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Influence of developmental stage, habitat, and captivity on thecutaneous bacterial communities of Eastern Hellbenders (Cryptobranchus Alleganiensis Alleganiensis) in West Virginia

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INFLUENCE OF DEVELOPMENTAL STAGE, HABITAT, AND CAPTIVITY ON THE CUTANEOUS BACTERIAL COMMUNITIES OF EASTERN HELLBENDERS (*CRYPTOBRANCHUS ALLEGANIENSIS ALLEGANIENSIS*) IN WEST VIRGINIA.

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In
Biological Sciences
by
Rachel Fern Arrick
Approved by
Dr. Jennifer Mosher, Committee Chairperson
Dr. Jayme Waldron
Dr. Shane Welch
Dr. Catherine Johnson

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APPROVAL OF THESIS

We, the faculty supervising the work of Rachel Arrick, affirm that the thesis, *Influence of developmental stage, habitat, and captivity on the cutaneous bacterial communities of Eastern hellbenders (Cryptobranchus alleganiensis alleganiensis) in West Virginia*, meets the high academic standards for original scholarship and creative work established by the Program of Biological Sciences and the College of Science. This work also conforms to the editorial standards of our discipline and the Graduate College of Marshall University. With our signatures, we approve the manuscript for publication.

Dr. Jennifer Mosher, Department of Biological Sciences   Committee Chairperson   Date

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ABSTRACT

Microbes inhabit virtually all surfaces of multicellular animal hosts, with microbial cells outnumbering the hosts' own cells 10:1. Symbiont microbes, collectively referred to as the microbiome, can have profound impacts on the metabolism, development, behavior, and disease resistance of their multicellular hosts. Because the community structure of symbiont bacteria can influence host health, the characterization of amphibian microbiomes is becoming an increasingly important tool for future conservation in the face of global amphibian declines. Eastern hellbenders are good candidates for a microbiome study because they have seen substantial declines in recent decades and learning more about the environmental and physiological drivers of the hellbender microbiome could inform management decisions. Previous studies have explored the cutaneous microbial communities of hellbenders. However, none have compared the microbiomes of various age classes to look for an ontogenetic shift. Additionally, previous studies did not include the comparison of captive hellbenders to those of wild populations. We obtained samples from hellbenders within 5 rivers across the Monongahela National Forest, West Virginia from April to September 2017 and from a captive, juvenile population. Results suggest an ontogenetic shift in cutaneous bacterial community structure could take place as hellbenders age from larvae to adults. There were also differences between captive and wild individuals although studies with greater replication of captive populations would need to be done to further support this. No significant variation among microbiomes was observed between wild sampling locations throughout sampling sites in West Virginia. Additionally, water flow rates and water temperature were found to significantly influence bacterial community divergence.

CHAPTER 1

INTRODUCTION

Microbes inhabit virtually all surfaces of multicellular animal hosts, with microbial cells sometimes outnumbering the hosts' own cells 10:1 (Costello et al. 2009). Due to their ubiquity, symbiont microbes, collectively referred to as the microbiome, can affect their host through the formation of complex associations, with interactions ranging from beneficial to detrimental. Microbiomes can provide essential functions in the life history and health of the host, influencing digestion and metabolism (Turnbaugh et al. 2009; Zhu et al. 2011), disease dynamics (Meyer, Paul, and Teplitski 2014; Woodhams et al. 2014), and development (Sampson and Mazmanian 2015; Wong et al. 2016; McFall-Ngai et al. 2013; Warne, Kirschman, and Zeglin 2017). The functions provided to hosts can depend greatly on the species composition of the microbiome (*e.g.*, species diversity and presence of key symbionts) (Chang et al. 2008; Warne, Kirschman, and Zeglin 2017; Woodhams et al. 2007). Therefore, understanding the drivers of microbiome structure, whether physiological or environmental, and how they influence the host is important because it will elucidate some of the many complexities of host-microbe interactions.

The skin serves as a barrier between the host and its environment and is home to a diverse community of microorganisms that can fluctuate and shift over the lifetime of the host due to combinations of environmental and physiological influence. In comparison to other vertebrate taxa, amphibian skin is especially important to many physiological processes, including respiration, osmoregulation, thermoregulation, chemical communication, and protection from pathogens (Campbell et al. 2012; Harris et al. 2009; Kueneman et al. 2014, 2015; Rollins-Smith et al. 2005). Amphibian skin harbors a diverse array of symbiotic microbes, and the microbiome has profound impacts on the health of its multicellular host (Woodhams et

al. 2014). One of the most important impacts symbionts have on amphibian health is through disease resistance. Although there are many factors that influence amphibian disease dynamics, such as host genetics, immunology, or skin peptides, resistance to pathogens can be tied to the cutaneous microbiome of amphibians as well as bacterial isolates found on amphibian skin (Harris, Lauer, et al. 2009).

Both environmental and physiological conditions can shape the amphibian microbiome, and we are only beginning to discover and interpret the complex associations between hosts, their environment, and microbes. The microbiome, and its effect on its host, can be structured by selective pressures experienced by both the host and the microbes (Ley, Peterson, and Gordon 2006). Pressures can include abiotic, environmental conditions (Becker et al. 2017; Becker et al. 2015; Kueneman et al. 2014; Longo et al. 2015; Longo and Zamudio 2017), interactions with other multicellular organisms (*e.g*., horizontal transfer or parent to offspring microbe transfer through live birth, parental care, egg deposition, etc.) (Estes et al. 2013; Mueller et al. 2015; Peralta-Sánchez et al. 2012; Zhou et al. 2017), interactions with other microbes (Meyer et al. 2015), the hosts' immune system and physiological processes (Herkrath 2015), the hosts' age or developmental stage (Kohl et al. 2013), innate host species-specific traits (McKenzie et al. 2012) or behaviors (Cook et al. 2005; Peralta-Sánchez et al. 2012; Walke et al. 2014), or a combination of the factors. Studying the intraspecific variation of the cutaneous microbiome can provide insight to the physiological mechanisms and environmental conditions that influence overall microbial community structure and diversity (Zilber-Rosenberg and Rosenberg 2008). In turn, understanding the intraspecific variation of the cutaneous microbiome could be widely applicable to amphibian conservation and management efforts in the face of global declines due to disease, habitat loss, and climate change.

In this study, I investigated intraspecific variation in the cutaneous bacterial communities of eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*; Daudin 1850), an imperiled amphibian native to the eastern United States. Eastern hellbenders are giant (up to 74 cm in length), fully aquatic salamanders, dependent on specific stream conditions (i.e., large, rocky substrates, heavily-vegetated riparian areas, and cool, swiftly-flowing waters) and prey items (i.e., crayfish) (Humphries and Pauley 2005). They have experienced declines and lack of recruitment throughout much of their range due to habitat loss and degradation (Burgmeier et al. 2011; Foster, McMillan, and Roblee 2009; Wheeler et al. 2003; Jachowski and Hopkins 2018; Freake and DePerno 2017). Therefore, they require extensive conservation and management actions, such as translocations, captive-breeding programs, reintroductions, and additional protections, to sustain current populations (Bodinof et al. 2012; Bodinof 2010). Hellbenders are a suitable model organism for this study because learning more about the factors important in shaping the hellbender microbiome could aid in devising more effective conservation and management strategies for hellbenders and potentially other amphibians in the future (Hernández-Gómez et al. 2017; Jiménez and Sommer 2017; Redford et al. 2012). Additionally, studying the hellbender microbiome could elucidate some of the factors that influence the interactions between hosts and microbes which will contribute to our growing understanding of the complexities of host-associated microbiomes.

Hellbender bacterial communities can vary geographically, along the body, and between subspecies (Hernández-Gómez et al. 2017; Hernández-Gómez, Hoverman, and Williams 2017; Nickerson et al. 2011). Although past research has highlighted some variables important for determining the microbial community structure of hellbenders, the influence of many drivers of the hellbender microbiome remain unexplored. For example, ontogenetic shifts in microbial

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communities, variation in the microbiome between body parts, and differences among captively-reared and wild hellbenders could be of significance.

The exploration of the effects of development and age class in shaping the hellbender microbiome could be important as the cutaneous bacterial communities of amphibians can vary ontogenetically. For example, the microbial community composition of frogs can shift as they develop, which could be attributed to a combination of changes in life history strategies and physiology (Kohl et al. 2013; Longo et al. 2015). Hellbenders undergo drastic changes in life history strategies due to their rapid shift in size, displaying an ontogenetic shift in diet and habitat selection. Adult hellbenders consume primarily crayfish (Peterson, Wiggs-Reed, and Wilkinson 1989), whereas larval hellbenders eat smaller freshwater invertebrates, such as mayfly and caddisfly nymphs (Hecht, Nickerson, and Colclough 2017). Although little information exists on larval hellbenders, some studies have observed a significant positive linear relationship between hellbender body size and rock shelter size (Nickerson et al 2003; Freake and DePerno 2017; Hecht et al. 2017). In our study, larvae were found primarily in the interstitial spaces of cobble-beds, and adult hellbenders were typically found sheltering under larger rocks. Due to these shifts in physiology, diet, and habitat use, it is possible hellbenders could exhibit shifts in microbial community composition as well.

Sampling both captively-raised and wild amphibians could allow for the differentiation between cutaneous bacterial communities among captive and wild hellbenders, which could be an important consideration for future reintroduction programs. There is some concern that captivity could alter the community structure of the microbiome due to changes in diet, environmental conditions, or social conditions. Alteration of microbial diversity could result in a subsequent loss of digestive or immunological functions provided by microbes, which could

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impact host survival after reintroduction to the wild (Kohl, Skopec, and Dearing 2014; Redford et al. 2012; Antwis et al. 2014). Current hellbender repatriation efforts use wild-collected eggs that are raised until development, re-released, and tracked via telemetry (Bodinof 2010; Boerner 2014). This approach highlights the importance of comparing captively-reared hellbender microbiomes to those of hellbenders in the wild. There is evidence that cutaneous bacterial communities differ between captive and wild amphibians (Becker et al. 2014), and the elimination of natural sources of symbiont microbes could impact hellbender adaptability and immunity (Hernández-Gómez, Hoverman, and Williams 2017).

Additionally, it is important to explore regional variation in bacterial communities on the body of hellbenders to provide an accurate representation of symbiont microbes associated with hellbenders. Skin is structurally heterogeneous; therefore, animal skin can provide a unique habitat for microbes to colonize. Physiological characteristics unique to each bodily region may select for unique microbes. The presence of unique bacterial communities present on each body region is well-documented in humans (Huttenhower et al. 2012), but only a few studies have investigated regional microbial variation in amphibians. Species with differences in texture and toxin secretion have been observed having higher species richness and phylogenetic diversity in areas of the body that do not secrete toxins (Bataille et al. 2016; Sabino-Pinto et al. 2016).

My study goals were to (1) characterize the core microbiome of eastern hellbenders within the Monongahela National Forest in West Virginia, (2) look for an ontogenetic shift by comparing the cutaneous communities of larvae, juveniles, and adults, (3) examine skin microbiome heterogeneity among individuals of different locations (rivers), (4) compare the differences in the microbes associated with body regions (*e.g.,* dorsal and ventral surfaces, cloaca, and feet), (5) compare bacterial communities between wild and captively-reared

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hellbenders, and (6) explore the significance of both physiological and environmental drivers in the overall structure of the hellbender microbiome.

First, I hypothesized that captive and wild individuals would exhibit cutaneous bacterial communities that differed in structure. I expected environmental differences between captive and wild habitats would be drivers for hellbender-associated microbial community structure. Second, I hypothesized that the use of different niches and the physiological differences between wild hellbenders of different age classes would result in distinct microbial communities associated with larvae and adults. Lastly, I expected to detect variation in bacterial taxa represented among body parts sampled.

METHODS

Field Methods

I sampled eastern hellbenders in the Monongahela National Forest, West Virginia from April 2017 to October 2017. Individuals were located through a combination of snorkeling, rock-turning, and nocturnal visual encounter surveys. All captured hellbenders were handled following an approved protocol from Marshall University Animal Care and Use Committee (IACUC 957095-2), the United States Forest Service (USFS), and WV DNR.

Once located and captured, I kept adult and subadult hellbenders in a mesh net submersed in the water. I kept larvae in a sterile plastic container, which I refilled with new river water every few minutes. Using clean gloves, I lifted hellbenders from their container and swabbed them for 10-20 seconds using a sterile cotton-tipped swab. I swabbed adult hellbenders on the dorsal and ventral surfaces, cloaca, and feet. I swabbed juveniles on their dorsal and ventral surfaces. I swabbed larvae only on their dorsal surface because they were too small to accurately swab each body part. Immediately after collection, I placed swabs in individual sterile centrifuge

tubes, put them on dry ice, and later stored them in a -80∘C freezer until DNA extraction could

occur. In addition to swabbing, I weighed, measured, and PIT-tagged (if greater than 20 cm) all

captured hellbenders. Then, I recorded water quality parameters at each site. I used a

multiparameter probe (Hanna Instruments HI98196) to collect pH, conductivity, flow, turbidity,

water temperature, dissolved oxygen, total dissolved solids, and salinity to include in redundancy

analysis. The details of sampled individuals and locations are as follows and are noted in Figure

1 and Table 1.
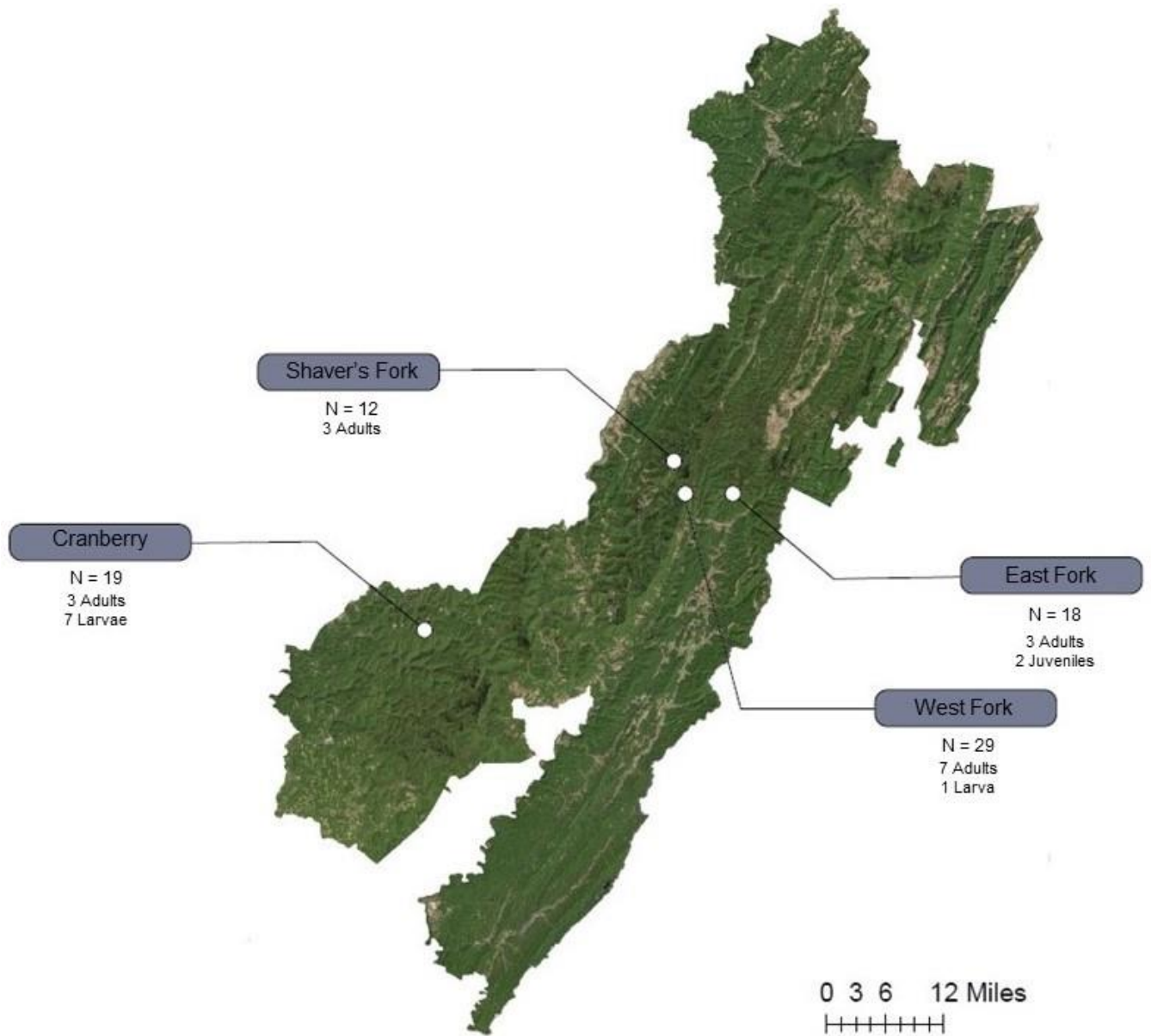
Figure 1. Sample and study site overview. Sampling scheme of sites within the Monongahela National Forest. Table 1 lists the body regions swabbed from each individual included in the study (N = 27). One adult hellbender was captured at the Back Fork, and it is not included in Figure 1 because the site was a short distance outside of the MNF proclamation boundary (on a public access site).

Table 1. Description of samples. Description of samples taken from 27 wild hellbenders, varying in age. Body region swabbed depended on the size of the captured individual. Larvae were swabbed only on their dorsal surfaces. Juveniles were swabbed on dorsal and ventral surfaces. Adults were swabbed in four locations on the body.

Location	Hellbenders Swabbed	Cloacal Samples	Dorsal Samples	Ventral Samples	Foot Samples
Cranberry	10	3	10	3	3
Shaver's Fork	3	3	3	3	3
East Fork	5	4	5	5	4
West Fork	8	7	8	7	7
Back Fork	1	1	1	1	1

(1) I captured wild Eastern hellbenders from five rivers within the Monongahela National Forest in West Virginia. In total, I swabbed 27 hellbenders including: eight larvae, two juveniles, and 17 adults. Larvae were entirely black in color and below 10 cm in size. They were collected predominantly at the Cranberry River, but one was captured at the West Fork. Two juveniles were swabbed, with one found in the East Fork and one located in the West Fork. 17 adults were captured throughout all five rivers sampled for this study (Figure 1, Table 1).

(2) I sampled captive Eastern hellbenders, approximately 4 years old, at the SWiM Lab at West Liberty University, which were hatched and raised there. The eggs were collected from northeastern West Virginia. I obtained dorsal and cloacal swabs from 13 individuals. I viewed the captive samples with *a priori* prejudice because the captive hellbenders were kept in the same tank leading to pseudo-replication of the population.

Laboratory Methods

I extracted DNA from 95 hellbender swabs using a PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA). The standard MoBio protocol was used. I modified the final solution concentration of C6 to account for low amounts of DNA. I sent samples to BioAnalytical Services Lab, University of Maryland Center for Environmental Science Institute,

Baltimore, Maryland, where 16S rRNA was amplified for bacteria using a PCR primer which targeted the V3-V4 region of 16s rRNA.

Sequence Analysis

Amplicons were sequenced on an Illumina MiSeq lane yielding 10,657,308 paired-end reads. I used QIIME2 (Quantitative Insights into Microbial Ecology 2) to combine the paired end reads for sequences and to reduce sequencing and PCR errors by evaluating quality scores. Afterwards, I removed any sequences with ambiguous bases or with a length of greater than 300 base-pairs. I processed the improved sequences by using a pipeline called DADA2. Filtering resulted in 5.3 million sequences, which were then processed in QIIME2. All following analyses were completed with QIIME2 unless otherwise stated. I clustered the 5.3 million sequences into operational taxonomic units (OTUs) based on a 97% similarity. The GreenGenes database was used to assign taxonomy to the resulting 55,115 OTUs, with the most abundant read per OTU acting as the reference sequence (Rideout et al. 2014). If sequences did not match the reference database, OTUs were clustered *de novo*. I rarefied samples to 3,500 reads per sample. A rarefaction depth of 3,500 allowed for the inclusion of all but one sample while capturing a large amount of bacterial diversity. Lastly, I created phylogenetic tree to analyze phylogeny-based alpha and beta diversity measures. The abundance of bacterial phyla per category were calculated by dividing the number of sequences of each OTU by the total number of reads per category (*e.g.,* wild, captive, larvae, adult) and multiplying by 100 to obtain a percentage. Phyla representing over 1% of the category were added to a bar plot.

Statistical Analysis

The overall data were divided into the following sets to allow comparisons to meet the project objectives: (1) *a priori* examination of all samples together, which allowed for general

comparisons of abundant phyla, patterns among alpha diversity metrics, and clustering within ordination plots of beta diversity distance matrices between groups, (2) comparison of dorsal and cloacal samples from captive individuals to assess the effects of body region in captivity, (3) comparison of body region among wild samples from the same river to examine the effects of body region in the wild, (4) comparison of samples grouped by body part to explore how geographic location effects cutaneous bacteria, and (5) comparison of various age classes from the same location to elucidate the impacts of age class on microbial assemblages.

I calculated alpha diversity measures (OTU richness, Chao1, Shannon Diversity Index, and Faith's Phylogenetic Diversity) for each sample. OTU richness is the number of OTUs observed per sample. Chao1 is a non-parametric measure of richness that incorporates the number of rare classes per sample into the calculation (Chao 1984). Faith's phylogenetic diversity measures biodiversity and is calculated by using phylogenetic differences between OTUs with taxonomic unit branch lengths (Faith 1992). Shannon Diversity Index observes both richness and diversity, accounting for abundance and evenness of species present. Resulting alpha diversity values were assessed for normality using the shapiro.test() function in R. Based on normality results, nonparametric Kruskal-Wallis tests were used to statistically compare alpha diversity values if more than 3 samples per category were available. If overall Kruskal-Wallis results were significant, pairwise Kruskal-Wallis tests were subsequently completed. Then, adjusted p-values were used to assess the significance of pairwise Kruskal-Wallis tests. The adjusted p-value approach helped correct for multiple significance testing (Benjamini and Hochberg 1995).

For each group of interest (*e.g.,* captive vs. wild, body region, and developmental stage), beta diversity was calculated among the samples using Bray Curtis Dissimilarity, Jaccard Index,

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Weighted UniFrac, and Unweighted UniFrac. Bray Curtis Dissimilarity uses OTU abundance to calculate dissimilarity between samples (Bray and Curtis 1957). Jaccard uses the presence and absence of OTUs to calculate community distances. Unweighted UniFrac uses phylogenetic distances between observed OTUs in each community to calculate distance between communities but does not consider taxonomic abundance. Weighted UniFrac uses phylogenetic distances, but also incorporates taxonomic abundance into the calculation of distances (Lozupone and Knight 2005). The resulting distance matrices were plotted using Principal Coordinate Analysis (PCoA) using the package phyloseq (McMurdie et al. 2018) in R and tested for significance using Permutational Multivariate Analysis of Variance (PERMANOVA) with 999 permutations. Pairwise PERMANOVAS were subsequently completed and referenced if groups of interest had shown significant variation. PERMDISP was checked in R. If PERMANOVAS were significant in QIIME2, I used each distance matrix to test for homogeneity of dispersions using the betadisper function in the vegan package in R (Okasen et al. 2016). Examining for homogeneity of variance ensured our significant results were not due to heterogeneity of dispersions within our sampled groups (Anderson and Walsh 2013). In addition, I completed redundancy analysis (RDA) using PC-ORD to find patterns of community variation when geochemical data was available for samples. Specifically, it identified what bacterial taxa characterized samples and what water quality parameters (*e.g.*, pH, conductivity, flow, turbidity, water temperature, dissolved oxygen, total dissolved solids, and salinity) significantly influenced the bacterial community composition (Mosher et al. 2012).

Using the microbiome package (Lahti and Shetty 2018) in R, I identified the core microbiome, defined as OTUs present on at least 60% of the samples per group. Venn diagrams were created in R to display the taxa shared between groups using the package VennDiagram

(Chen 2018). Lastly, I used the Antifungal Isolates Database developed by Woodhams et al. 2015 separately on captive and wild samples to see if any OTUs matched bacterial species that exhibit antifungal properties. I filtered our list of OTUs to include only those that matched the Woodhams et al. 2015 database. To check our OTU matched the antifungal isolates from the database, phylogenetic trees were made using MEGA X to assess genetic similarity.

RESULTS

Sequencing

The sequencing of 95 cutaneous swabs from 40 individual hellbenders resulted in 10,657,308 reads representing 55,115 OTUs. One sample (dorsal swab from an adult hellbender from the Cranberry River) was eliminated from analysis due to the low number of reads (n = 57).

General Cutaneous Bacterial Composition and Structure of *C. alleganiensis alleganiensis*

Cutaneous bacterial communities of all groups of hellbenders from this study were composed of Proteobacteria (44%), Bacteroidetes (21%), Cyanobacteria (9%), Actinobacteria (4%), Firmicutes (3%), Verrucomicrobia (3%), and Acidobacteria (1%). Remaining bacteria represented <1% of reads or had unknown taxonomy. Cutaneous bacteria structure differed between captive and wild hellbenders. For captive hellbenders, the cutaneous bacteria were represented by Bacteroidetes (62%), Proteobacteria (23%), Firmicutes (5%), and Cyanobacteria (3%). Remaining bacteria represented <1% of reads or had unknown taxonomy. For wild hellbenders (of all age classes), bacterial communities were dominated by Proteobacteria (49%), Bacteroidetes (12%), Cyanobacteria (9%), Actinobacteria (4%), Verrucomicrobia (4%), Firmicutes (3%), Acidobacteria (2%), Chloroflexi (1%), and Plancomycetes (1%). Remaining bacteria represented <1% of reads or had unknown taxonomy.

The cutaneous microbiome of adult and larval hellbenders found from the same location

(the Cranberry River) differed in structure. Bacterial communities found on wild larval

hellbenders were composed of Proteobacteria (73%), Bacteroidetes (8%), Cyanobacteria (3%),

and Actinobacteria (1%). Remaining bacteria represented <1% of reads or had unknown

taxonomy. Cutaneous bacteria of wild adults were represented by Proteobacteria (52%),

Bacteroidetes (14%), Cyanobacteria (7%), Firmicutes (6%), Actinobacteria (4%),

Verrucomicrobia (3%), Acidobacteria (2%), and Planctomycetes (1%). Remaining bacteria

represented <1% of reads or had unknown taxonomy. Abundance information for the listed

groups is summarized in Figure 2.
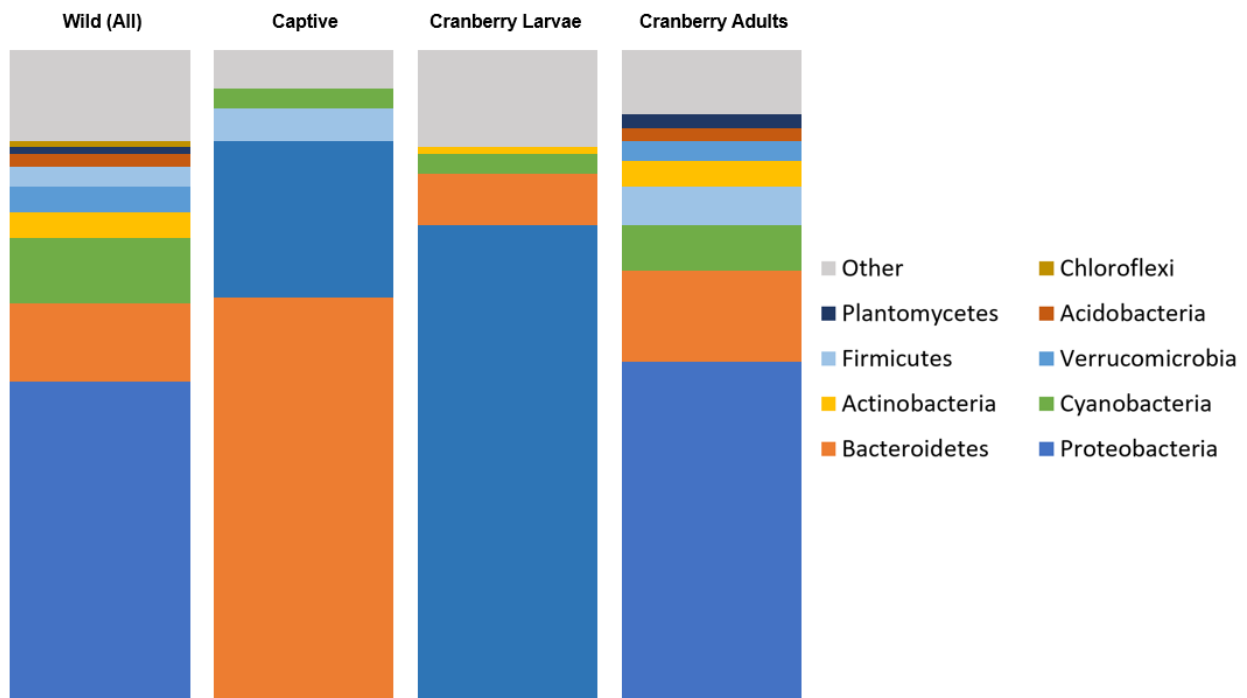
Figure 2. Most abundant bacterial phyla. Summary of most abundant phyla present on wild (n = 27) and captive (n = 13) hellbenders and on larvae (n = 7) and adults (n = 3) from the Cranberry River. Multiple body regions were swabbed on each hellbender, and all body regions were included for this summary.

Alpha Diversity Summary of All Samples

Through *a priori* inspection of general trends, we detected variability among cutaneous

microbiota among the 95 samples from all individuals (n = 40). The number of OTUs ranged

from 16 to 847, Chao1 ranged from 57 to 5941, Faith's Phylogenetic Diversity (FPD) ranged

from 3.9 to 91.3, and Shannon's Index ranged from 1.4 to 9.6 (Table 1). Also, Shapiro-Wilk tests

revealed alpha diversity metrics did not have a normal distribution ($p < 0.05$). Generally, captive

individuals exhibited lower alpha diversity measures than hellbenders captured in the wild and

wild larvae had lower alpha diversity than wild adults (Table 2). Significance between captive

and wild alpha diversity measures was not examined because captive samples were taken from

one tank.

Table 2. Alpha diversity comparison. Comparison of mean alpha diversity indices of cutaneous
bacterial communities between captive and wild individuals. For wild hellbenders, the mean and
standard deviations for adults and larvae from all locations were considered. Values are mean
and standard deviation.

Sample category	Individuals	Chao1 index	OTU richness	Faith's PD	Shannon index
C. alleganiensis wild adults	17	2666 ± 1150	389.5 ± 211.3	52.0 ± 21.3	6.7 ± 1.9
C. alleganiensis wild larvae	8	487.9 ± 296.9	115.1 ± 54.1	23.7 ± 7.8	3.2 ± 1.6
C. alleganiensis captive juveniles	13	358.3 ± 232.7	119.6 ± 66.6	19.1 ± 9.7	4.3 ± 1.5

Overall Beta Diversity Summary

A priori inspection of general trends in principal coordinates ordination of both

phylogenetic (Weighted and Unweighted UniFrac) and non-phylogenetic (Bray Curtis and

Jaccard) beta diversity distance matrices revealed that captive hellbenders showed a distinct

pattern of separation from wild individuals in all measures but Weighted UniFrac. Larval

samples clustered closely together, away from other samples, in non-phylogenetic ordination

plots. In the Weighted UniFrac plot, larval samples clustered closely among other samples.

However, in the Unweighted UniFrac ordination plot, there was no discernible clustering among

larval samples. No distinct clustering patterns exist among wild locations (Figure 3). In addition,

cloacal and dorsal samples clustered separately among captive samples in the overall ordination.

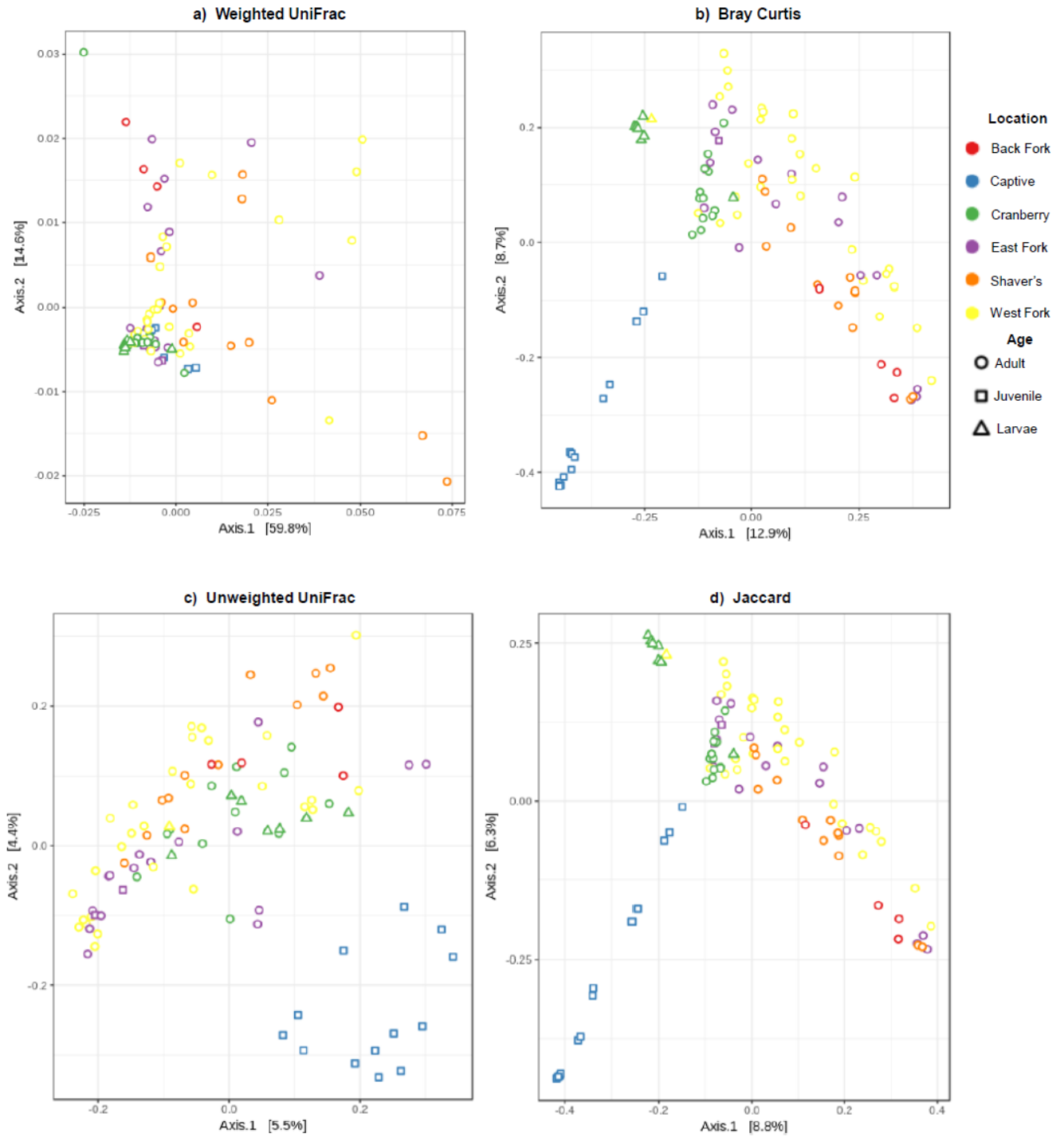Among wild samples, body region did not cause patterns in the ordination.

Figure 3. Overall beta diversity ordination. Principal coordinates ordination of Bray Curtis, Jaccard, Unweighted UniFrac, and Weighted UniFrac distances including all samples (n = 95) and displayed by location and age class. Each symbol represents a sample.
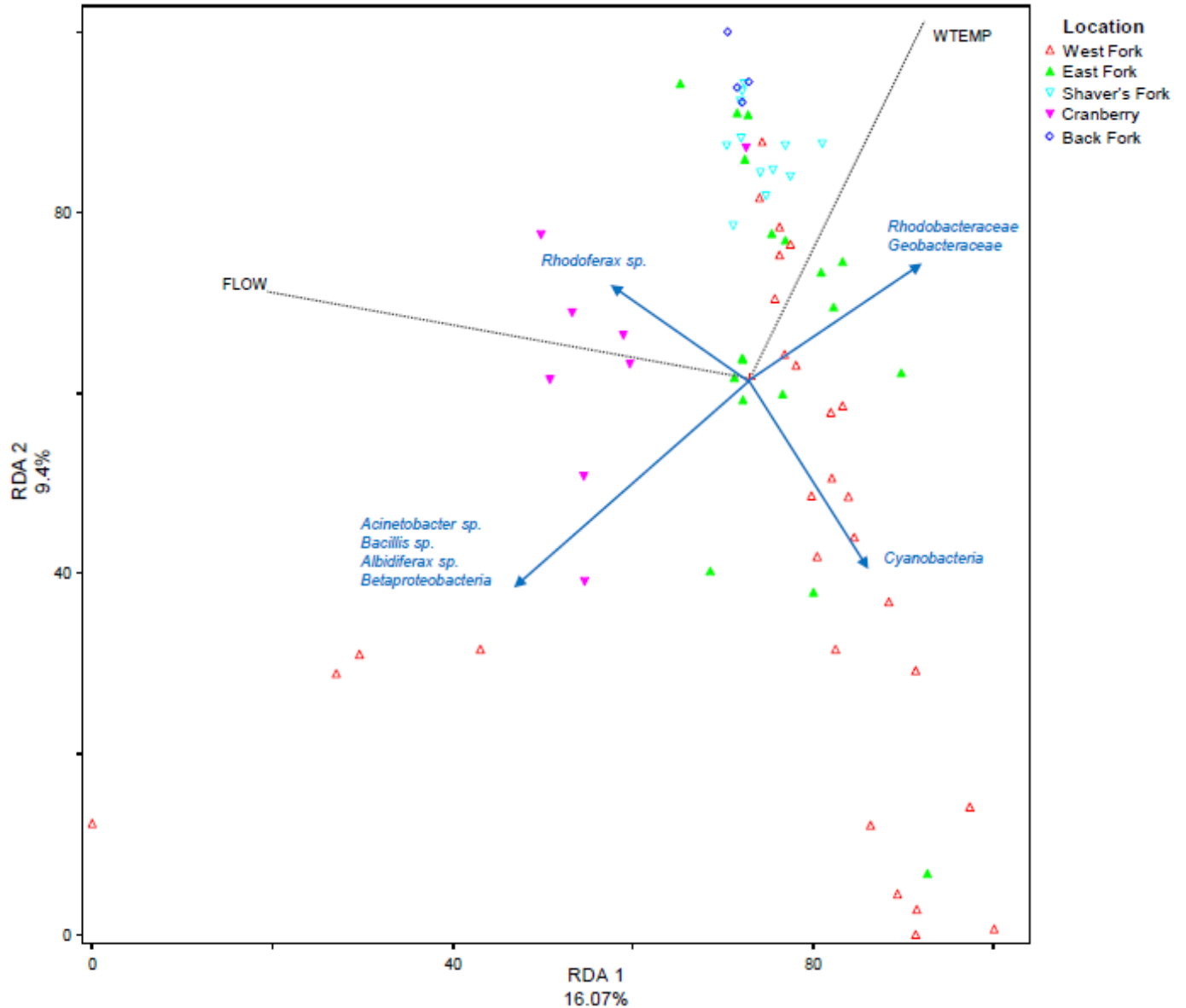
Figure 4. Redundancy analysis of water quality parameters and bacterial taxa. Plot of Redundancy Analysis showing the bacterial communities or water quality parameters that accounted for significant variation in bacterial communities between samples. Dashed lines represent the predictor variables significantly associated ($p < 0.05$) with variation among bacterial community composition. Solid lines represent the bacterial taxa that significantly associated with variation between samples. Samples are displayed by location. Bacterial taxa that were significant contributors are plotted.

Redundancy Analysis of Samples

The RDA analysis was performed with the 70 swabs that had associated water quality data – only adults were included. In the RDA analysis, 16.07% of the variation was described by

RDA Axis 1 and 9.4% of the variation was described by RDA Axis 2. Samples appeared to cluster slightly based on sampling location, with the Back Fork, Shaver's Fork, and Cranberry samples grouping together. West Fork samples were dispersed widely throughout the plot, with East Fork samples showing a similar but less extreme pattern of dispersion.

Water temperature and water flow were detected to be significant contributors to community variation observed in samples. Bacteria important for community variation were: *Rhodoferax* spp., *Acinetobacter* spp., *Bacillus* spp., *Albidiferax* spp., Betaproteobactera, Cyanobacteria, Rhodobacteraceae, and Geobacteraceae. The difference among water flow contributed to Cranberry River samples grouping separately, with *Rhodoferax* spp., *Acinetobacter* spp., *Bacillis* spp., *Albidiferax* spp., and Betaproteobacteria accounting for the taxonomic variation. Water temperature, along with the presence of Rhodobacteraceae and Geobacteraceae seemed to be the main influence for samples from the Shaver's Fork and the Back Fork (Figure 4).

Comparison of Cutaneous Bacterial Communities among Hellbender Body Region

Captive dorsal and cloacal samples were used to explore the differences in cutaneous microbiota among eastern hellbenders. Captive individuals exhibited significant differences in alpha diversity between sampled body regions; all alpha diversity measures were significantly higher in cloacal samples than dorsal samples (Chao1: $p = 0.002$, $H = 9.0$; OTU richness: $p = 0.004$, $H = 8.16$; Faith's PD: $p = 0.015$, $H = 5.90$; Shannon Index: $p = 0.003$, $H = 9.0$) (Figure 5). Additionally, PERMANOVA tests showed there were significant differences between cloacal and dorsal bacterial communities of captive hellbenders for all tested beta-diversity metrics (PERMANOVA Bray Curtis: Pseudo-$F = 6.14$, $p = 0.003$; Weighted UniFrac: Pseudo-$F = 9.33$, $p = 0.002$; Jaccard: Pseudo-$F = 2.80$, $p = 0.002$; Unweighted UniFrac: Pseudo-$F =$

3.71, p = 0.002). However, homogeneity of dispersion tests detected significant differences in dispersion between dorsal and cloacal samples (BETADISPER Bray Curtis: $F_{1,11} = 5.06$, p = 0.023; Weighted UniFrac: $F_{1,11} = 11.701$, p = 0.005; Unweighted UniFrac: $F_{1,11} = 4.56$, p = 0.052; Jaccard: $F_{1,11} = 3.84$, p = 0.045) except in Unweighted UniFrac. PCoA ordination showed patterns of separation between samples, with slight grouping of cloacal and dorsal samples respectively (Figure 4).
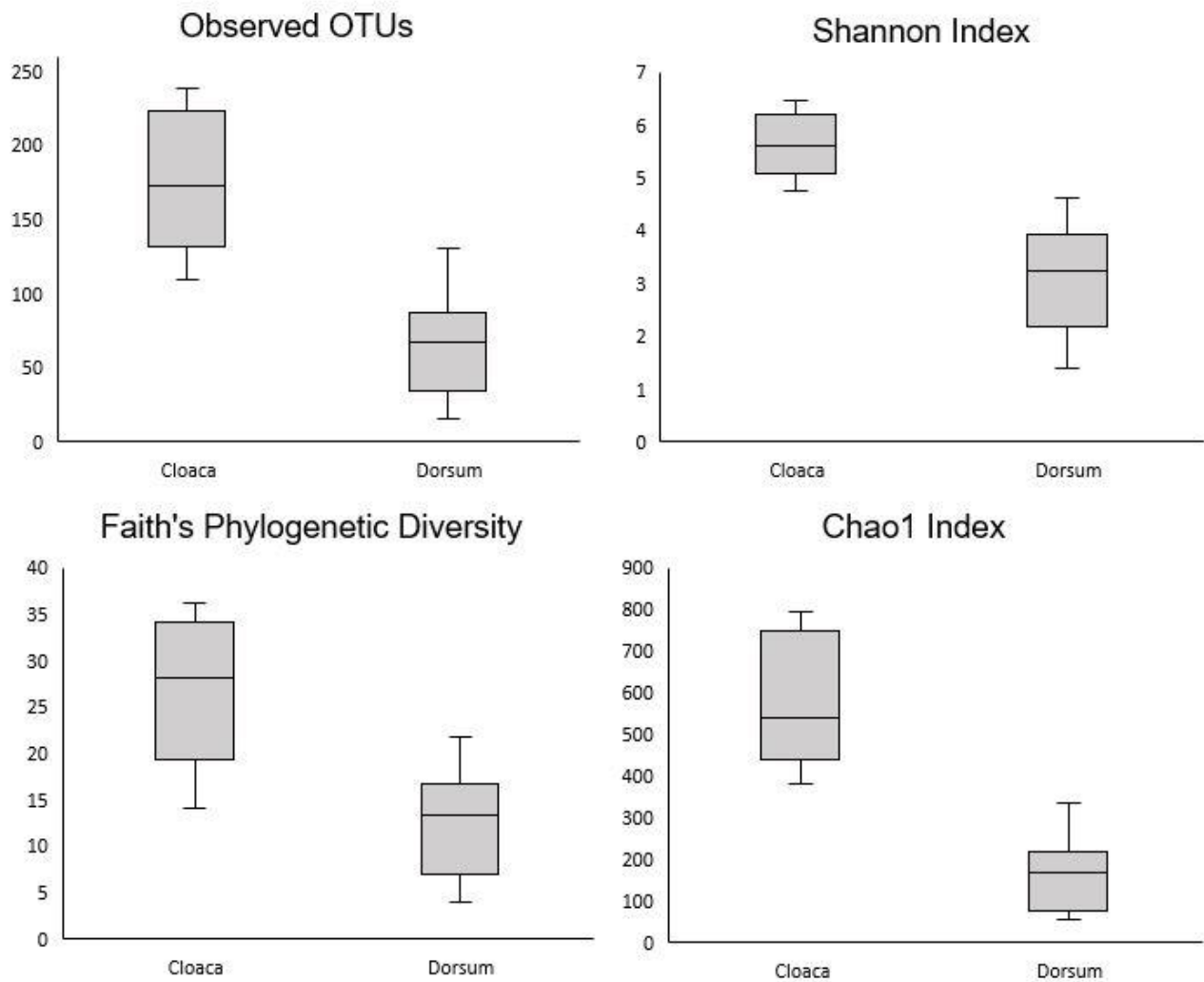

Figure 5. Alpha diversity boxplots comparing body regions among captive samples. Alpha diversity metrics of samples for captive hellbenders (n = 13). Alpha diversity was significantly higher in cloacal swabs (n = 6) when compared to dorsal swabs (n = 7).
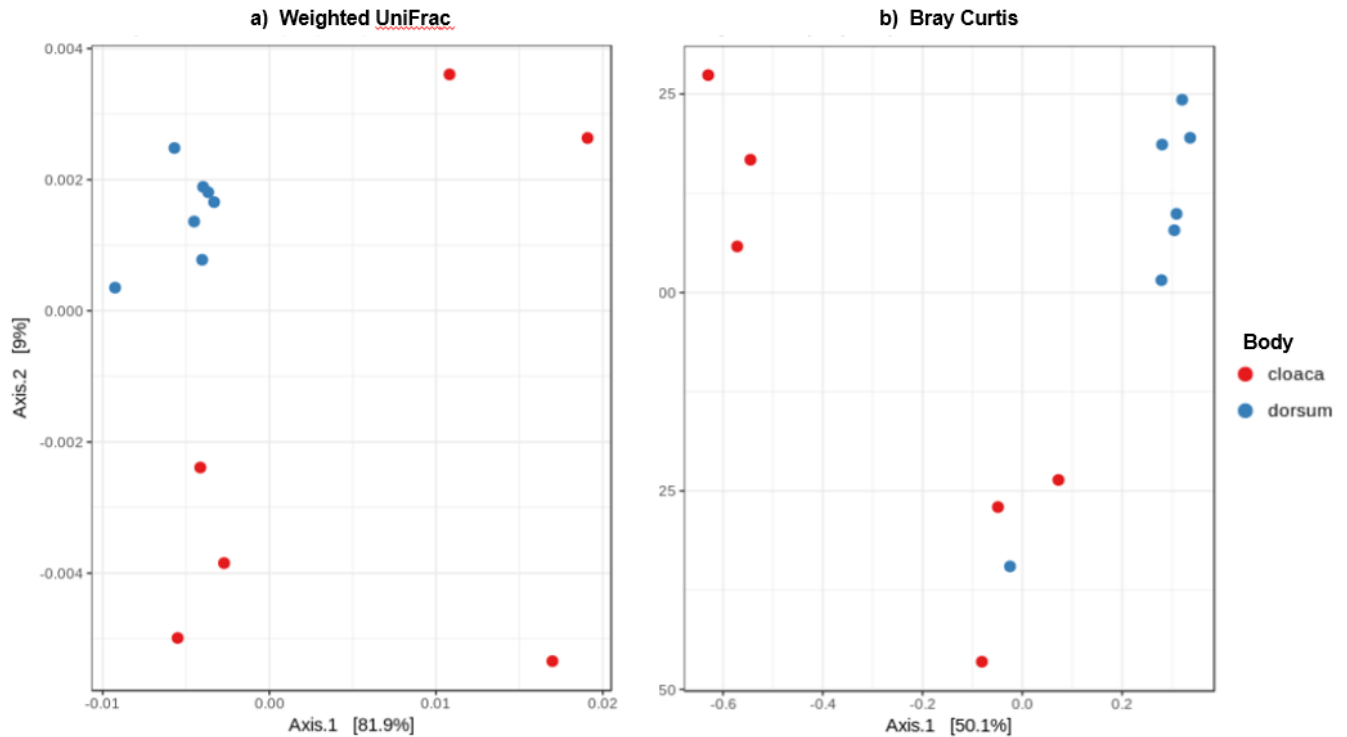
Figure 6. Beta diversity ordination comparing body regions among captive samples. PCoA ordination of Weighted UniFrac and Bray Curtis distance matrices comparing cloacal and dorsal swabs of captive hellbenders (n = 13). Ordination resulted in cloacal and dorsal swabs clustering separately in both phylogenetic (Weighted UniFrac) and non-phylogenetic (Bray Curtis) beta diversity metrics.

Overall Kruskal-Wallis tests failed to detect differences in alpha diversity between all body parts among wild adults from the West Fork (n = 28; Observed OTUs: p = 0.47, H = 2.50; Faith's PD: p = 0.53, H = 2.22; Shannon's Index: p = 0.27, H = 3.89) or East Fork (n = 16; Chao1: p = 0.66, H = 1.61; Observed OTUs: p = 0.51, H = 2.29; Faith's PD: p = 0.69, H = 1.48; Shannon's Index: p = 0.56, H = 3.07). Furthermore, subsequent pairwise Kruskal-Wallis tests failed to detect significant differences in alpha diversity between all the body parts (all p > 0.05).

No significant differences in community structure between body regions were detected in the West Fork (PERMANOVA Bray Curtis: Pseudo-F = 0.97, p = 0.526; Weighted UniFrac: Pseudo-F = 1.06, p = 0.342; Jaccard: Pseudo-F = 0.949, p = 0.956; Unweighted UniFrac: Pseudo-F = 0.996, p = 0.453) or the East Fork (PERMANOVA Bray Curtis: Pseudo-F = 0.879,

p = 0.793; Weighted UniFrac: Pseudo-F = 0.599 p = 0.926; Jaccard: Pseudo-F = 0.941, p = 0.975; Unweighted UniFrac: Pseudo-F = 0.737, p = 0.988). The subsequent pairwise PERMANOVAs for both the East Fork and West Fork did not show significant differences in community structure between body regions (all p > 0.05). Dispersion tests revealed there was not significant dispersion from the centroid in samples from the East Fork (BETADISPER Bray Curtis: $F_{3,14}$ = 0.765, p = 0.536; Weighted UniFrac: $F_{3,14}$ = 0.72, p = 0.561; Unweighted UniFrac: $F_{3,14}$ = 0.194, p = 0.906; Jaccard: $F_{3,14}$ = 0.879, p = 0.905) and the West Fork (BETADISPER Bray Curtis: $F_{3,15}$ = 0.314, p = 0.806; Weighted UniFrac: $F_{3,25}$ = 2.178, p = 0.122; Unweighted UniFrac: $F_{3,25}$ = 0.845, p = 0.459; Jaccard: $F_{3,25}$ = 0.331, p = 0.807).

Comparison of Cutaneous Bacterial Communities among Location in Wild

To assess the effect of geographic location on the cutaneous microbial assemblages of wild hellbenders, I compared swabs of the same body region of adults by their respective location. Kruskal-Wallis tests showed no significant differences in alpha diversity by location in the wild among foot samples collected from adult hellbenders of the five wild populations (n = 18; Chao1: p = 0.50, H = 3.34; Faith's PD: p = 0.57, H = 2.93; Observed OTUs: p = 0.46, H = 3.65; Shannon's Index: p = 0.25, H = 5.39) and cloacal samples (n = 18; Chao1: p = 0.33, H = 4.62; Faith's PD: p = 0.43, H = 3.8; Observed OTUs: p = 0.22, H = 5.72; Shannon's Index: p = 0.17, H = 6.36). In addition, pairwise Kruskal-Wallis tests yielded no significant results (all p > 0.05).

In community composition comparisons of all geographic locations, PERMANOVA tests revealed significant differences between overall sampling among cloacal swabs (Bray Curtis: Pseudo-F = 1.34, p = 0.02; Weighted UniFrac: Pseudo-F = 2.17, p = 0.004; Jaccard: Pseudo-F = 1.09, p = 0.001; Unweighted UniFrac: Pseudo-F = 3.71, p = 0.002) and foot

swabs (Bray Curtis: Pseudo-F = 1.26, p = 0.027; Weighted UniFrac: Pseudo-F = 1.50, p = 0.058; Jaccard: Pseudo-F = 1.09, p = 0.001; Unweighted UniFrac: Pseudo-F = 1.22, p = 0.029). Subsequent pairwise PERMANOVAS failed to detect significant differences between the locations when referring to the adjusted p-value (all p > 0.05). Furthermore, PCoA of beta diversity revealed no strong clustering patterns by location (Figure 4). In addition, dispersion among beta diversity measures was significant between locations within the cloacal swabs besides in Unweighted UniFrac (BETADISPER Bray Curtis: $F_{5,19}$ = 4.97, p = 0.006; Weighted UniFrac: $F_{5,19}$ = 0.949, p = 0.46; Unweighted UniFrac: $F_{5,19}$ = 5.91, p = 0.005; Jaccard: $F_{5,19}$ = 8.89, p = 0.001). For foot swabs, dispersion was significant for every measure besides unweighted UniFrac (BETADISPER Bray Curtis: $F_{4,13}$ = 19.78, p = 0.001; Weighted UniFrac: $F_{4,13}$ = 1.23, p = 0.307; Unweighted UniFrac: $F_{4,13}$ = 153.27, p = 0.001; Jaccard: $F_{4,13}$ = 64.36, p = 0.001).

Comparison of Cutaneous Bacterial Communities among Age Classes from the Same River

To begin our comparison of various age classes from the same sampling location, I first tested the role of body region on alpha and beta diversity measures. Significant differences in alpha diversity were shown in the Kruskal-Wallis comparison of all groups in the body region comparison (Chao1: H = 8.31, p = 0.04; Faith's PD: H = 9.41, p = 0.02; Observed OTUs: H = 10.0, p = 0.02; Shannon: H = 7.87, p = 0.05). Subsequent pairwise Kruskal-Wallis tests had no significant results (p > 0.05 between all body regions). PERMANOVAS comparing the difference between body region detected significant differences in community composition between samples (Bray Curtis: Pseudo-F = 2.07, p = 0.02; Weighted UniFrac: Pseudo-F = 2.31, p = 0.005; Jaccard: Pseudo-F = 1.17, p = 0.006; Unweighted UniFrac: Pseudo-F =

1.30, p = 0.023). Pairwise PERMANOVA comparison detected no significant variation between body regions (p > 0.05 between all body regions).

After no significant differences in alpha and beta diversity measures were revealed between pairs of adult body regions in the Cranberry River, all adult swabs from the site were included in analysis. Kruskal-Wallis tests among hellbenders from the Cranberry River observed that all alpha diversity metrics except for Chao1 were significantly higher in adult samples (n = 11) than in larval samples (n = 7) (Chao1: H = 1.61, p = 0.20; Faith's PD: H = 10.93, p = 0.00095; Observed OTUs: H = 10.93, p = 0.00095; Shannon's Index: H = 9.76, p = 0.0018) (Figure 5). PERMANOVA tests showed there were significant differences between adult and larval samples for all tested beta-diversity metrics (Bray Curtis: Pseudo-F = 5.7, p = 0.001; Weighted UniFrac: Pseudo-F = 7.86, p = 0.001; Jaccard: Pseudo-F = 1.74, p = 0.001; Unweighted UniFrac: Pseudo-F = 2.66, p = 0.001). Further, PCoA ordination showed patterns of separation between adult and larval individuals (Figure 4). Dispersion was significantly different in every measure except for the Weighted UniFrac (BETADISPER Bray Curtis: $F_{1,17}$ = 14.81, p = 0.001; Weighted UniFrac: $F_{1,17}$ = 1.77, p = 0.249; Unweighted UniFrac: $F_{1,17}$ = 6.45, p = 0.011; Jaccard: $F_{1,17}$ = 14.21, p = 0.001).
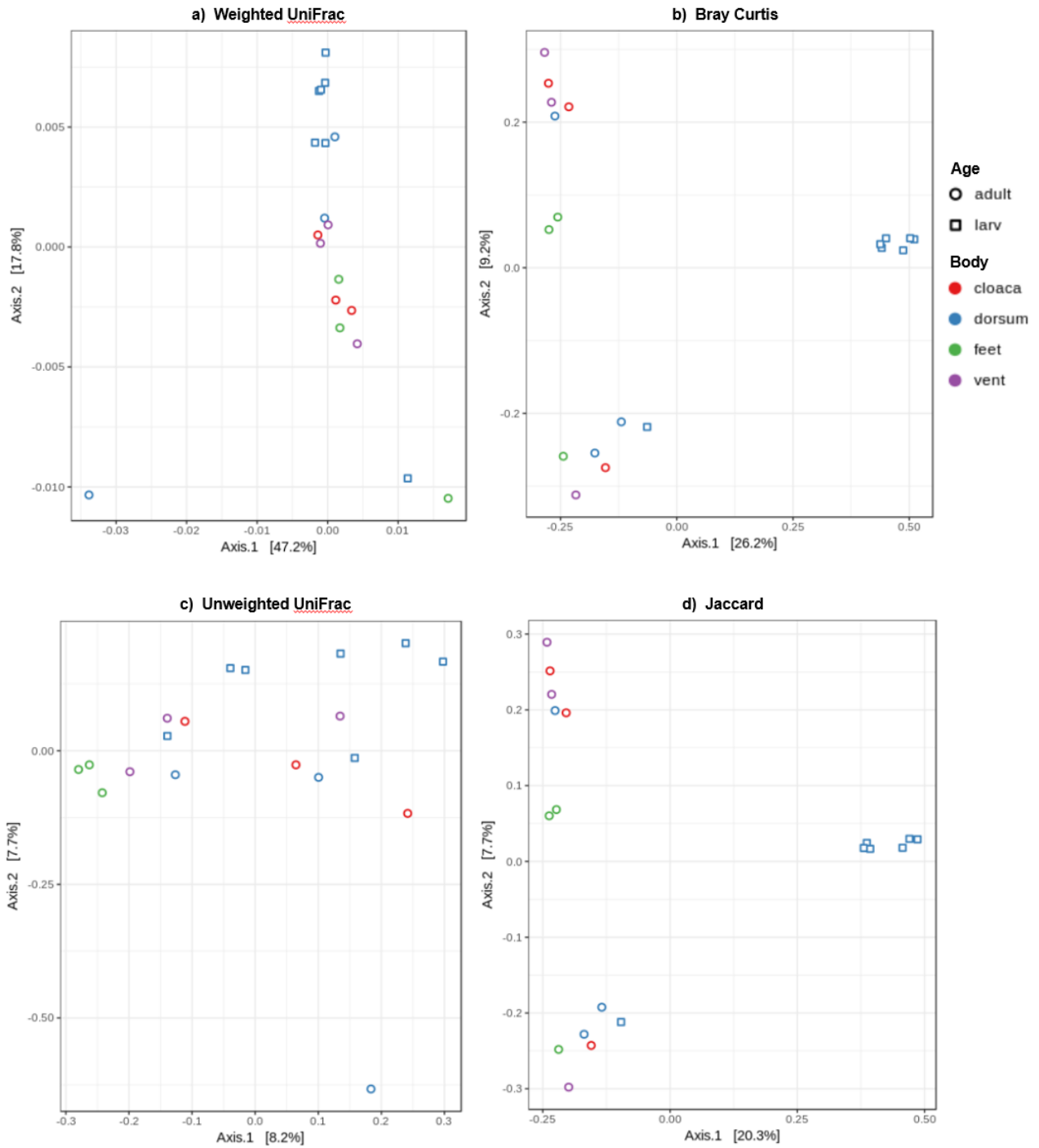
Figure 7. Beta diversity ordination comparing age classes among Cranberry River samples.
PCoA ordination showing patterns of beta diversity between hellbenders in the Cranberry River, WV.
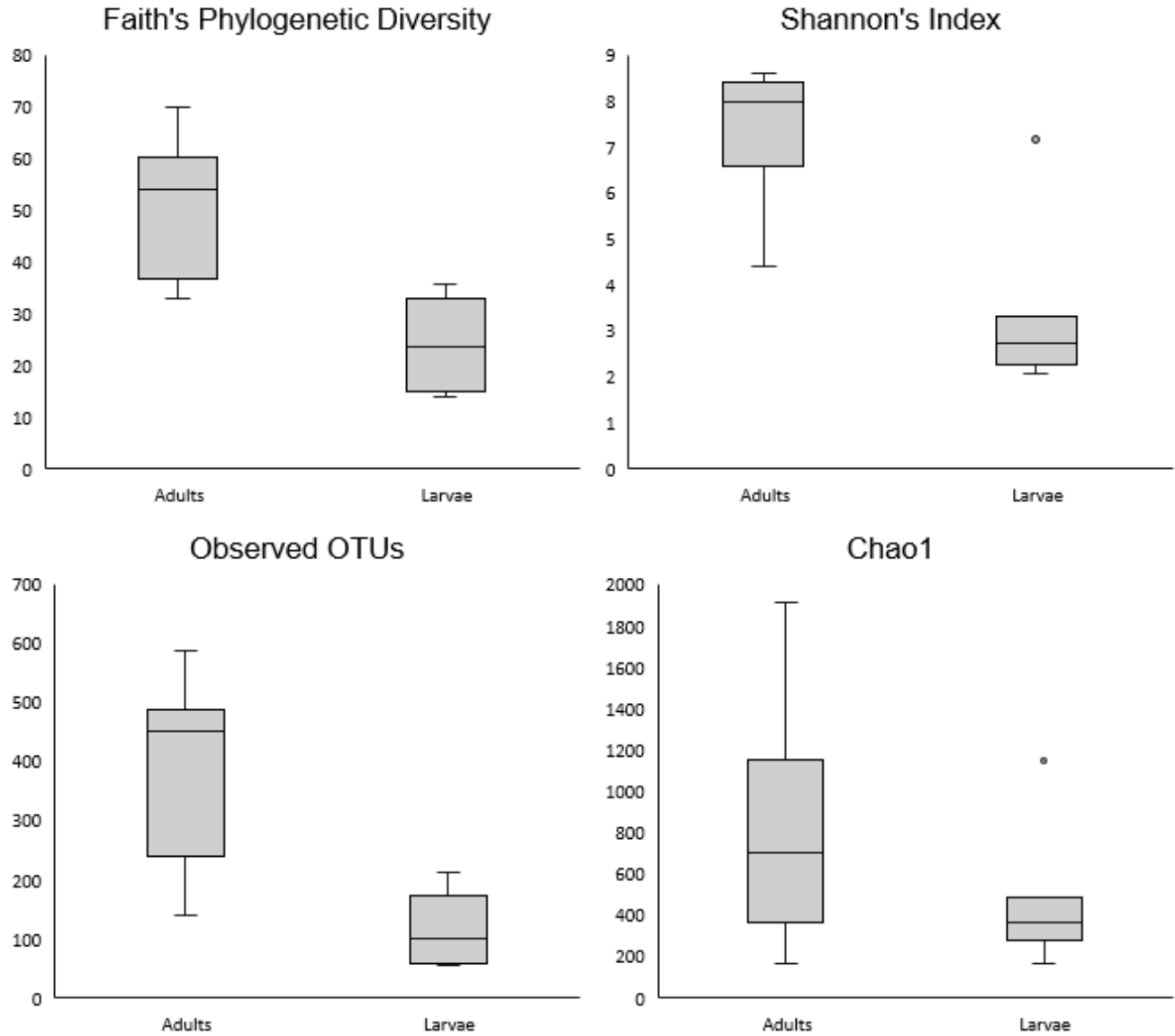
Figure 8. Alpha diversity boxplots comparing age classes among Cranberry River samples. Alpha diversity metrics of adult and larval hellbenders from the Cranberry River, WV. Larval microbial communities were significantly difference in all alpha diversity measures when compared to adult samples except for Chao1.

Core Microbiome of Wild Individuals

The core microbiome of all samples from wild individuals (n = 27) was defined as the set of taxa detected in 60% of the samples. Among all wild samples, 10 core OTUs were detected. One had unknown taxonomy, and the remaining nine were grouped into seven unique taxonomic groups (Table 2). Core OTUs of wild hellbenders belonged to the phyla Proteobacteria, Actinobacteria, and Cyanobacteria, which was consistent with the most abundant phyla observed among wild adults (Figure 2). Additionally, two core OTUs were identified as antifungal isolates. *Novosphingobium stygium* was matched at the genus-level, and *Acinetobacter venetianus* was matched at the species-level (Table 3, Figure 9).

Table 3. Core OTUs of all wild samples. Classification of OTUs present on 60% of all wild samples. OTUs highlighted in yellow exhibited antifungal properties on amphibian skin. Relative abundance is displayed in Figure 6.

Phylum	Class	Order	Family	Genus, Species
Cyanobacteria	Chloroplast	Stramenopiles	Unknown	Unknown
Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	*Propionibacterium*
Proteobacteria	Alphaproteobacteria	Unknown	Unknown	Unknown
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	*Novosphingobium* stygium
Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	*Acineto bacter venetianus*
Proteobacteria	Betaproteobacteria	Unknown	Unknown	Unknown
Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Unknown

Core Microbiome of Adults from the Cranberry River

The core microbiome (with 60% prevalence) was calculated for adult hellbenders from

the Cranberry River, and 19 unique OTUs were detected. Core OTUs of adult hellbenders from

the Cranberry River were dominated by Proteobacteria, Cyanobacteria, Bacteroidetes, and

Actinobacteria, which was consistent with the most abundant phyla overall in samples from

Cranberry adults (Figure 1). The same two OTUs identified as antifungal isolates in the pooled

wild samples were observed in the core microbiome of adults from the Cranberry River (genus

Novosphingobium and *Acinetobacter venetianus*) (Table 4, Figure 9).

Table 4. Core OTUs of Cranberry River adults. Classification of OTUs present on 60% of all
adult samples from the Cranberry River. OTUs highlighted in yellow exhibited antifungal
properties on amphibian skin.

Phylum	Class	Order	Family	Genus, Species
Cyanobacteria	Oscillatoriophycideae	Oscillatoriales	Phormidiaceae	*Phormidium*
Cyanobacteria	Chloroplast	Stramenopiles	Unknown	Unknown
Cyanobacteria	Synechococcophycideae	Synechococcales	Chamaesiphonaceae	Unknown
Bacteroidetes	Unknown	Unknown	Unknown	Unknown
Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	*Propionibacterium*
Proteobacteria	Alphaproteobacteria	Rhizobiales	Unknown	Unknown
Proteobacteria	Alphaproteobacteria	Unknown	Unknown	Unknown
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	*Novosphingobium* stygium
Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	*Acinetobacter venetianus*
Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	*Massilia*
Proteobacteria	Betaproteobacteria	Unknown	Unknown	Unknown
Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	*Polynucleobacter*
Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Unknown

Core Microbiome of Wild Larval Hellbenders from the Cranberry River

The core microbiome (with 60% prevalence) was calculated for larval hellbenders from the Cranberry River. The most abundant phyla within the core microbiome of larvae were Proteobacteria, Cyanobacteria, and Bacteroidetes. These bacterial phyla mirrored the results of the most abundant phyla detected in wild larvae overall (Figure 1). Two genus-level matches were made to the antifungal database; however, they were not the same matches observed in the core microbiome of wild samples overall or in the core microbiome of wild adults from the Cranberry River (Table 5, Figure 9).

Table 5. Core OTUs of Cranberry River larvae. Classification of OTUs present on 60% of all larval samples from the Cranberry River. OTUs highlighted in yellow exhibited antifungal properties on amphibian skin.

Phylum	Class	Order	Family	Genus, Species
Cyanobacteria	Gloeobacterophycideae	Gloeobacterales	Gloeobacteraceae	*Gloeobacter violaceus*
Cyanobacteria	Synechococcophycideae	Unknown	Unknown	Unknown
Cyanobacteria	Chloroplast	Stramenopiles	Unknown	Unknown
Cyanobacteria	Nostocophycideae	Nostocales	Nostocaceae	Unknown
Cyanobacteria	Unknown	Unknown	Unknown	Unknown
Bacteroidetes	Unknown	Unknown	Unknown	Unknown
Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Unknown
Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Sporocytophaga
Actinobacteria	Unknown	Unknown	Unknown	Unknown
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Rhodobacter capsulatus*
Proteobacteria	Alphaproteobacteria	Unknown	Unknown	Unknown
Proteobacteria	Alphaproteobacteria	Rhodospirillales	Unknown	Unknown
Proteobacteria	Betaproteobacteria	Unknown	Unknown	Unknown
Proteobacteria	Betaproteobacteria	Methylophilales	Unknown	Unknown
Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	*Acidovorax facilus*
Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	*Acidovorax*
Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Unknown
Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Unknown

Core Microbiome of Captive Hellbenders

The core microbiome of captive hellbenders included 82 OTUs. *Acinetobacter venetianus* was still present among the core taxa found in captive samples. Overall, captive samples shared 3 common core OTUs with wild samples (Figure 9).

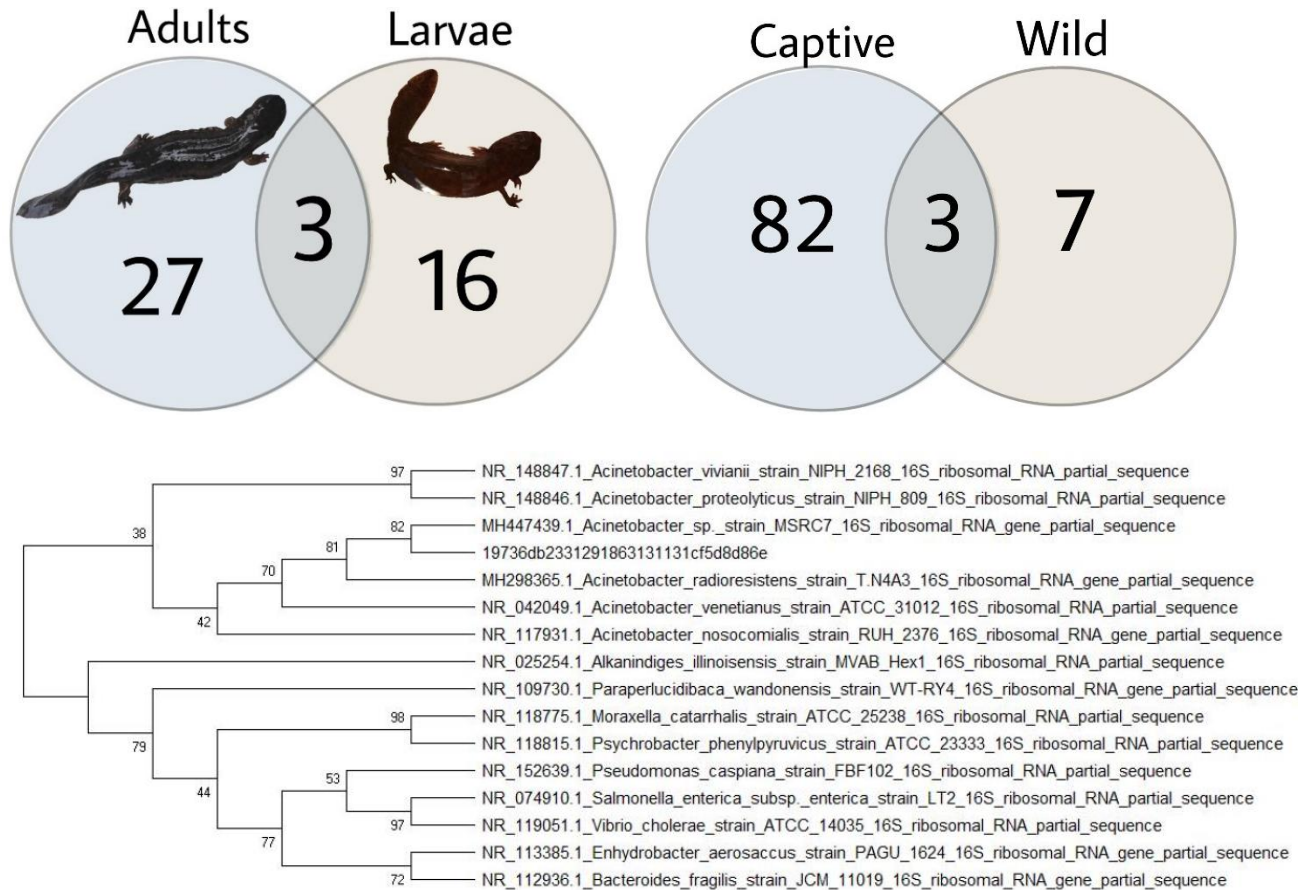**Figure 9. Venn diagram comparing core OTUs.** Venn diagrams showing the shared core OTUs among wild adults and larvae from the same site (Cranberry River) and between all captive and all wild samples. Below is a phylogenetic tree for the antifungal isolate, *Acinetobacter venetianus*, which supports that the core OTU detected among sampled hellbenders matches the species in the antifungal isolates database.

DISCUSSION

My study provided a greater understanding of the physiological and environmental factors that influence the cutaneous bacterial communities of eastern hellbenders, an imperiled, long-lived, fully-aquatic salamander. I examined the intraspecific variation of the skin microbiome of the hellbender, across different regions of the body, age classes, and locations throughout West Virginia, including wild and captive populations. Additionally, I compared cutaneous bacterial communities to available water quality data to see if those parameters are correlated with a shift in bacterial communities. Overall, I discovered that wild individuals throughout the Monongahela National Forest exhibit similar microbiomes despite being isolated geographically by river. However, each population was characterized by unique proportions of OTUs. I detected the bacterial phyla most abundant in our samples to be comparable to previous eastern hellbender microbiome studies. I observed differences among wild and captive samples, although I observed the retention of many bacterial taxa in captive conditions. Lastly, I detected variation between wild larvae and adults and between body regions among our captive samples.

Captive samples were taken from hellbenders from the same isolated enclosure; therefore, I did not use these samples when analyzing the significance of location (captive vs. wild) on alpha or beta diversity of skin bacterial communities. Despite this limitation, I generally observed lower alpha diversity among captive samples when compared to wild adults, but not when compared to wild larvae (Table 2). Although the alteration of microbial diversity in captivity is supported by other studies (Becker et al. 2014; Sabino-Pinto et al. 2016; Loudon et al. 2014), I think this observation should not be used to generalize the effects of captivity on hellbenders or amphibians overall because of the lack of variability in my sampling scheme and my subsequent inability to test for significance. Another caveat is that age of the individual could

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be an influence in the community structure between captive and wild samples as all captive samples were taken from subadults (4-5 years old) while most samples collected from the wild were taken from adults. The degree to which life history stage impacts the amphibian microbiome varies by study, but age class can influence the cutaneous microbiome of amphibians (Kueneman et al. 2014; Prado-Irwin et al. 2017), which remains an important point as I begin my discussion of the effects of age further below.

Notably, captive individuals in our study maintained the presence of the most abundant bacterial phyla observed in wild individuals despite shifting in overall structure, composition, and abundance. The slight change in community structure was also supported by captive samples clustering together – away from wild samples in our initial ordination plots. The differences between captive and wild samples is consistent with the (Bataille et al. 2016) study, which noted a shift in the relative abundance of major phyla between wild and captive fire-bellied toads. The (Bataille et al. 2016) study also has a small sample size, with two captive populations kept in similar conditions, which was not adequate to make general conclusions regarding the impacts of captivity on the amphibian microbiome (Bataille et al. 2016). Despite the change in bacterial community structure in my study, the bacterial phyla present on captive hellbenders was comparable to both wild hellbenders within our study and in the (Hernández-Gómez et al. 2017; Hernández-Gómez, Hoverman, and Williams 2017) study on hellbender bacterial communities, which were composed primarily of Bacteroidetes, Proteobacteria, Firmicutes, and Cyanobacteria. Similarly, a 2014 study on the effect of captivity on the cutaneous bacteria of the Panamanian golden frog saw captivity was correlated with significantly more homogenous skin microbiota, but captive frogs still shared 70% of their microbial community with their wild counterparts after eight years of living in captivity (Becker et al. 2014).

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I swabbed captive individuals either on their cloaca or dorsum and was therefore able to use those samples to assess how body region influences the structure and composition of the microbiome. Cloacal swabs exhibited significantly higher alpha diversity than dorsal swabs by every metric tested. Initially, principal coordinate ordination and PERMANOVAs supported this result. However, the homogeneity of dispersion tests were significant; therefore, the results could be due to dispersion between our samples. Although I expected bacterial richness and diversity to vary based on body region, I thought that would hold true when assessing bacterial communities on wild samples as well.

To compare the effect of body region on wild samples, I examined adult individuals of the same sites, at both the East Fork and the West Fork. Among wild samples, I observed higher average cloacal alpha diversity metrics in comparison to dorsal samples; however, pairwise Kruskal-Wallis tests between those body parts did not detect significant differences among samples. Although these results did not align with my initial hypothesis, similar results were observed in a 2016 study on fire-bellied toads. Bacterial richness was higher in ventral samples when compared to dorsal samples for captive toads, but not for wild toads (Bataille et al. 2016). Bataille et al. 2016 proposes that physiological differences between the dorsal and ventral surfaces of the toad could potentially account for the observed differences between the body regions of captive individuals. In some studies, cloacal swabs serve as a proxy for assessing gut microbiota in birds (Flammer and Drewes 1988; van Dongen et al. 2013), and the gut exhibits comparatively greater diversity (Quigley 2013). Cloacal bacterial communities of birds are influenced by environmental factors as well (Lucas and Heeb 2005), so the communities observed on the hellbender cloaca are likely a combination of bacteria originating from the gut as well as those obtained from environmental reservoirs.

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Previous hellbender microbiome studies observed that bacterial communities did not differ significantly between the dorsum or feet of wild hellbenders, but instead differed significantly between individuals (Hernández-Gómez et al. 2017). The failure to detect significantly different bacterial communities between wild body regions in previous hellbender microbiome studies aligns with my observations, but still raises the question of why I observed bacterial communities that were significantly different between body regions in captivity, but not in the wild. If individuals were considered, that could be where significance lies; perhaps a less complex environment in captivity ultimately leads to less variability between individuals. The consideration of individuals could allow the detection of comparatively subtle differences in bacterial community composition when looking for variation between regions in the body.

After examining the effects of captivity and body region on eastern hellbenders, I examined how location in the wild would alter the skin bacteria. Although I did not detect differences between body region impacting the cutaneous microbiota of hellbenders in the wild, I observed patterns of higher average diversity among the same body parts. Therefore, I opted to compare adult samples from only the same body part to eliminate as much potential variation as possible. Among all compared body parts, I did not detect differences between the bacterial communities of adult hellbenders between the five locations I sampled, which was consistent with a study exploring intraspecific variation of the microbiome of a terrestrial, direct-developing plethodontid salamander subspecies (Prado-Irwin et al. 2017). Although geographically and genetically isolated, the salamanders in this study were found to harbor similar cutaneous bacteria. Later work building off the Prado-Irwin et al. 2017 study that compared the microbiome of two subspecies concluded environmental factors can play a greater role in salamander cutaneous microbiome than host phylogeny (Bird et al. 2018). Therefore, the extremely

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specialized habitat required by eastern hellbenders could result in the presence of similar bacteria although populations are isolated.

My findings conflicted slightly with a previous study on the variation of hellbender microbiomes by population (Hernández-Gómez, Hoverman, and Williams 2017), which noted unique bacterial communities for each population and observed a positive correlation with the divergence of cutaneous bacteria and genetic divergence by population. Although I did observe slight variation in overall bacterial structure, I did not see significance between alpha and beta diversity measures between rivers. Failing to detect differences between bacterial communities between hellbenders of different rivers could be attributed to the scale of the two studies: my study was limited to southeastern West Virginia, while their study sampled eastern hellbenders in northern West Virginia, Indiana, Tennessee, North Carolina, and Georgia.

To examine the effects of age on the cutaneous bacterial communities of eastern hellbenders, I compared samples from individuals within the same river. Because body region did not have a significant effect on bacterial communities, I chose to compare larval swabs that were taken from the dorsum to adult samples of all body regions. Comparing swabs from different body regions was necessary because I lost an adult dorsal sample from the Cranberry River after rarefaction, leaving me with only two samples in that category. In result, I observed significant differences between larval and adult hellbenders in the Cranberry River. As mentioned previously, the degree to which age effects the amphibian microbiome varies depending on the study. In a 2013 study on frogs, which undergo complete metamorphosis and dramatic shifts in physiology and therefore life history strategies as they age, age was correlated with bacterial community structure and composition. Bacterial communities of post-metamorphic stages did not differ significantly from those of adults, but tadpoles were significantly different from both post-

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metamorphic frogs and adults (Kueneman et al. 2014). In contrast, a study on terrestrial, direct-developing plethodontid salamanders did not detect a significant change in bacterial community assemblages between ages (Prado-Irwin et al. 2017). The major takeaway from comparing these two studies is that the presence of a larval stage (and metamorphosis) resulted in the detection of significant differences in bacterial richness and diversity between age classes whereas the absence of a larval stage did not.

Anuran metamorphosis from tadpole to adult results in both dramatic physiological and life-history related changes. When applying this comparison to hellbenders, I encountered a few problems. Although hellbenders undergo metamorphosis, it is considered "partial metamorphosis" (Petranka 1998). Hellbenders remain completely aquatic throughout their lives (Petranka 1998), unlike frogs who can move rapidly from aquatic to terrestrial environments after undergoing metamorphosis. There are also difficulties comparing hellbenders directly to the terrestrial, direct-developing plethodontid salamander because of the extreme differences in habitat, life history, and physiology. No other studies have compared the differences between hellbender larvae, so the next best comparison is within the Family Cryptobranchidae. *Andrias japonicus* exhibits extremely similar life-history characteristics when compared to hellbenders; they undergo partial metamorphosis and remain entirely aquatic throughout their lives (Petranka 1998). A 2017 study compared the microbiota of *Andrias japonicus* larvae and adults and observed similar results when compared to our study. Bletz et al. 2017 noted that adult and larval individuals shared similar phyla with slightly different taxonomic proportions but differed significantly in some measures of bacterial richness as well as overall community structure (Bletz et al. 2017).

There was also a similarity in the most abundant phyla when comparing eastern hellbenders to other giant salamanders: Bletz et al. 2017 discovered that Proteobacteria, Bacteroidetes, Actinobacteria, Firmicutes, and Verrumicrobia were the most abundant phyla in *A. japonicus* larvae and adults. This study also compared the cutaneous microbiota of *A. japonicus* to co-occurring fish and amphibian species and observed bacterial communities of *A. japonicus* to be structurally different in comparison to the other species. This study attributed the unique microbiome structure to the unique mucus composition and antimicrobial peptides possessed by giant salamanders (Li et al. 2015, Rollins-Smith 2009, Bletz et al. 2017). These observations raise interesting questions: how much does the unique structure of the skin of giant salamanders contribute to the overall structure and diversity of their microbiome, and does it contribute to a "core microbiome" that is similar among cryptobranchids?

A core microbiome is defined as the shared members of microbial assemblages across a group (Shade and Handelsman 2012). The core microbiome is thought to provide important functions that greatly influence the health and physiological function of the host; therefore, it is important to identify OTUs shared between all groups within a study. The identification of a core microbiome of a group provides us insight into the host-microbe relationship (Shade and Handelsman 2012). In my study, I identified the core microbiome of captive individuals, wild individuals overall, adults from the Cranberry River, and larvae from the Cranberry River. I detected 10 core OTUs present in 60% of our samples overall. Among that list, one OTU, identified to species (*Acinetobacter venetianus*), was matched with an isolate from the Woodhams et al. 2015 database of antifungal isolates from amphibian skin. Detecting core bacteria with antifungal properties is noteworthy because disease is a major conservation concern for amphibians worldwide. Although amphibian pathogens, fungal infections, and skin lesions

have been detected on hellbenders at various times and locations (Bales et al. 2015; C. Bodinof et al. 2011; Souza et al. 2012; Tominaga et al. 2013; Williams and Groves 2014), it remains unclear to what extent disease has negatively impacted these giant salamanders. However, the presence of pathogens and skin lesions present on hellbenders warrants concern. Nineteen OTUs were identified as core taxa among wild adults from the Cranberry River, and the core OTUs maintained similarity to those identified from all wild samples. Additionally, the antifungal isolate identified as a core OTU for wild samples remained as a core OTU for wild adults from the Cranberry River.

Furthermore, captive individuals in my study contained overall similar core microbiota as those in Hernández-Gómez et al. 2017, including members of family Comanmonadaceae and order Stramenopiles, although I did see some loss in richness and less diverse community composition. Loss of bacterial richness is supported in the Becker et al. 2014 study on Panamanian golden frogs – researchers observed that bacterial communities were altered, but captive individuals still retain most bacteria that originated from their wild ancestors, even after multiple generations in captivity. The presence of similar bacterial phyla between captive and wild individuals in combination with the retention of bacterial taxa with anti-fungal properties, may bode well for concerns about the potential captive-rearing has to alter the amphibian microbiome and result in a weakened immune system before reintroduction. It remains unclear how a less diverse microbiome could influence survival after captive reintroductions. Studies in the future could benefit from monitoring the cutaneous microbial communities and overall host health both pre- and post-translocation to ameliorate these concerns. In addition, scientists could experiment with introducing probiotics to captive environments; some studies suggest that

introducing environmental substrates to captive populations could help mitigate the loss of bacterial diversity while in captivity.

In addition to the exploration of the effects of physiology and geographic location influencing the hellbender microbiome, redundancy analysis on available water quality parameters helped me distinguish more specific environmental factors that significantly impacted bacterial community composition among hellbenders in our study. I detected that temperature had a significant effect on bacterial communities. Other studies have obtained similar results: Kohl and Yahn 2016 found that temperature significantly impacted the tadpole gut microbiome. Tadpoles raised in cool temperatures (18 °C) exhibited greater amounts of Proteobacteria and less Actinobacteria and Planctomycetes than tadpoles kept in warm temperatures (28 °C). It is unclear whether these changes in the gut microbiome were caused by the change in temperature or physiological changes in the host resulting from the temperature. Although the exact cause of the alteration is unclear, the implications remain the same. The change of the amphibian microbiome because of greater fluctuations in water temperatures could pose a threat to many species as global climate change becomes an increasing threat (Beebee and Griffiths 2005; Foden et al. 2013; Lips et al. 2008; Westhoff and Paukert 2014). On that note, future studies should strive to learn more of the changes in amphibian microbiomes when faced with temperatures that mimic climate change projections and what that could mean for the overall health of the organism.

Another environmental factor that influenced the bacterial communities among eastern hellbenders was the velocity of water flow. To my knowledge, no studies exist where water flow was correlated with the structure of the amphibian microbiome. Other studies do point to the alteration of stream microbial biofilms because of water velocity; biofilms that developed under

higher flows were structurally different than those that were exposed to lower velocities (Battin et al. 2003). In addition to considering the importance of environmental variables, redundancy analysis picked out bacterial taxa that were significantly different between hellbender samples. Although the taxonomic resolution of some significant bacteria from the redundancy analysis was too large to draw any meaningful conclusions, some taxa were identified to be closely related to: *Acinetobacter, Bacillus, Albidiferax,* and *Rhodoferax. Acinetobacter* was identified as the genus of one of the antifungal isolates identified from our samples, *Acinetobacter venetianus*.

Amphibians are a highly threatened taxa, with disease, climate change, and habitat destruction acting as major threats. Host-associated microbes, or microbiomes, are extremely important to the overall health of their hosts. We know little about the environmental and physiological factors that influence the structure and composition of the microbiome. We also know little about how microbial taxa interact with and impact their multicellular hosts. For future studies, it is imperative to tease out the intricacies of complex interactions between host, environment, and microbes. Discovering more about host-microbiome interactions is especially important for amphibians as past research has enumerated the many ways in which their microbiome can influence health, disease resistance, and development. Eastern hellbenders rely totally on freshwater systems, which are one of the earth's most imperiled. In result, hellbenders are experiencing widespread declines and are listed as threatened on the IUCN Redlist. Because of this, hellbenders are in need of conservation and management throughout their range, making them good candidates for microbiome studies (Hernández-Gómez et al. 2017). My study began to fill in some of the gaps in our knowledge regarding the physiological and environmental drivers of variation in hellbender-associated bacteria; however, it raises future research questions. I suggest future studies incorporate greater replication to compare captive and wild

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populations, research how introducing environmental substrates to captive population may change the microbiome and influence reintroduction success, and monitor how microbiomes may change in response to reintroductions. Additionally, I suggest that future studies pursue how temperature may impact the symbiont bacterial communities of eastern hellbenders as this could be important for conservation in the face of global climate change.

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APPENDIX A: OFFICE OF RESEARCH INTEGRITY APPROVAL LETTER

MARSHALL UNIVERSITY
www.marshall.edu

Animal Resource Facility

DATE: November 8, 2016

TO: Jayme Waldron, PhD
FROM: Marshall University IACUC

IACUC #:
PROJECT TITLE: [957095-2] Evaluating the use of environmental DNA (eDNA) as a
                         supplemental method of detecting Eastern Hellbenders (Cryptobranchus a.
                         alleghaniensis)
SUBMISSION TYPE: New Project

ACTION: APPROVED
APPROVAL DATE: November 8, 2016
EXPIRATION DATE: June 1, 2018
REVIEW TYPE: Full and Designated Member Review

Thank you for your submission of Revised materials materials for this research project. The Marshall University IACUC has APPROVED your submission. All research must be conducted in accordance with this approved submission.

This submission has received Full and Designated Member Review.

Please note that any revision to previously approved materials must be approved by this committee prior to initiation. Please use the appropriate revision forms for this procedure.

Please report all NON-COMPLIANCE issues regarding this project to this committee.

This project requires Continuing Review by this office on an annual basis. Please use the appropriate renewal forms for this procedure.

If you have any questions, please contact Monica Valentovic at (304) 696-7332 or valentov@marshall.edu. Please include your project title and reference number in all correspondence with this committee.

Monica A. Valentovic, Ph.D.
Chairperson, IACUC