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Nutritional Analysis of Diet Items Available to Captive and Free-ranging Hellbenders (*Cryptobranchus alleganiensis*)

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Zoological collections provide unique opportunities to conduct wildlife research in controlled settings. However, providing captive animals with a nutritionally complete diet can be challenging, particularly for amphibians and other species for which nutritional requirements are poorly understood. To overcome this challenge, captive diets can be formulated to closely mimic natural diets. Yet for carnivorous amphibians, providing a naturalistic diet can be logistically challenging and may introduce health risks. For example, certain prey items (e.g., snails and crayfish) of appropriate size are not consistently available from commercial sources, and collecting these items from the wild requires significant time and resources (in addition to collection permits). Breeding colonies of prey animals can provide a consistent food source, but establishing these colonies can be resource intensive. Furthermore, bringing live prey into a captive animal facility (whether for direct feeding or to establish a colony) presents disease risks (Anderson 1988; Densmore and Green 2007). These risks are not always lessened by obtaining prey from commercial sources because many of these companies collect animals from the wild and do not screen for disease. Lastly, captive prey typically have fewer escape options and potentially

more limited food sources than their wild counterparts, and therefore may be aggressive towards their potential predators (Pough 2007; Warwick 1990).

The Hellbender, *Cryptobranchus alleganiensis*, is a large, aquatic salamander reaching lengths up to 74 cm (Fitch 1947; Petranka 1998). It requires cool, flowing water (Smith 1907; Wheeler et al. 2003) and large slabs of rock for cover and nesting sites (Bishop 1941). There are currently two recognized subspecies: the Eastern Hellbender, *C. a. alleganiensis*, and the Ozark Hellbender, *C. a. bishopi*. Their biology and natural history are similar (Ettling et al. 2013). Wild populations of *C. alleganiensis* are declining throughout the species' range, likely due to siltation, damming of streams, and pollution (Trauth et al. 1992; Wheeler et al. 2003; Hiler et al. 2005; Briggler et al. 2007; Nickerson and Briggler 2007; Foster et al. 2009; Nickerson et al. 2009; Burgmeier et al. 2011). This species has become increasingly popular in zoological collections and rearing facilities as conservation efforts focus on research, reintroduction programs, and public education (International Species Information System 2014).

Meeting the nutritional demands of *C. alleganiensis* in captivity is challenging because little is known about the diet of larvae and juveniles. Stomach content analysis has revealed that adult *C. alleganiensis* primarily feed on crayfish and small fishes in the wild (Smith 1907; Netting 1929; Peterson et al. 1989), although a variety of other prey may be consumed (Smith 1907; Petranka 1998; Humphries et al. 2005; Hecht-Kardasz and Nickerson 2013; Groves and Williams 2014; Irwin et al. 2014). Salamander larvae and juveniles have similar mouth parts and digestive systems as adults (McWilliams 2008), suggesting that immature *C. alleganiensis* consume some of the same prey as adult specimens but are constrained by prey size. Larval *C. alleganiensis* are known to consume insects from the orders Trichoptera, Plecoptera, and Ephemeroptera (Hecht-Kardasz 2011), but they likely feed on a wider array of aquatic macroinvertebrates (Alexander 1927; Nickerson and Mays 1973; Pitt and Nickerson 2006). With the exception of dissecting museum specimens, stomach content analysis of juvenile *C. alleganiensis* is challenging because of their small size and the inaccuracies that can result from more digestible items (soft-bodied prey) being underrepresented compared to less digestible items (hard-bodied prey). Furthermore, capturing wild larvae and juveniles is difficult because poor recruitment has been observed in many of the remaining populations and immature individuals are often rare (Gates et al. 1985; Pfingsten 1989; Mayasich et al. 2003; Nickerson et al. 2003; Wheeler et al. 2003).

Given that immature *C. alleganiensis* are difficult to sample in the wild, a sampling of aquatic invertebrates in the species' habitat can provide information about the diversity and nutritional composition of prey species available *in-situ*. Although several studies have analyzed the nutritional composition of invertebrates (Bernard et al. 1997; Barker et al. 1998; Finke 2002; Finke 2012; Oonincx and Dierenfeld 2012), there are few comparisons of captive diets and wild prey items for amphibians (Dierenfeld et al. 2009). By identifying the most appropriate captive diet for *C. alleganiensis* and determining the primary reasons institutions are unable to offer these food items, we can start to develop strategies to provide the species with better nutrition in captivity. The objectives of this study were to determine 1) what food items are commonly fed to *C. alleganiensis* in captivity, and 2) how the nutritional content of a typical captive diet compares to wild-caught invertebrate prey available *in-situ*.

Methods

Zoological surveys.—To identify diets offered to *C. alleganiensis* in captivity, a survey was distributed to 16 zoological institutions housing *C. alleganiensis* in April 2014.

Sample collection.—Seven known *C. alleganiensis* sites in Pennsylvania, Virginia, and New York were surveyed for invertebrates (including crayfish) from May to August 2012 using dip net (D-net) sampling. Collections were taken between sites of recent (i.e., within the last 30 days) *C. alleganiensis* captures, to ensure the salamanders were present in the sampling area. The sites varied from slow-moving waters with high sediment to clear, cool, flowing streams, so sample area varied as well (riffle, pool, etc.). Dip nets were held firmly against the streambed and positioned with the net opening facing upstream. The substrate directly upstream from the D-net (approximately 1 m^2) was disturbed by moving and turning the rocks to allow any macroinvertebrates in the sample area to flow into the net. This process was repeated until approximately 50 g (dry weight) of macroinvertebrates were obtained. Invertebrates were identified to the taxonomic order, transported on ice, and frozen (-20°C) within 24 h of collection. Commercially sourced food items were obtained from G & G Aquatics (Lorton, Virginia, USA) and Mike's Wholesale Bait Co. Inc (Gambrills, Maryland, USA) and included both frozen (krill) and live (crayfish, black worms, ghost shrimp, and earth worms) invertebrates. Samples (50 g, dry weight) of each commercially sourced food item were frozen until analysis.

Nutritional analysis.—For comparison, all macroinvertebrates from each source (i.e., commercial or wild caught) were pooled

Table 1. Occurrence of taxa represented in captive diets* at *Cryptobranchus alleganiensis* field sites**.

Class	Order	Percent of sites
Insecta	Plecoptera	100
Insecta	Trichoptera	100
Crustacea	Decopoda(Crayfish)	86
Insecta	Ephemeroptera	86
Insecta	Megaloptera	86
Insecta	Odonata	86
Insecta	Diptera	57
Various	*Fish	43
Insecta	Coleoptera	29
Gastropoda		29
Oligochaeta	Opisthopora	29
Crustacea	Decopoda	0
Malacostraca	Euphausiacea	0
Oligochaeta	Haplotaxida	0
Insecta	Hemiptera	0
Crustacea	Isopoda	0
Insecta	Lepidoptera	0
Clitellata	Lumbriculidae	0
Malacostraca	Mysida	0
Insecta	Orthoptera	0
Mammalia	Rodentia	0

* Based on survey responses from 16 AZA institutions.
** Total sites (N = 7) in New York, Pennsylvania, and Virginia.

for analysis. The wild caught macroinvertebrates from each site were identified before being pooled for analysis to determine the proportion in each sample (Table 1). Because of their larger size, crayfish were analyzed separately. Thus, nutritional analysis was conducted on four prey groups: 1) commercially sourced macroinvertebrates, 2) commercially sourced crayfish, 3) wild caught macroinvertebrates, and 4) wild caught crayfish. Assays included dry matter, crude fat, crude protein, available crude protein, chitin, neutral detergent fiber (NDF), acid detergent fiber (ADF), ash, and gross energy. Percent dry matter was calculated as the ratio of dry weight to starting weight (AOAC 1990). Crude fat was measured by sequential extractions with petroleum ether using an Ankom XT15 Extraction Analyzer (Ankom Technology, Macedon, New York, USA). The Van Soest Method (Van Soest 1963) was used to determine NDF and ADF. Total nitrogen was determined in dried samples using a carbon, hydrogen, and nitrogen (CHN) elemental gas analyzer (Model 2400, Perkin Elmer; Norwalk, Connecticut). The obtained total nitrogen value was multiplied by 6.25 to determine the amount of crude protein in the samples (Jones 1931; AOAC 1990). The whole-sample nitrogen values were corrected for the amount of nitrogen in the ADF residue to obtain an estimate of available protein. Chitin was estimated by multiplying the amount of nitrogen in the ADF by 6.7 and the percent ADF in the sample (Stemlock et al. 1985; Finke 2007). Ash was determined by weighing a subsample, placing in a muffle furnace at 550°C for 6 h, and then reweighing. Gross energy was directly measured by adiabatic bomb calorimetry (Model 1241, Parr Instrument Co., Moline, Illinois, USA). Samples were formed into a pellet and dried in a forced air convection oven prior to adiabatic bomb calorimetry.

Statistical analysis.—Data were analyzed using the Plyr and base packages in R Statistical Software (R Development Core Team 2008). For each nutrient, means were compared among prey groups using Tukey's test. Data are presented as means ± standard deviation.

Results

Zoo surveys.—Thirteen of the sixteen institutions surveyed housed juvenile *C. alleganiensis*, whereas the remaining three housed only adults (Table 2). Collectively, captive diets included chordates, annelids, and arthropods, and represented thirteen orders from six animal classes (Oligochaeta: Opisthopora and Haplotaxida; Malacostraca: Euphausiacea and Mysida; Clitellata: Lumbriculidae; Crustacea: Decopoda and Isopoda; Insecta: Orthoptera, Coleoptera and Lepidoptera; Mammalia: Rodentia; Table 3). Fish were not identified to the class or order level, and one institution fed fish analog, a manufactured diet from Mazuri® (Richmond, Indiana, USA). On average, institutions provided *C. alleganiensis* with six different food items as part of a regular diet, although this number ranged from one to thirteen. Captive diets included some items that are unlikely to be available to the species *in-situ*; specifically, rodents, lepidopterans, and lumbriculidans. Crayfish, a main food source for wild *C. alleganiensis*, were not included in regular diets for most (75%) institutions. Of the institutions that do not feed

Table 2. Responses from16 institutions surveyed.

Institution	Age class of *C. alleganiensis*	Number of diet items offered	Crayfish offered	Reason for omitting crayfish
1	Juveniles	3	Yes	N/A
2	Juveniles	5	No	Disease risk
2	Adults	2	No	Disease risk
3	Juveniles	3	No	Expensive
4	Larvae	3	No	Disease risk
4	Juveniles	5	No	Disease risk
4	Adults	1	No	Disease risk
5	Juveniles	13	No	Disease risk
6	Juveniles	2	No	Difficult to procure; disease risk
6	Adults	3	No	Difficult to procure; disease risk
7	Juveniles	4	No	Expensive
7	Adults	2	No	Expensive
8	Juveniles	2	No	Difficult to procure
9	Adults	2	Yes	N/A
10	Juveniles	5	No	Disease risk
11	Adults	1	No	Difficult to procure; disease risk
12	Juveniles	3	No	Difficult to procure; disease risk
12	Adults	3	No	Difficult to procure; disease risk
13	Adults	3	No	Disease risk
14	Juveniles	3	Yes	N/A
15	Larva	7	Yes	N/A
15	Juvenile	6	Yes	N/A
15	Adults	3	Yes	N/A
16 (NZP)	Juveniles	5	Yes	N/A

* Fish were counted as one type of diet, although some institutions feed different species of fish.

TABLE 3. The number of samples (institutions and D-net samples) containing the taxa listed. There were seven different field sites and sixteen institutional *Cryptobranchus alleganiensis* diets.

Class	Order	# of institutional diets	# of D-net samples
Insecta	Coleoptera	2	2
Crustacea	Decopoda	4	0
Crustacea	Decopoda(Crayfish)	3	6
Insecta	Diptera	0	4
Insecta	Ephemeroptera	0	6
Malacostraca	Euphausiacea	8	0
Gastropoda		0	2
Oligochaeta	Haplotaxida	1	0
Insecta	Hemiptera	0	0
Crustacea	Isopoda	1	0
Insecta	Lepidoptera	1	0
Clitellata	Lumbriculidae	7	0
Insecta	Megaloptera	0	6
Malacostraca	Mysida	3	0
Insecta	Odonata	0	6
Oligochaeta	Opisthopora	10	2
Insecta	Orthoptera	3	0
Polychaeta	Glyceridae	1	0
Insecta	Plecoptera	0	7
Mammalia	Rodentia	3	0
Insecta	Trichoptera	0	7
	*Fish	10	3
	* Fish analog diet (Mazuri)	1	0

*Unknown fish species were collected in three D-net samples (one fish per sample).
**One institution fed fish analog diet.

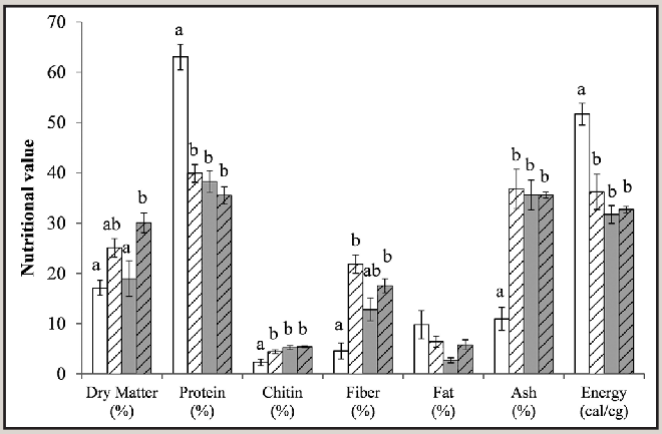

FIG. 1. Nutritional profiles of macroinvertebrates (white bars) and crayfish (grey bars) obtained from commercial sources (solid bars) or caught in Hellbender habitat (lined bars). Protein and fiber represent available protein and neutral detergent fiber, respectively. Energy is reported in cal/cg to conform to the scale of other nutrients. Within nutrients, bars with different superscripts differ ($P < 0.05$). No differences in percent fat were observed among animal groups.

crayfish, 73% stated that the risk of spreading diseases, such as *Batrachochytrium dendrobatidis* (*Bd*) (McMahon et al. 2013), was the primary reason for not feeding crayfish. Other identified reasons included the difficulty and expense of obtaining crayfish (Table 2).

Wild-caught prey species.—D-net surveys identified insects from eight orders (Ephemeroptera, Plecoptera, Trichoptera, Coleoptera, Megaloptera, Diptera, Odonata, and Hemiptera) and crustaceans from three orders (Amphipoda, Decapoda, and Isopoda). The number of invertebrate orders identified at each site ranged from two to eleven. Six out of seven sites had Ephemeroptera, Decopoda, Megaloptera, Odonata, and all seven sites had insects from the orders Plecoptera, and Trichoptera. Less frequently observed were species of Coleoptera, Diptera, Gastropoda, and Opisthopora (Table 3). It is important to note that, despite similarities at the level of taxonomic orders, the species composition of macroinvertebrate samples differed between commercial and wild sources.

Nutritional analysis (dry matter basis).—Commercially sourced macroinvertebrates contained more ($P < 0.005$) energy and protein (crude and available), but less chitin and ash ($P < 0.005$) per unit of mass, compared to all other prey groups (Fig. 1, Table 4). In contrast, fat content was similar among all prey groups (Fig. 1). Although the source of prey (i.e., commercial or wild caught) did not influence dry matter or fiber content, these nutrients differed between species groups. Specifically, wild caught crayfish had greater percentages of dry matter compared to wild-caught macroinvertebrates, and commercially sourced crayfish had a higher NDF content compared to commercially sourced macroinvertebrates (Fig. 1). Noteworthy is the high percent of crude fat in commercially sourced krill (14.26%) and black worms (15.08%) as well as the large amount of gross energy in commercially sourced krill (5451.20 cal/g), black worms (5623.74 cal/g) and earthworms (4873.15 cal/g) when compared to the other samples (Fig.1).

DISCUSSION

This study was the first to evaluate species composition and nutritional content of captive diets for *C. alleganiensis* relative to prey available *in-situ*. This study yielded three major findings. First, few (25%) of the AZA institutions surveyed provide crayfish to *C. alleganiensis*, primarily due to the perceived risk of disease transmission. Second, wild caught and commercially sourced crayfish had similar nutritional profiles. Given that crayfish are a primary food source for wild *C. alleganiensis*, these commercial sources may help provide captive individuals with a diet comparable to that in the wild, particularly if disease risks can be minimized. Finally, commercially sourced macroinvertebrates differed from all other prey groups with respect to five out of eight nutritional parameters (specifically, protein, ash, chitin, fiber, and energy), which may have important consequences for maintaining juvenile *C. alleganiensis* in captivity.

Although the number of institutions housing captive *C. alleganiensis* has increased in the last decade (International Species Information System, 2014), little is known about the nutritional requirements of this species. Understanding these requirements is important because inadequate nutrition is a leading cause of disease and death in captive amphibians (Boutilier et al. 1992; Campbell 1995) and numerous nutritional diseases are poorly understood in these species (Wright and Whitaker 2001). Knowledge about a species' natural history, including available prey items, is an important aspect of husbandry and may help improve captive diets. This study

TABLE 4. Mean and Standard Error of the nutritional parameters for each sample on a dry matter basis. Energy is reported in cal/cg to conform to the scale of other nutrients.

Nutritional parameter (dry matter basis)	Commercially-sourced macro-invertebrates	Commercially-sourced crayfish	Wild-caught macro-invertebrates	Wild-caught crayfish
% Dry Matter	17.12 ± 1.49	18.98 ± 3.56	25.09 ± 1.83	30.05 ± 1.93
% Crude Protein	63.92 ± 2.25	42.77 ± 1.72	46.53 ± 2.84	40.41 ± 1.52
Available % Crude Protein	63.07 ± 2.54	38.23 ± 2.14	39.87 ± 1.75	35.47 ± 1.74
Mean % Chitin	2.29 ± 0.65	5.22 ± 0.39	4.42 ± 0.37	5.37 ± 0.18
% Neutral Detergent Fiber	4.57 ± 1.61	12.82 ± 2.29	21.85 ± 1.83	17.57 ± 1.41
% Acid Detergent Fiber	4.66 ± 1.73	11.07 ± 1.19	17.81 ± 2.23	12.81 ± 0.33
% Crude Fat	9.80 ± 2.82	2.70 ± 0.51	6.39 ± 1.14	5.78 ± 0.99
% Ash	10.97 ± 2.30	35.57 ± 2.96	36.80 ± 3.92	35.57 ± 0.60
Gross Energy (cal/cg)	51.69 ± 2.17	31.69 ± 1.74	36.19 ± 3.52	32.69 ± 0.60

revealed that a majority of institutions surveyed do not include crayfish in their captive diets, due to perceived concerns about disease transmission. Crayfish are known to carry the widespread fungal pathogen, *Bd* (McMahon et al. 2013) and their potential to transmit other amphibian diseases (e.g., ranavirus) is unknown. Because there is evidence of *Bd* susceptibility in *C. alleganiensis* (Junge 2012), precautions should be taken to prevent introducing this pathogen into captive populations. Furthermore, wild caught prey items may carry amphibian pathogens that have not yet been described, a risk that is highlighted by the recent discovery of a second *Batrachochytrium* species (Martel et al. 2013). Nonetheless, crayfish are an important component of this species' diet *in-situ*, representing 70–100% (by mass) of prey consumed by adult hellbenders (Peterson et al. 1989). Future research should focus on developing effective treatments to eliminate disease risk from crayfish that are sourced outside of zoological institutions.

Commercially sourced crayfish were nutritionally similar to wild caught counterparts, providing a convenient alternative for institutions that do not have the time or resources to collect this prey item from the wild. However, it is important to realize that commercial sourcing probably does not reduce the risk of disease transmission from crayfish. Most (if not all) crayfish suppliers obtain their animals directly from the wild, without testing for disease. Furthermore, commercial companies are often unable to provide a steady supply of crayfish, resulting in the need to provide captive *C. alleganiensis* with alternate food items. Although significant resources are required to establish and maintain captive crayfish colonies, this may be the only effective option for ensuring a constant supply of disease-free food items.

Carnivorous amphibians rely primarily on fat and protein as energy sources (Donoghue 1998). Commercially sourced macroinvertebrates contain higher percentages of energy and protein compared to all other sample groups. The protein content of these macroinvertebrates exceeded the suggested concentrations for captive amphibians (30–60% of calories; Donoghue 1998). While high energy content is sometimes considered beneficial in captive diets, it is important to realize that consumption of excess calories or protein may carry health risks. Although rarely reported in amphibians, obesity is a health concern that may arise from diets containing a surplus of energy (Densmore and Green 2007; Donoghue 1996). Animals that grow at an abnormally fast rate or become obese can have decreased fecundity (Wallach 1971), high instances of arthritis and other joint problems (Irwin 2013) and possibly even a reduction in life expectancy. Although the effect of excess protein in salamander diets is poorly understood, feeding a variety of insects to avoid possible nutritional imbalances has been recommended for all insectivorous species (Bernard et al. 1997; Oonincx and Dierenfeld 2012).

Chitin is a carbohydrate that constitutes the exoskeletons of arthropods (Gooday 1998; Robyt 1998). In crayfish, chitin is found in a matrix with protein and minerals (No et al. 1989), but in most insects the chitin is found in a matrix with cuticular proteins, lipids, and other compounds (Kramer et al. 1995). For this reason, chitin can be assessed using portions of the crude fiber (CF), ADF, and NDF of a sample. Most species with a chitin-rich diet have the ability to secrete chitinase (Micha et al. 1973). It is likely that *C. alleganiensis* produce chitinolytic enzymes, as crayfish, the primary diet of adults, have a significant amount of chitin. The commercially sourced macroinvertebrates analyzed had significantly lower dietary chitin, compared to crayfish, which may have health consequences for captive *C. alleganiensis*. However, this deficiency may be the result of the particular macroinvertebrate analyzed (e.g., black worms, earthworms, shrimp). Species with exoskeletons (e.g., beetles, mealworms, crickets) may provide comparable percentages of chitin to an *in-situ* diet. Furthermore CF, ADF, and NDF analyses also include N (scleritized/ bound; Finke 2002) and performing a protein analysis on the ADF or NDF fraction allows determination of how much N may be bound in the chitin. This bound N is more important in determining the hardness of the cuticle than chitin alone, and may impact digestibility among items that have otherwise similar chitin values (Finke 2002).

Although individual vitamin and mineral levels were not directly examined in this study, ash provides a measure of the total amount of minerals within a food. Dierenfeld et al. (2009) found wild-caught crayfish contain more ash compared to fishery-reared counterparts. However, no difference in ash between wild-caught crayfish and commercially sourced crayfish was found in this study. This highlights the potential importance of sourcing for diets containing crayfish.

CONCLUSION

This research demonstrates the importance of investigating the nutritional parameters of *in-situ* and *ex-situ* diets as a critical part of captive husbandry. A varied diet that includes macroinvertebrates and size-appropriate crayfish is recommended for *C. alleganiensis* juveniles in captivity. Analyses in this study revealed key differences in nutrient composition

among available prey items (macroinvertebrates versus crayfish) and sources (wild-caught or commercially-sourced). Because the nutritional requirements of *C. alleganiensis* are unknown, captive diets should initially attempt to represent the spectrum and proportions of nutrients available in nature. Our findings suggest that crayfish should be included in captive diets to not only familiarize animals slated for reintroduction with a wild type diet item, but also to provide the chitin, fiber, and ash that may be lacking in commercially sourced macroinvertebrates. Yet regardless of their source (commercial or wild caught), crayfish represent a significant disease risk. Understanding and mitigating this risk is a high priority for future research. Furthermore, the potential for other food items (particularly macroinvertebrates) to introduce disease into captive *C. alleganiensis* populations should be investigated.

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