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Two is better than one: Coupling DNA metabarcoding and stable isotope analysis improves dietary characterizations for a riparian-obligate, migratory songbird

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Abstract

While an increasing number of studies are adopting molecular and chemical methods for dietary characterization, these studies often employ only one of these laboratory-based techniques; this approach may yield an incomplete, or even biased, understanding of diet due to each method's inherent limitations. To explore the utility of coupling molecular and chemical techniques for dietary characterizations, we applied DNA metabarcoding alongside stable isotope analysis to characterize the dietary niche of breeding Louisiana waterthrush (*Parkesia motacilla*), a migratory songbird hypothesized to preferentially provision its offspring with pollution-intolerant, aquatic arthropod prey. While DNA metabarcoding was unable to determine if waterthrush provision aquatic and terrestrial prey in different abundances, we found that specific aquatic taxa were more likely to be detected in successive seasons than their terrestrial counterparts, thus supporting the aquatic specialization hypothesis. Our isotopic analysis added greater context to this hypothesis by concluding that breeding waterthrush provisioned Ephemeroptera and Plecoptera, two pollution-intolerant, aquatic orders, in higher quantities than other prey groups, and expanded their functional trophic niche when such prey were not abundantly provisioned. Finally, we found that the dietary characterizations from each approach were often uncorrelated, indicating that the results gleaned from a diet study can be particularly sensitive to the applied methodologies. Our findings contribute to a growing body of work indicating the importance of high-quality, aquatic habitats for both consumers and their pollution-intolerant prey, while also demonstrating how the application of multiple, laboratory-based techniques can provide insights not offered by either technique alone.

KEYWORDS
arthropods, diet, DNA metabarcoding, mixing models, stable isotope analysis, trophic niche

1 | INTRODUCTION

Studying a species' diet is an essential step in characterizing its ecological niche (Eaton, 1958) and may provide important insights into the species' long-term conservation (Burin et al., 2016). The vast majority of diet studies to date have characterized a consumer's prey using morphological methodologies, such as identification of prey during feeding events (Stenzel et al., 1976) or of prey parts collected from stomach samples (Sherry, 1984), which are often unable to detect particular prey items, let alone classify them with high taxonomic

precision (Parrish, 1997; Ralph et al., 1985). Recently, ecologists have gained more complete prey identifications through genetic techniques, such as DNA metabarcoding (Pompanon et al., 2012), which detect and identify prey using taxon-specific DNA molecules rather than diagnostic prey tissues (Hebert et al., 2003). However, while DNA metabarcoding yields improved prey classification and taxonomic dietary diversity estimates, it is often faulted for detecting only the presence, but not the abundance, of each taxon (Piñol et al., 2015); this is particularly so in taxonomically rich samples (e.g., insectivores; Jusino et al., 2019).

To reveal the more quantitative and functional aspects of a species' trophic niche, researchers often turn to stable isotope analysis of consumer tissues which can provide information about a species' dietary niche breadth (Newsome et al., 2007) or be used in mixing models to quantify a consumer's prey composition (Phillips, 2001). However, because stable isotope ratios are determined by life-history traits (e.g., diet or locale; Schoeninger, 2010), ecologically similar, but phylogenetically distinct, prey taxa (e.g., aquatic detritivores in different taxonomic orders) may have indistinguishable stable isotope ratios. This means that researchers must derive taxonomic prey composition, a prerequisite for stable isotope mixing models (Phillips, 2001), from other dietary techniques. The vast majority of previous work has determined which prey to include in mixing models using morphological dietary studies; however, because DNA-based methods have proven more effective at detecting and identifying prey than morphological techniques (Braley et al., 2010), it appears that the most informed mixing models will be those supported by DNA-based evidence (Chiaradia et al., 2014). Furthermore, interpreting the results of DNA-based and isotopic techniques together will probably yield a better understanding than either method alone as their coupled application can provide both qualitative (e.g., the prey species consumed and taxonomic diversity) and quantitative (e.g., prey proportion and functional niche breadth) information about a species' trophic niche. However, even as researchers continue to stress the importance of combining these methods for dietary studies (Hoenig, Snider, et al., 2021; Matley et al., 2018; Nielsen et al., 2018; Whitaker et al., 2019), it is still exceedingly rare to see both methods applied side-by-side (but see; Cordone et al., 2022; Génier et al., 2021).

To examine the dietary insights offered by DNA metabarcoding and stable isotope analysis and to highlight the robust inferences resulting from their coupled application, we used each of these techniques to better understand the arthropod prey composition and dietary niche dynamics of nestling Louisiana waterthrush (*Parkesia motacilla*; hereafter, waterthrush). Waterthrush are riparian-obligate, migratory wood-warblers (family: Parulidae) which are hypothesized to specialize on pollution-intolerant aquatic arthropod prey, (e.g., Ephemeroptera and Plecoptera) due to their preference for the high-quality breeding territories that support these prey (Mulvihill et al., 2008) and the frequent detection of these taxa in their diets (Trevelline et al., 2016; Trevelline, Nuttle, Hoenig, et al., 2018; Trevelline, Nuttle, Porter, et al., 2018). However, recent DNA-based evidence has found that terrestrial prey groups, such as Lepidoptera, are detected in nestling waterthrush diets as frequently as pollution-intolerant, aquatic prey (Trevelline et al., 2016; Trevelline, Nuttle, Hoenig, et al., 2018; Trevelline, Nuttle, Porter, et al., 2018), causing some to question if waterthrush are more generalist than once believed (Bryant et al., 2020). Because deviations from a species' fundamental dietary niche can have lasting impacts on reproductive success and population stability (Narango et al., 2018), effective species conservation may be reliant upon a strong understanding of species' prey preferences—especially for avian insectivores, a group undergoing unprecedented population declines (Rosenberg et al., 2019), probably in response to equally drastic population declines of their arthropod prey (Hallmann et al., 2017).

In this study, we used the taxonomic resolution offered by DNA metabarcoding to determine if adults provision the same aquatic-derived taxa, but variable terrestrial-derived taxa, in two distinct breeding seasons, as such behaviour would suggest specialization on specific aquatic prey (Sherry, 1990). Using the quantitative insights afforded by stable isotope analysis, we also tested if adult waterthrush provision pollution-intolerant aquatic prey in higher amounts than other prey groups and if their dietary niche breadth expands when pollution-intolerant taxa are consumed in lower quantities. Finally, we determined how the choice of DNA metabarcoding or stable isotope analysis shaped our understanding of nestling waterthrush diet by comparing the measurements of dietary niche breadth and prey composition offered by each method. Our findings highlight how the combination of DNA metabarcoding and stable isotope analysis can be used to overcome the methodological shortcomings of each approach and provide novel insights about a species' trophic niche, life history and long-term conservation.

2 | MATERIALS AND METHODS

2.1 | Sample collection

Waterthrush nests were located by monitoring behavioural cues of adults on four headwater streams in the Laurel Highlands of Pennsylvania, USA (Camp Run, Linn Run, Loyalhanna Creek and Powdermill Run; see Mulvihill et al., 2008 for site descriptions), from mid-April to mid-July in the 2016 and 2017 breeding seasons. In both breeding seasons, nestling faecal samples were collected on up to two occasions and stored in 20 ml of absolute ethanol at -20°C until DNA extraction up to 3 months later (2016, $n = 101$; 2017, $n = 72$). In the 2017 breeding season, blood samples ($n = 72$) were collected from the brachial vein in 100 µl heparinized capillary tubes when nestlings were ~ 7 days post-hatch to ensure the nestlings were large enough to safely collect blood (>10 g; Fair & Jones, 2010; McGuill & Rowan, 1989). Whole blood samples were kept chilled on ice packs (<3 h) until centrifugation to separate red blood cells from plasma, and red blood cells were kept frozen at −20°C until stable isotope analysis. Commonly detected dietary aquatic arthropod taxa were collected directly from Powdermill Run via D-net sampling in February 2020, while terrestrial arthropods

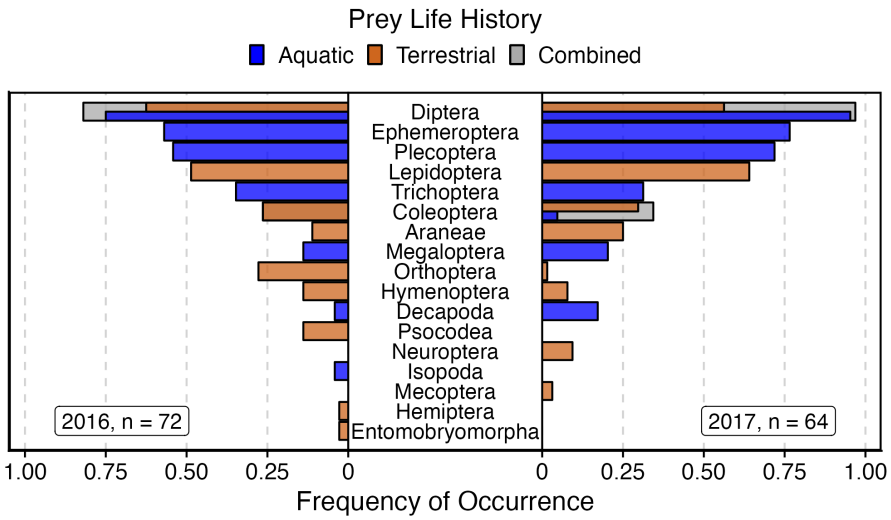

FIGURE 1 Dietary DNA metabarcoding suggests that major Louisiana waterthrush prey groups are represented by both aquatic and terrestrial taxa and exhibit little annual variation in frequency of occurrence. For each year, frequency of occurrence was calculated by dividing the number of nestling faecal samples in which each prey order was detected by the total number of faecal samples returning sequencing data. The "combined" bars account for the presence of aquatic and terrestrial taxa in the Diptera and Coleoptera prey orders [Colour figure can be viewed at wileyonlinelibrary.com]

were collected opportunistically from 2017 to 2019 using light traps stationed ~100 m from Powdermill Run. Aquatic arthropods were stored at −20°C while terrestrial arthropods were pinned and stored at room temperature before being prepared for stable isotope analysis.

2.2 | DNA-based methods

DNA was extracted from individual nestling faecal samples following Trevelline et al. (2016), and the samples from individual nests were processed separately after thorough bleaching of equipment and bench surfaces to reduce the potential for contamination among nests (Trevelline, Nuttle, Hoenig, et al., 2018). Resulting total DNA extracts were subjected to a polymerase chain reaction (PCR) protocol following Hoenig, Trevelline, et al. (2021) to amplify a hypervariable region of the arthropod cytochrome *c* oxidase I gene using the general arthropod minibarcoding primers ZBJ-ArtF1c and ZBJ-ArtR2c (Zeale et al., 2011); primers were first appended with 5′ adapter sequences complementary to the Nextera XT indexing primers required for sequencing on the Illumina MiSeq Platform (Trevelline et al., 2016). PCRs for each sample were repeated two further times, and the resulting triplicate reactions were pooled and indexed following Illumina's recommended indexing reaction protocol. Positive (*Acroneuria carolinensis*; order: Plecoptera) and negative (DNA-free water) controls were included in all PCR batches and were pooled and indexed in the same manner as the nestling samples. Indexed amplicons were pooled to equimolar concentrations and sequenced on 250-bp paired-end V2 Illumina MiSeq sequencing runs at the Genomics Facility of Biotechnology Resource Center at Cornell University (2016 nestling samples) and the Department of Biological Sciences at Duquesne University (2017 nestling samples). Sequencing was performed with loading concentrations of 8 pM (2016 samples) and 12 pM (2017 samples) and included a 15% (2016 samples) or 20% (2017 samples) PhiX "spike-in" to increase the complexity of the libraries and improve the quality of the sequencing runs.

Resulting DNA sequencing reads were demultiplexed into separate FastQ files and only forward reads were retained for further analysis as the 250-bp reads fully encompassed the DNA barcoding region and flanking primer sequences (~212 bp). Using QIIME2 (Bolyen et al., 2019), sequencing reads were deposited into a shared file using the *tools import* function and forward and reverse primer sequences were removed using the *cutadapt trim-single* function; reads not containing both primer sequences in their entirety were removed from downstream analyses. The remaining sequencing reads were denoised and dereplicated using the DADA2 plug-in's *dada2 denoise-single* function, leaving only unique, nonchimeric exact sequence variants (ESVs; Callahan et al., 2016). ESVs that appeared in fewer than two distinct samples (i.e., singletons) or were represented by fewer than 10 sequencing reads across all samples were removed to reduce the possibility of erroneous sequencing reads being retained in downstream analyses. A naïve Bayes classifier was trained on all North American arthropods within the Barcode of Life Data System (BOLD; Ratnasingham & Hebert, 2007) using the *feature-classifier fit-classifier-naïve-bayes* function. Using the classifier and the *scikit classify-sklearn* function with default arguments, ESVs were matched to the lowest possible taxonomic unit, and if an ESV matched multiple taxa with high confidence (e.g., two species), the lowest shared taxonomic classification was used to classify this sequence (i.e., genus level); ESVs found in either the negative or the positive control were removed from downstream analyses. The resulting taxonomic classifications were associated with the sequences and samples from which they were derived, and the community data set was transformed to a presence–absence data set.

2.3 | Stable isotope methods

Nestling blood samples were analysed at the Cornell University Isotope Laboratory (COIL) where they were freeze-dried, ground and homogenized before ~1 mg of the sample was loaded into tin capsules. Representative samples from the five most commonly detected dietary prey groups in this study (e.g., Ephemeroptera,

Plecoptera, Aquatic Diptera, Terrestrial Diptera and Lepidoptera; Figure 1) were dried in open glass vials at 60°C for 72 hr, ground with a mortar and pestle, and sent to COIL before being loaded into tin capsules. Samples were analysed for $\delta^{13}C$ (an indicator of basal nutrient source; DeNiro & Epstein, 1978) and $\delta^{15}N$ (an indicator of trophic position; DeNiro & Epstein, 1981) on a Thermo Scientific Delta V Advantage Isotope Ratio Mass Spectrometer coupled with a PN150 autosampler and a Carlo Erba NC2500 elemental analyser. Isotope ratios were expressed in parts per mil deviations from each standard (‰) which were Vienna PeeDee Belemnite (VPDB and atmospheric air for $\delta^{13}C$ and $\delta^{15}N$, respectively. Per cent composition of carbon and nitrogen were included and used in downstream isotopic mixing models (Pearson et al., 2003; Phillips & Koch, 2002). COIL also analysed deer tissue as an internal standard for quality assurance and reported the standard deviation for measurement as 0.06‰ and 0.07‰ for $\delta^{13}C$ and $\delta^{15}N$, respectively.

Prior to downstream mixing model analyses, nestling isotopic ratios were corrected with trophic discrimination factors (TDFs) to account for the difference in isotopic signature between predator and prey resulting from biochemical processes during nutrient assimilation (Macko et al., 1986). To determine appropriate TDFs, we used Bayesian linear models (Goodrich et al., 2020; function *stan_lm*, package *rstanarm*, version 2.21.3) which included the isotope ratio of one element (i.e., $\delta^{13}C$ or $\delta^{15}N$) as the response variable and sample type (i.e., nestling or arthropod) as the predictor variable. The TDFs for each element were defined as the slope coefficients from each model. Finally, to ensure that the findings of this study were robust and not heavily influenced by any single TDF value, we calculated 90% credible intervals around each slope coefficient and integrated these intervals into TDF estimates in downstream mixing model analysis. Models were run with four chains each with 2000 iterations, standard uniform priors specified by the *stan_lm* function and the target average proposal acceptance probability set to 0.999 to eliminate divergent transitions. The TDF for $\delta^{13}C$ was 2.35‰ [1.90‰–2.81‰], similar to the expected value based on the literature (Pearson et al., 2003; Post, 2002) and was used in all downstream analyses. The TDF for $\delta^{15}N$ was 0.645‰ [0.012‰–1.278‰], about 2.7‰ lower than what is generally found in studies of birds (Post, 2002). However, all downstream analyses used the modelled $\delta^{15}N$ TDF value for four reasons: (i) using a typical $\delta^{15}N$ TDF value (3.4‰) suggested, impossibly, that the trophic level of nestling waterthrush was below that of their known dietary taxa; (ii) in over 4100 nest site observations (B. Hoenig, unpublished data), very rarely (15 occurrences) did nestlings consume prey other than arthropods (i.e., salamanders), but never primary producers; (iii) all nestling isotope values were within the minimum convex polygon outlined by the prey isotope values; and (iv) the slopes for each sample type (i.e., nestling vs. arthropod) were statistically similar; these all suggested that shared trends in predator and prey isotopic signature were the result of prey consumption and not erroneous TDFs. Furthermore, studies have shown that ontogeny has an effect on $\delta^{15}N$ TDFs, with younger, rapidly developing birds exhibiting lower TDFs in controlled studies (Bearhop et al., 2000; Micklem et al., 2021; Sears et al., 2009), suggesting that such an effect would be expected in this study of nestling songbirds.

Because the isotopes found in consumer tissues constitute a mixture of the isotopes derived from their prey ("animals are what they eat …"; DeNiro & Epstein, 1976), stable isotope mixing models can be used to assess the relative contributions of each prey source to a consumer's assimilated diet (Phillips, 2001). Using the arthropod $\delta^{13}C$ and $\delta^{15}N$ values as sources, nestling values as mixtures, and the calculated TDFs and their credible intervals as mean correction factors and correction errors, respectively, we applied Bayesian stable isotope mixing models (BSIMMs) to determine the relative contribution of each potential prey group to the diets of all nestlings in the 2017 season (i.e., community-level), all nestlings found on the same stream (i.e., stream-level) and all nestlings within the same brood (i.e., nest-level). Because Ephemeroptera and Plecoptera had highly overlapping $\delta^{13}C$ and $\delta^{15}N$ values and share many life-history traits (Brittain, 1990), they were grouped into "Ephemeroptera and Plecoptera." BSIMMs were performed using the *simmr_mcmc* function within the *SIMMR* package (Parnell et al., 2010) with diffuse priors and varied iterations, burn-in and thinning based on the groups of interest (community-wide, iterations = 1000,000, burn-in = 10,000, thinning = 100; stream-wide, iterations = 1000,000, burn-in = 100,000, thinning = 1000; nest-wide, iterations = 100,000, burn-in = 10,000, thinning = 100). The standard ellipse area (a proxy for isotopic niche breadth; Newsome et al., 2007) of each nest was calculated using the functions within the *SIBER* package (Jackson et al., 2011), which follows a similar Bayesian framework as *SIMMR* (diffuse priors, iterations = 100,000, burn-in = 1000, thinning = 10).

2.4 | Statistical analyses

All statistical analyses were performed in R (version 4.1.0; R Core Team, 2021) and summary statistics are presented as the mean ± SD. Frequencies of occurrence (FOO) for the DNA-based methods are the result of dividing the number of diets in which a particular taxon was detected by the total number of diets sampled in a group of interest (e.g., all birds within a season). Tests of equal or given proportions (function *prop. test* package *base*) were performed to determine differences in FOO between breeding seasons, and permutational multivariate analysis (PERMANOVA; function *adonis2*, package *vegan*; Oksanen et al., 2013) and nonmetric multidimensional scaling (NMDS; function *metaMDS*, package *vegan*; Oksanen et al., 2013) using the Jaccard index (Jaccard, 1908) were used to identify annual differences in prey composition; one sample from the 2016 breeding season did not return any species-level prey classifications and was therefore removed from this analysis. For samples collected in 2017, stream-by-stream comparisons of family-level taxonomic richness of different prey groups were performed using an analysis of variance (ANOVA) for each major prey group followed by a post hoc Tukey's test to determine the degree to which streams differed. Similar comparisons using dietary contribution of each prey group

from our isotopic analyses were performed by comparing probabilities of one prey group contributing more than the other being compared (function *compare_groups* package *SIMMR*; Parnell et al., 2010). We calculated the Shannon diversity index (Shannon, 1948) and the standardized Levins' diversity index (Levins, 2020; Trevelline, Nuttle, Hoenig, et al., 2018) using all identified prey found in the samples collected from the 2017 breeding season. Using Bayesian linear models with standard uniform priors and target average proposal acceptance probability set to 0.999 (Goodrich et al., 2020; function *stan_lm*, package *rstanarm*, version 2.21.3), we compared these values to the mean isotopic niche breadth of each nest containing at least three nestling isotopic samples ($n = 11$). To determine the relationship between isotopic niche breadth and the relative contribution of the four common prey groups for each nest, we used Bayesian linear mixed effect models with default priors, the mean isotopic niche breadth as the response variable, the mean contribution estimate of each prey group as the predictor variable, and random intercepts among the streams (LMMs; Goodrich et al., 2020; function *stan_lmer*, package *rstanarm*, version 2.21.3). Effect sizes are presented with either 95% confidence intervals or 90% credible intervals for Bayesian statistics, and test values and *p*-values were calculated at $\alpha = 0.05$.

3 | RESULTS

3.1 | Dietary DNA metabarcoding

High-throughput sequencing returned data for 71.3% (72/101) and 88.9% (64/72) of nestlings sampled in 2016 and 2017, respectively, resulting in 5,554,895 sequencing reads (2016: 2,704,097 reads; 2017: 2,850,798 reads) and 321 unique ESVs after sequence processing. Diptera was the most frequently detected prey order (89.0% FOO across all nestling diets) with Aquatic Diptera present in 84.6% of diets and Terrestrial Diptera in 59.6% of diets. Ephemeroptera (66.2%) and Plecoptera (62.5%) were the next two most frequently detected orders followed by Lepidoptera (55.9%), Trichoptera (33.1%) and Coleoptera (30.1%); however, Coleoptera of terrestrial origin (27.9%) were detected more frequently than those of aquatic origin (2.2%).

While the prey taxa consumed were generally similar between the two breeding seasons, our analysis found that diets did show annual differences at the order-level (Figure 1). The prey orders Decapoda (Difference: 0.13 [0.012–0.248], $p = .027$) and Ephemeroptera (Difference: 0.196 [0.027–0.365], $p = .025$) were detected significantly more often in 2017 than in 2016, while Orthoptera (Difference: 0.262 [0.140–0.385], $p < .001$) was more frequently detected in 2016. Diptera were detected significantly more often in 2017 than in 2016 (Difference: 0.149 [0.036–0.263], $p = .012$), though this effect was driven by differences in Aquatic Diptera (Difference: 0.203 [0.076–0.331], $p = .002$) and not Terrestrial Diptera (0.063 [−0.117 – to 0.242], $p = .57$). We also found that prey composition among nestlings differed between years (PERMANOVA; Pseudo-$F_{1,132} = 7.85$, Standardized Effect Size = 3.10, $p < .001$). NMDS visualization of the first major axis suggested that these differences were driven by consumption of terrestrial prey as aquatic prey scores were centred on the overlapping area of each year's nestling points while terrestrial prey scores showed two distinct peaks centred over each year's nestling points (Figure 2). However, we did not observe significant differences in terrestrial prey richness between years (2016: 63 total terrestrial taxa; 2017: 60 total terrestrial taxa) or in the average number of terrestrial taxa detected in individual nestling samples (2016: 3.17 ± 2.52; 2017: 2.62 ± 1.51). This suggested that differences were driven by consumption of unique terrestrial taxa (14 shared between 2016 and 2017, 25 unique to 2016, and 24 unique to 2017), as opposed to aquatic taxa (24 shared between 2016 and 2017, 19 unique to 2016, and 17 unique to 2017). Among streams, we found significant differences in richness of both Lepidoptera (ANOVA; $F_{3,132} = 2.896$, $p = .038$; Figure 3a) and Ephemeroptera and Plecoptera ($F_{3,132} = 3.514$, $p = .017$; Figure 3a). Nestlings raised on Loyalhanna Creek exhibited a greater dietary richness of Ephemeroptera and Plecoptera taxa when compared to those on Camp Run (Tukey test; Difference: 1.189 [0.064–2.313], adjusted-$p = .034$), Linn Run (Difference: 1.047 [0.023–2.066], adjusted-$p = .042$) and Powdermill Run (Difference: 1.308 [0.191–2.426], adjusted-$p = .015$) as well as a lower dietary richness of Lepidoptera than those nestlings found on Linn Run (Difference: −0.860 [−1.726 to −0.004], adjusted-$p = .048$). We found no significant difference among streams in the average taxonomic richness of Aquatic Diptera ($F_{3,132} = 0.238$, $p = .87$) or Terrestrial Diptera ($F_{3,132} = 1.383$, $p = .251$).

3.2 | Dietary stable isotope analysis

Stable isotope analysis returned data for 53 of the 72 nestlings (73.6%) sampled in the 2017 breeding season with carbon ($\delta^{13}C$) and nitrogen isotopic ($\delta^{15}N$) ratios ranging from −29.95‰ to −24.37‰ and 3.75‰ to 7.61‰, respectively (Figure 4a). Isotopic data were also returned for the four prey groups: Aquatic Diptera (range; $\delta^{13}C$: −28.38‰ to −24.31‰; $\delta^{15}N$: 0.99‰ to 14.17‰), Terrestrial Diptera ($\delta^{13}C$: −25.57‰ to −25.17‰; $\delta^{15}N$: 3.65‰ to 13.00‰), Ephemeroptera and Plecoptera ($\delta^{13}C$: −31.06‰ to −25.27‰; $\delta^{15}N$: 0.25‰ to 7.41‰) and Lepidoptera ($\delta^{13}C$: −30.89‰ to −25.97‰; $\delta^{15}N$: 0.24‰ to 9.36‰; Figure 4a).

BSIMMs performed at the community-level (Figure 4b) indicated that Ephemeroptera and Plecoptera made up the greatest proportion of assimilated waterthrush nestling diet (0.598 ± 0.087) followed by Aquatic Diptera (0.172 ± 0.069), Lepidoptera (0.139 ± 0.057) and Terrestrial Diptera (0.091 ± 0.037). When investigating how prey composition differed among nestlings on each of the study streams, BSIMMs indicated that nestlings on Loyalhanna Creek assimilated a lower proportion of dietary Ephemeroptera and Plecoptera and a higher proportion of Terrestrial Diptera than the other study streams in nearly every mixing model iteration (Figure 3b). Furthermore, nestlings on Loyalhanna Creek were found to assimilate fewer Lepidoptera-derived nutrients than nestlings on Linn Run in over 90% of mixing model iterations (Figure 3b).

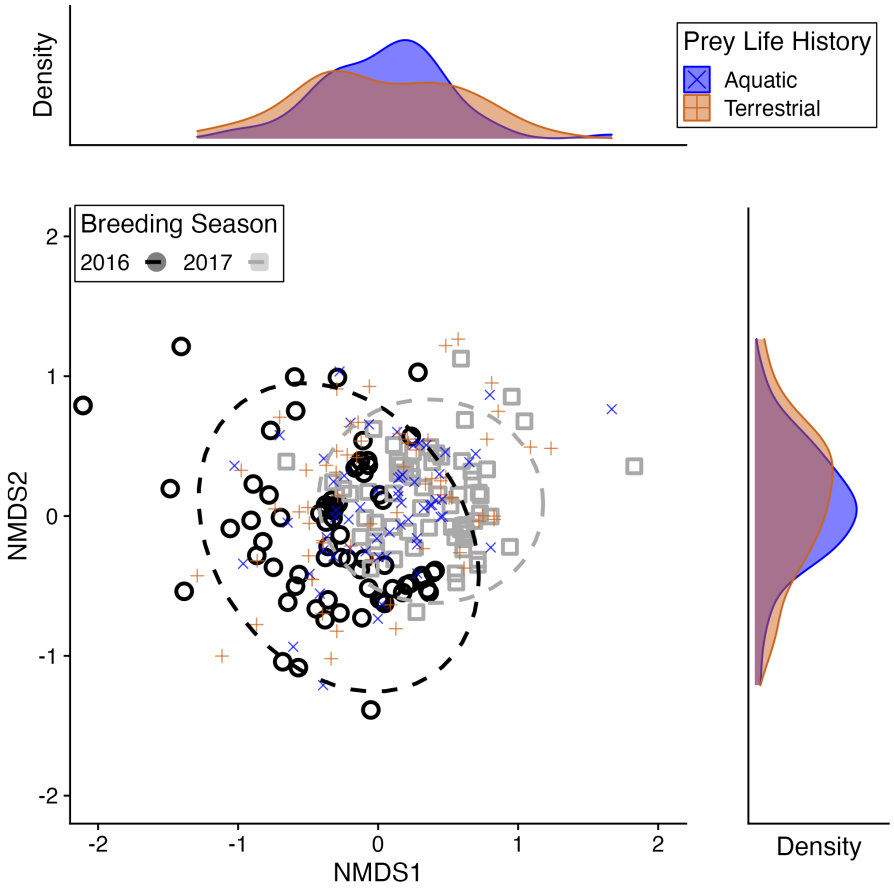

FIGURE 2 DNA-based dietary characterizations highlight the consumption of overlapping aquatic prey taxa, but distinct terrestrial prey taxa, between consecutive breeding seasons. Open shapes from nonmetric multidimensional scaling (stress: 0.210) represent the dietary composition of each nestling in each sampling year, while coloured crosses represent the prey species used in ordination. Ellipses were drawn at 95% confidence for each breeding season. Density diagrams indicate the relative density of prey with either aquatic or terrestrial developmental stages in each dimension [Colour figure can be viewed at wileyonlinelibrary.com]

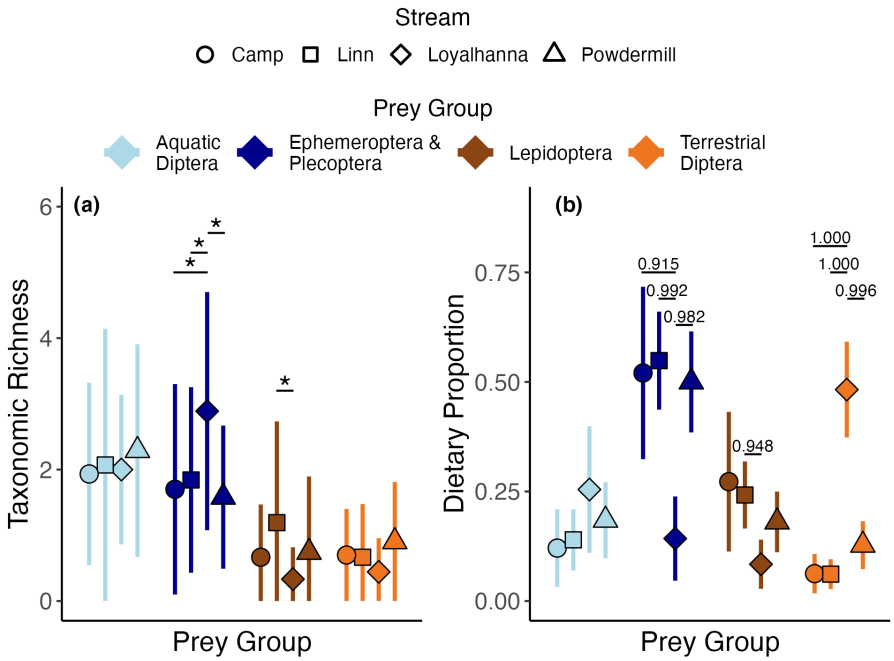

FIGURE 3 DNA metabarcoding (a) and stable isotope analysis (b) reveal different information about nestling Louisiana waterthrush prey composition. Points and lines represent the mean and one standard deviation of the estimated richness or proportion, respectively. (a) Analyses of variance with post hoc Tukey's tests were used to determine significance (*, $p < .05$) in the richness of each prey group between nestlings on different streams. (b) The probabilities of interstream differences in the consumption of a specific prey group were derived from the frequency of BSIMM iterations in which the resulting model predicted one-sided dietary differences greater than 90% of the time [Colour figure can be viewed at wileyonlinelibrary.com]

3.3 | Investigating dietary niche dynamics

To better understand the relationship between DNA-based and isotopic indicators of niche breadth, we performed and compared niche breadth analyses common in such dietary studies; however, we did not find a significant relationship between average isotopic niche breadth and either Levins' ($b = -0.0002$ [−0.0053 to 0.0041] Figure 5a) or the Shannon index ($b = 0.026$ [−0.047 to 0.096] Figure 5b). We also investigated the relationship between the mean dietary proportion of the four main prey groups and the taxonomic niche breadth analyses for each of the nests returning both DNA and isotopic data. We found that decreases in Aquatic Diptera

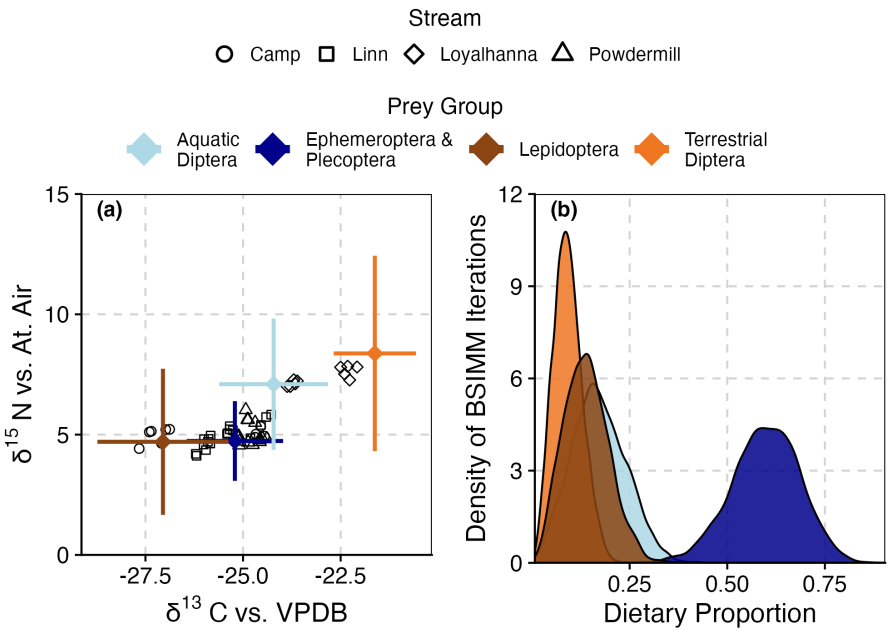

FIGURE 4 Stable isotope mixing models highlight the importance of aquatic arthropod prey for nestling Louisiana waterthrush but reveal dietary variability among nestlings raised on different streams. Open shapes represent the isotopic value of each nestling after correcting for trophic discrimination. Coloured points and lines indicate the mean and one standard deviation for arthropod $\delta^{13}C$ and $\delta^{15}N$ values. Density diagrams show the frequency of BSIMM iterations estimating a dietary proportion for each prey group [Colour figure can be viewed at wileyonlinelibrary.com]

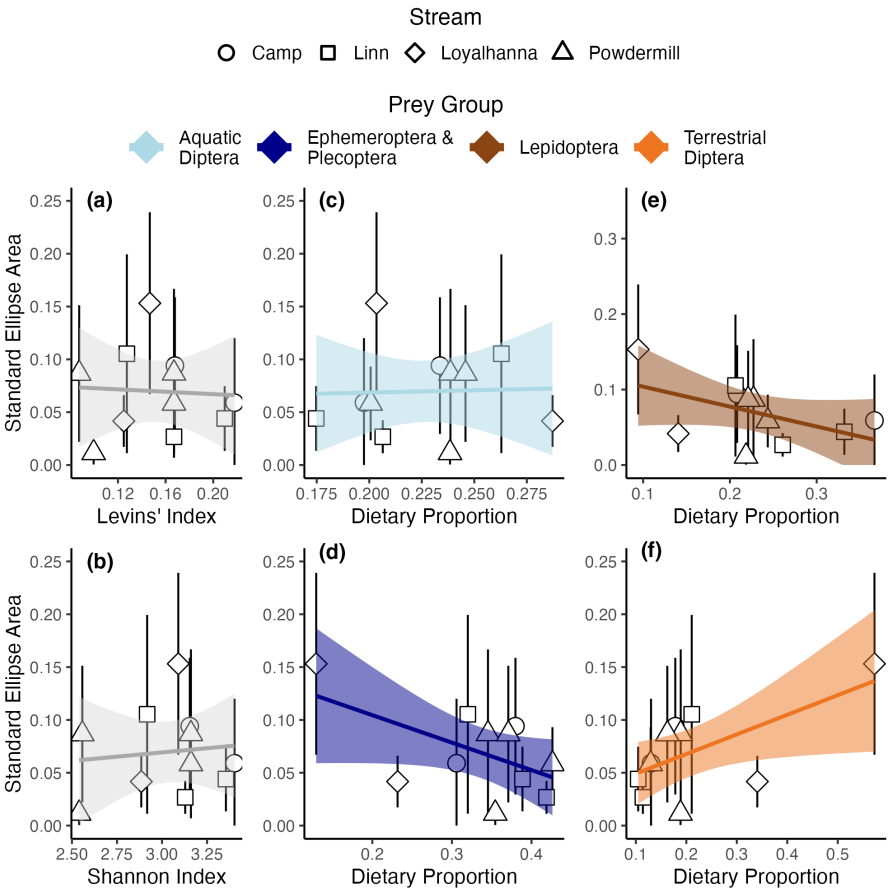

FIGURE 5 Isotopic dietary niche space of Louisiana waterthrush broods are not correlated with taxonomic dietary diversity estimates (a, b) but exhibit significant correlations with quantitative increases in Ephemeroptera and Plecoptera (d) and Terrestrial Diptera prey groups (f). Points indicate the dietary diversity (a, b) or dietary proportion estimate (c–f; *x*-axis) for each Louisiana waterthrush brood with three or more nestlings ($n = 11$) returning isotopic data as well as the mean and one standard deviation around the mean standard ellipse area (*y*-axis). Shading displays the 95% confidence interval around the line of best fit [Colour figure can be viewed at wileyonlinelibrary.com]

($b = -102.10$ [−166.20 to −39.73]) and increases in Lepidoptera ($b = 45.02$ [13.60–77.42]) were strongly associated with higher Levins' index values; however, we did not observe strong effects for Ephemeroptera and Plecoptera ($b = 10.49$ [−27.40 to 50.31]) or Terrestrial Diptera ($b = -11.16$ [−36.49 to 12.81]). Using the Shannon index, Aquatic Diptera prey contribution did show a strong negative correlation with dietary diversity ($b = -5.23$ [−8.91 to −1.48]). However, we did not observe strong effects of prey contribution for Ephemeroptera and Plecoptera (− = 0.27 [−1.82 to 2.41]), Lepidoptera ($b = 1.86$ [−0.12 to 3.95]) or Terrestrial Diptera ($b = -0.33$ [−1.77 to 1.05]) on the Shannon index for each nest.

Finally, we investigated the relationship between the dietary contribution of each prey group and the isotopic niche breadth for each nest. The isotopic niche breadth among nests decreased

as Ephemeroptera and Plecoptera dietary proportion increased (b = −0.277 [−0.548 to −0.009]; Figure 5d), while increases in Terrestrial Diptera prey contribution were correlated with increases in isotopic niche breadth (b = 0.215 [0.051–0.428]; Figure 5f). We did not find a strong relationship between isotopic niche breadth and dietary proportion for Aquatic Diptera (b = −0.021 [−0.716 to 0.727]; Figure 5c), though a weak negative relationship was observed for Lepidoptera (b = −0.273 [−0.589 to 0.019]; Figure 5e).

4 | DISCUSSION

To test how strongly methodology can drive inference in dietary studies, we investigated how the application of two laboratory-based techniques, DNA metabarcoding and stable isotope analysis, shaped our understanding of the prey composition and dietary niche dynamics of a riparian-obligate songbird, the Louisiana waterthrush (*Parkesia motacilla*). Species-level dietary characterizations offered by DNA metabarcoding indicated that specific aquatic-derived prey taxa were more consistently detected between successive seasons than terrestrial-derived taxa. However, as major terrestrial and aquatic prey groups were detected at near equal frequencies, the solely qualitative results returned by DNA metabarcoding could not definitively support the hypothesis that waterthrush specialize on aquatic arthropods. Though unable to provide taxonomic information about waterthrush diet, the quantitative results afforded by stable isotope analysis revealed that aquatic prey contributed to over 7% of the waterthrush's dietary niche and this was largely driven by a high contribution of pollution-intolerant Ephemeroptera and Plecoptera prey.

In addition to quantitative information about prey composition, stable isotope analysis also revealed that the functional dietary niche breadth of waterthrush shrunk as Ephemeroptera and Plecoptera prey contribution increased but expanded when Terrestrial Diptera were consumed in higher amounts; these results suggest specialization when preferred, aquatic prey are available, but generalization when such prey are consumed in lower amounts. Finally, although we found that common prey composition and niche breadth analyses offered by molecular and isotopic approaches were at times uncorrelated, there were also instances where apparently conflicting results led to more informed hypotheses about Louisiana waterthrush dietary ecology. Our results empirically support the recent calls for coupling DNA-based and isotopic approaches in dietary studies (Hoenig, Snider, et al., 2021; Nielsen et al., 2018) and highlight the novel insights that result from combining multiple methods within a single dietary study.

4.1 | Comparing DNA-based and isotopic prey composition estimates

While our DNA-based and isotopic analyses each independently support the waterthrush's reliance upon aquatic prey taxa, we found that the results of DNA metabarcoding and stable isotope analysis, taken together, provided greater insights than what was offered by either technique alone. For example, nestlings raised on Loyalhanna Creek, a stream previously found to have territories nearly devoid of pollution-intolerant taxa (Trevelline, Nuttle, Porter, et al., 2018), exhibited the highest average dietary richness of Ephemeroptera and Plecoptera taxa. An increased dietary taxonomic richness of Ephemeroptera and Plecoptera would seemingly suggest that a greater richness (Birkhofer & Wolters, 2012), and, as a result, abundance (Southwood et al., 1982), of these taxa is available in the environment. However, our isotopic analyses indicated that the dietary contribution of Ephemeroptera and Plecoptera for nestlings on Loyalhanna Creek was actually three times lower than of the other study streams, suggesting that an increase in dietary taxonomic richness for Loyalhanna nestlings may have been a result of prey limitations and not a surplus. These data support the hypothesis that waterthrush on acidified streams occupy territories up to three times greater in length than those on high-quality streams (Mulvihill et al., 2008) and frequently forage in unacidified tributaries (Mulvihill, 1999) in an attempt to provision pollution-intolerant prey not available in the impacted main channel. As a result, waterthrush breeding on impacted streams may be more likely to provision a higher diversity of prey than their counterparts foraging in the main stem of high-quality territories as the likelihood of encountering unique taxa is expected to be positively correlated with the number of unique foraging locations.

Though it has not yet been studied in the waterthrush, research on other altricial species found that utilizing novel foraging substrates (Hollander et al., 2013) and increasing foraging distance (Tremblay et al., 2005) often left nests unoccupied for longer durations and young more vulnerable to predators (Martindale, 1982) or brood parasites (T. E. Martin et al., 2000). Longer foraging trips may also increase the reproductive costs for breeding adults (Stauss et al., 2005) and could explain why acidified streams tend to fledge just over half as many young per kilometre as circumneutral streams (Mulvihill et al., 2008) and why some adults exhibit deleterious carry-over effects on the wintering grounds (Latta et al., 2016). In addition to the impacts that adults may incur, limitations in aquatic arthropod prey may also explain why nestlings raised on streams devoid of pollution-intolerant aquatic arthropods exhibited stunted physiological development (Mulvihill et al., 2008). Recent studies have shown that aquatic arthropod prey possess much higher concentrations of long-chain polyunsaturated fatty acids (Hixson et al., 2015), and that consumption of these aquatic-derived fatty acids are critical for the rapid development of nestling songbirds (Twining et al., 2016, 2018). As prey composition represents an important facet of a species' life history with far-reaching consequences, our findings suggest an imperative need for researchers to study both the qualitative and the quantitative aspects of a species' diet to ensure our understanding of a species' trophic ecology is as complete as possible.

4.2 | Comparing DNA-based and isotopic niche breadth measurements

In addition to understanding how method choice influenced our understanding of waterthrush prey composition, we compared common dietary niche breadth metrics returned by DNA-based and isotopic methods to determine how method selection could impact our understanding of dietary niche dynamics. Surprisingly, we found that the isotopic niche breadth showed no correlation with either Levins' or Shannon diversity indices. Although we expected a positive correlation between isotopic niche breadth and taxonomic niche breadth, this does not necessarily need to be the case. Hypothetically, a species that consumes only aquatic arthropod detritivores, a prey group with hundreds of species spanning multiple taxonomic orders (Merritt et al., 2017), would probably exhibit a high degree of *taxonomic* dietary diversity, but display almost no *functional* dietary diversity as these phylogenetically distinct prey still derive their isotopic signatures from the same substrates.

Similarly, when determining how niche breadth is affected by the contribution of each common prey group, the method of niche breadth analysis greatly influenced the interpretation of our results. For example, although nest-wide comparisons of the isotopic niche breadth to either Aquatic Diptera or Lepidoptera prey dietary contribution showed no significant correlations, comparing the mean dietary contribution of Aquatic Diptera and Lepidoptera to Levins' index exhibited significant negative and positive correlations, respectively. At first glance, this would seem to further support the hypothesis that waterthrush do specialize on aquatic prey when it is available but generalize on terrestrial prey when it is not. However, as this relationship was not observed with the main aquatic prey source, Ephemeroptera and Plecoptera, it is also possible that these correlations bear little ecological consequence and are instead a spurious result of taxonomically rich (Diptera: >120,000 species, Lepidoptera: >150,000 species; Grimaldi & Engel, 2005), but functionally uniform, prey groups. As the taxonomic classification of prey is of little importance to the consumers being studied ("[consumers] do not eat Latin binomials"; Janzen, 1979), future work should strive to incorporate functional or trait-based prey measurements into dietary characterizations (e.g., Arrizabalaga-Escudero et al., 2019), while considering how much information can be gleaned from taxonomic richness alone (Fordyce et al., 2016) and being cautious not to misinterpret results that may be statistically significant but have questionable ecological relevance.

4.3 | Waterthrush prey composition and functional niche dynamics

In this study, we also sought to better understand how each brood's functional dietary niche breadth was influenced by its quantitative prey composition. Although we did not find a significant correlation between isotopic niche breadth and either Aquatic Diptera or Lepidoptera prey contribution, we did observe significant correlations for both the Ephemeroptera and Plecoptera and Terrestrial Diptera prey groups, in negative and positive directions, respectively. The observed shrinking of functional dietary niche breadth in response to increased Ephemeroptera and Plecoptera prey contribution and niche expansion in response to an increase in Terrestrial Diptera prey contribution provides further evidence that breeding waterthrush preferentially provision pollution-intolerant aquatic prey to their young but widen their trophic niche when such prey are not readily available (Trevelline, Nuttle, Porter, et al., 2018).

Numerous studies have documented similar shifts in a consumer's functional dietary niche breadth in response to prey availability. For example, Gulka et al. (2017) found that the isotopic niche breadth of five marine predators decreased significantly as the abundance of a dominant forage fish, the capelin (*Mallotus villosus*), increased, while a study of great and sooty shearwaters (*Ardenna gravis* and *A. grisea*, respectively) found that each of these species increases its isotopic niche and relies on alternative prey when the abundance of capelin is limited (Carvalho & Davoren, 2020). In these studies, prey limitations were caused by regular seasonal prey fluctuations, though similar results were observed in martens (*Martes americana*), whose prey were limited by the simultaneous collapse of various prey populations in response to extreme cold weather and increased snow retention (Thompson & Colgan, 1990). While the prey limitations observed in these studies stemmed from natural, yet often unpredictable, processes, detrimental prey limitations for waterthrush and other ecological specialists will probably be anthropogenic in nature (Clavel et al., 2011) due their reliance on unimpacted habitats (Julliard et al., 2006) and the prey that these habitats support (Morelli et al., 2021). For waterthrush, these anthropogenic effects often come as a result of direct impacts, such as abandoned mine discharge (Mulvihill et al., 2008) or shale gas development (Frantz et al., 2018). However, climate change and other persistent, large-scale disturbances point to the essential need to characterize how anthropogenic impacts alter trophic interactions so that we may identify the prey groups of highest conservation concern, such as those that sustain the greatest diversity of consumer taxa (Rosenberg et al., 2019; Wagner et al., 2021).

4.4 | Methodological considerations

Although the combination of DNA-based and stable isotope approaches greatly increased our ability to characterize waterthrush diets, our methods still present limitations worth considering. Because the DNA-based description of diet was based on a single primer set targeting only arthropod taxa, it is almost certain that some prey taxa went undetected (Elbrecht & Leese, 2017b; Forsman et al., 2021). The intentional nondetection of nontarget prey taxa (i.e., salamanders) is often necessary in DNA metabarcoding as it is unlikely that any single primer set can be sufficiently generalized to amplify taxon-specific sequences for all putative target taxa (Elbrecht & Leese, 2015, 2017a) while simultaneously being sufficiently specialized to not amplify nontarget DNA (Vestheim &

Jarman, 2008). While the missed detection of nontarget prey would not alter the conclusions of a study and can be resolved by targeted single-species sequencing (Shokralla et al., 2014) or novel CRISPR-based applications (Williams et al., 2019), the nondetection of target prey taxa (i.e., false negatives) poses much larger issues (Zinger et al., 2019) and may leave a lasting impact on our understanding of a species' dietary niche (Forsman et al., 2021). Although the present study was limited to a single primer set (ZBJ; Zeale et al., 2011), we agree with the growing body of work recommending that researchers use multiple primer sets (Forsman et al., 2021), potentially targeting multiple genes (Alberdi et al., 2018), as such approaches tend to detect a greater number of prey taxa and provide more taxonomically precise prey characterizations. Nevertheless, as our DNA-based analyses were focused on broad taxonomic classification (i.e., family or order level) and our results largely agree with past work employing both DNA-based (Trevelline et al., 2016; Trevelline, Nuttle, Hoenig, et al., 2018; Trevelline, Nuttle, Porter, et al., 2018) and morphological methods (Eaton, 1958; though see Craig, 1987), we are confident that the present study provides an accurate taxonomic representation of the arthropod diet of nestling waterthrush.

In addition to those drawbacks common to DNA-based studies, there were limitations to our isotopic dietary description. Although prey DNA recovered from diet samples is often degraded, in terms of sequence it is fundamentally unchanged, providing a direct link between DNA sequence and taxonomic classification. However, as a result of consumer metabolism, stable isotope ratios are often altered before assimilation into consumer tissues in a process termed trophic discrimination (Macko et al., 1986). Because the accuracy of mixing model analyses can be sensitive to trophic discrimination (Bond & Diamond, 2011), researchers often derive corrections from controlled-feeding studies or relevant published literature to apply before mixing model analysis (Martínez del Rio et al., 2009). However, the degree of trophic discrimination for a consumer can result from a number of factors (reviewed in Caut et al., 2009), meaning it is unlikely that any published TDFs are perfectly applicable to any study system (Bond & Diamond, 2011). Due to a lack of published TDFs for nestling songbirds, this study derived TDFs from Bayesian linear models and found that, while the model-derived $\delta^{13}C$ TDF was close to that found in other insectivorous birds (Pearson et al., 2003), the model-derived $\delta^{15}N$ TDF was considerably lower (−0.645‰) than suggested by most literature (3‰–3.5‰; Post, 2002). Although we are confident the TDFs applied in this study are appropriate for reasons outlined above (see Stable isotope methods), we recognize the importance of empirically identifying the role of ontogeny in trophic discrimination and encourage future research to not only investigate these effects, but also incorporate them into existing models used to estimate trophic discrimination factors (Healy et al., 2018).

4.5 | Future directions

Our findings suggest that single-methodology studies, even those employing highly sensitive molecular and chemical techniques, may be hampered by technique-specific limitations, thus leaving our understanding of a species' dietary ecology either incomplete, or worse, incorrect. To mitigate the biases associated with any single technique, we recommend that researchers critically consider the limitations of the study's proposed methodologies, attempt to employ a supplementary method to answer trophic questions not possible with the primary study method, and better contextualize the findings of their work by explicitly stating the dietary variable being studied. As molecular dietary characterizations are in their infancy compared to traditional and chemical techniques, there is still debate over the boundaries of DNA-based applications, with prey quantification being of the most contentious. Though the prospect of DNA-based quantification continues to become more attractive with advances in computational modelling (Piñol et al., 2019) and DNA amplification approaches (e.g., digital-droplet PCR), the biases inherent to molecular techniques currently leave only a weak and imprecise relationship between prey sequence count and biomass (Lamb et al., 2019). While the present study used stable isotope analysis to overcome this prey quantification gap, researchers have supplemented DNA-based studies with morphology-based prey identification (Martin et al., 2021) and other chemical-based techniques (fatty acid analysis; Génier et al., 2021) to get a better understanding of prey amounts. With that said, not all studies will have the instrumentation, personnel or financial capabilities to perform–essentially–two separate dietary studies. In situations where only a single approach can be used, we recommend that researchers be transparent about the limitations of their chosen approach and specific about the results being discussed. Though trophic ecological terms, such as *dietary niche breadth* or *prey composition*, are often treated as interchangeable between studies, the present study indicates a need for contextualization of these results to ensure that the difference between quantitative/qualitative and taxonomic/functional results are well understood.

4.6 | Conclusions

Though DNA-based and isotopic dietary characterizations, independently, have shown marked improvements over those stemming from morphological techniques, the application of DNA metabarcoding and stable isotope analysis, together, presents an avenue for researchers to obtain insights into a species' trophic ecology not possible with either technique alone. In this study, the use of either method, independent of the other, supported previous work suggesting that waterthrush preferentially provision pollution-intolerant aquatic arthropods to their young. However, the use of these methods in concert indicated that adult waterthrush expand their functional trophic niche when pollution-intolerant prey are provisioned less frequently and highlighted a disconnect between the common measurements of niche breadth and prey composition afforded by each method. This study strongly suggests that the application of multiple techniques within a single dietary study will yield the most comprehensive

understanding of a species' diet (Hoenig, Snider, et al., 2021; Nielsen et al., 2018), and we encourage future researchers to incorporate multiple approaches to adequately address the most pressing questions in trophic ecology.

AUTHOR CONTRIBUTIONS

Brandon D. Hoenig, Steven C. Latta and Brady A. Porter conceived and designed the study. Brandon D. Hoenig collected all nestling samples (with Brian K. Trevelline), and Andrea Kautz (with Brandon D. Hoenig) collected and taxonomically classified all arthropod samples. Brandon D. Hoenig conducted all laboratory (with Brian K. Trevelline), bioinformatic and statistical analyses. Brandon D. Hoenig wrote the manuscript in the laboratory of Brady A. Porter, which was improved by contributions from Brian K. Trevelline, Andrea Kautz, Steven C. Latta and Brady A. Porter.

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CONFLICT OF INTEREST

S.C.L. serves as Director of Conservation and Field Research at the National Aviary and A.K. serves as a Research Entomologist at Powdermill Nature Reserve (Carnegie Museum of Natural History).

OPEN RESEARCH BADGES

This article has earned an Open Data Badge for making publicly available the digitally-shareable data necessary to reproduce the reported results. The data is available at [provided https://doi.org/10.5061/dryad.rv15dv48z].

DATA AVAILABILITY STATEMENT

The data and analytical pipelines required to reproduce the analyses in this paper are archived and can be found at https://doi.org/10.5061/dryad.rv15dv48z.

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