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Macroinvertebrate Communities and Body Condition of Larval Eastern Hellbender Salamanders (*Cryptobranchus alleganiensis*) in North Carolina

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Salamanders are important biological indicators of freshwater aquatic ecosystems. The Eastern Hellbender, *Cryptobranchus alleganiensis*, is found primarily in streams across the southeastern, midwestern, and eastern portions of the United States. However, this unique large aquatic salamander is facing numerous threats and declines across its geographic range, including in Appalachia. Moreover, little is known regarding the early life history stages (gilled larvae), particularly regarding food availability (aquatic insects present in streams) and body condition. In this study, we assessed the macroinvertebrate communities of streams sampled for larval *C. alleganiensis* and report on the body condition index within western North Carolina streams. We found varying levels of diversity across sample locations for macroinvertebrates (total sampled = 3,619, representing over 30 genera), with the most prevalent insects from Trichoptera order, with an overall high percent of Ephemeroptera, Plecoptera, and Trichoptera (EPT) in all streams, ranging from 68.5% to 90.7%. Functional Feeding Groups included 40.5% filterers, 24.3% predators, 17.6% gatherers, 10.7% shredders, and 6.9% scrapers. Shannon Diversity Index in sample streams ranged from 2.101 to 2.698. Body condition or SMI (scaled mass index) ranged from 1.5 to 3.3 across sites with a largely consistent and strong linear relationship between log mass and total length (r^2 = 0.910). Our results add to the body of knowledge on the larval ecology of this North American salamander and may aid in future management of hellbender stream habitats.

Key words: *Cryptobranchus alleganiensis*, Aquatic Insects, Water Quality, Salamander, Cryptobranchidae.

BACKGROUND

Salamanders are key members of ecological communities in freshwater streams (Davic and Welsh 2004). Salamander larvae can exert strong trophic control on macroinvertebrate taxa (Holomuzki et al. 1994). The Eastern hellbender salamander is found across the midwestern, northeastern, and southeastern United States (Petranka 1998), but is facing declines in population (Wheeler et al. 2003). Subsequently, little is known regarding important early life history stages (gilled larvae) of this unique fully aquatic species. Previous surveys of this species have historically consisted of rock lifting surveys sampling primarily adults. However, a limited number of studies on larval biology have identified macroinvertebrates as

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important food items for larval Eastern Hellbenders (Hecht-Kardasz and Nickerson 2013; Unger et al. 2020a). Therefore, further work is needed to quantify macroinvertebrates across streams as an important environmental factor for larval presence and persistence.

Benthic macroinvertebrates, primarily aquatic insects, can be used to assess varying levels of ecological health of streams, as some are less tolerant of pollution, particularly members of Ephemoptera, Plecoptera, and Trichoptera (Dudgeon 1999). Sampling the diversity of benthic macroinvertebrates provides a method to assess both water quality and dietary items. Food availability can not only ensure the survival of larval salamanders but also allow for proper development (Vaissi and Sharifi 2016; Beachy 2018), determine the size at metamorphosis (Warburg 2009), and correlate with long-term adult fitness (Semlitsch et al. 1988) and the overall health of individuals. Larval stream salamanders are known to impact several functional feeding groups of macroinvertebrates from the insect orders Ephemeroptera, Plecoptera, Trichoptera and Diptera (Trice et al. 2015) and actively predate on a variety of macroinvertebrates (Schultheis and Batzer 2005). Moreover, the relationship between salamander presence and macroinvertebrate prey is likely affected by the level of development or disturbance (Barrett et al. 2012). Therefore, further data is needed on the aquatic insect communities present in streams where gilled larval hellbenders are present to highlight potential relationships between overall body condition and thus the health of larval salamanders.

Body condition can be an important predictor of healthy individual salamanders and thus a good measure of long term population stability (Lunghi et al. 2018). Body condition in salamanders may indicate the overall health of individuals and whether they can move spatially within streams (Lowe et al. 2006). Moreover, body condition varies across species with some showing both positive and negative relationships between forest type and other environmental variables (Welsh and Hodgson 2013). Poor nutritional conditions can even alter the behavior of early developing larval salamanders (Krause et al. 2011). However, there is still more to learn about the body condition of early life stages of the eastern hellbender, a species of conservation concern. The goals of this study were to assess the larval ecology of this unique salamander by focusing on potential prey items for larvae, which can inform both the body condition and overall stream health as biological indicators of stream quality. In this study we assessed the food availability and macroinvertebrate community diversity and body condition of gilled larval Eastern Hellbenders in streams in North Carolina, USA. More specifically we tested the following hypotheses: a) salamander body condition correlates to EPT richness and b) EPT richness differs across natural and disturbed stream sites.

MATERIALS AND METHODS

Hellbender sampling

Cryptobranchus alleganiensis gilled larvae were sampled May to September of 2016–2017 in western North Carolina, USA. Two watersheds, the French Broad River and Hiawassee River, were selected based on previous surveys indicating that adult salamanders were present and there was a high likelihood of finding larvae. We sampled 10 total streams, each with two separate 50 meter transects that were at least 200 meters from each other within the same stream (Fig. 1). Site (river) names are withheld due to the risk of illegal collecting but are on file with the North Carolina Division of Wildlife Resources. Surveys consisted of authors snorkeling the stream sections until a salamander was captured, then collecting biological data/measurements. Each site was surveyed on a total of three occasions. Sites were selected across both natural and disturbed sites. Sites were a minimum of 200 meters apart and in most cases at least 600 meters to keep from sampling the same individuals and we grouped sites within stream reaches to reflect this, as gilled larvae are fully aquatic and constrained to stream reaches.

Macroinvertebrate sampling

We collected macroinvertebrate samples from each stream (one sample per stream) using standard sample methodologies in primarily run and riffle habitats between transects. At each site we assessed the in-stream food availability of larvae (potential macroinvertebrate food items) by collecting 10 Surber samples (Surber 1970) using a Wildco® 500-µm mesh, stream bottom sampler (Wildco Supply Comany, Yulee, Florida, USA) and a stainless steel, mesh sieve set. We targeted runs and riffles to include potential diverse food items and macroinvertebrates, then combined samples after identification. All macroinvertebrate surveys were conducted following completion of snorkel surveys to minimize the disturbance to the habitat. In brief, we sampled streams following Kincaid et al. (2018), which involved surveying both riffle and run habitats within 100 m of one randomly selected reach for each stream, collected all aquatic insects, preserved them in 90% ethanol, and identified them in the laboratory.

Body condition

We collected standard biological data for individual larval salamanders captured during surveys, including total length (TL) (cm) and snout-vent length (SVL) (cm) and mass to the nearest 0.1 g. Each larvae was confirmed to be a larval salamander by the presence of gills. We compared scaled mass index (SMI) across sites by using the log of mass and SVL (Pieg and Green 2009; Liles et al. 2017) and a reduced major axis regression in the program RMA (Version 1.17) (Bohonak and van der Linde 2004). The use of SMI has been applied to salamanders by Morgan et al. (2014), Costa et al. (2015), and Lacking et al. (2017). We further examined the mass and TL relationship using a standard linear regression as an indicator of overall health of larvae. We were unable to directly sample the diet of the macroinvertebrate taxa from individual larvae sampled in this study due to their relative small size and a lack of permitting.

Statistical analyses

We compared aquatic insect and body condition using descriptive statistics. We further compared macroinvertebrate community diversity by calculating the Shannon Diversity Index and Hilsenhoff Biotic Index (Hilsenhoff 1987; Lenat 1993); the latter incorporates water quality tolerance values of each macroinvertebrate taxonomic grouping. We further characterized all aquatic insects identified to functional ecological feeding groups (percent scrapers, predators, gatherers, filterers, and shredders) (Merritt et al. 2019). The EPT richness was calculated by identifying the macroinvertebrates present across all streams collected in the field during the middle of sampling periods for each year, then identifying them to the lowest taxonomic level (typically genus). We conducted a Kruskal-Wallis test to compare EPT richness across sample sites (N = 20). We reported the larval mean SMI for body condition of salamanders across streams, based on our limited

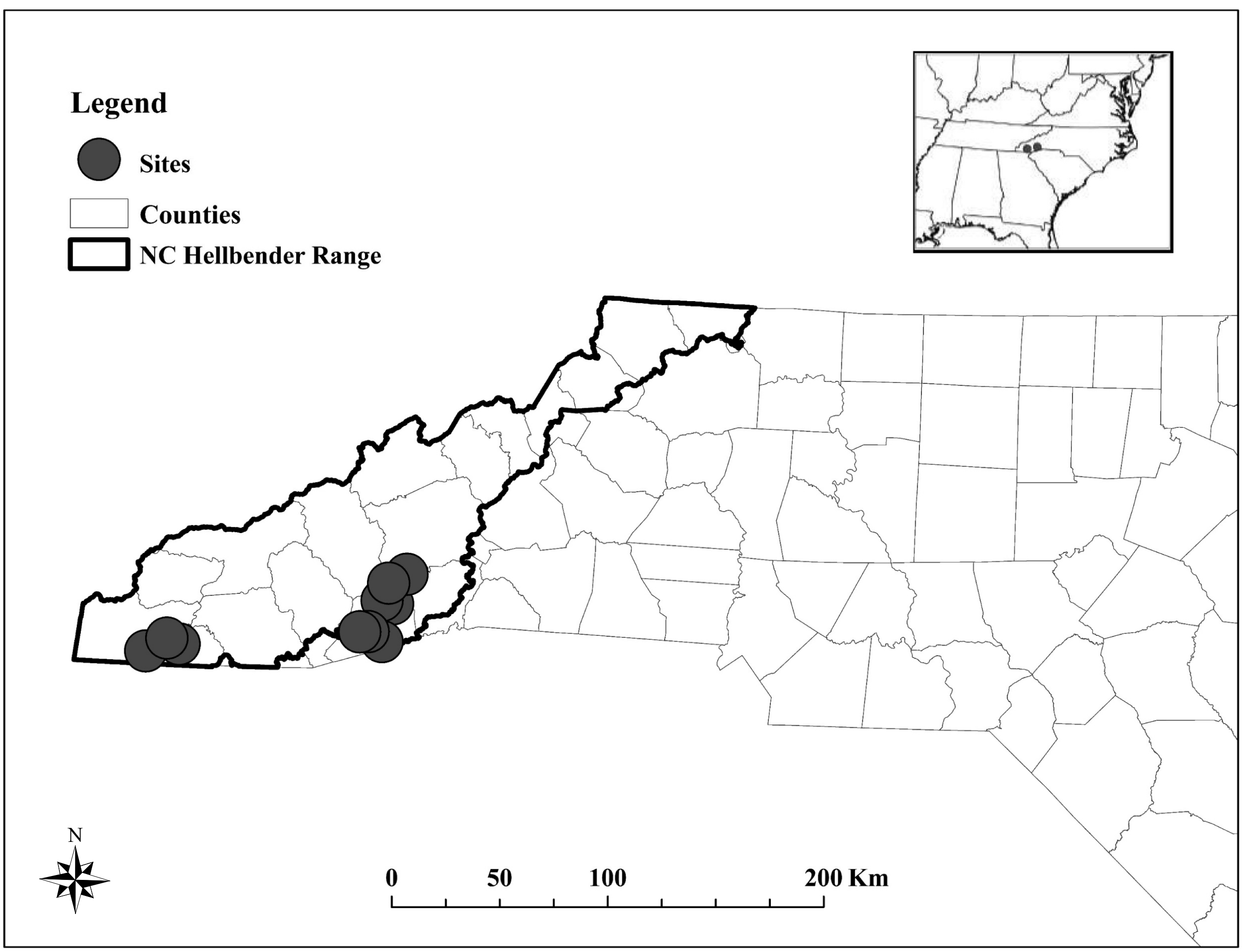

Fig. 1. Map of sample locations in western North Carolina, USA. Circles represent sites across two watersheds within the range of the Eastern Hellbender *Cryptobranchus alleganiensis* in the state indicated by dark outline.

sample size. To investigate the relationship between total EPT richness and body condition index for sites in which we captured larvae, we ran a Pearson Correlation Analysis. We further compared EPT richness to the level of site disturbance based on previous research by authors (Unger et al. 2020b) and by assigning sites into either natural (NAT) or disturbed (DIST) categories. Natural sites occurring primarily in National Forest with little evidence of stream disturbance included sites 3,4; 5,6; 7,8; 9,10; 11,12; 17,18; and 19,20. Disturbed sites with clear evidence of human visitation (stacking of rocks, alteration of habitat, and combination of private land surrounding streams) included sites 1,2; 13,14; and 15,16. We further used ClustVIS (Metsalu and Vilo 2015), a multivariate analysis, to generate a PCA of disturbed versus natural sites across EPT groupings and a heatmap.

RESULTS

We found that the most prevalent macroinvertebrates were from the insect order Trichoptera, including the caddisflies *Hydropsyche* sp., *Brachycentrus* sp., and *Dolophilodes* sp. (Table 1). The percent of insect orders Ephemeroptera, Plecoptera, and Trichoptera (EPT) across stream reaches was relatively high and ranged from 68.5% (site 19 and 20 in the Hiwassee River sub-basin) to 90.7% (site 1 and 2 in the Upper French Broad River sub-basin). Functional feeding groups for

Table 1. Relative diversity of Ephemeroptera, Plecoptera, and Trichoptera (EPT) from sites (n = 20) sampled for larval Eastern Hellbenders (*Cryptobranchus alleganiensis*) across 10 streams in the Hiwassee and Upper French Broad River sub-basins, western North Carolina, USA

Organism	Family	EPT	Sites 1, 2	Sites 3, 4	Sites 5, 6	Sites 7, 8	Sites 9, 10	Sites 11, 12	Sites 13, 14	Sites 15, 16	Sites 17, 18	Sites 19, 20	Total
Baetis sp.	Baetidae	E	10	49	19	17	26	2	6	36	4	6	175
Isonychia sp.	Isonychiidae	E	0	0	4	3	1	0	0	0	0	0	8
Homoleptohyphes sp.	Leptohyphidae	E	0	31	39	12	26	2	5	5	16	1	137
Maccafertium sp.	Heptageniidae	E	19	8	72	30	2	5	15	6	6	9	172
Leptohyphes sp.	Leptohyphidae	E	0	5	0	1	0	2	0	0	0	4	12
Neoephemera sp.	Neophemeridae	E	3	14	27	30	0	0	0	5	2	0	81
Ephemera sp.	Ephemeridae	E	0	0	2	0	5	0	0	0	3	0	10
Belonuria sp.	Perlidae	P	25	10	16	26	14	18	29	0	19	21	178
Viehoperla ada	Peltoperlidae	P	5	0	1	10	1	10	1	0	4	1	33
Perlesta sp.	Perlidae	P	4	130	60	4	34	0	9	2	1	23	267
Leuctra sp.	Leuctridae	P	12	25	31	26	41	5	0	1	35	22	198
Tallaperla sp.	Peltoperlidae	P	0	0	0	7	1	3	0	0	0	5	16
Dolophilodes sp.	Philopotamidae	T	28	84	60	61	34	8	9	1	19	19	323
Hydropsyche sp.	Hydropsychidae	T	77	20	196	28	44	24	81	77	61	10	618
Baraea sp.	Baraeidae	T	0	5	7	0	2	1	2	0	0	0	17
Rhyecophila sp.	Rhyacophilidae	T	0	5	9	9	0	0	0	0	0	1	24
Brachycentrus sp.	Brachycentridae	T	17	36	17	167	16	89	4	0	15	1	362
Glossosoma sp.	Glossosomatidae	T	5	18	13	9	0	12	0	0	1	0	58
Lepidostoma sp.	Lepidostomatidae	T	0	7	1	2	2	2	0	4	27	3	48
Helicopsyche sp.	Helicopsychidae	T	0	2	0	1	0	0	0	0	0	0	3
Micrasema sp.	Brachycentridae	T	0	5	1	15	2	0	0	9	2	1	35
Pseudogoera sp.	Odontoceridae	T	0	0	0	0	7	23	0	2	16	1	49
Psephenus sp.	Psephinidae		3	0	88	13	27	8	8	1	11	12	171
Corydalus sp.	Corydalidae		13	11	8	14	10	8	15	24	1	19	123
Greniera sp.	Simuliidae		1	15	0	2	3	3	2	16	9	0	51
Paramerina sp.	Chironimidae		0	23	0	5	1	0	0	0	0	3	32
Dasyhelea sp.	Ceratopogonidae		0	102	8	13	22	1	0	2	4	3	155
Progomphus sp.	Gomphidae		1	0	6	2	2	8	7	0	4	1	31
Tipula sp.	Tipulidae		0	0	61	2	20	0	0	0	13	2	98
Atherix sp.	Athericidae		3	3	7	41	2	35	16	6	0	19	132
Erpetogomphus sp.	Gomphidae		0	0	1	1	0	0	0	0	0	0	2
	Total		266	608	754	551	345	269	209	197	273	187	3619
	EPT Richness		11	17	19	19	17	15	10	11	16	16	
	(% EPT)		(90.7)	(74.7)	(76.3)	(83.1)	(74.8)	(76.6)	(77)	(75.1)	(84.6)	(68.5)	

macroinvertebrates identified across sites, consisted of a diverse series of ecologically relevant insects with 40.5% filterers, 24.3% predators, 17.6% gatherers, 10.7% shredders, and 6.9% scrapers. Percent EPT (mean ± one standard deviation [SD]) for occupied sites was slightly higher (79.1% ± 7.2) than in unoccupied sites (75.8% ± 1.1). The most frequently identified pollution sensitive genera within EPT were *Baetis* sp., *Perlesta* sp., and *Hydropsyche* sp. Shannon Diversity Index estimates showed slight variation, ranging from 2.101 to 2.698, with Hilsenhoff Index ranging from 1.49 to 3.57. Kruskal-Wallis test indicated a significant difference among EPT richness across sites, H = 19.314, $p < 0.0001$. PCA analysis indicated that PC1 explained 33.8% of the variation and PC2 19.7% of the variation, with some evidence of clustering across the data on EPT richness (Fig. 2). We detected a trend across EPT richness and natural versus disturbed sites with our heatmap analysis visualization with a higher relationship for specific EPT groups in natural sites including *Lepidostoma* sp., *Hydropsyche* sp., *Maccafertium* sp., *Perlesta* sp., *Helicopsyche* sp., and *Brachycetrus* sp. (Fig. 3). *Belonuria* sp. and *Leuctra* sp. were associated with disturbed sites based on heat map analysis.

In total we found 27 gilled larvae across all streams, two from sites 3,4; one from sites 11,12; six from site 19,20; five from sites 19,20; six from sites 9,10; three site 7,8; one from sites 17,18; and four from sites 5,6. Average mass (± SE) and TL of larvae were 2.7 g (± 0.1) and 6.9 cm (± 0.2), respectively. The log mass versus log TL relationship showed slight variation but was largely consistent across stream reaches with a strong linear trend ($r^2 = 0.910$; Fig. 4). SMI (mean ± SE) varied across sites where larvae were encountered (2.5 ± 0.2), ranging from 1.5 for sites 17 and 18 from the same Hiwassee River sub-basin stream to 3.3 for sites 7 and 8 from the same Upper French Broad River sub-basin stream (Fig. 5). We found a moderate positive correlation between EPT richness and body condition index, which was significant, R = 0.603, p = 0.134, N = 16. However, salamanders with the highest SMI corresponded with NAT3 (sites 7,8), NAT6 (sites 11,12), NAT2 (sites 5,6), and NAT4 (sites 9,8), but the pattern was not consistent across SMI scores or all sites or categories.

DISCUSSION

This is the first report of body condition indexes and macroinvertebrate communities in sites with varying levels of gilled larval hellbender presence in North Carolina. We found a positive correlation across sample sites between EPT richness and body condition, indicating that sites containing a greater diversity of EPT relative abundance had a higher overall body condition. We further observed diverse taxonomic groups of macroinvertebrates across all sites, indicating that the majority of streams presently provide a wide variety of potential food items for hellbender salamanders. Specific relationships across disturbed and natural sites for EPT richness were also present; however, it is unknown to what extent these trends reflect body condition, as we did not directly sample diet from larval

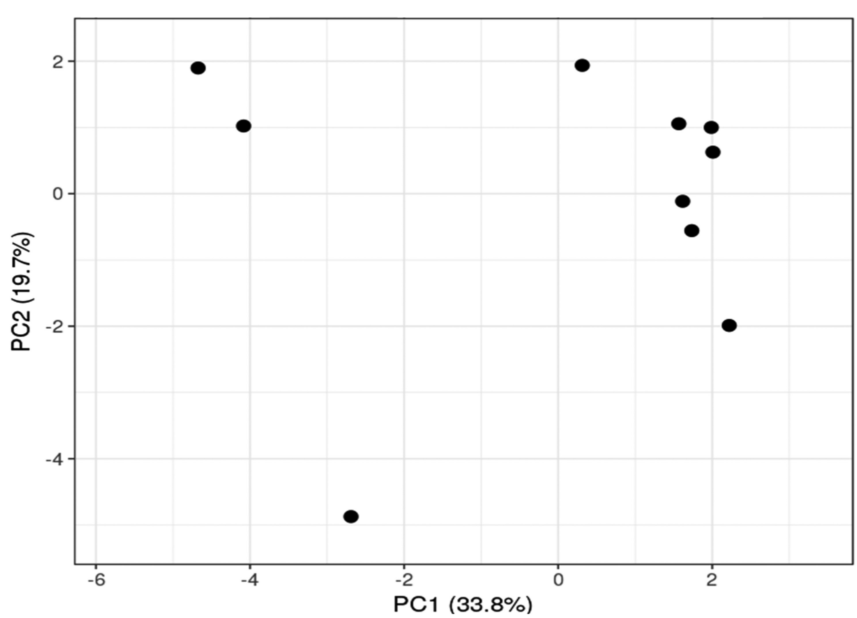

Fig. 2. PCA analysis of EPT groupings in this study. Only EPT taxa sampled and identified to nearest genus used in analysis.

salamanders. The presence of insect orders EPT has previously been associated with "high water quality" as they are pollution-intolerant indicator taxa (Rosenburg et al. 1993; Cushing and Allan 2001) that concomitantly provide hellbender larvae with a food source (Hecht et al. 2017). Our estimates for the body condition of larvae were consistent with other published studies (Nickerson et al. 2002), indicating that gilled larvae appear healthy across streams. EPT richness is associated with increases in occupied sites in amphibians (Watson et al. 2017) and provides a potential indicator variable for other studies of occupancy in stream dwelling salamanders, as larval salamanders as predators have an important ecological role in structuring aquatic macroinvertebrate communities (Keitzer and Goforth 2013). Future work could focus on diet or even captive

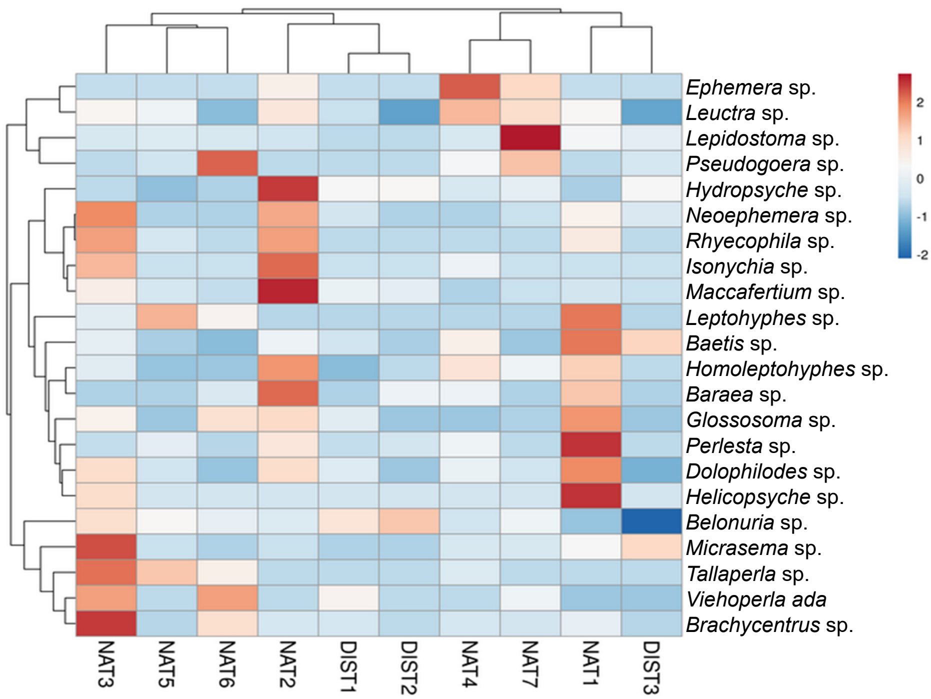

Fig. 3. Heat map of natural (NAT) versus disturbed (DIST) sites. Corresponding site codes are as follows: NAT1 (sites 3,4), NAT2 (sites5,6), NAT3 (sites7,8), NAT4 (sites9,10), NAT5 (sites 19,20), NAT6 (sites 11,12), NAT7 (sites 17,18); DIST1 (sites1,2), DIST2 (sites13,14), and DIST3 (sites 15,16). Note: High prevalence of various EPT taxa for NAT sites versus DIST sites.

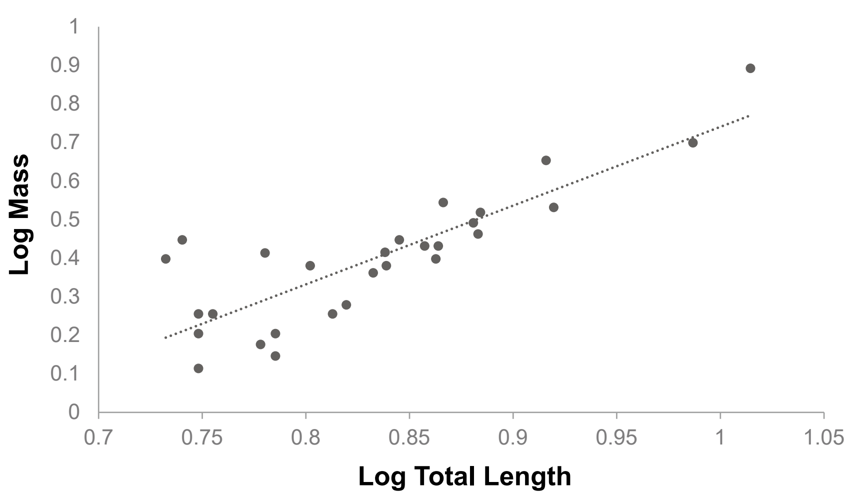

Fig. 4. Log mass (g) versus total length (cm) relationship of gilled, larval Eastern Hellbenders (*Cryptobranchus alleganiensis*) measured in the Hiwassee and Upper French Broad River sub-basins, western North Carolina, USA.

rearing experiments assessing temperature variation on the body condition of juveniles, as has been conducted for closely related Chinese giant salamanders *Andrias* (Zhang et al. 2014).

The higher overall relative frequency of EPT taxa is consistent with historical surveys of macroinvertebrates in the same watersheds (Penrose et al. 1982) and suggests that the majority of streams sampled still provide habitats for representative southern Appalachian, pollution sensitive macroinvertebrates. Among the most commonly encountered genera included Trichopterans *Hydropsyche* sp. (618 enumerated across sites), or the net-spinning caddisfly, and *Brachycentrus* sp. (362 enumerated across sites) or the American Grannom, which is an important stream filtering caddisfly sensitive to temperature (Gallepp 1977). Moreover, we sampled 323 *Dolophilodes* sp., net-spinning caddisflies, which are important feeders of fine particulate detritus material (Malas and Wallace 1977). We also noted a low relative abundance of *Psephenus* sp. (water penny beetle), which is found primarily in riffles but also in runs (Murvosh 1971), as well as *Corydalus* sp. (dobsonfly), an important predator insect in many lotic ecosystems (Mangan 1992). In addition, we sampled *Dasyhelea* sp. (biting midges) and *Tipula* sp. (crane fly), which are generalists found in more slow moving water habitats (Merritt et al. 2019). However, the high relative abundance of EPT macroinvertebrate taxa we observed are known to utilize interstitial spaces within the stream substrate, the same microhabitat as *C. alleganiensis* larvae (Pitt et al. 2016), and should therefore be incorporated into conservation and monitoring programs. Finally, several of the taxa we identified were recently confirmed to be included in the diet of larval eastern hellbenders including *Baetis* sp. and *Perlesta* sp., *Maccaffertium* sp. using DNA barcoding (Unger et al. 2020a), which can potentially be used in future studies comparing body condition.

Juvenile life history stages of amphibians, including salamanders, should be considered as an important measure of ecosystem health and recruitment into populations. Moreover, the scaled mass index we utilized for body condition allowed us to compare across the streams sampled in this study over standard regression comparisons. This index has been utilized to some extent in other herpetofauna, including snakes (Frank and Dudas 2018), bullfrogs, and newts (MacCracken and Stebbings 2012), yet it has seldom been applied to a specific life history stage in salamanders. Future research should use scaled mass index to give researchers the ability to compare multiple populations for a species and even life history stages of amphibians. However further work should be conducted to assure that these fully aquatic gilled individual salamanders do not move between sites or stream reaches, possibly by marking with visible implant elastomer (VIE) or other methods. Also, laboratory studies on body condition and diet could help further elucidate the correlation between diet and body condition. However, as this species is of special conservation concern and rarely encountered in the wild, this may prove challenging.

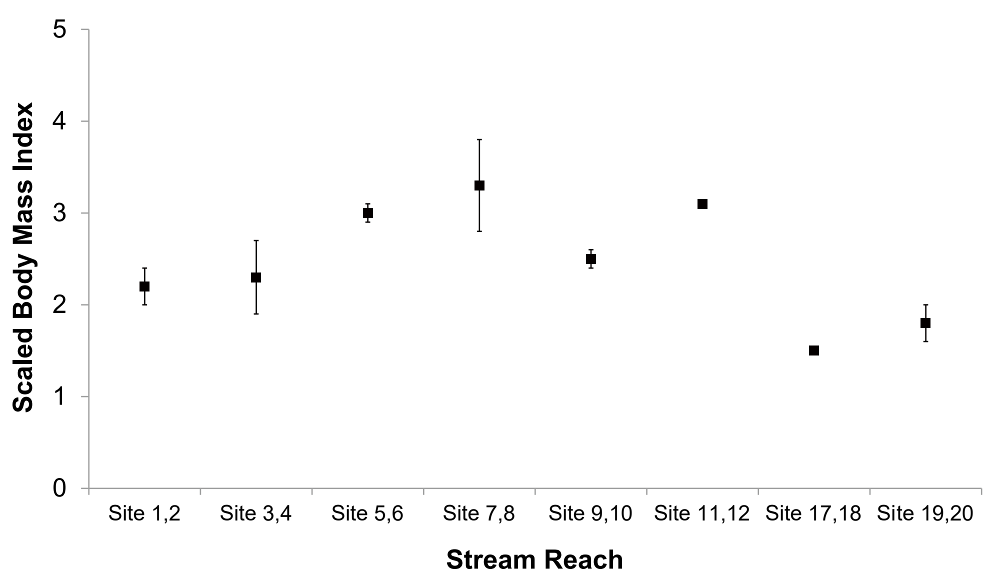

Fig. 5. Scaled mass index of individual Eastern Hellbender (*Cryptobranchus alleganiensis*) larvae captured across sites (n = 20); sites 13–16 had no captures. Error bars denote standard error of the mean and are not shown for sites 11, 12 and 17, 18 as only one larva was measured at those sites. Sites 1–12 were from the Upper French Broad River sub-basin, and sites 17–20 were from the Hiwassee River sub-basin of western North Carolina, USA.

CONCLUSIONS

This study is the first to document dominant aquatic insects available as potential food items in North Carolina across several watersheds and correlate specific EPT taxa with level of disturbance for the eastern hellbender larval salamander, a species of special conservation concern. As expected, we found high levels of EPT taxa in most streams, which are positively correlated with the body condition of the larvae. Our study confirms that streams occupied by hellbender gilled larvae contain a diversity of macroinvertebrate communities with individual salamanders showing a variety of body conditions. We found differences across sites which may lead to further research addressing environmental factors that may impact both macroinvertebrates and larval salamander body condition across western North Carolina and the species geographic range. Management of these streams should incorporate conservation of stream habitats, which protect both macroinvertebrate communities as well as salamanders and riparian biodiversity across Appalachia.

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Availability of data and materials: Not applicable.

Competing interests: The authors declare no conflict of interest.

Ethics approval consent to participate: Not applicable.

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