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Short communication

# DNA barcoding to assess diet of larval eastern hellbenders in North Carolina

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ABSTRACT

Freshwater macro-invertebrates are integral components of stream ecosystems, for example, as prey for many aquatic vertebrates. Identification of smaller aquatic macro-invertebrate prey items presents challenges to researchers due to size, taxonomic skill required to properly classify species, or lack of complete diet specimens. Therefore, many traditional methods of morphological identification may not reveal cryptic biodiversity of diet available to vertebrates, such as small larval salamanders, in lotic environments. We used DNA barcoding to identify prey items obtained from gastric lavage of ten small, larval eastern hellbender salamanders, *Cryptobranchus alleganiensis*, allowing for greater taxonomic resolution than in previous studies. The eastern hellbender is a species of conservation concern in many areas throughout its geographic range, with little ecological knowledge presently available for juvenile life stages. We identified twelve taxonomic groups of invertebrate prey, including *Nixe spinosa*, *Baetis intercalaris*, *Baetis flavistriga*, *Baetis sp.*, *Lumbriculus rubellus*, *Thienemannimyia sp.*, *Perlesta nelsoni*, *Maccaffertium ithaca*, *Iswaeon anoka*, *Isoperla dicalas*, *Epeorus vitreus*, and *Maccaffertium pudicum*. Our data illustrate the utility of this emerging, affordable method to further inform traditional food web studies which researchers can use to manage aquatic ecosystems.



## 1. Introduction

Freshwater ecosystems are increasingly threatened with potential loss of biodiversity, much of which may be unknown to biologists, representing potentially important ecological food web interactions (Barnosky et al., 2011; Senapati et al., 2018). Macroinvertebrates are a foundational component of aquatic food webs. For example, many aquatic insects play a key role in nutrient cycling as they break down allochthonous input (e.g., leaf litter), and serve as prey for higher trophic levels. As such, macroinvertebrates are widely used as biological indicators for aquatic biomonitoring of watershed health (Rosenberg and Resh, 1993; Wallace and Webster, 1996). Macro-invertebrates are common prey species for a variety of larval and adult salamanders, some of which are the most dominant vertebrates (by biomass) inhabiting Appalachian streams (Parker, 1994; Petranka, 1998; Peterman et al., 2007). Moreover, headwater streams represent vital habitats for many populations of salamanders.

The eastern hellbender, *Cryptobranchus alleganiensis*, is one example of a fully aquatic salamander that depends heavily on headwater streams; it is experiencing dramatic declines throughout its geographic range (Wheeler et al., 2003). Importantly, declines in hellbender abundance are often concurrent with a shift in age class structure of populations, indicative of low recruitment to the adult age class (Bodinof Jachowski and Hopkins, 2018). These patterns suggest that low reproductive success or low survivorship of young age classes may often function as the demographic driver of hellbender population declines. Habitat requirements of young hellbenders have been poorly studied, and there is a dearth of knowledge on the diet of larval eastern hellbenders in particular. Of the two studies that have addressed larval hellbender diet from visual inspection of stomach contents, both were only able to identify prey items to taxonomic Order (Pitt and Nickerson, 2006; Hecht et al., 2017). Improving our understanding of larval hellbender diet can improve our ability to evaluate hellbender habitat quality and may assist in identifying factors that limit recruitment of young hellbenders to older age classes.

Application of DNA barcoding to stomach content analyses may be useful for improving our understanding of animal diets. Visually identifying prey species obtained from small bodied predators can be difficult if only a small portion (e.g., exoskeleton, leg fragment) of the organism remains or is partially digested. Emerging technologies which incorporate small segments of DNA have facilitated species identification when only small samples are available (Soininen et al., 2009; Horreo et al., 2015; Koltz et al., 2019). Briefly, DNA barcoding relies on the

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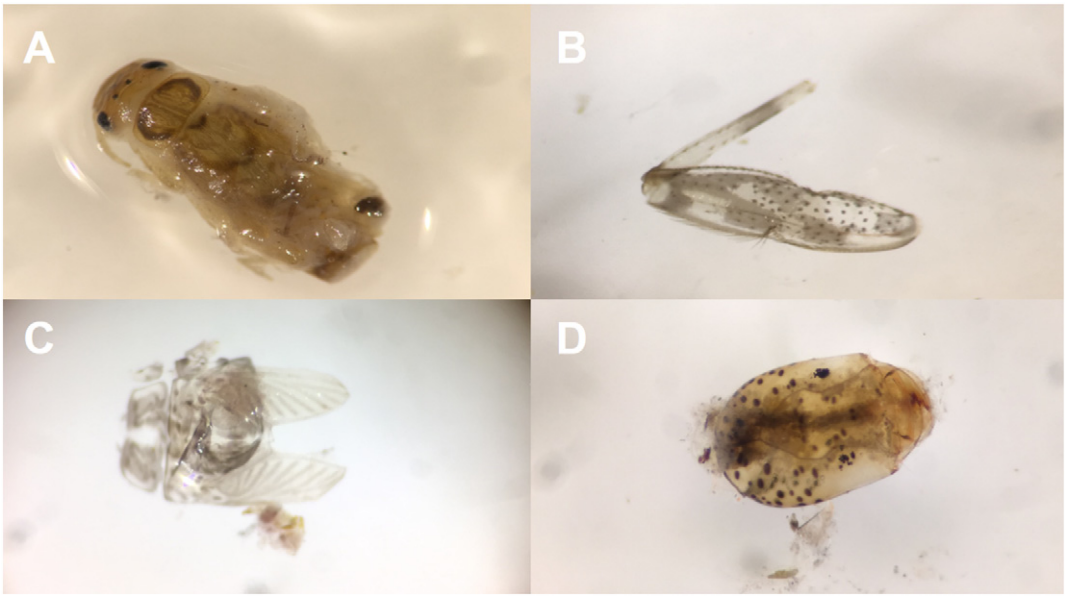


**Fig. 1.** Diet items, including partially-digested *Perlesta nelsoni* pale stone mayfly (top left), small minnow mayfly *Baetis intercalaris* leg (top right), exoskeleton portion of small minnow mayfly *Iswaeon anoka* (bottom left), and anterior head section of flat headed mayfly *Maccaffertium pudicum* (bottom right). Note many of these items may normally be difficult to identify past insect Order using solely morphological identification, as dichotomous keys require multiple specific body structures (e.g., gill covers, abdominal projections, bands or rings on tail, etc.).

mitochondrial COI gene as a unique biomarker or barcode for species resolution and identification of organisms when only a small quantity of tissue, and thus DNA, is present (Hebert et al., 2003; Jarman et al., 2004). Only a few studies have utilized DNA barcoding to investigate larval amphibian diet, but these approaches have become increasingly affordable and require less time, little genomic experience, and less overall financial investment than traditional methods (Ficetola et al., 2019). Traditional methods for species-level identification of aquatic macro-invertebrate prey and salamander diet is challenging, with ~ 50% reporting genus, not species, as the lowest taxonomic level of identification (Felix and Pauley, 2006; Sweeney et al., 2011). Specific identification is difficult as many early life stages lack diagnostic features (Jones, 2008; Orlofske and Baird, 2013). Moreover, DNA barcoding allows for greater taxonomic resolution, as well as a more precise identification technique for dietary analysis and macroinvertebrate identification over traditional morphological visual inspection of prey (Carreon-Martinez et al., 2011; Kress et al., 2015; Jackson et al., 2014), and can account for identification of both larval and adult aquatic insect life stages (Packer et al., 2009). The objective of this study was to test the use of DNA barcoding to identify prey items of larval eastern hellbenders in North Carolina, and to provide a brief description of this method for other researchers interested in utilizing this tool to investigate food web dynamics.

## 2. Materials and methods

We opportunistically sampled larval hellbenders during July and August 2019 in two watersheds (French Broad and Hiwassee Rivers) in North Carolina (locality information on file with North Carolina Wildlife Resources Commission; not included to protect collection locations). We anesthetized individuals prior to gastric lavage (stomach flushing) using a 1 g/L mixture of Orajel® into de-chlorinated distilled water following Cecala et al. (2007a). Individual salamanders were held in small plastic containers with this solution for ~7–10 min or until they showed no signs of movement tested by gently tapping their tail. Gastric lavage was conducted following Cecala et al. (2007b), Bondi et al. (2015), and Hutton et al. (2019), which involved attaching a Cole-Parmer PTFE 1/32" Industrial tubing to a Norm-Ject 1 mL graduated sterile syringe, carefully inserting the tube into the salamander's mouth, slowly flushing, and then removing to obtain diet items. Performing gastric lavage on a single larva took under 3 min in all instances. For each gastric lavage, we used separate sterile tubing, syringes, and gloves when handling. All containers used for collection, and fine-point forceps used for picking out stomach contents, were cleaned with 5% bleach solution and ethanol to prevent contamination. Immediately following gastric lavage, we returned salamanders to a recovery container under shade in the stream to ensure proper water temperature, increased oxygen

**Table 1**
Diet items identified using DNA barcoding of larval *Cryptobranchus alleganiensis* from North Carolina, including taxonomic identification level, common name, mean DNA sequence length (barcode), and percent match in the BOLD database. All organisms are in Class Insecta, with the exception of Annelida worms in Class Clitellata.

| Order | Family | Genus | Species | Common name | DNA sequence length (mean) | Percent match in BOLD (%) | Relative abundance |
|---|---|---|---|---|---|---|---|
| Ephemeroptera | Baetidae | *Baetis* | *intercalaris* | Minnow mayfly | 524.1 | 82.9–99.4 | 7 |
| | | *Baetis* | *flavistriga* | Minnow mayfly | 636 | 94.8 | 1 |
| | | *Baetis* | *sp.* | Mayfly | 345 | 83.2–90.6 | 3 |
| | | *Iswaeon* | *anoka* | Blue mayfly | 306.5 | 97–99.8 | 2 |
| | Heptageniidae | *Maccaffertium* | *pudicum* | Flathead mayfly | 280.8 | 98.1–100 | 5 |
| | | *Maccaffertium* | *ithaca* | Flathead mayfly | 326 | 100 | 1 |
| | | *Nixe* | *spinosa* | Mayfly | 232.5 | 100 | 2 |
| | | *Epeorus* | *vitreus* | Sulfur mayfly | 267 | 99.6 | 1 |
| Plecoptera | Perlidae | *Perlesta* | *nelsoni* | Pale stonefly | 255 | 100 | 2 |
| | Perlodidae | *Isoperla* | *dicala* | Stripetail stonefly | 383 | 98.1 | 1 |
| Diptera | Chironomidae | *Thienemannimya* | *sp.* | Midge | 465 | 91.8 | 1 |
| Haplotaxida | Lumbricidae | *Lumbricus* | *rubellus* | Red earthworm | 419 | 99.5 | 1 |

uptake, and to facilitate monitoring until full recovery or normal movement (~15 min). We returned individuals to their point of capture upon full recovery. We concluded all individuals were first-year larvae based on presence of gills and total lengths <100 mm.

We placed all pieces of dietary samples in 95% ethanol in sterile 1.5 mL microcentrifuge tubes. In the laboratory, we used a Motic SMZ-168 series dissecting microscope with 50× magnification to categorize and enumerate diet samples for each individual. We selected three representative subsamples per individual to place into separate 8 × 1.5 cm vials similar to Sikes et al. (2016). Samples were submitted to Lifescanner (http://lifescanner.net/) for extraction and sequencing. The Lifescanner kit and submission enables affordable processing of samples for citizen science DNA barcoding. Samples submitted ranged ~1–5 mm in total length, and in many cases were barely visible to the naked eye. DNA barcode sequences were uploaded to NCBI BLAST (National Center for Biotechnology Information Basic Local Alignment Search Tool) and queried using standard nucleotide blast (BLASTN; Zheng et al., 2000; Morgulis et al., 2008) and the MegaBlast function (available at blast.ncbi.nlm.nih.gov) to identify likely identity of unknown diet samples (i.e., portions of insects, insect legs, exoskeleton fragments; Fig. 1). We matched and aligned sequences with the highest probable match by sorting for highest percent identity with query cover >97–100%, which represents the percentage of nucleotide sequence that overlaps a known reference sample. We selected species identity based on the highest percent identity score for sequences generated on NCBI BLAST. We performed a Pearson correlation analysis to investigate the relationship between DNA sequence length obtained with percent identity in program R.

## 3. Results and discussion

We collected 10 larval hellbenders during surveys. In total we submitted 27 total items for DNA barcoding, as samples from three individuals only contained enough partial insects to submit two subsample specimens. We were able to successfully identify 100% of submitted food items to some level of taxonomic identification below order or family (85.2% to species, and 14.8% to genus).

Across all samples, we identified twelve species from nine distinct genera in six families and four orders. The highest proportion of prey items identified was *Baetis intercalaris* mayfly, followed by *Maccaffertium pudicum* mayfly (Table 1), with most prey items from the insect order Ephemeroptera (88.8%). Average nucleotide sequence length used for identification was 377.5. We found only a weak negative correlation for sequence length and percent identity $r(25) = -0.212$, $p = 0.291$; this supported a high likelihood of identification across DNA sequence lengths used in this study (Table 1). Individual salamanders had between one and three species identified from gastric lavage, with a mean of 1.7 species identified per individual across salamanders.

Several of the macro-invertebrate prey groups we identified have been shown to be important prey species for other Appalachian larval stream salamanders, *Desmognathus* and *Eurycea* (Trice et al., 2015). Our results using DNA barcoding are somewhat consistent with previous studies of larval hellbender diet, reporting Ephemeroptera and Trichoptera as the most prevalent aquatic insects identified from the Little River, Tennessee (Hecht et al., 2017), in addition to Diptera pupae, Megaloptera, and several aquatic insects unable to be identified by manual examination (Pitt and Nickerson, 2006). While our data include food items from the orders Diptera and Plecoptera, they also include an aquatic worm from the order Haplotaxida.

This highlights the potential ease for collecting baseline samples from small larval salamanders with a higher range of resolution, potentially identifying cryptic diversity over more traditional identification methods or in situations where only partial food items are obtained from gastric lavage of smaller animals (Fig. 1). We identified macro-invertebrate genera comprising a variety of functional feeding groups, including collectors, gatherers, scrapers, and predators, which are determined at either the family or genera level for most macro-invertebrates (Merritt and Cummins, 1996). Further research on salamander diet could sample larval eastern hellbenders across temporal periods, as many insects are aquatic and then become terrestrial or aerial following summer emergence.

Researchers face a trade-off between limitations of traditional approaches and balancing these challenges with DNA barcoding in terms of experience, cost, and resources available for identification. However, this method using Lifescanner, the first commercially-available citizen science DNA barcoding kit, is affordable (~$10 US per sample), requires only small portions of an insect (~1 mm length or less in some cases as only an insect leg portion was submitted), and provides potentially greater taxonomic identification than only Family or Genus level noted in many dichotomous keys.

Our findings highlight the need for future work using DNA barcoding to study freshwater food webs to elucidate taxonomic identification in smaller aquatic organisms. Future studies could compare traditional methods of morphological identification with this method. In addition, DNA barcoding could be used as quality control of traditional morphological identifications in food web studies. One of the challenges for freshwater systems is the lower likelihood of direct observation of feeding interactions (Sousa et al., 2019), which is remedied by DNA barcoding.

Larval salamanders are important predators in stream food webs (Johnson and Wallace, 2005), but there are limited studies using DNA sequencing for dietary studies from gut contents in herpetofauna (Reilly et al., 2010). We encourage researchers studying diet of smaller amphibians, reptiles, and mammals to utilize DNA barcoding to increase resolution of prey identification. Researchers should also carefully evaluate results from DNA barcoding analysis, dependent upon the taxonomic resolution available for their species identified, as many insect groups in the BOLD database may only allow for either genus or family as the lowest level of identification (Trebitz et al., 2015). However, we expect this database to increase as more reference samples are verified, and as affordable genomic methods become more accessible to researchers.

## Declaration of competing interest

The authors have no conflict of interest to declare for this manuscript

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