

Article

Stable Isotope Analysis of Ozark Hellbender (*Cryptobranchus alleganiensis bishopi*) Living and Preserved Museum Tissue Reveals a Shift in Their Generalist Diet Composition

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Abstract: Ozark hellbenders (*Cryptobranchus alleganiensis bishopi*) have undergone marked population declines across their entire distribution. A variety of ecological life history research has been conducted to determine the cause(s) of the declines. Historically, hellbender diet studies used stomach content examination methods; however, alternative approaches such as less intrusive stable isotope analyses are now options for researchers. The goals of our study were to conduct stable isotope analysis on live and formalin-preserved museum specimen Ozark hellbender tissues to identify diet composition in the Eleven Point and Spring rivers, Arkansas. Also, we used stable isotope analysis to investigate if Spring River hellbender diets have changed over time. We sampled fish, live hellbenders (non-destructively), and formalin-preserved hellbender tissues from museum collections for stable isotope analysis. We sampled crayfish for assemblage composition and stable isotope analysis. The results of our stable isotope study revealed three main findings: (1) there were no statistically significant differences between hellbender $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values among sites and hellbender stable C and N isotopes were correlated with body length; (2) traditional $\delta^{13}\text{C}$ versus $\delta^{15}\text{N}$ bi-plots and trophic discrimination values did not provide complete discernment in hellbender diets; however, Bayesian MixSIAR models revealed hellbenders to be generalists, and (3) the use of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values adjusted historic formalin-fixed and ethanol preserved hellbenders matched well with current crayfish and fish stable isotope values based on Bayesian MixSIAR models. These findings provide important diet information and a possible tool to examine dietary patterns from preserved specimens that may be used for hellbender conservation and management.

Keywords: amphibian declines; diet analysis; ontogenic diet change; Bayesian mixing models

1. Introduction

The Ozark hellbender (*Cryptobranchus alleganiensis bishopi*) is a large, long-lived (20+ years) benthic salamander that has undergone marked declines over the past five decades [1–3]. These declines resulted in the placement of the Ozark hellbender onto the endangered species list as of October 2011 [4]. A variety of ecological life history research has been conducted to determine the cause(s) of the decline [5–11]. One important area of study for the conservation and management of Ozark hellbenders is to understand their diet and food habits.

The feeding kinetics of adult hellbenders was described in detail by Cundall et al. [12], who found that hellbenders exhibit asymmetric movements of the lower jaw and hyoid apparatus during suction feeding. Suction feeding explains the extraneous debris (i.e., leaves, gravel, and snails) sometimes found within the gut of the hellbender. Few studies have examined larval hellbender diets, but one study found larval diets are primarily comprised of Ephemeroptera and Trichoptera invertebrate nymphs and suggested that hellbenders may undergo an ontogenetic dietary shift from these small macro-invertebrates as larvae to larger prey items into adulthood [13] and Unger et al. [14] confirmed a primary invertebrate diet for larval hellbenders. A large number of adult hellbender diet studies reported crayfish as primary prey [15–20]. Peterson et al. [19] examined the seasonal prey items of Ozark hellbenders in the Spring River (Fulton, AR, USA) using gut contents, and documented crayfish as comprising at least 99% of hellbender diet in 7 months of the year. Despite the high incidence of crayfish consumption, many cases of alternate food sources also have been documented in Ozark hellbenders. Peterson et al. [19] found that in several months, crayfish comprised ~80% of hellbender stomach contents with small fish species making up the difference. Nickerson et al. [21] preserved a regurgitated lamprey from a Big Piney River (Texas, MO, USA) hellbender. Nickerson and Mays [22] found 38 snails inside the gastrointestinal tract of a female North Fork River (Ozark, MO, USA) hellbender. Furthermore, Gall and Mathis [11] found that small (<9.6 cm) *Cottus caroliniae* exhibit avoidance behavior when exposed to hellbender alarm cues, suggesting a coevolutionary relationship between predator and fish prey. Additionally, limited observations of Eastern hellbender (*C. a. alleganiensis*) report cannibalism [23–26] and scavenging behavior [27]. These findings support an alternative diet explanation that hellbenders undergo ontogenetic shifts in their diet and as adults are opportunistic feeders, which use not only a variety of congeners and conspecifics as food resources but also utilize other taxonomic groups inhabiting the benthic habitat of hellbenders, such as *Cottus* and *Etheostoma*. Therefore, hellbenders may be eating crayfish in proportion to their availability in their benthic habitat; and simply supplementing with accessible food resources.

Hellbender food habits have been investigated historically by using an intrusive process of post-mortem stomach content examination [15–17,19]. A post-mortem examination is no longer an acceptable means of studying the trophic interactions of Ozark hellbenders due to the invasive nature of the procedure and the conservation status of Ozark hellbenders. Furthermore, stomach content examination also may overrepresent the total percentage of the hellbender diet made up of crayfish because a large amount of chitin present in the exoskeletons of crayfish [5]. Dierenfeld [20] suggested chitin is hard to digest, and subsequently, crayfish parts spend more time in the digestive tracts of hellbenders than the body parts of fishes, which are primarily made up of soft tissues [20,28].

An alternate and non-invasive diet analysis can be achieved through stable isotope analysis. Stable isotope analysis has become increasingly important to ecologists, enabling the quantification of nutrient flow through food webs. Carbon and nitrogen stable isotopes are commonly used when examining ecological food webs [29,30], where nitrogen isotope ratios determine the trophic position of food web components, and carbon isotopes trace the flow of organic matter through organisms within food webs [31]. The use of stable isotope analysis for exploring dietary interactions hinges on the fact that the isotopic composition of a consumer's tissues closely resembles the isotopic composition of the resource pool used by the consumer over the time of tissue generation (after adjusting for trophic discrimination) [32,33].

Furthermore, an informative approach to understanding historic diets and food webs is to determine the efficacy and effectiveness of conducting stable isotope analysis on formalin-fixed and alcohol preserved museum specimens. Edwards et al. [31] investigated the short and long term effects on fixation and preservation on stable isotope values of fluid-preserved museum fish specimens and found that there is predictability in the changes due to preservation and that the values of the preserved tissue samples should be adjusted -1.1% for $\delta^{13}\text{C}$ and $+0.5\%$ for $\delta^{15}\text{N}$. Edwards et al. [31] further noted that while the

magnitude of change in $\delta^{13}\text{C}$ depended on the isotopic composition of the formalin used, the duration of fixation (short versus long) or lipid extraction did not influence the isotopic composition. These results were further supported by Arrington and Winemiller [34], who reported that the magnitude of change was small and the directionality uniform in formalin and ethanol preserved fish tissues with average changes of -1.12‰ $\delta^{13}\text{C}$ and $+0.62\text{‰}$ $\delta^{15}\text{N}$ changes. However, Arrington and Winemiller [34] reported inconsistencies in results when comparing their findings to published works on other taxonomic groups such as invertebrates [35,36] and birds [37]. This suggests caution should be used while extending findings across taxonomic groups and that conducting taxon-specific experiments is likely to provide more accurate results [38].

While stable isotopes are considered a powerful tool for ecologists studying diets, food webs, and nutrient cycling, the utility of stable isotopes becomes challenging if and when food sources overlap in isotope values or are not sampled. In these cases, simple two-member bi-plots (e.g., $\delta^{15}\text{N}$ versus $\delta^{13}\text{C}$) and the use of trophic discrimination factors do not adequately represent the diet and feeding relationships, and more complex analysis techniques are recommended [39–42]. Phillips et al. [42] suggested that studies with $n + 1$ (n = number of isotopic tracers) sources use mixing models to find a unique set of assimilated diet proportions. Mixing models that use Bayesian statistics are gaining widespread use because of their characteristics of incorporating prior information, integrating across sources of uncertainty, and explicitly comparing the strength of support for competing models or parameter values [39,42].

The goals of our study were to conduct stable isotope analysis on live and formalin-preserved museum specimen Ozark hellbender tissues to identify diet composition in the Eleven Point and Spring rivers, AR, USA, and to use stable isotope analysis to investigate if Spring River hellbender diets have changed over time (1970–1975 to early 2000's). To achieve these goals, we sampled crayfish to determine assemblage composition and for stable isotope analysis, sampled fish from the Eleven Point River for stable isotope analysis, non-destructively (tissue plugs) sampled live Ozark hellbender tissues at sampling stations for stable isotope analysis, sampled (tissue plugs) historic collections of Ozark hellbender tissues from Spring River formalin preserved museum specimens, and analyzed stable isotope values using traditional two-member isotope ($\delta^{15}\text{N}$ versus $\delta^{13}\text{C}$) plots and Bayesian mixing models. We expected that Ozark hellbenders (1) have similar isotope values among sites within a river (2) will show an ontogenic shift in their diet, (3) are diet generalist (i.e., eat a variety of taxa at multiple trophic levels), and (4) had a temporal diet composition shift in the Spring River due to changes in the relative abundance of prey items since the 1970s.

2. Materials and Methods

2.1. Field-Site Description

The sampling stations of this study are located in the Eleven Point and Spring rivers of the Ozark Highlands Ecoregion of northeastern Arkansas, USA (Figure 1). The exact locations of the stations are not disclosed herein due to the state and federal endangered status of hellbenders. Both rivers are characterized by approximate channel widths of ~15–20 m, mesoscale habitats of shallow riffles, runs, and shallow to moderate depth lateral and mid-channel pools, and substrates comprised of sand, silt, gravel, cobble, and boulders overlying solid bedrock.

The Eleven Point River is a 222 km long spring-fed river located in southeastern Missouri and northeastern Arkansas, that originates near Willow Springs, MO, USA, and flows into the Spring River near Black Rock, AR, USA (Figure 1). Three study stations were chosen from the lower reaches of the Eleven Point River in Randolph County, Arkansas based on prior knowledge of existing hellbender populations. Station EP1 was the most upstream locale and station EP3 the most downstream. Hellbender and crayfish samples were collected in 2005. However, because initial stable isotope analysis did not reveal sufficient dietary composition based on crayfish alone, we collected fish in the summer of 2011

from a fourth station (EP4) just upstream of the Arkansas Game and Fish Commission's Dalton Landing river access site. This station is located between stations EP2 and EP3 and was chosen to avoid disturbing the hellbender mesoscale unit habitat while collecting fish samples.

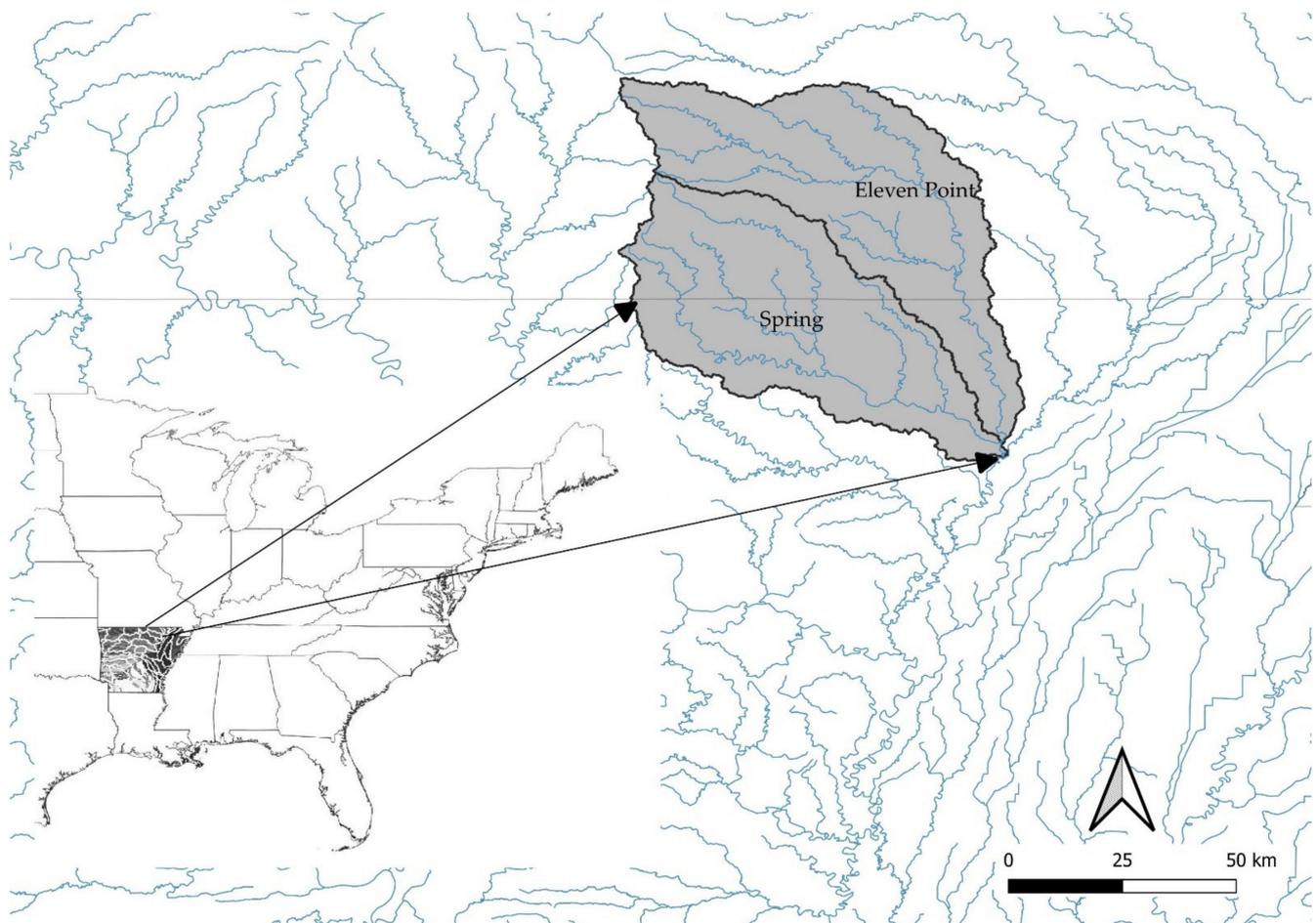


Figure 1. Spring and Eleven Point River watersheds (gray filled) of southeast Missouri and northeast Arkansas, USA (light gray horizontal line). Insert map is of southeastern United States with Arkansas (gray-scale shade) as reference.

The Spring River proper is a spring-fed river originating in Mammoth Spring, AR, USA, in which over 37,000 m³ per hour of water flow out of the spring to form the Spring River (Figure 1). The Spring River flows into the Black River near Black Rock, AR, USA. The lone Spring River station (SR1), was located approximately 7 km downstream of Mammoth Spring in Fulton County, AR, USA. Historically, this site possessed a robust hellbender population, which Peterson et al. [43] estimated to be comprised of 442 individuals based on data collected between 1980–1982. However, the Spring River hellbender populations have steadily declined over the past 40+ years [3]. Our study site was one of only two remaining sites on the river which hellbenders were known to occur at the time of sampling [44].

2.2. Crayfish Sampling

To determine crayfish assemblage richness and relative abundance and to collect samples for stable isotope analysis, crayfish were collected by hand by two SCUBA divers using timed 1-h crayfish surveys at each study station. An attempt was made to collect every crayfish encountered within that time period. Crayfish habitat searched directly overlapped with typical hellbender habitat [22]. Crayfish whole-body samples were taken from each study station with efforts made to collect an encompassing cohort; however, collections were limited by the relative abundance of each species within a study site.

Crayfish were kept alive until individuals were identified to species using a dichotomous key [45] and the total length recorded, after which they were placed in an ultra-cold freezer at -70°C . A total of 50 crayfish were analyzed for stable isotopes from three Eleven Point River stations, and 38 crayfish were examined from the lone Spring River station. Any crayfish not used in the stable isotope analysis were preserved in 70% ethanol and deposited in the Arkansas State University Museum of Zoology-Aquatic Macro-Invertebrate Collection.

2.3. Live and Formalin-Preserved Museum Hellbender Sampling

Hellbenders were located using standard rock-flipping techniques by divers utilizing SCUBA or skin-diving equipment [22]. Nine Eleven Point River Ozark Hellbenders were captured (two from EP1, five from site EP2, and two from EP3) during a one-month period in the winter of 2004 (16 November–17 December). Tissue samples were taken from three Spring River hellbenders captured between October 2004–March 2005. Hellbenders were scanned for PIT tags and measured for total length and mass. Non-lethal tissue plugs (~3 mm diameter) from individual hellbenders were extracted from the dorsal caudal musculature and included skin and muscle tissue. Once extracted, tissue sample plugs were individually placed into sterile deep freeze vials and frozen in liquid nitrogen. Hellbenders were immediately released after tissue samples were taken.

Tissue samples taken from 2000s Spring River specimens were collected from specimens that were captured in extremely morbid states and were subsequently removed from the river for study. One specimen contained multiple epidermal papillomas and died during transport to the laboratory for tumor biopsy. Another individual was floating in a backwater area of the Spring River amongst woody debris and exhibited severe gashes on its dorsum that had been invaded by fungus. Historical hellbender tissue samples were obtained from museum holdings (Milwaukee Public Museum, Milwaukee, WI, USA) of 39 Spring River hellbenders collected during a 5-year period beginning in 1970 and ending in 1975. To our knowledge all of the hellbenders were collected near the SP1 station; however, in 1975, 13 animals were collected with no specific location data other than simply “Spring River”. All of the tissue samples taken from museum specimens were fixed in 10% formalin and preserved in 70% ethanol. Tissue sample plugs from the museum specimens were collected in the same way and body location as the live hellbender tissue plugs but were immediately placed into individual labeled sterile vials containing 100% ethanol and housed at ambient temperatures upon transport back to Arkansas State University.

2.4. Fish Sampling

Fish were collected in both rivers by divers using hand nets and collection bags. At the Spring River station, fish (three *Cottus* sp.; sculpins) were collected in 2005 at the same time as hellbender and crayfish. However, Eleven Point River fish were collected in the summer of 2011 at EP4 station. The discrepancy in the collection timing in the Eleven Point River was due to not observing any benthic fish at the time of crayfish. Due to targeting benthic fish, the size of the river at this site, and limitations of sampling gear, no attempt was made to estimate fish richness and relative abundance for either 2005 Spring River or 2011 Eleven Point River fish sampling efforts.

2.5. Laboratory Processing and Stable Isotope Analysis

Hellbender tissue samples, entire crayfish, and entire fish were placed in a drying oven and dehydrated between 80 – 100°C . Lipids were not removed from any of our tissues due to no clear consensus on the efficacy of lipid extraction at the time of our laboratory processing [46]. Desiccated hellbender tissues, entire crayfish, and fish muscle tissues were ground into a fine powder using a mortar and pestle. However, museum samples were powdered using a dental amalgamator instead of a mortar and pestle. Dried powdered samples were placed in tin cups, weighed in milligrams, folded into cubes, and placed into

96-well plates. Sample-filled 96-well plates were labeled and stored in a desiccator until transported to the stable isotope facility.

Stable isotope analyses of the crayfish and hellbenders were conducted at the University of Arkansas Stable Isotope Laboratory or the University of Georgia Analytical Laboratory and used a Carlo Erba NC2500 elemental analyzer coupled with a Finnegan Delta+ isotope ratio mass spectrometer. Stable isotope analysis of Eleven Point River fish was conducted at the University of Massachusetts Boston's Environmental Analytical Facility used a Thermo Delta V Isotope Ratio Mass Spectrometer (IRMS) with samples being combusted with a Costech EA 4100. Isotope ratios were expressed in the $\delta^{13}\text{C}$ or $\delta^{15}\text{N}$ notation = $(R_{\text{sample}}/R_{\text{standard}}) \times 1000$, where $R = {}^{13}\text{C}/{}^{12}\text{C}$ or ${}^{15}\text{N}/{}^{14}\text{N}$ using international standards. Data were expressed in parts per mil (‰).

2.6. Statistical Analyses

A Kruskal-Wallis non-parametric test was performed for pair-wise comparisons between stations on crayfish assemblages (MINITAB 13.32). To determine if there was a difference in hellbender $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values among the three Eleven Point River stations, we used two ANCOVA tests with the main effects of the station and the covariate of length (MINITAB 13.32). To determine if hellbender length was correlated with either $\delta^{13}\text{C}$ or $\delta^{15}\text{N}$ values, we used regression analysis (MINITAB 13.32).

2.7. Bayesian Stable Isotope Mixing Model

A Bayesian mixing model, MixSIAR [47], was used at each site to estimate the contribution of each prey to the biomass of *C. a. bishopi* [39]. This program incorporates source and model uncertainty, in addition, it takes into account trophic discrimination factors associated with the predator [39]. The Markov Chain Monte Carlo parameters were set at the long test length (chain length = 1,000,000, burn = 700,000, thin = 300, chains = 3). The consumer data inputted contained the stable isotope composition ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) of each individual Ozark hellbender. The stable isotope composition ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) for historical consumer data that were corrected for the preservation effects of formalin using methods described in Gonzalez-Bergonzoni et al. [38]. The source data contained the mean isotopic values ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) of each prey and the standard deviations for those groups. The discrimination values were set at $\delta^{13}\text{C} = 0.8\% \pm 0$ and $\delta^{15}\text{N} = 3.4\% \pm 0$. Discrimination values account for the biomass an organism assimilates from its food sources [40,41].

3. Results

3.1. Crayfish Assemblages

During the hour-long timed crayfish surveys, 92 crayfish were collected from EP1, 24 from EP2, 39 from EP3, and 81 crayfish from SR1 (Table 1). Crayfish were represented by the following species: (1) *Cambarus hubbsi* (Hubbs Crayfish), (2) *Orconectes eupunctus* (Coldwater Crayfish), and (3) *Orconectes ozarkae* (Ozark Crayfish). *Cambarus hubbsi* was the dominant species at all stations except at EP2, where *O. eupunctus* shared a similar percentage of the total sample. *O. ozarkae* comprised the smallest fraction of crayfish from each of the Eleven Point River stations, while *O. eupunctus* was the least abundant at the Spring River station (Table 1). A Kruskal-Wallis test of crayfish community composition showed no significant differences between species or among study sites ($p = 0.443$).

3.2. Among Station Variation in Hellbender Isotopes

Hellbender $\delta^{13}\text{C}$ values from the three Eleven Point River stations were not significantly different from each other (ANCOVA, $df = 3.5$, $r^2 = 0.561$, $F = 4.4172$, $p = 0.0717$), with the main effects of station ($p = 0.4233$, $F = 1.0261$) and the covariate of length ($F = 0.5889$, $p = 0.4775$) not being significant. Hellbender $\delta^{15}\text{N}$ values from the three Eleven Point River stations were significantly different from each other (ANCOVA, $df = 3.5$, $r^2 = 0.744$, $F = 8.7710$, $p = 0.0195$); however, neither the main effect of station ($F = 3.157$, $p = 0.1298$) or the covariate of length ($F = 1.1720$, $p = 0.3283$) were significant. Stable isotope analysis

of Eleven Point River hellbender tissue revealed a large amount of variation between individuals; however, there was a significant positive association with increasing total length for both $\delta^{15}\text{N}$ (Regression, $n = 9$, $df = 1$, $r^2 = 0.64$, $F = 12.46$, $p = 0.01$; Figure 2) and $\delta^{13}\text{C}$ (Regression, $n = 9$, $df = 1$, $r^2 = 0.61$, $F = 11.07$, $p = 0.013$; Figure 2).

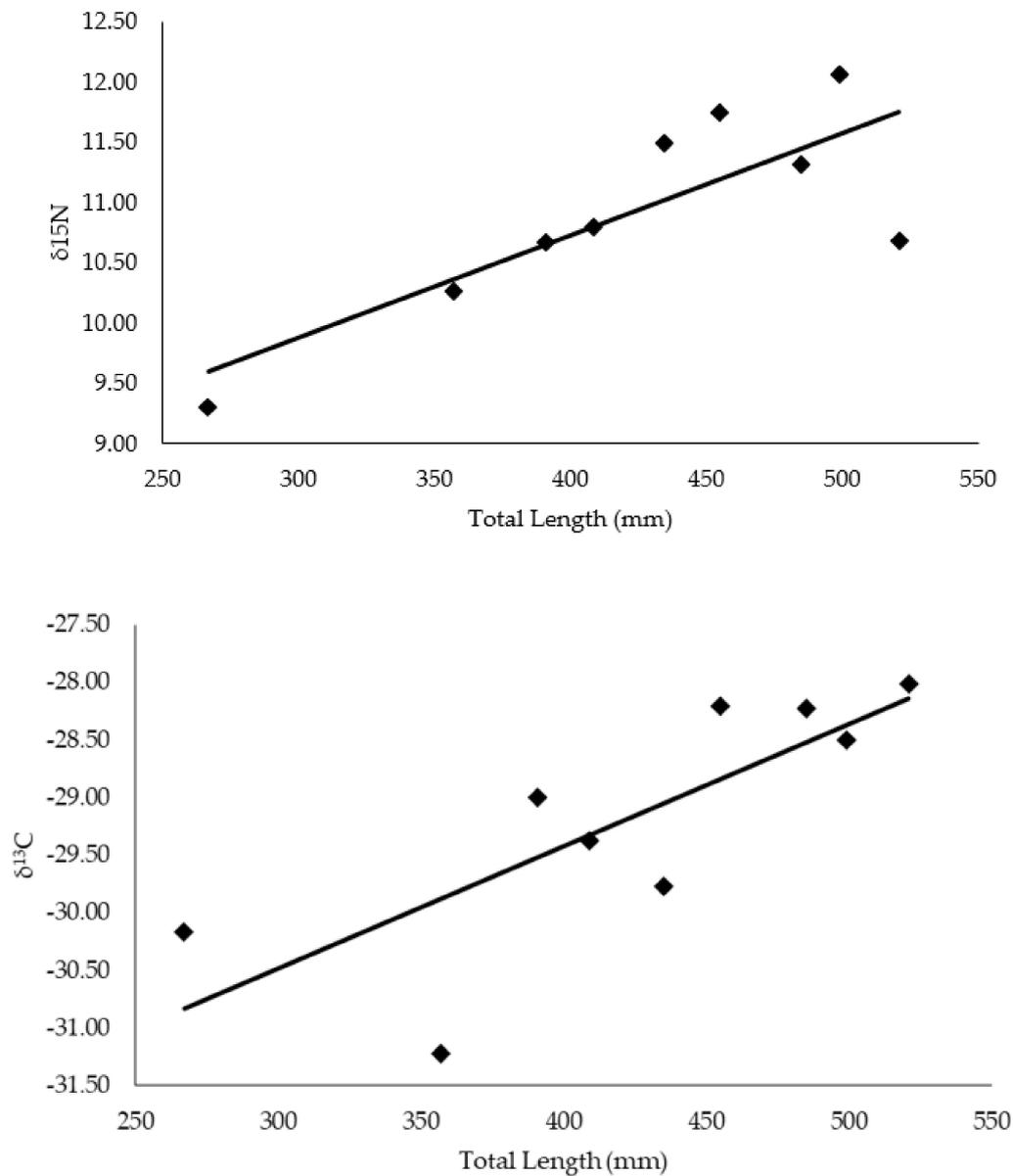


Figure 2. Eleven Point River hellbender $\delta^{15}\text{N}$ (top; $n = 9$, $r^2 = 0.64$; $y = 0.0085x + 7.3271$, $p < 0.05$) and $\delta^{13}\text{C}$ (bottom; $n = 9$, $r^2 = 0.61$; $y = 0.0106x - 33.67$, $p < 0.05$) values plotted with total length as a predictor. Isotope values are in ‰.

The same trend was exhibited by Spring River $\delta^{15}\text{N}$ values of museum samples, which appear to be correlated with increasing total length (Regression, $n = 39$, $df = 1$, $r^2 = 0.32$, $F = 17.39$, $p < 0.001$; Figure 3); however, the museum $\delta^{13}\text{C}$ values showed no correlation with total length (Regression, $n = 39$, $df = 1$, $r^2 = 0.01$, $df = 1$, $F = 0.47$, $p = 0.496$; Figure 3).

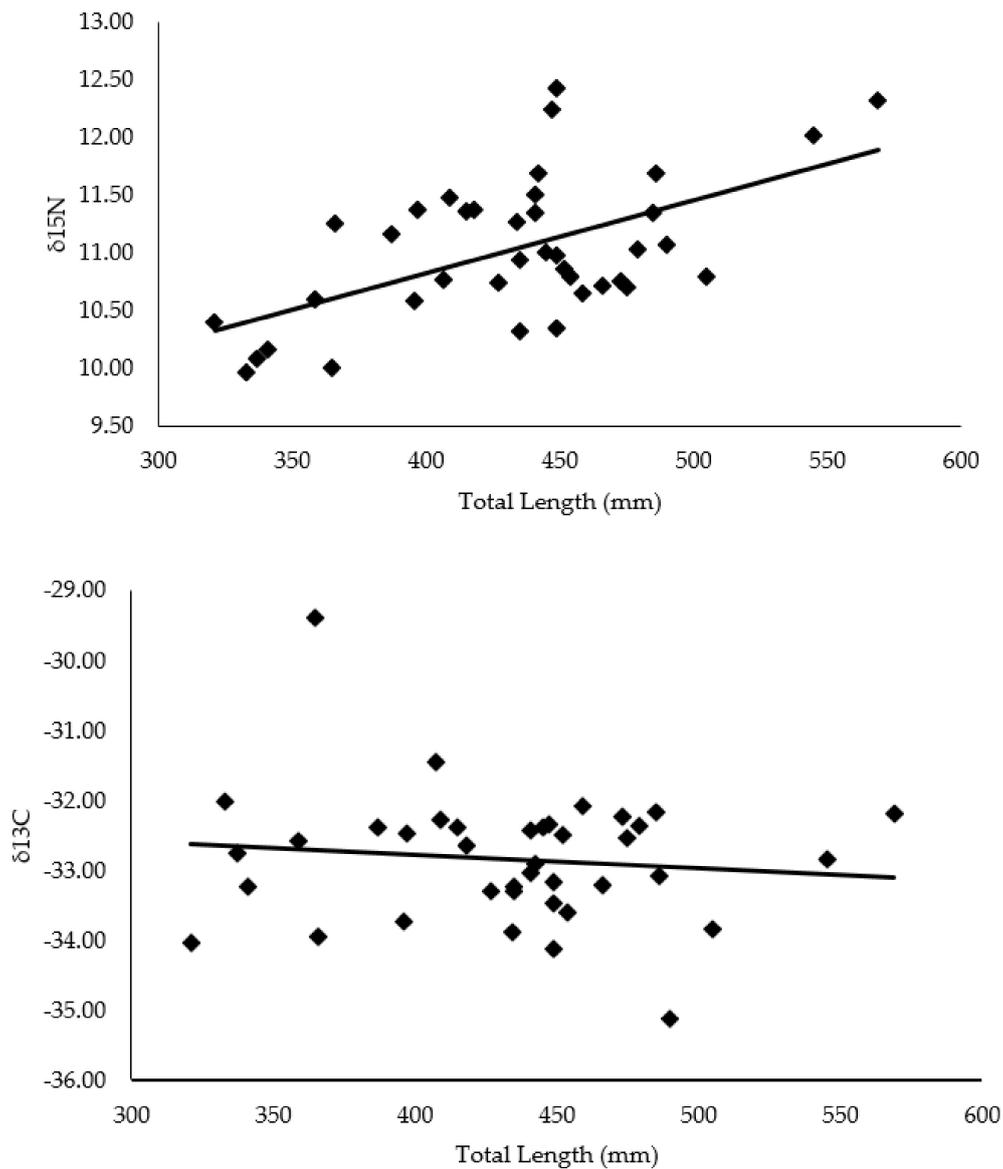


Figure 3. Spring River (1970–1975) hellbender $\delta^{15}\text{N}$ (**top**; $n = 39$, $r^2 = 0.32$; $y = 0.0063x + 8.3046$, $p < 0.05$) and $\delta^{13}\text{C}$ (**bottom**; $n = 39$, $r^2 = 0.01$, $y = -0.0019x - 32.018$, $p = 0.496$) values from preserved tissue samples plotted with total length as a predictor. Isotope values are in ‰.

Table 1. Crayfish relative abundance for each station collected during 1-h timed searches at each station from the Eleven Point and Spring Rivers, AR, USA.

River	Station	Species	<i>n</i>	Percent of Total
Eleven Point	EP1	<i>Cambarus hubbsi</i>	48	52.17
		<i>Orconectes eupunctus</i>	41	44.57
		<i>Orconectes ozarkae</i>	3	3.26
	EP2	<i>Cambarus hubbsi</i>	10	41.67
		<i>Orconectes eupunctus</i>	10	41.67
		<i>Orconectes ozarkae</i>	4	16.67
	EP3	<i>Cambarus hubbsi</i>	22	56.41
		<i>Orconectes eupunctus</i>	15	38.46
		<i>Orconectes ozarkae</i>	2	5.13
Spring	SR1	<i>Cambarus hubbsi</i>	40	49.38
		<i>Orconectes eupunctus</i>	18	22.22
		<i>Orconectes ozarkae</i>	23	28.40

3.3. $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ Values

Overall, Eleven Point River hellbenders and three fish taxa ranged from -27.85 to -32.36 $\delta^{13}\text{C}$ and 9.61 to 11.30 $\delta^{15}\text{N}$ and were higher in $\delta^{15}\text{N}$ compared to the three crayfish taxa from the Eleven Point River that ranged from -29.59 to -30.87 $\delta^{13}\text{C}$ and $+5.14$ to $+5.70$ $\delta^{15}\text{N}$ (Table 2; Figure 4). Based on mean δ values and using $\pm 1\%$ for $\delta^{13}\text{C}$ and $+3.5\%$ trophic discrimination values for $\delta^{15}\text{N}$, both *C. hubbsi* and *O. ozarkae* could be potential food sources for hellbenders; however, *C. eupunctus* and the three fish species are not likely food resources for hellbenders in the Eleven Point River.

Similarly, all Spring River hellbenders ranged from -29.98 to -31.91 $\delta^{13}\text{C}$ and $+11.31$ to $+12.15$ $\delta^{15}\text{N}$ and were higher in $\delta^{15}\text{N}$ compared to the three crayfish taxa from the Spring River, which ranged from -28.6 to -29.44 $\delta^{13}\text{C}$ and $+6.03$ to $+6.65$ $\delta^{15}\text{N}$, with one fish taxon that had a $\delta^{13}\text{C}$ of -31.07 and a $\delta^{15}\text{N}$ of $+8.15$ (Figure 4). Based on mean δ values and using $\pm 1\%$ for $\delta^{13}\text{C}$ and $+3.5\%$ trophic discrimination values for $\delta^{15}\text{N}$, all three species of crayfish along with the *Cottus* sp. sampled from SR1 are potential food sources for the current hellbender sample. Our adjusted mean $\delta^{13}\text{C}$ value of the historic hellbender samples restricts the potential of all three crayfish species as food sources; however, there is a close relationship with the *Cottus* sp. sample. The historic adjusted mean $\delta^{15}\text{N}$ value is nearly the same as the current SR1 hellbender sample (Figure 4).

Table 2. Mean $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of each species sampled from the Eleven Point and Spring Rivers, AR, USA. n = sample size and SD = standard deviation. Isotope values are in ‰.

Species	River	Station	n	Mean $\delta^{13}\text{C}$	SD	Mean $\delta^{15}\text{N}$	SD
<i>C. hubbsi</i>	Eleven Point	EP1	10	-30.02	1.04	+5.44	0.56
<i>O. eupunctus</i>	Eleven Point	EP1	10	-31.08	1.14	+4.92	0.33
<i>O. ozarkae</i>	Eleven Point	EP1	4	-30.05	0.59	+5.39	0.33
<i>C. a. bishopi</i>	Eleven Point	EP1	2	-30.69	0.75	+9.79	0.68
<i>C. hubbsi</i>	Eleven Point	EP2	10	-28.94	0.62	+5.80	0.42
<i>O. eupunctus</i>	Eleven Point	EP2	10	-30.28	0.77	+5.51	0.37
<i>O. ozarkae</i>	Eleven Point	EP2	4	-29.16	0.94	+5.16	0.23
<i>C. a. bishopi</i>	Eleven Point	EP2	5	-28.88	0.75	+10.99	0.39
<i>C. hubbsi</i>	Eleven Point	EP3	10	-29.81	0.75	+5.87	0.40
<i>O. eupunctus</i>	Eleven Point	EP3	10	-31.24	0.34	+4.99	0.34
<i>O. ozarkae</i>	Eleven Point	EP3	2	-30.40	1.62	+4.86	0.47
<i>C. a. bishopi</i>	Eleven Point	EP3	2	-28.35	0.21	+11.91	0.22
<i>C. hubbsi</i>	Eleven Point	Combined	30	-29.59	0.57	+5.70	0.09
<i>O. eupunctus</i>	Eleven Point	Combined	30	-30.87	0.51	+5.14	0.02
<i>O. ozarkae</i>	Eleven Point	Combined	10	-29.87	0.64	+5.14	0.12
<i>N. albater</i>	Eleven Point	EP4	6	-30.07	1.62	+10.29	0.05
<i>Etheostoma</i> sp.	Eleven Point	EP4	16	-30.70	0.79	+10.25	0.21
<i>C. carolinae</i>	Eleven Point	EP4	2	-27.85	1.69	+11.30	0.19
<i>C. hypselurus</i>	Eleven Point	EP4	14	-32.36	0.90	+9.61	0.13
<i>C. a. bishopi</i>	Eleven Point	Combined	9	-29.31	1.23	+10.90	1.07
<i>C. hubbsi</i>	Spring	SR1	15	-29.01	1.19	+6.65	0.55
<i>O. eupunctus</i>	Spring	SR1	10	-29.44	1.30	+6.05	0.50
<i>O. ozarkae</i>	Spring	SR1	10	-28.60	0.93	+6.03	0.96
<i>Cottus</i> sp.	Spring	SR1	3	-31.07	0.53	+8.15	0.13
<i>C. a. bishopi</i>	Spring	SR1	3	-29.98	2.77	+11.31	0.52
<i>C. a. bishopi</i> 1970's	Spring	SR1	39	-31.91	0.93	+11.37	0.61
<i>C. a. bishopi</i> 2000's	Spring	SR1	10	-31.06	1.61	+12.15	3.38

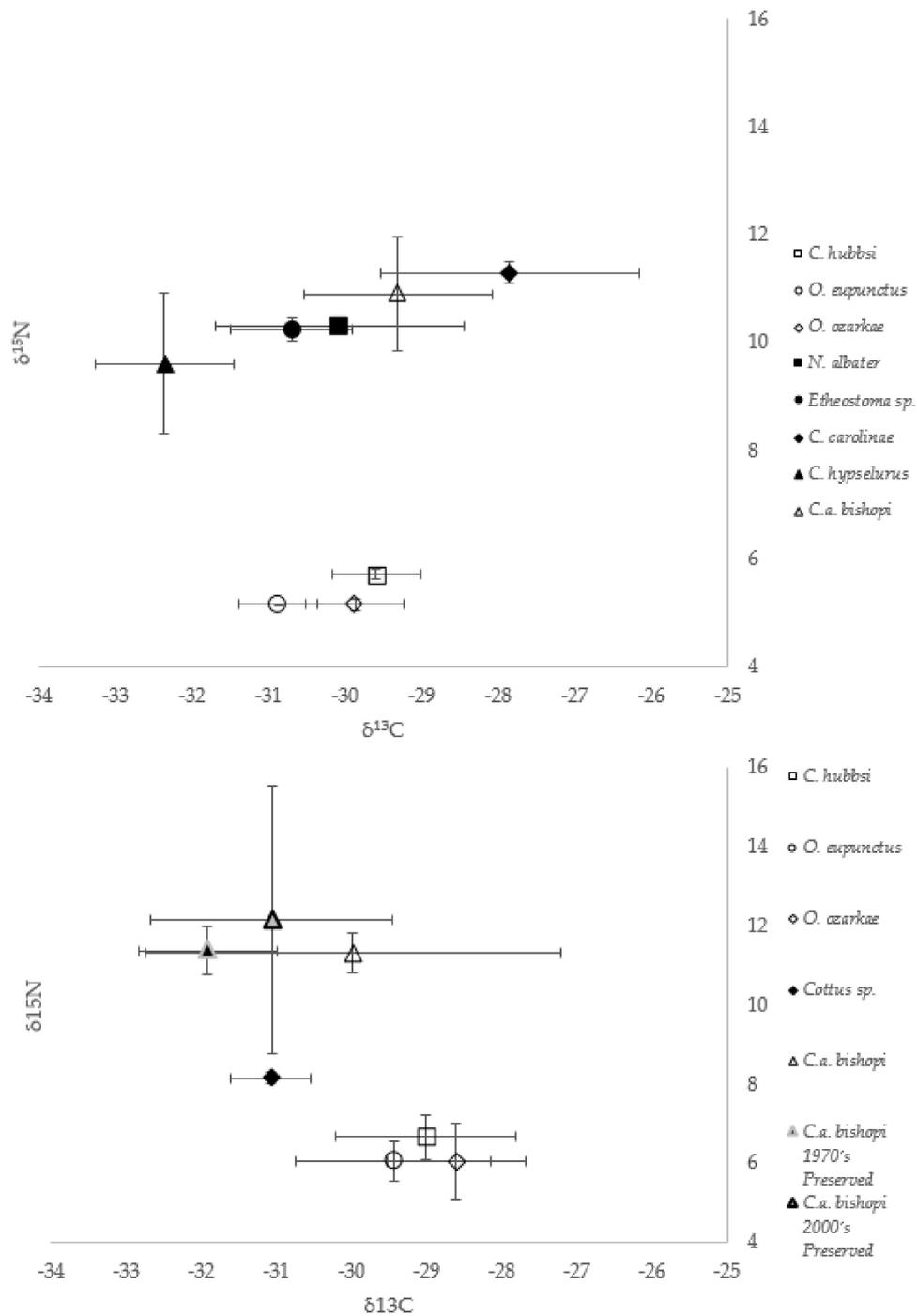


Figure 4. Mean $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ (\pm SD) for pooled contemporary and preserved tissue of *C. alleganiensis* and prey from the Eleven Point (top) and Spring Rivers (bottom), AR, USA. Isotope values are in ‰.

3.4. Isotopic Mixing Model

Our Bayesian Mixing Model showed contrasting results compared to traditional $\delta^{13}\text{C}$ versus $\delta^{15}\text{N}$ bi-plots (Table 3). For example, the Eleven Point River Bayesian Mixing Model estimated fish and crayfish contributed relatively evenly to hellbender tissue (herein described as diet) with fish ranging from 9.1 to 24.1% of hellbender diets, while crayfish contributed 8.6 to 21.0% of hellbender diets. The Eleven Point River models indicated *C. hubbsi* was always the highest crayfish contributor, with *O. ozarkae* then *O. eupunctus* second and fish taxa *C. hypselurus* always being the least contributing, and *Etheostoma* being the second-lowest contributor while *C. carolinae* was often, but not always, the highest fish

contributor (Table 3). Lastly, models using historical data suggest fish comprised a higher proportion of hellbender diets in the 1970s, and a more omnivorous diet of crayfish and fish is indicative of the models beginning in the 2000s.

Table 3. Mean percent contribution (SD; 95% CI) of prey items of contemporary and historical *C. a. bishopi* at three locations in the Eleven Point and Spring Rivers, AR, USA derived from MixSIAR models.

River	Station	Time	Prey	Mean Contribution (%)	SD	95% CI			
Eleven Point	EP1	Present	Crayfish	<i>Cambarus hubbsi</i>	14.2	10.0	32.1		
				<i>Orconectes eupunctus</i>	10.8	8.3	27.1		
				<i>Orconectes ozarkae</i>	14.0	10.0	32.5		
			Fish	<i>Noturus albater</i>	15.7	12.9	41.9		
				<i>Etheostoma</i> sp.	12.2	10.3	33.0		
				<i>Cottus carolinae</i>	24.1	11.8	42.5		
	EP2	Present	Crayfish	<i>Cottus hypselurus</i>	9.10	8.5	25.9		
				<i>Cambarus hubbsi</i>	21.0	14.9	48.2		
				<i>Orconectes eupunctus</i>	17.1	13.2	42.9		
			Fish	<i>Orconectes ozarkae</i>	18.6	13.0	42.6		
				<i>Noturus albater</i>	13.1	9.5	31.0		
				<i>Etheostoma</i> sp.	10.9	8.5	27.6		
				<i>Cottus carolinae</i>	10.2	7.1	23.4		
				<i>Cottus hypselurus</i>	9.20	7.3	23.0		
				<i>Cambarus hubbsi</i>	15.0	10.8	35.3		
			EP3	Present	Crayfish	<i>Orconectes eupunctus</i>	11.3	8.6	27.9
						<i>Orconectes ozarkae</i>	12.5	8.9	28.9
					Fish	<i>Noturus albater</i>	15.2	12.4	40.2
<i>Etheostoma</i> sp.	12.6	10.4				33.2			
<i>Cottus carolinae</i>	23.7	11.8				42.6			
<i>Cottus hypselurus</i>	9.70	9.2				28.3			
Spring	SR1	Present	Crayfish	<i>Cambarus hubbsi</i>	16.1	15.0	46.5		
				<i>Orconectes eupunctus</i>	13.7	13.4	41.0		
				<i>Orconectes ozarkae</i>	12.8	12.3	38.0		
			Fish	<i>Cottus</i> sp.	57.4	20.9	85.2		
				<i>Cambarus hubbsi</i>	20.9	19.0	60.8		
				<i>Orconectes eupunctus</i>	31.5	21.2	68.8		
		Historical 2000's	Crayfish	<i>Orconectes ozarkae</i>	22.3	18.2	57.6		
				<i>Cottus</i> sp.	25.3	13.2	48.1		
				<i>Cambarus hubbsi</i>	3.40	3.9	10.9		
			Historical 1970's	<i>Orconectes eupunctus</i>	21.9	10.4	38.1		
				<i>Orconectes ozarkae</i>	4.4	4.9	15.2		
				<i>Cottus</i> sp.	70.4	9.1	86.7		
		Both Historical sets of samples	Crayfish	<i>Cambarus hubbsi</i>	3.30	3.7	9.8		
				<i>Orconectes eupunctus</i>	33.4	9.6	47.1		
				<i>Orconectes ozarkae</i>	3.80	4.6	12.7		
			Fish	<i>Cottus</i> sp.	59.6	7.2	71.8		

4. Discussion

The results of our stable isotope study revealed three main findings related to our expectations. First, we found that there were no statistically significant differences between hellbender $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values among sites, and, consistent with our expectations, hellbender stable C and N isotopes were correlated with body length. Second, we found that contrary to our expectation, traditional $\delta^{13}\text{C}$ versus $\delta^{15}\text{N}$ bi-plots and trophic discrimination values didn't provide complete discernment in hellbender diets; however, Bayesian MixSIAR models revealed hellbenders to have generalist diets of multiple taxa of crayfish and fish, but that these relative contributions were not necessarily related to the observed relative abundance of crayfish. Third, we found, as expected, that the use of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values adjusted historic formalin-fixed and ethanol preserved hellbenders matched well with current crayfish and fish stable isotope values, in which Bayesian MixSIAR

models were able to reasonably estimate the percent contribution of crayfish and fish to hellbender diets. The MixSIAR results provisionally suggest a hellbender shift in diet composition since the 1970's. Combined, these findings provide some important evidence about hellbender diet composition and a potential tool to assess historical dietary habits for this imperiled taxon.

4.1. Hellbender Length Correlation with C and N Isotope Values

The observed shift in $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values as total length increases (Figures 2 and 3), assuming length can be interpreted as an indicator of relative age, supports our expectation that hellbender isotopic composition changes through time and there is an ontogenetic change in hellbender diet from juvenile stages into adulthood. Our finding supports the ontogenetic diet change documented in larval hellbenders (<125 mm) [13,14]. Further, our finding is consistent with observed ontogenetic changes in diets of other vertebrates such as birds [37] and fish [48]. With increasing size, hellbender $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values move accordingly, moving closer to a midpoint average of all prey items consumed. This change can be attributed, in part, to an expanded diet (i.e., as hellbender size and age increase) through the greater availability of prey items due to changes in maximum gape size [49]. This physical change due to growth enables hellbenders to exploit the most readily available food source or at least allows for diversification of prey.

4.2. Contemporary Hellbender Diets

Our traditional two-member bi-plot isotope analyses and Bayesian MixSIAR models of hellbender and crayfish tissues loosely support our second expectation and findings reported in past studies that hellbenders are opportunistic prey generalists [15–17,19,22]. Our data show a relationship between hellbenders and crayfish and illustrates a high potential for opportunism.

Our isotope values from each site were consistent with crayfish being the most dominant food item of the hellbender; however, the variation in mean $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values observed between sites suggest a spatial and prey consumption variability and that hellbenders do not depend solely on one species or group of organisms. All three species of crayfish collected were previously known to inhabit both river systems as well as the meso unit habitats surveyed [45,50]; therefore, gaps in mean δ values between hellbenders and crayfish species sampled are not due to the recent exposure of one species to another. Furthermore, the distribution of mean crayfish $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values, grouped according to the study site, share overlapping distributions. The mean $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ value for *Cottus* sp. at SR1 indicates *Cottus* sp. could be potential prey even with a 35-year hiatus. Peterson et al. [19] did find *Cottus* sp. in the stomachs of hellbenders in several months of their one-year study; however, as a group *Cottus* sp. never comprised more than 15% of hellbender diets. The mean current hellbender isotope data suggest that all three species of crayfish represent a potential prey item; however, there is a relatively large amount of $\delta^{13}\text{C}$ variance.

Our Eleven Point River stable isotope results revealed that hellbenders from EP1 and EP2 are potentially consuming all three species of crayfish observed in this study. Meanwhile, data from EP3 exhibits the least amount of hellbender $\delta^{13}\text{C}$ variation; yet the mean value is skewed away from that of the local crayfish isotopic composition. Potential explanations include the following: (1) an abundance in the availability of alternative prey items, which are just as or more accessible than crayfish; or (2) our small sample size which does not depict an accurate representation of the hellbender population at EP3; which combined could clarify the data.

While traditional two-member bi-plot isotope analyses of hellbender and crayfish tissues were somewhat informative, it was our Bayesian MixSIAR models that provided greater resolution and estimated that hellbenders were consuming a mix of crayfish and fish. Furthermore, there was some indication of a slight preference for *C. hubbsi* crayfish and *C. hypselurus* fish in hellbender diets at a majority of stations. This mixed diet com-

position is in contrast with other studies that have used gut content as evidence of diet composition [15–17,20]. However, these findings are in support of previous studies that have found seasonal gut analysis evidence of fish [19] or combinations of crayfish, snails, and fish [22].

4.3. Historical Hellbender Diets

Our results from the Spring River station indicated provisional support for our third expectation and may be explained as either a potential shift in crayfish isotopic composition over the last 35 years or that isotope values have been compromised through the standard fixation and preservation process. If the first explanation is an accurate portrayal of the data, then there also must have been a shift in isotope composition originating in trophic levels below that of crayfish. A shift at the lowest trophic levels (i.e., primary producers and primary consumers) could eventually affect higher levels and cause a noticeable shift in isotope ratios over a 35-year period. If there has been a shift at the lowest trophic levels, the cause remains speculative; however, Trauth et al. [1] documented a noticeable shift in the habitat at SR1 over a 10-year period, with an increased silt load and increased rooted vegetation. The variation in the current hellbender $\delta^{13}\text{C}$ values from Spring River also may support this idea. Hellbenders are long-lived organisms (with longevity records of over 40 years), and all Spring River hellbenders sampled from the 2000s were old individuals (>500 mm); consequently, it may be possible that wide variation in isotope ratios are due to the consumption of many generations of shorter-lived species that exhibit a wide range of values. The variation could also be the result of a small sample size; yet, the state of the Spring River hellbender population would not permit a larger number of samples to be taken. If the second explanation is accurate, which states that the tissue samples were compromised during fixation and preservation, then future studies should be wary of the potential problems of using this technique. However, the preserved 1970s samples mean $\delta^{15}\text{N}$ value was similar to the 2000s sample mean, even with the 35-year temporal gap. Also, an association between total length and $\delta^{15}\text{N}$ comparable to that of the Eleven Point River trend was observed; this suggests that the second explanation may not be the case.

5. Conclusions

Our study represents a stable isotope approach to studying the food habits of hellbenders using both fresh tissue and formalin-fixed tissues and adds to our understanding of hellbender food habits. The spatial consistency of the crayfish community composition across study sites in the Eleven Point River suggests food availability and stability within the river system. These data can serve as baseline information for future studies involving crayfish inhabiting the Eleven Point River. Furthermore, a lack of prey items caused by a change in benthic habitat seems to be an unlikely influence of the Spring River hellbender decline due to the overlap in species composition among all study sites. Additionally, the mean crayfish $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values between species and study sites suggest that crayfish diet patterns are similar both spatially within and across river systems. Natural history collections may be a source of untapped data for many aspects of research including trophic interactions and diet analysis using stable isotope analysis. However, a definitive interpretation of these data, especially for the formalin-fixed and ethanol preserved data, should be used with caution until further studies are conducted. Management and conservation of imperiled species can have a positive effect on many species or even the entire ecosystem [51]. Therefore, the conservation of the Ozark hellbender should be an important goal for preserving sensitive ecosystems (e.g., rivers and streams). Consequently, the results of our study could be used to support conservation efforts directed toward understanding potential causes of hellbender decline.

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W.H., S.E.T., A.J., and A.D.C.; writing—review and editing, W.H., S.E.T., B.W., A.J., M.R., J.R.M., and A.D.C.; visualization, W.H., J.R.M., M.R., and A.D.C.; supervision, S.E.T., J.R.M., and A.D.C.; project administration, S.E.T. and A.D.C.; funding acquisition, S.E.T. and A.D.C. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: At the time of fish and fresh hellbender tissue, 2005, vertebrate wildlife protocols were not subject to IACUC review at Arkansas State University, however, samples were collected under an Arkansas Game and Fish Commission permits under the supervision of ADC and SET and we used best ethical practices to sample fish and hellbender tissues. Crayfish are invertebrates and thus are not subject to vertebrate IACUC protocols. The 2011 Eleven Point River fish samples were collected under the supervision of ADC's University of Massachusetts Boston IACUC protocol number IACUC2013005.

Informed Consent Statement: Not applicable.

Data Availability Statement: None of the data are publicly available, however do reside with the authors on backup hard drives and requests for data may be made with the authors.

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