3	
			Helotropha	*leucostigma*	8.3	
			Lithophane	*unimoda*		16.0
			Metaxaglaea	*inulta*		5.3
				sp.	10.4	
			Morrisonia	sp.	12.5	
			Mythimna	*unipuncta*		1.3
			Orthosia	*garmani*	27.1	
			Orthosia	*hibisci*	10.4	2.7
			Sunira	*bicolorago*	8.3	2.7
			Xestia	sp.	8.3	
		Notodontidae	*Ellida*	*caniplaga*	4.2	4.0
			Heterocampa	*guttivitta*	2.1	
			Lochmaeus	*bilineata*	8.3	
				sp.	33.3	
			Nadata	*gibbosa*		6.7
		Tortricidae	*Acleris*	*nigrolinea*		1.3
			Phaecasiophora	*confixana*		1.3
			Pseudexentera	*oregonana*	2.1	
	Megaloptera	Corydalidae	*Nigronia*	*fasciatus*	16.7	20.0
		Sialidae	*Sialis*	sp.	6.3	6.7
	Orthoptera	Rhaphidophoridae	*Euhadenoecus*	*puteanus*		1.3
	Plecoptera	Perlidae	*Acroneuria*	sp.		6.7
		Perlodidae	*Isoperla*	*similis*		6.7
	Psocoptera	Peripsocidae	*Peripsocus*	*subfasciatus*		1.3
	Trichoptera	Limnephilidae	*Platycentropus*	*radiatus*		1.3

DNA Extraction, Amplification, and Sequencing

We successfully extracted DNA and amplified the COI barcode from all 130 Louisiana Waterthrush nestling fecal samples. Template DNA concentrations ranged between 0.5 and 142.9 ng μL^{-1} with a mean of $\sim$20 ng μL^{-1}. We successfully recovered sequence data from 123 fecal samples (95%). After quality trimming and the exclusion of infrequent haplotypes, we recovered 91,765 sequences that clustered into 125 (Arkansas) and 166 (Pennsylvania) MOTUs. Representative sequences were compared to the BOLD reference library, which resulted in a 100% match to a reference sequence for 132 MOTUs (51,175 of recovered sequences) and 107 unique taxa (Table 1). Among these unique taxa, 83% were assigned to the species level and the remaining 17% to genus level (Table 1). We rejected 5 MOTUs because they were identified as Lepidoptera that

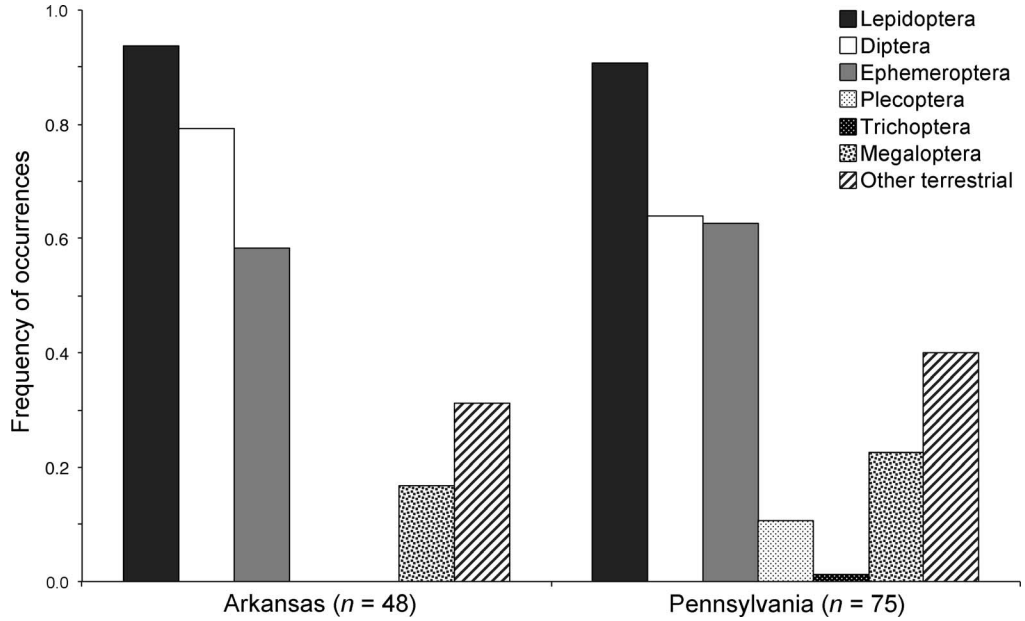

FIGURE 2. Frequency of occurrence of identified prey in the diet of Louisiana Waterthrush nestlings in Arkansas and Pennsylvania. The orders Lepidoptera (92%) and Diptera (70%) were the most common across waterthrush nestling fecal samples in both study regions. The order Ephemeroptera (60%) was detected frequently in both study regions while Plecoptera (7%) and Trichoptera (1%) were rarely detected. Frequency of occurrence = number of fecal samples (from a study region) in which an order was detected divided by the total number of fecal samples (from the same study region).

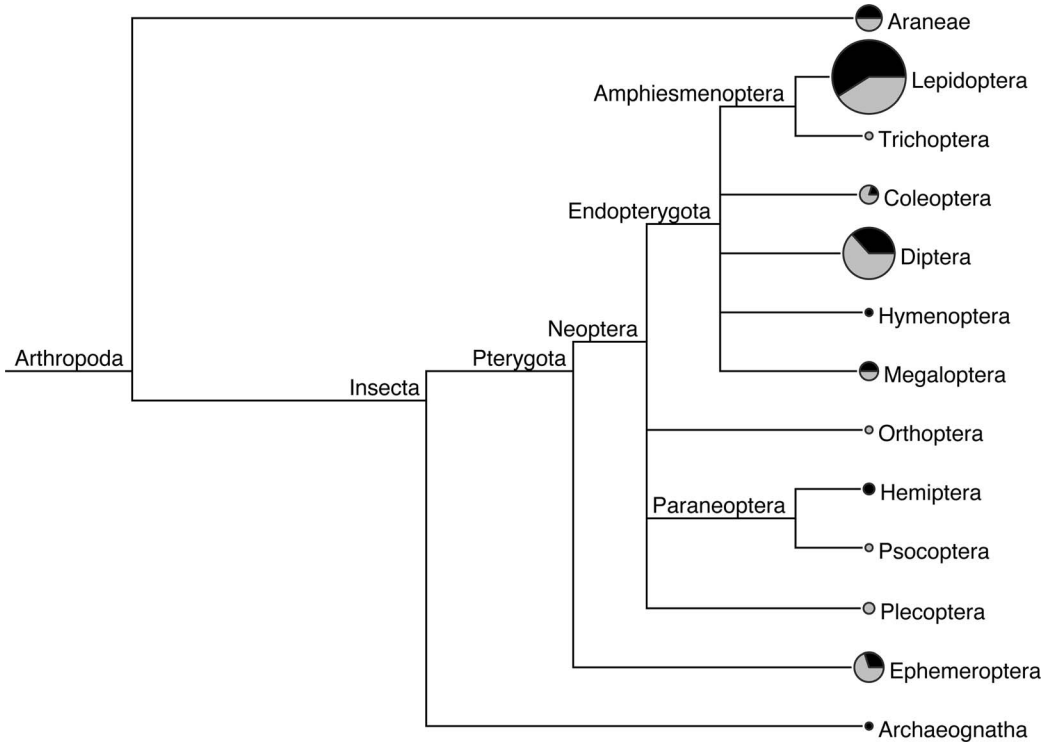

FIGURE 3. Order-level summary of Louisiana Waterthrush nestling diet in Arkansas and Pennsylvania. Tree includes MOTUs that exhibit 100% similarity to a reference sequence in BOLD for Louisiana Waterthrush fecal samples collected from Arkansas (black) and Pennsylvania (gray). Node size scaled to represent the number of identified MOTUs within a given order.

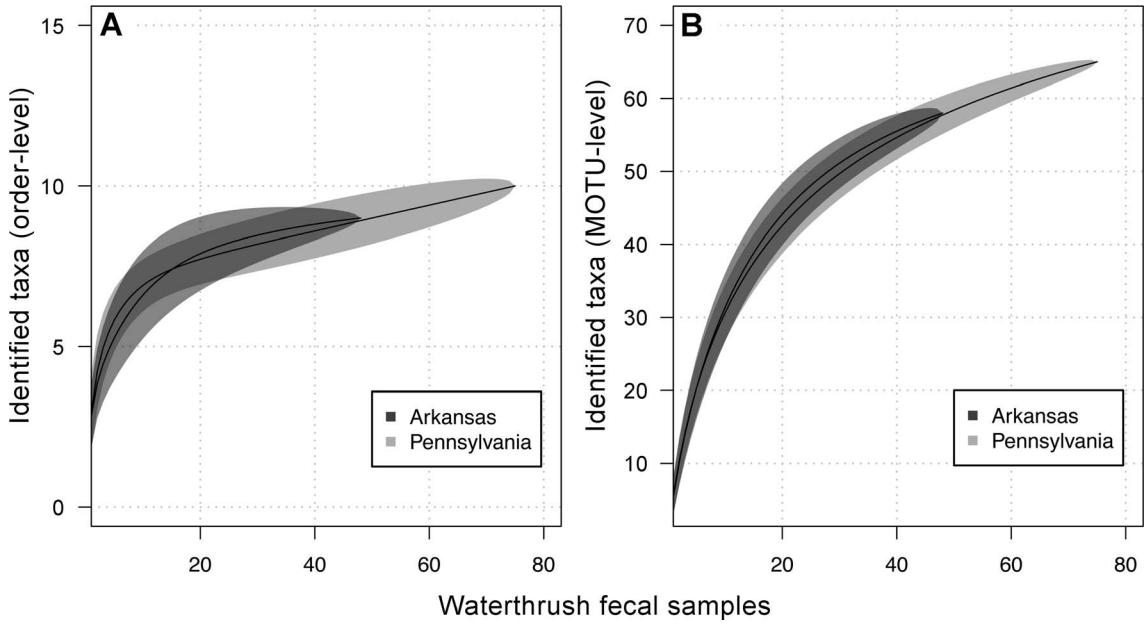

FIGURE 4. Species accumulation curves for the diversity of identified prey consumed by Louisiana Waterthrush nestlings at the (**A**) order-level and (**B**) MOTU-level. Lines represent mean estimates of taxon richness and shading represents standard deviation.

do not occur in eastern North America (Supplemental Material Table S1; J. Rawlins personal communication). The order-level taxonomic richness of Louisiana Waterthrush nestling diet was similar in both Arkansas (9) and Pennsylvania (10; Figure 4A). By contrast, Arkansas waterthrush nestling diet exhibited substantially fewer MOTUs (58) compared to the diet of waterthrush nestlings in Pennsylvania (65; Figure 4B). Asymptotic species richness estimates at the MOTU-level suggest that the analysis of additional fecal samples may result in the identification of further prey taxa in both Arkansas (7 MOTUs) and Pennsylvania (14 MOTUs).

Waterthrush Nestling Diet

The terrestrial order Lepidoptera was detected in 92% of Louisiana Waterthrush nestling fecal samples and was significantly more common than all other orders except Diptera in Arkansas ($\chi^2 = 14.64$, df = 1, $p < 0.001$) and all other orders in Pennsylvania ($\chi^2 = 13.73$, df = 1, $p < 0.001$; Figure 2). Orders Diptera (70%) and Ephemeroptera (61%) were also frequently detected in both study regions (Figure 2). Among EPT taxa, Ephemeroptera was by far the most abundant, contributing to 93% of EPT MOTUs in samples collected from both study regions combined (Table 1, Figure 3). The mayfly family Heptageniidae was particularly well represented across fecal samples from both Arkansas (58%) and Pennsylvania (61%) and was the only family of Ephemeroptera detected in the diet of waterthrush nestlings in Arkansas (Table 1). By contrast, 4 families of Ephemeroptera were found in waterthrush nestling diet in Pennsylvania: Ameletidae (13%), Baetidae (3%), Ephemerellidae (1%), and Heptageniidae (61%; Table 1). Orders Plecoptera (7%) and Trichoptera (1%) were detected in only 9 waterthrush fecal samples from Pennsylvania and were not detected in any fecal samples collected from Arkansas. Relaxing our conservative 100% similarity requirement to a less stringent ≥98% (Appendix B) did not result in additional detections of Plecoptera or Trichoptera (Supplemental Material Table S1). In addition to the aquatic order Megaloptera (20%), several terrestrial orders were detected infrequently and analyzed as a group: Araneae, Archaeognatha, Coleoptera, Hemiptera, Hymenoptera, Orthoptera, and Psocoptera (Table 1, Figure 2).

Based on our general description of waterthrush nestling diet (Figures 2 and 3), we investigated potential changes in frequency of occurrence over the nesting period for the 3 most commonly detected dietary orders: Lepidoptera, Diptera, and Ephemeroptera. In fecal samples collected from Arkansas, the frequency of occurrence of Lepidoptera ($\chi^2 < 0.01$, df = 1, $p > 0.05$) and Ephemeroptera ($\chi^2 = 0.45$, df = 1, $p > 0.05$) did not change over the course of the nesting period (Figure 5A). By contrast, among fecal samples collected from Pennsylvania, frequency of occurrence of Lepidoptera and Ephemeroptera differed significantly within the time intervals of late-May ($\chi^2 = 13.29$, df = 1, $p < 0.001$) and early-June ($\chi^2 = 9.67$, df = 1, $p < 0.01$). Furthermore, the frequency of occurrence for Ephemeroptera differed significantly ($\chi^2 = 6.82$, df = 1, $p < 0.01$) over the course

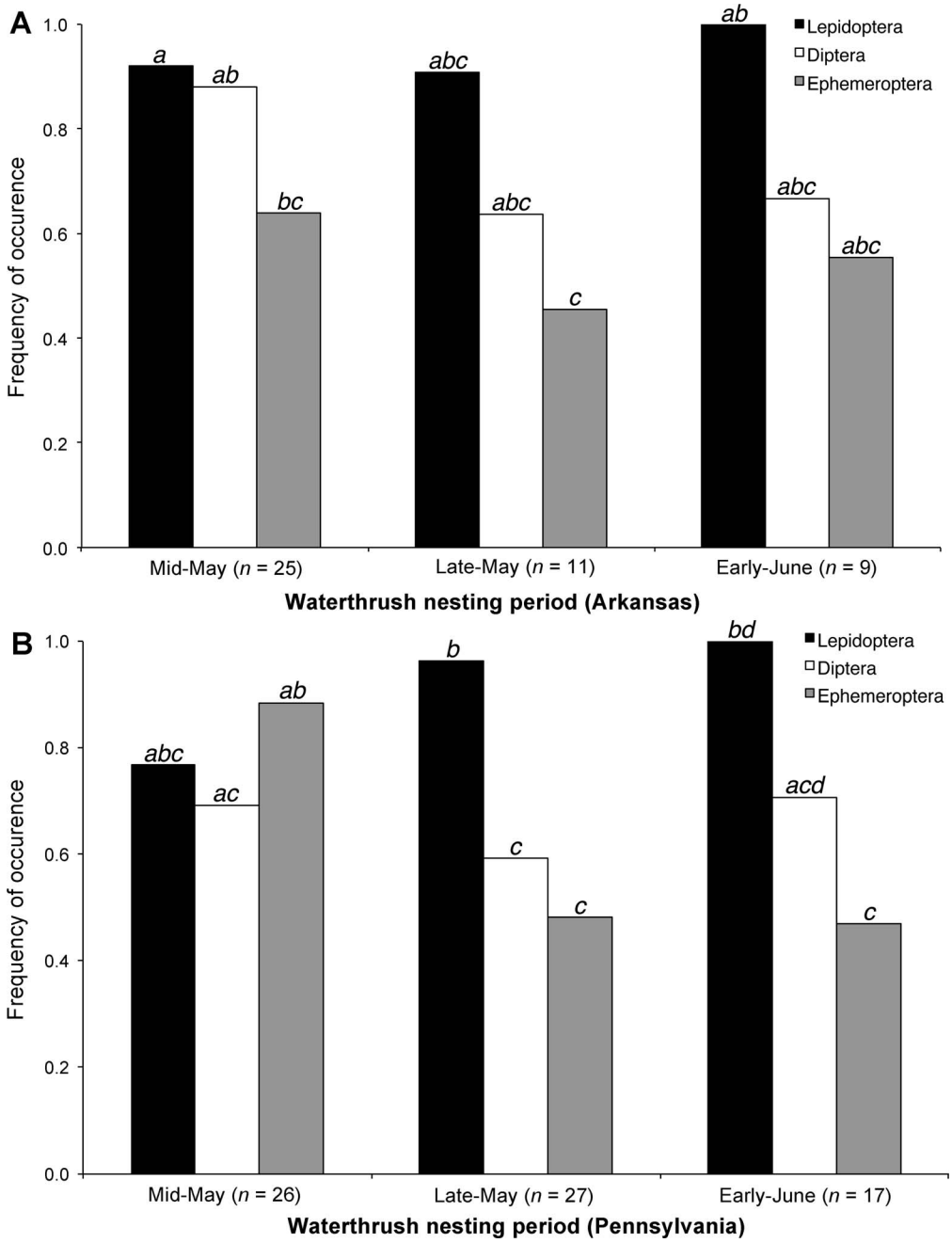

FIGURE 5. Frequency of occurrence of Lepidoptera, Diptera, and Ephemeroptera in the diet of Louisiana Waterthrush nestlings over the course of the nesting period in Arkansas and Pennsylvania. (**A**) In Arkansas, the frequency of occurrence of Lepidoptera and Ephemeroptera did not differ significantly over the course of the breeding season ($p > 0.05$). (**B**) In Pennsylvania, the frequency of occurrence of Lepidoptera and Ephemeroptera differed significantly within the late-May ($p < 0.001$) and early-June ($p < 0.01$) time intervals and over the course of the nesting period ($p < 0.01$). The order Diptera did not differ significantly over the nesting period in Arkansas or Pennsylvania ($p > 0.05$). Same letters above bars indicate no significant difference ($p > 0.05$). Frequency of occurrence = number of fecal samples (from a time interval) in which an order was detected divided by the total number of fecal samples (from the same time interval).

of the nesting period in Pennsylvania (Figure 5B). The order Diptera was also analyzed over these time intervals but did not differ significantly over the nesting period in Arkansas ($\chi^2 = 1.55$, df = 1, $p > 0.05$) or Pennsylvania ($\chi^2 = 0.22$, df = 1, $p > 0.05$; Figure 5).

DISCUSSION

We applied a next-generation sequencing approach to successfully identify Louisiana Waterthrush prey taxa to the genus or species level and elucidated the nestling diet

of this Neotropical migrant. We found that waterthrush nestlings frequently consumed terrestrial Lepidoptera and Diptera in both study regions, contrary to the long-standing assertion that this species relies heavily on pollution-sensitive aquatic insects throughout its breeding range (Mattsson et al. 2009). The frequent detection of Lepidoptera and Diptera suggests that adult Louisiana Waterthrush target terrestrial taxa regularly, and that soft-bodied prey may have been overlooked in previous diet studies. Contrary to our hypothesis that EPT taxa would dominate waterthrush nestling diet, only the order Ephemeroptera was detected frequently. Plecoptera and Trichoptera were poorly represented despite their availability throughout waterthrush foraging territories in both Arkansas and Pennsylvania (Supplemental Material Table S3 and S4), suggesting these taxa may not be important prey during the post-incubation period. These results were remarkably similar between study regions, which are ~1,300 km apart and on opposite extremes of the Louisiana Waterthrush breeding range (Figure 1).

The description of Louisiana Waterthrush diet presented here represents an account of prey taxa targeted by adults during the post-incubation period. Given previous research on waterthrush foraging behavior (Eaton 1958, Craig 1984, Mattsson et al. 2009), the large proportion of nestlings that consumed Lepidoptera (92%) and Diptera (70%) was unexpected. However, Louisiana Waterthrush have been observed to feed larval and adult Lepidoptera to nestlings at several of our study sites in Pennsylvania (R. Mulvihill personal communication). Although differentiating between larval and adult life stages based solely on insect DNA is impossible, previous observational studies have reported that ~11% of Louisiana Waterthrush foraging was directed at riparian foliage during the post-incubation period (Mattsson et al. 2009). Foliage serves as a host for larval Lepidoptera, which have been suggested as an important food item for the nestlings of other Neotropical migrants (Holmes et al. 1979). Clearly, the high frequency of detection for orders Lepidoptera and Diptera suggests that non-EPT taxa may be more important to Louisiana Waterthrush than previously thought. This finding emphasizes the need for improved understanding of Louisiana Waterthrush foraging ecology and how changes in the availability of non-EPT taxa influence both nestlings and adults.

In Pennsylvania, we found that Louisiana Waterthrush nestling diet changed over the course of the nesting period. This shift in diet resulted from a significant reduction in the detection of dietary Ephemeroptera and an increased detection of Lepidoptera in the later stages of the nesting period, suggesting that a reduction in the availability of Ephemeroptera or an increased availability of Lepidoptera may be driving the change in diet. Louisiana Waterthrush may therefore target Ephemeroptera in the early season but switch to Lepidoptera as they become available later in the breeding season. This shift was not observed in the diet of waterthrush nestlings in Arkansas, which may be partly explained by the phenology of waterthrush. Neotropical migrants are believed to rely on photoperiod cues to determine date of departure from the wintering grounds (Hagan et al. 1991) to maximize phenological synchrony and the availability of insects during chick rearing (Perrins 1970, Lany et al. 2015). Yet latitudinal and climatic differences across the Louisiana Waterthrush breeding range affect the timing of leaf expansion and Lepidoptera prey abundance (e.g., Parry et al. 1998, Butler and Strazanac 2000). Therefore, we might expect Lepidoptera to be available prey earlier in the breeding season for waterthrush in Arkansas than for conspecifics nesting in Pennsylvania. Our findings suggest that the availability of terrestrial prey such as Lepidoptera and Diptera may be important to Louisiana Waterthrush during the post-incubation period and should be a priority for future research. These results also emphasize the plasticity of waterthrush diet, but whether changes in the orders of prey insects consumed affect waterthrush nest success or other vital rates remains unknown.

Despite the frequent detection of Lepidoptera in nestling diet, previous studies have convincingly demonstrated that Louisiana Waterthrush respond negatively to reductions in EPT availability (Mattsson and Cooper 2006, Mulvihill et al. 2008, 2009, Wood et al. 2016). EPT taxa are also reliable indicators of overall riparian quality (Hilsenhoff 1977, Barbour et al. 1999) and reflect several factors that impact the suitability of waterthrush breeding territories (e.g., bank erosion, anthropogenic land use, and stream order; Brooks et al. 1998, Prosser and Brooks 1998, Mattsson and Cooper 2006). Therefore, EPT taxa may be a reliable indicator of waterthrush site occupancy but may not completely reflect their foraging ecology. As predicted by a previous study (Mulvihill et al. 2008), we found that Ephemeroptera (61%) were particularly well-represented across Louisiana Waterthrush diets. Whether those prey individuals were larval (aquatic) or adult (terrestrial) Ephemeroptera remains unknown and represents an important limitation of molecular diet analyses. Regardless, the frequency of occurrence of a single family of Ephemeroptera (Heptageniidae) in waterthrush nestling fecal samples (60%) is particularly interesting because it contains several of the most pollution-sensitive aquatic insects in eastern North America (Barbour et al. 1999). Reliance on Heptageniidae raises considerable conservation concern as anthropogenic impacts to water quality continue throughout the Louisiana Waterthrush breeding range (Drohan et al. 2012, Wood et al. 2016).

Our results were derived using a single primer set designed to amplify a small fragment (157 bp) of a single barcode marker (COI) and should not be considered a comprehensive description of Louisiana Waterthrush nest-

ling diet. To confidently identify all dietary insects, our methodology should be expanded to include multiple primer sets or additional barcoding genes, which may capture a greater variety of prey taxa (e.g., Hajibabaei et al. 2012, Bowser et al. 2013). Unfortunately, the potential advantages of alternative barcoding markers for insectivores are hindered by a relatively limited barcode library compared to that currently available for COI. Furthermore, the arthropod COI barcode library managed by BOLD is ideal because of strict vouchering requirements that reduce the risk of misidentification (Ratnasingham and Hebert 2007). The application of a single primer set is not expected to have biased our results however, as demonstrated by several studies that also identified EPT taxa using the primer set developed by Zeale et al. (2011; e.g., Clare et al. 2009, 2011, Razgour et al. 2011, Vesterinen et al. 2013); therefore, the use of a single primer set and genetic marker should not diminish the conclusions of this study.

Until now, our understanding of Louisiana Waterthrush nestling diet was limited to studies that used morphological identification (Eaton 1958) and foraging observations of adults (Craig 1984). We now understand that waterthrush nestling diet is broader than previously thought and includes non-EPT taxa such as terrestrial Diptera and Lepidoptera. Although most of our analyses were collapsed to the order-level, we identified soft-bodied prey taxa (orders Diptera and Lepidoptera) that may have escaped detection using morphological identification techniques. These findings demonstrate the advantages of DNA-based techniques for studying the diet of Neotropical migrants and emphasize the need for its widespread application. Our results may be particularly interesting to ecologists studying species with similar foraging specialties or limited dietary information. The incomplete understanding of Neotropical migrant diet is a pervasive problem, but with the advent of DNA-based approaches, ornithologists are now able to investigate some of the most elusive questions regarding the importance of diet throughout the annual cycle.

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APPENDIX A

DNA extraction from avian fecal material using Qiagen QIAamp DNA Stool Kit (Cat. #: 51504). Adapted from Zeale et al. (2011) and Qiagen Handbook August 2001: Protocol for Isolation of DNA from stool for Pathogen Detection

This protocol is designed to maximize extraction of insect prey DNA from bird feces stored in ethanol. It does not prevent or exclude the extraction of bird, bacterial, fungal, or other non-prey DNA from fecal samples.

DAY 1

1. Transfer fecal sample (including preservative ethanol) into a sterile weigh boat.
2. Homogenize fecal sample using a sterile, DNA-free instrument (e.g., pipette tip) to permit complete ethanol evaporation.
3. Incubate fecal sample in weigh boat using a slide warmer set to medium heat. Incubate until sample is

completely dry and all ethanol has evaporated (~1 hr). Residual ethanol will interfere with DNA extraction.
4. Carefully transfer as much of the dried fecal material as possible to a sterile 2 mL microcentrifuge tube. Add 1.4 mL ASL buffer to the weigh boat to transfer any remaining fecal material. Continuously vortex the sample for 10 min.
5. Incubate the suspension overnight at 70°C, vortexing occasionally.

DAY 2

6. Vortex continuously for 10 min and centrifuge at full speed (~20,000 × g) for 1 min at room temperature to pellet fecal particulate.
7. Pipet 1.2 mL of the supernatant into a new 2 mL centrifuge tube.
8. Add 1 InhibitEX tablet to the sample and vortex immediately and continuously for 3 min or until completely suspended. Incubate suspension for 5 min at room temperature to allow inhibitors to absorb to the InhibitEX matrix.
9. Centrifuge sample at full speed for 3 min to pellet InhibitEX matrix.
10. Transfer 600 μL of supernatant into a new 1.5 mL centrifuge tube and discard the pellet.
11. Add 40 μL Proteinase K to the supernatant and mix thoroughly by vortexing.
12. Add 600 μL Buffer AL and vortex for 15 s. Incubate overnight at 70°C.

DAY 3

13. Remove sample from incubation and vortex continuously for 1 min.
14. Add 600 μL of 100% ethanol to the lysate and mix by vortexing.
15. Add 600 μL of the lysate to a QIAmp spin column. Centrifuge at full speed for 1 min. Place spin column in a new collection tube and discard the tube containing the filtrate.
16. Repeat step 13 to load the remaining aliquots of the lysate to the spin column.
17. Add 500 μL Buffer AW1. Centrifuge at full speed for 1 min. Place spin column in a new collection tube and discard the tube containing the filtrate.
18. Add 500 μL Buffer AW2. Centrifuge at full speed for 3 min. Place spin column in into a new 1.5 mL centrifuge tube and discard the tube containing the filtrate
19. Pipette 50 μL of pre-warmed (70°C) Buffer AE directly onto the spin column membrane. Incubate for 5 min at room temperature then centrifuge at full speed for 1 min to elute DNA.
20. Transfer the eluted DNA from step 19 onto the spin column membrane to concentrate the DNA sample. Incubate for 2 min and centrifuge at full speed for 1 min.

APPENDIX B

MOTU identification criteria using the BOLD search tool (species-level barcode records), adapted from Razgour et al. (2011)

1. 100% match to one species–species-level assignment; 100% match to more than one species in the same genus–genus-level assignment
2. ≥98% match to one species–species-level assignment; ≥ 98% match to more than one species in the same genus–genus-level assignment
3. ≥98% match to one or more taxa (genus or species) in the same family–family-level assignment
4. ≥98% match to one or more taxa (genus, species, or family) to in the same order–order-level assignment.
5. <98% match to one or more taxa–top match.
6. No match in BOLD.