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Evaluation of Fecal Metabarcoding for Studying the Diets of Sympatric Dusky Salamanders (*Desmognathus*)

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Abstract.—**Amphibian diet studies often rely on visual identification of prey obtained through forced regurgitation or dissection. These approaches are somewhat invasive and often lack taxonomic specificity, which can discourage diet studies involving at-risk species and limit fine-scale investigations of diet composition. Here, we employ and assess a non-invasive molecular technique to characterize the diets of three co-occurring stream-dwelling salamander species, Ocoee Salamanders (*Desmognathus ocoee*), Seal Salamanders (*Desmognathus monticola*), and Nantahala Black-bellied Salamanders (*Desmognathus amphileucus*) in northern Georgia, USA, and we investigate possible dietary partitioning within and among species. We used DNA metabarcoding to classify the arthropod prey communities from fecal samples of field-collected salamanders and investigated associations with predator species and snout-vent length (SVL). Of 200 salamanders captured and held for 24 h, 38 (19%) produced fecal samples containing arthropod DNA. We identified 53 prey taxa, of which we could classify 27 to species, 12 only to genus, 10 only to family, and four only to order. We found no evidence of dietary partitioning among species or by SVL. Individual fecal samples generally contained few taxa, and few taxa were shared among samples, suggesting that our sample size likely limited the power of our inference. Our results support the utility of fecal metabarcoding as a non-invasive and taxonomically precise alternative to traditional diet analysis techniques. Researchers should also consider the challenges associated with fecal metabarcoding (e.g., infrequent defecation by study organisms, inability to identify life stage of prey), however, before using it to complement more traditional methods.**

Key Words.—arthropod; competition; genetic; next-generation sequencing; Plethodontidae

Introduction

Dietary studies allow biologists to investigate broad questions about ecology and evolution and can have practical use for managing species of conservation concern (Anderson 1991; Solé and Rödder 2009). The scope of these studies is sometimes limited by the feasibility, reliability, and resolution of the methods used, however. Traditional methods used to investigate the diet of amphibians include direct observation of *in-situ* feeding, analysis of stable isotopes, and visual identification of consumed prey via gastric lavage, fecal dissection, or lethal dissection (Solé and Rödder 2009). Although observation of *in-situ* feeding provides direct evidence of amphibian diet composition, the secretive behavior of many amphibians makes it impractical for widespread use in field studies. Stable isotope analyses can reveal broad dietary patterns and the structure of food webs, but they may require exhaustive tissue sampling of all potential prey taxa to reliably identify and distinguish between similar species (Whiles et al. 2006; Gillespie 2013; Schriever and Williams 2013; Arribas et al. 2015). Gastric lavage and lethal dissection techniques are more invasive and may be biased against the identification of soft-bodied prey, which are digested more quickly and underrepresented in feces (Marques et al. 2022), and fecal samples are often too small and degraded for comprehensive and accurate visual identification of consumed prey (Crovetto et al. 2012; Costa et al. 2014). Furthermore, these dissection-based methods often fail to identify prey beyond taxonomic order (Homyack et al. 2010; Strain et al. 2014; Hutton 2019). Therefore, investigations of fine-scale dietary differentiation among individuals, sexes, or species with relatively similar diets (i.e., mostly consisting of the same broad taxonomic groups) are often not possible using only traditional techniques. One alternative is barcoding (i.e., DNA sequencing) of individual prey items isolated from gastric lavage or lethal dissection (e.g., Unger et al. 2019). Although this method may provide greater taxonomic resolution, it still requires somewhat invasive procedures and may be cost-prohibitive for many samples and diverse prey communities.

In the last decade, the decreasing cost of high-throughput DNA sequencing has made the application of DNA metabarcoding a realistic alternative for dietary studies. This next-generation sequencing adaptation of traditional barcoding allows for identification of various taxonomic groups in mixed, degraded community samples (Taberlet et al. 2012). Typically, metabarcoding approaches minimally consist of three steps: (1) DNA extraction from a mixed community (e.g., a fecal sample); (2) PCR amplification and sequencing of a specific barcoding locus (for animals, often the mitochondrial cytochrome c oxidase [COI]; Hebert et al. 2003; Deiner et al. 2017); and (3) identification of amplified sequences (e.g., by comparison to a reference database; Taberlet et al. 2012; Fig. 1). Dietary analyses based on fecal metabarcoding are now common in various taxa, including mammals (Trevelline et al. 2018; Harper et al. 2020; Sonsthagen 2020; Ingala et al. 2021; Roffler et al. 2021), birds (Shutt et al. 2020; Garfinkel et al. 2022; Snider et al. 2022), and fish (Guillerault et al. 2017; Johnson et al. 2021; Villsen et al. 2022). In the last two years, fecal metabarcoding has been used in diet studies of amphibians (Pereira et al. 2021; Wang et al. 2021; Marques et al. 2022), and it may provide an avenue for the investigation of dietary preferences and differentiation at a finer taxonomic resolution than previously possible.

Diverse communities of dusky salamanders (genus *Desmognathus*) in the southeastern U.S. provide an opportunity to test the feasibility and utility of fecal metabarcoding for amphibian diet studies while investigating novel questions about the relationship between microhabitat, body size differences, and fine-scale diet differentiation. Typically, sympatric species of *Desmognathus* exhibit a gradient in size and microhabitat association (Krzysik 1979; Hairston 1980). Larger species are usually more aquatic than smaller species, which are typically more terrestrial (Krzysik 1979; Hairston 1980). This apparent niche partitioning is likely the result of competition and other interspecific interactions (e.g., predation) that structure these communities (Krzysik 1979; Hairston 1980; Bruce 2011). *Desmognathus* salamanders are often considered opportunistic generalist insectivores (Krzysik 1979) and the few studies that have examined diets at broad taxonomic resolution (e.g., by identifying prey to order or family) find substantial dietary overlap among sympatric species (Krzysik 1979; Holomuzki 1980), with limited differentiation putatively explained by differences in gape size and microhabitat preference (Krzysik 1979). Because previous studies rarely identified prey beyond taxonomic order or family, sympatric species of *Desmognathus* provide a valuable opportunity to test the merits of fecal metabarcoding for *in-situ* diet analyses of amphibians.

Salamander communities in the Upper Tallulah River of northern Georgia, USA, include as many as six species of *Desmognathus* (Rothermel et al. 2013), several of which belong to groups that have undergone taxonomic revisions during our study (Pyron and Beamer 2022a,b; Pyron et al. 2022). The three most common species are Nantahala Black-bellied Salamanders (*D. amphileucus*), Seal Salamanders (*D. monticola*), and Ocoee Salamanders (*D. ocoee*). Of the three, *D. amphileucus* is the largest and most aquatic, *D. ocoee* is the smallest and most terrestrial, and *D. monticola* is intermediate in size and terrestriality (Petranka 1998). Other *Desmognathus* spp. in this community include Shovel-nosed Salamanders (*D. marmoratus*; *marmoratus* B sensu Beamer and Lamb 2020), Dwarf Black-bellied Salamanders (*D. folkertsi*), and Seepage Salamanders (*D. aeneus*). Here, we refine a protocol

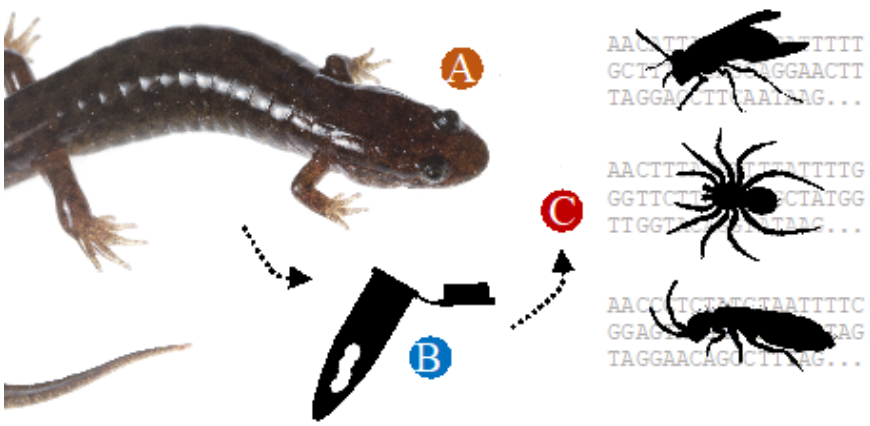

Figure 1. A simplified visual schematic highlighting the major steps of a fecal metabarcoding study: (A) location of the study species or sample source (in this case, a Seal Salamander, *Desmognathus monticola*); (B) fecal sample collection, DNA extraction, and library preparation; and (C) sequencing and bioinformatic assignment of sequence reads from prey DNA.

for the field collection and metabarcoding analysis of amphibian fecal samples (Fig. 1) and assess potential species- and size-associated dietary partitioning among *Desmognathus*. We discuss the potential utility and pitfalls of metabarcoding-based approaches for amphibian diet studies and hope that our research serves as a proof of concept and a roadmap for future investigators.

Materials and Methods

Fecal sample collection.—We conducted our study along a short reach of the Tallulah River and two of its tributaries (Beech Creek and Burnt Cabin Branch) at the Charles H. Wharton Conservation Center in the Chattahoochee National Forest of northern Georgia, USA (34.9899, -83.5567). During a 3-d period in early October 2019 and a 3-d period in late September 2020, we captured 200 salamanders in and immediately adjacent to these streams. We identified 83 as *D. monticola*, 54 as *D. ocoee*, and 63 as *D. amphileucus* (formerly a taxonomically unresolved member of the *D. quadramaculatus* complex; Pyron et al. 2022a) using traditional gross morphological diagnostic characteristics including coloration, dorsal and ventral pattern, and tail shape (Petranka 1998; see Pyron et al. 2022a for diagnostic characteristics specific to *D. amphileucus*). After capture, we placed each salamander in a single-use plastic bag, marked the site of each capture with construction flagging, and transported salamanders to a central, temperature-controlled location on-site. We then housed salamanders individually in sterile plastic sandwich containers with moist paper towel substrates for 24 h. During the housing period, we checked for feces every 3–6 h and transferred each observed fecal sample to a labeled 1.5 mL microcentrifuge tube containing 95% EtOH. After 24 h, we measured the snout-vent length (SVL) of each salamander, released it at the site of capture, and removed all construction flagging. We stored fecal samples at -20° C prior to DNA extraction.

DNA extraction and metabarcoding library preparation.—We extracted DNA from full fecal samples using a Quick-DNA Fecal/Soil Microbe Miniprep kit (Zymo Research, Irvine, California, USA) following the recommended protocol of the manufacturer. To identify potential contamination associated with molecular work, we conducted a single negative control extraction and carried it through all subsequent steps. We amplified DNA using iTru fusion (Glenn et al. 2019b) versions of ANML primers designed for approximately 230 base pairs (bp) of the mitochondrial cytochrome oxidase I (COI) gene (Jusino et al. 2019). These primers were designed for use in barcoding diverse arthropods (Jusino et al. 2019) and have notable advantages over other COI primers (e.g., detection of more prey taxa in feces from predators fed a standardized diet and increased detection of known arthropod species in field trials relative to other common arthropod COI primer sets; Jusino et al. 2019). They also have been used in many previous diet studies that rely on metabarcoding of feces from species that produce small and degraded fecal samples (e.g., Novella-Fernandez et al. 2020; Whitby et al. 2020; Forsman et al. 2022; Stillman et al. 2022). We used a Kapa HiFi HotStart PCR kit (Kapa Biosystems, Woburn, Massachusetts, USA) and set up 25 µL reactions containing the following: 5 µL 5X HiFi Buffer, 0.75 µL dNTPs (final concentration = 0.3 µM), 11.75 µL H2O, 0.5 µL Kapa HiFi DNA Polymerase (final concentration = 0.02 unit/µL), 1 µL each iTru fusion ANML forward and reverse primers (final concentration = 0.4 µM each), and 5 µL extracted DNA. We conducted PCR using the following thermocycler conditions: 95° C for 3 min; then, 35 cycles of 98° C for 20 s, 55° C for 30 s, and 72° C for 15 s; then, 72° C for 1 min.

Following the first PCR, we cleaned PCR products with SpeedBeads (Sigma-Aldrich, Burlington, Massachusetts, USA) using a 1.5:1 SpeedBeads:PCR product volume ratio and resuspended DNA in 25 µL H2O. To add indexes for multiplexing and to create full-length Illumina libraries, we then conducted a second PCR using iTru5 and iTru7 primers (Glenn et al. 2019a). We again used a Kapa HiFi HotStart PCR kit and set up 25 µL reactions containing the following reagents: 5 µL 5X HiFi Buffer, 0.75 µL dNTPs (10 µM), 8.75 µL H2O, 0.5 µL Kapa HiFi DNA Polymerase (1 unit/µL), 2.5 µL each of iTru5 and iTru7 primers, and 5 uL of cleaned product from the first PCR. We conducted a limited-cycle PCR using the following thermocycler conditions: 95° C for 2 min; then, 5 cycles of 98° C for 20 s, 60° C for 15 s, and 72° C for 30 s; then, 72° C for 5 min. Following the second PCR, we cleaned PCR products with a 1:1 SpeedBeads:PCR product volume ratio and resuspended DNA in 25 µL H2O. We then quantified products with a Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA), pooled proportionately for approximately 40 ng DNA per sample, and combined these libraries with those from unrelated projects to target a total of approximately 1.6 million PE150 reads on two Illumina MiSeq Nano sequencing runs, each with about 5% of reads dedicated to PhiX.

Bioinformatic analyses.—We demultiplexed reads using iTru7 and iTru5 indexes and entered reads in the DADA2 (Callahan et al. 2016) bioinformatic pipeline in QIIME 2 2019.10 (Bolyen et al. 2019). After quality filtering, trimming, merging, and denoising paired reads, we detected 437 amplicon sequence variants (ASVs).

We then implemented a naive Bayes classifier in QIIME 2 2019.10 using scikit-learn (Pedregosa et al. 2011) with a 70% confidence threshold to assign taxonomy to retained arthropod ASVs. We conducted this taxonomic assignment with a pre-trained COI classifier for ANML amplicons (bold_full_ArthOnly_classifier.qza; https://osf.io/jxtek) that was trained from sequences on BOLD (Ratnasingham and Hebert 2007) and created using RESCRIPt (O'Rourke et al. 2020; Robeson et al. 2021). We then filtered our dataset to retain only those ASVs identified at least to taxonomic order and exported tables with taxonomy collapsed to order, family, and species. Because these reads were already denoised in DADA2, we did not further exclude arthropod ASVs with low read counts from subsequent analyses. For ASVs that had many raw reads but were not retained following taxonomic classification, we conducted a GenBank BLAST (Altschul et al. 1990; Clark et al. 2015) search to determine putative sources. All sequence reads are available from the NCBI SRA (PRJNA795787), and all code and results are available from the Dryad Digital Repository (https://doi.org/10.5061/dryad.2rbnzs7r0).

Statistical analyses.—We first tested whether the probability of defecation (i.e., 0 = no fecal samples with arthropod ASVs; 1 = at least one fecal sample with an arthropod ASV) was predicted by salamander species, SVL, or year of collection. Because we could not conclusively determine whether samples that contained no arthropod ASVs were other organic material or feces containing DNA only from non-arthropod prey (see Results), we only counted putative fecal samples that included at least one ASV assigned to an arthropod as positive evidence of defecation. We fit a Generalized Linear Model with a binomial error term using the function glm in R v4.1.2 (R Core Team 2021) to evaluate these relationships, and included salamander species, year of collection, and SVL as predictors. We assessed statistical significance of predictors using Likelihood-ratio Chi-squared tests ($\alpha = 0.05$) with the function Anova in the package car v3.0-12 (Fox and Weisberg 2019).

We evaluated whether the presence of common prey taxonomic orders and families were associated with salamander species or SVL to assess dietary partitioning within and among sympatric species of *Desmognathus*. We included only the five orders (Araneae, Diptera, Hemiptera, Hymenoptera, and Lepidoptera) and four families (Cecidomyiidae, Limoniidae, Aphididae, and Erebidae) of arthropods that were found in at least five fecal samples to avoid spurious associations. To assess these relationships, we again fit Generalized Linear models with a binomial error term using the function glm in R v4.1.2 (R Development Core Team 2021) and included source-individual species and SVL as predictors. We evaluated statistical significance of predictors using Likelihood-ratio Chi-squared tests with the function Anova in the package car v3.0-12 (Fox and Weisberg 2019) with a Bonferroni correction for multiple comparisons (adjusted $\alpha = 0.01$).

Results

For many fecal samples (especially those from *D. amphileucus* and *D. monticola*), most assembled reads were assigned to ASVs matching sequences of *Desmognathus* sp. available on GenBank. This demonstrates the non-specificity of primers (which were designed for arthropods) and non-target amplification of DNA from the source of the fecal sample. In most cases, all or the large majority of reads were assigned to an ASV corresponding to a single species of *Desmognathus* (e.g., a fecal sample from quad_24 had 15,529 reads assigned to an ASV matching *D. amphileucus* and only 55 reads assigned to an ASV matching *D. monticola*). This BLAST comparison of *Desmognathus* ASVs to sequence data available on GenBank corroborated most of our identifications from the field, but it also helped identify a few discrepancies. For example, one sample we originally identified as *D. amphileucus* (monticola_84) had most reads assigned to an ASV that matched *D. monticola,* and two samples we originally identified as *D. amphileucus* (quad_51 and quad_52) had most reads assigned to an ASV that differed from those found in all other putative *D. amphileucus* samples and instead closely matched sequence data available for Dwarf Black-bellied Salamanders (*D. folkertsi*), a closely related and morphologically similar species that is sometimes difficult to differentiate in the field. For simplicity, and because we had a limited sample size for these two species (which are close relatives and ecologically similar; Camp et al. 2013), we included these two samples with those from *D. amphileucus* for all subsequent analyses.

We collected 51 fecal samples from 49 individuals (25 *D. monticola*, 10 *D. ocoee*, 14 *D. amphileucus/folkertsi*). Of these samples, 11 (21.57%) did not yield amplified DNA that could be assigned to at least one arthropod order, suggesting that either the sample consisted of some organic material other than feces (e.g., shed skin or detritus that accidentally was placed into the container with the salamander), or that prey were not arthropods. Thus, of the 200 total salamanders in our study, at least 38 (19%) produced fecal samples with amplifiable arthropod DNA. These included 21 fecal samples from *D. monticola,* 11 fecal samples from *D. amphileucus* and *D. folkertsi*, and eight fecal samples from *D. ocoee*. No sequence reads from the negative control were assigned to arthropod ASVs; however, a small number of reads (21) in the negative

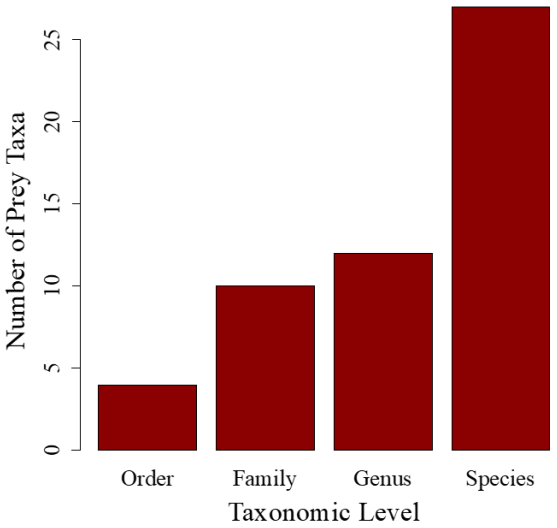

Figure 2. Total number of prey amplicon sequence variants (ASVs) from our study of Ocoee Salamander (*Desmognathus ocoee*), Seal Salamander (*D.monticola*), Nantahala Black-bellied Salamander (*D. amphileucus*), and Dwarf Black-bellied Salamander (*D. folkertsi*) feces that terminated at the four represented taxonomic levels (i.e., ASVs in the Genus category were able to be identified to order, family, and genus, but not species). Successful classification to species was more common than termination at any other taxonomic level.

control were assigned to an ASV that matched *D. amphileucus*, perhaps indicating low levels of laboratory contamination or index-hopping in the sequencing run.

The probability of producing one of these fecal samples did not differ by species (Likelihood-ratio Chi-squared [*LR*] = 3.338, *P* = 0.189; SVL, *LR* = 0.212, *P* = 0.645; or year *LR* = 0.786, *P* = 0.375). After filtering and classification, fecal samples yielded an average of 2,458 arthropod-assigned reads per sample (range, 2–14,829), from which we identified 53 arthropod prey taxa in eight orders. Of these taxa, we could identify most to species (n = 27) and relatively few only to genus (n = 12), family (n = 10), or order (n = 4; Fig. 2). Fecal samples from *Desmognathus monticola* contained 33 arthropod taxa (Table 3), those from *D. ocoee* contained 18 taxa (Table 1), and those from *D. amphileucus/folkertsi* contained 15 taxa (Table 2). Individual fecal samples generally contained few classified taxa (median = 1.5; range, 1–6), and each classified taxon was often present in few fecal samples (median = 1; range, 1–8). We found no evidence that the presence of the five most common prey orders differed among species (*LR* = 0.791–4.651, *P* = 0.098–0.673) or by SVL (*LR* = 0.0155–4.059, *P* = 0.044–0.901) or that the presence of the four most common families differed among species (*LR* = 0.488–4.162, *P* = 0.125–0.784) or by SVL (*LR* = 0.016–3.842, *P* = 0.050–0.899). For the full model outputs, refer to the materials in the Dryad Digital Repository (https://doi.org/10.5061/dryad.2rbnzs7r0).

Discussion

Our data illustrate both the promise and pitfalls of the use of fecal metabarcoding for dietary analysis. One major advantage of this approach is its improved taxonomic resolution. We were able to identify 51% of prey items to the species level and nearly 75% to genus, indicating that metabarcoding can provide a level of taxonomic resolution that is often not possible using traditional methods (Homyack et al. 2010; Strain et al. 2014; Hutton 2019). This specificity may be useful to researchers interested in fine-scale dietary partitioning among species, populations, or individuals. We only used primers that targeted arthropod prey, but future researchers could use additional primers to characterize

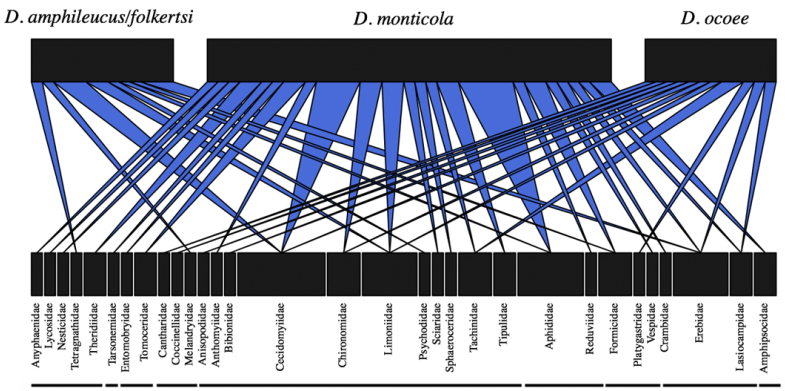

Figure 3. Interaction web depicting predation of arthropod families by either Nantahala Black-bellied Salamanders or Dwarf Black-bellied Salamanders (*Desmognathus amphileucus/folkertsi*), Seal Salamanders (*D. monticola*), and Ocoee Salamanders (*D. ocoee*). Arthropod families are sorted by taxonomic order and listed alphabetically. Bars below family names represent arthropod orders detected in salamander feces (refer to Tables 1–3, Fig. 4) in alphabetical order. Line area represents the relative number of fecal samples from *Desmognathus* sp. containing each family (relative number = n*i*/Σ*i*=1; n*i* = number of *Desmognathus* sp. fecal samples containing family *i*). Most arthropod families detected in more than one fecal sample were preyed on by multiple species of *Desmognathus* salamanders.

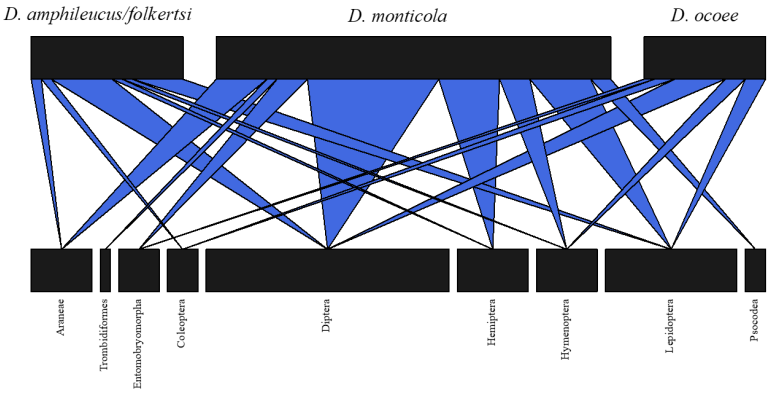

Figure 4. Interaction web depicting predation of arthropod orders by Nantahala Black-bellied or Dwarf Black-bellied Salamanders (*Desmognathus amphileucus/folkertsi*), Seal Salamanders (*D. monticola*), and Ocoee Salamanders (*D. ocoee*). Line area represents the relative number of fecal samples of *Desmognathus* sp. containing each order (relative number = $n_i/\Sigma_i=1$; n_i = number of *Desmognathus* sp. fecal samples containing order *i*). Most arthropod orders detected in more than one fecal sample were preyed on by multiple species of *Desmognathus* salamanders.

other components of salamander diets, including groups that are especially prone to be overlooked by traditional methods (e.g., annelids, some mollusks, other soft-bodied prey).

Although fecal metabarcoding offers increased taxonomic resolution, this benefit must be weighed against a loss of other relevant information. For example, our data alone cannot assess the size or life stage of consumed prey. Many common arthropods have distinct life stages that differ in size and microhabitat association. If dietary partitioning among amphibians is related to prey size or microhabitat, the information needed to understand these processes is absent from metabarcoding analyses. For example, we found DNA from chironomid midges in feces from both *D. ocoee* and *D. monticola*. Given that chironomid larvae are aquatic and that adults are terrestrial, it is possible that the more aquatic *D. monticola* prey on midge larvae, and that *D. ocoee* consume adults. This kind of information is absent from metabarcoding data but easily obtained from more traditional methods. Investigators must consider their specific research question, the dynamics of their system, and their biological intuitions regarding if and how partitioning may occur when assessing the potential utility of metabarcoding as a primary methodological approach or complement to traditional diet analysis techniques.

Table 1. Taxonomic identifications of prey from fecal samples of Ocoee Salamanders (*Desmognathus ocoee*) from northern Georgia, USA. Asterisks indicate the inability to classify amplicon sequence variants at that taxonomic level.

Order	Family	Genus and Species	# samples
Entomobyromorpha	*	*	1
Coleoptera	Cantharidae	*Rhagonycha hirticula*	1
	Coccinellidae	*Harmonia axyridis*	1
Diptera	*	*	2
	Bibionidae	*	1
	Cecidomyiidae	*Asteromyia* sp.	1
		*	1
	Anisopodidae	*Sylvicola alternatus*	1
	Chironomidae	*Limnophyes* sp.	1
		*	1
	Limoniidae	*Metalimnobia triocellata*	1
	Tachinidae	*Lespesia aletiae*	1
		Lespesia sp.	1
		*	1
Hymenoptera	*	*	1
	Platygastridae	*Leptacis* sp.	1
Lepidoptera	Erebidae	*Hydrillodes* sp.	1
	Lasiocampidae	*Artace anula*	1

Table 2. Taxonomic identifications of prey from fecal samples of Nantahala Black-bellied Salamanders (*Desmognathus amphileucus*) and Dwarf Black-bellied Salamanders (*D. folkertsi*) from northern Georgia, USA. Asterisks indicate the inability to classify amplicon sequence variants at that taxonomic level.

Order	Family	Genus and Species	# samples
Aranea	Tetragnathidae	*Tetragnatha elongata*	1
Coleoptera	Melandryidae	*Orchesia castanea*	2
Diptera	*	*	2
	Cecidomyiidae	*	3
	Limoniidae	*Epiphragma fasciapenne*	1
		Limonia immatura	1
		Limonia indigena	1
		Lespesia aletiae	1
		Molophilus sp.	1
	Psychodidae	*	1
Hemiptera	Aphididae	*Drepanaphis* sp.	1
Hymenoptera	Formicidae	*Stenamma* sp.	1
Lepidoptera	*	*	4
	Erebidae	*	1
		Hyphantria cunea	1
		Parallelia bistriaris	1

Table 3. Taxonomic identifications of prey from fecal samples of Seal Salamanders (*Desmognathus monticola*) from northern Georgia, USA. Asterisks indicate the inability to classify amplicon sequence variants at that taxonomic level.

Order	Family	Genus and Species	# samples
Aranea	Anyphaenidae	*Anyphaena pectorosa*	1
	Lycosidae	*Pirata sedentarius*	1
	Nesticidae	*Eidmannella pallida*	1
	Theridiidae	*Thymoites unimaculatus*	2
Trombidiformes	Tarsonemidae	*	1
Entomobyromorpha	*	*	1
	Entomobryidae	*Homidia sinensis*	1
	Tomoceridae	*Tomocerus* sp.	2
Diptera	*	*	4
	Anthomyiidae	*	1
	Chironomidae	*	2
	Cecidomyiidae	*	3
		Asteromyia sp.	1
	Limoniidae	*Epiphragma solatrix*	1
		Geranomyia sp.	1
	Sciaridae	*Dichopygina* sp.	1
	Sphaeroceridae	*Terrilimosina schmitzi*	1
	Tachinidae	*Lespesia aletiae*	1
	Tipulidae	*	1
		Tipula sp.	1
Hemiptera	Aphididae	*	2
		Aphis decepta	1
		Calaphis betulaecolens	1
		Drepanaphis sp.	1
	Reduviidae	*Rhiginia* sp.	1
Hymenoptera	Formicidae	*Prenolepis imparis*	2
	Vespidae	*Vespula flavopilosa*	1
Lepidoptera	*	*	3
	Erebidae	*	1
	Crambidae	*Microcrambus biguttellus*	1
	Lasiocampidae	*Artace anula*	1
Psocodea	Amphipsocidae	*Polypsocus corruptus*	2

Another advantage of fecal metabarcoding is that it does not necessitate invasive or lethal procedures. Although typically safe (Bondi et al. 2015; Hutton et al. 2021), gastric lavage can injure research subjects (Barbour et al. 2012), and lethal dissection requires euthanasia or collection of deceased individuals. These constraints may preclude diet studies concerning threatened and endangered amphibian species (Gillespie 2013; Pereira et al. 2021), and the use of metabarcoding could alleviate these concerns; however, fecal metabarcoding comes with its own practical challenges. Because amphibians are small and cryptic, researchers cannot reliably locate feces in the field (e.g., unlike some mammals; Solé and Rödder 2009). Instead, we attempted to collect feces from animals held in captivity for a short period of time (24 h), but we found that a relatively low proportion of captured salamanders defecated within this timeframe and defecation probability did not differ among species or by SVL. This suggests that fecal yields may be consistently low in studies concerning semi-aquatic plethodontids. Although longer sequestration periods may increase fecal yields, permitting and animal welfare concerns (e.g., disease transmission risk, post-sequestration survival) often discourage the release of captured individuals following a more prolonged period in captivity (Beaupre et al. 2004; Alworth and Harvey 2007). In fishes, methods to induce defecation (e.g., gentle massage or submersion in a warm water bath; Vandenberg and De La Noüe 2001) or to collect analogous samples (e.g., cloacal swabs; van Zinnicq Bergmann et al. 2021) have proven useful. These methods (or other alternatives) should be explored in amphibians, as they could greatly improve the practicality of diet metabarcoding studies in wild populations.

A major challenge in our study was the non-specific amplification of predator DNA from fecal samples. In a pilot study using fecal samples collected from Blue Ridge Two-Lined Salamanders (*Eurycea* cf. *wilderae*), ANML primers amplified arthropod DNA without non-target amplification of salamander DNA (Alexander T. Funk et al., unpubl. data), suggesting sufficient primer specificity for studies of *Eurycea* diets from fecal samples. In contrast, a large proportion of the sequencing reads from this current study matched *Desmognathus* ASVs, which suggests that ANML primers are ill-suited for diet studies concerning *Desmognathus* species. An unintended benefit of this mistake was that it provided us with genetic verification of field identifications and allowed for differentiation between morphologically cryptic species (e.g., *D. amphileucus* and *D. folkertsi*). *Desmognathus* sequences effectively wasted many sequence reads, however, reducing the depth of sequencing for target arthropod DNA. Primer selection is crucial to effective metabarcoding studies, and future studies concerning diet partitioning in *Desmognathus* should explore alternative arthropod COI primers (and/or blocking primers) that would alleviate this problem.

We did not find evidence that diet composition is predicted by species or SVL among sympatric species of *Desmognathus*, and most identified arthropod orders and families detected in multiple fecal samples were consumed by more than one species. Most fecal samples contained relatively few prey taxa, and relatively few prey taxa were present in more than one fecal sample. Our analyses were also affected by an inability to sample during multiple seasons. This is problematic because prey availability may differ seasonally. Given these constraints, we believe that our sampling effort was not robust enough for a conclusive characterization of diet. Our study should be viewed as a proof of concept rather than a definitive answer regarding the degree of dietary partitioning in this community. Future researchers using a fecal metabarcoding approach for diet analysis should collect more samples, sample throughout the year, and obtain better sequencing coverage (e.g., by using more specific primers) to increase statistical power. The use of more specific primers will increase sequencing

coverage by reducing amplification of predator ASVs, thereby increasing yield of prey ASVs with the same overall sequencing depth. This increase in yield, however, is still insufficient to increase statistical power if the fecal samples analyzed do not contain the full suite of prey consumed by individuals of different species or sizes. Given the diversity of potential prey items in any area and the fact that fecal samples provide only a brief history of recent food habits, large sample sizes are crucial for accurate assessment and comparison of diets at a narrow taxonomic scale.

Our results support the utility of fecal metabarcoding for investigations of novel questions about amphibian ecology and evolution. Notable advantages of this method are the ability to detect soft-bodied prey, an increased taxonomic specificity, and the non-invasive nature of sample collection. Important challenges of this technique include the inefficiency of sample collection and the inability to discriminate among prey life stages and sizes. Although metabarcoding alone may be appropriate for some investigations (e.g., diet partitioning among species with similar broad taxonomic prey preferences, detailed diet analyses concerning at-risk species), we echo the conclusions of Durso et al. (2022) who further describe the utility and methodological constraints of stable isotope, gut content, and fecal metabarcoding approaches to diet analysis and encourage future researchers to consider the simultaneous use of multiple, complementary methods to assess diet.

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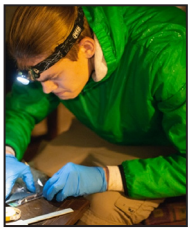

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