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HOST MICROBE INTERACTIONS

Captivity-Induced Changes in the Skin Microbial Communities of Hellbenders (*Cryptobranchus alleganiensis*)

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Abstract

Variation in environmental conditions can result in disparate associations between hosts and microbial symbionts. As such, it is imperative to evaluate how environmental variables (e.g., habitat quality) can influence host-associated microbiome composition. Within wildlife conservation programs, captive conditions can negatively influence the establishment and maintenance of "wild-type" microbiotas within a host. Alternative microbial communities can result in the proliferation of disease among captive stock or upon reintroduction. Hellbenders (*Cryptobranchus alleganiensis*) are a threatened salamander for which extensive captive management is currently employed. Using metabarcoding, we characterized the skin microbiota of wild and captive hellbenders from two subspecies in the state of Missouri, the eastern (*C. a. alleganiensis*) and the Ozark hellbender (*C. a. bishopi*). Both subspecies in our study included wild adults and captive juveniles that were collected from the wild as eggs. Our objectives were to investigate differences in the skin microbial communities' richness/diversity, composition, and functional profiles of microbes between wild and captive individuals. Captive eastern hellbenders possessed richer communities than wild cohorts, whereas the opposite pattern was observed within the Ozark subspecies. We found significant microbial community structure between wild and captive populations of both subspecies. Microbiota structure translated into differences in the predicted metagenome of wild and captive individuals as well. As such, we can expect captive hellbenders to experience alternative microbial structure and function upon reintroduction into the wild. Our study provides a baseline for the effect of captivity on the skin microbial communities of hellbenders, and highlights the need to incorporate microbiota management in current captive-rearing programs.

Keywords Ozark hellbender · Eastern hellbender · 16S rRNA · Amphibian conservation · Amphibian skin microbiota · Metabarcoding

Introduction

Advances in microbiology have resulted in the ability to characterize compositional and functional structure among host-associated microbial communities. Recent work has identified important contributions of microbial communities to host physiological processes including immune system activation, metabolism, energy uptake, host tissue differentiation, and pathogen defense [1–4]. In-depth analyses of microbiomes among hosts have characterized a high degree of covariation between community composition and function [5]; thus, evaluating compositional variation has become an important tool to predict functional variation among hosts [6]. Species identity is considered a major predictor of microbiota composition in wildlife [7, 8]. However, within populations of a species, environmental variation can also introduce variation in microbiota composition [9, 10]. For example, environmental

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variables such as habitat quality, diet, and presence of sympatric species can contribute to changes in associations between hosts and their symbionts [11–13]. To evaluate the effect of environmental heterogeneity on microbial composition and function, characterizing host-associated microbial communities across habitats is an important step.

Evaluating the effect of environmental variation on host-associated microbiomes has become an important concern within wildlife conservation programs. For example, captive conditions can hamper the establishment and maintenance of "wild-type" microbial communities within a host. Maladaptive changes to host-associated microbiotas can result from differences in diet [14, 15] and absence of natural bacterial reservoirs (e.g., substrate) [16]. Changes in the microbiota of captive-reared individuals can result in the proliferation of disease among captive stock or upon reintroduction to the wild [17]. Amphibians have become an important model to evaluate the effect of captive conditions on host-associated microbial communities, especially due to the contribution of skin microbiota to disease resistance [18]. Currently, extensive captive collections of amphibians exist around the world as a response to massive population declines [19]. Studies evaluating differences between wild and captive populations of amphibians have observed dramatic differences in richness/diversity and community composition between these two populations [14, 16, 20, 21]. In addition, a negative relationship between the presence of naturally occurring pathogen-inhibitory bacteria and time spent in captivity has been observed in captive amphibian populations, for example, in boreal toads (*Anaxyrus boreas*) [22]. As such, evaluating disparity in the microbial communities between wild and captive amphibians can be used to explore potential negative effects of captive rearing. This approach is particularly important for endangered amphibians, where captive rearing is now a critical management strategy.

Hellbenders are fully aquatic salamanders distributed throughout streams in the eastern USA. Two subspecies of hellbenders are recognized, the Ozark hellbender (*Cryptobranchus alleganiensis bishopi*), endemic to southern Missouri and northern Arkansas, and the eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*), found in the Appalachian Mountain region, Midwest, and an isolated population in central Missouri [23]. Both subspecies have experienced declines within the past 30 years [24], both are listed as state endangered in Missouri in 2003, and in 2011, the Ozark subspecies was listed as federal endangered [25]. In the state of Missouri, the Missouri Department of Conservation (MDC) and the St. Louis Zoo's Ron Goellner Center for Hellbender Conservation (RGCHC) maintain captive populations of both subspecies. The MDC and RGCHC were the first to establish captive-breeding programs for both subspecies, and possess large captive stock from captive-bred and wild-collected eggs with the intent to bolster wild populations [26–29]. Conservation management programs throughout the remaining range of the eastern hellbender currently maintain captive populations from wild-collected eggs, and have performed translocations among populations [30]. Current hellbender captive-rearing practices involve the use of mechanical, chemical, biological, and ultraviolet filters to clean water used in hellbender enclosures [26, 29]. In addition, captive individuals are often fed commercially obtained fish, shrimp, and worms that provide adequate nutrition, but do not match naturally occurring food resources [26, 29]. When hellbenders are fed wild caught prey, the prey items are quarantined and treated to remove bacteria and other potential infective agents (e.g., *Batrachochytrium dendrobatidis*). Given the obvious differences between captive and wild conditions, it is necessary to quantify variation in microbial community diversity and function between captive and wild hellbenders.

To evaluate if captivity influences the skin microbiota of hellbenders, we set out to characterize the cutaneous microbial communities of wild and captive Ozark and eastern hellbenders within the state of Missouri. Our objectives were to (1) investigate differences in the skin microbiota richness/diversity and composition between wild and captive individuals, (2) predict functionality of hellbender skin communities using established bioinformatics pipelines, and (3) evaluate whether functional profiles between wild and captive hellbenders differ. Because captive hellbenders are maintained in clean conditions throughout their development, we expected captive individuals to possess less rich and diverse communities than their wild counterparts. In addition, we predicted differences in the composition of the skin microbiota between wild and captive individuals. Finally, we anticipated to observe a similar pattern of change in the predicted functional profiles between wild and captive individuals.

Materials and Methods

Study Hellbender Populations

We swabbed the skin of wild adult hellbenders in the Eleven Point River (EPR-Ozark hellbenders) and the Big Piney River (BPR-eastern hellbenders). Both of these rivers are located within the Salem Plateau, a subdivision of the Ozark Plateau in southwestern Missouri. The Eleven Point River flows southeast and joins the Spring River in Arkansas, whereas the Big Piney River flows northeast and joins the Gasconade River in central Missouri. Both rivers possess similar riparian characteristics consisting of forest cover (EPR 65%, BP 68.3%), grassland/cropland cover (EPR 33.7%, BPR 31.1%), and urban cover (EPR 0.4%, BPR 0.2%) [31, 32]. Our study sites in both rivers were located in areas with fast-flowing water and abundant large cover rocks, as this is indicative of good hellbender habitat [23].

Captive individuals from the Ozark and eastern hellbender subspecies were collected from individual nests as eggs in 2010 and 2013, respectively, from the same rivers where we sampled the wild hellbenders. All captive eastern hellbenders were sampled from the same tank in the RGCHC; however, six captive Ozark hellbenders were sampled from a separate tank in the RGCHC and four from a display tank in the St. Louis Zoo's Charles H. Hoessle Herpetarium (CHH). Eastern hellbenders, RGCHC Ozark hellbenders, and CHH Ozark hellbenders are all kept under separate life support systems; however, environmental conditions among all hellbenders are similar (e.g., constant temperature ~ 14 °C, same diet, presence of tile/rock hides, use of gloves by staff before handling). Hellbenders in the RGCHC and the Ozark hellbenders in the CHH are cared for by separate teams of caretakers, and the CHH enclosure possesses gravel substrate while enclosures in the RGCHC do not.

Sample Collection

We swabbed the skin of wild adults and captive juveniles for both subspecies of hellbenders. Swab samples were obtained by swabbing the dorsum of each individual following the protocol of Hernández-Gómez et al. [33]. We swabbed the skin of wild hellbenders between August 25th and October 27th, 2015, from the Eleven Point River and the Big Piney River. We swabbed captive hellbenders from both subspecies at the St. Louis Zoo's RGCHC on December 2nd, 2015. We handled hellbenders following an approved protocol by the Purdue University Animal Care and Use Committee (PACUC protocol no. 14060011094).

DNA Extractions, Amplification, and Sequencing

We isolated DNA from skin swabs using the PowerSoil DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA) following the protocol described in Hernández-Gómez et al. [33]. We amplified the bacterial 16S rRNA V2 region using primer pair 27F/338R [34] with the attachment of connector sequences [33]. We ran each sample in triplicate, and each reaction consisted of 5 μL of template DNA, 12.5 μL of MyTaq Master Mix (Bioline, Tauton, MA), 1 μL of 10 mM forward and reverse primers, and 6.5 μL of PCR water (MoBio Laboratories Inc., Carlsbad, CA) for a total of 25 μL per reaction. PCR conditions consisted of 95 °C for 2 min, 30 cycles of 94 °C for 45 s, 50 °C for 60 s, and 72 °C for 90 s, followed by 72 °C for 10 min. We pooled amplicon triplicates and cleaned the products using the UltraClean PCR Clean-up kit (MoBio Laboratories Inc., Carlsbad, CA).

We performed a second PCR on microbiota amplicons to add-on dual-index barcodes connected to Illumina sequencing adaptors [33] to the ends of amplicons. The PCR consisted of 5 μL of clean amplicons, 12.5 μL of MyTaq Master Mix, 1 μL of forward and reverse barcode primers, and 6.5 μL of water for a total of 25 μL reactions. PCR conditions consisted of 95 °C for 2 min, 5 cycles of 94 °C for 45 s, 65 °C for 60 s, and 72 °C for 90 s, followed by 72 °C for 10 min. We quantified the PCR products using a Qubit Fluorometer (Invitrogen Corp, Carlsbad, CA), pooled samples in equimolar amounts, and cleaned the sample pool using the UltraClean PCR Clean-Up kit. The sample pool was sequenced on a MiSeq machine (Illumina Inc., San Diego, CA) using the Reagent Kit V2 to produce 250 bp paired-end reads.

Microbiota Sequence Analysis

We processed raw sequencing reads using Trimmomatic [35] to remove adapter sequences, bases below threshold quality of phred-20 from both ends of reads, and any resulting reads under 30 bp. We paired reads that passed initial quality control using PANDAseq [36]. Only reads that paired successfully were employed in subsequent analysis.

Our microbiota sequence analysis consisted of established sequence read processing pipelines to filter erroneous reads, cluster reads into operational taxonomic units (OTUs), and generate abundance-based OTU tables. We used a previously published custom Python program [33] to remove quality scores and rename reads with a name compatible with the chosen pipeline. We processed the resulting read file using the Quantitative Insights Into Microbial Ecology version 1.9.0 (QIIME) pipeline [37]. We clustered reads at the standard 97% similarity using the open-reference protocol and the Greengenes 13_5 reference database [38]. Reads that failed to cluster using the open-reference algorithm were clustered into *de novo* OTUs with UCLUST [39]. OTUs that clustered using the Greengenes database retained the accorded taxonomy, while de novo OTUs were assigned taxonomy using the RDP Classifier [40] at 80% confidence. We resolved any unassigned OTU taxonomies with an additional search of sequences in the RDP rRNA sequence database [41]. We aligned representative sequences to the pre-aligned Greengenes reference using PyNAST [42], and used the alignment to produce a phylogenetic tree through FastTree [43]. To avoid including any OTUs generated by sequencer error, such as base miscalls or chimeras, we performed additional quality filtration on the OTU table by removing OTUs that were represented by fewer than 0.005% of the total read count [44]. To standardize sequencing depth throughout all samples, we rarefied the OTU table to 3545 sequences per sample.

Metagenome Prediction

Because metagenome prediction requires the use of reference-based OTUs only, we filtered all de novo OTUs from the raw open-reference OTU table produced in QIIME to produce a close-reference OTU table. We proceeded to quality filter the

raw closed-reference OTU table as described above and standardized the sequencing depth throughout all samples to 656 sequences per sample. We implemented the bioinformatics software package Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt v. 1.1.1) to infer metagenome functions from the closed-reference OTU table [45]. The program normalizes the OTU table by dividing each OTU's frequency by its known 16S rRNA gene copy number, retrieves gene content for each OTU from the reference OTU tree, estimates per sample abundance of each gene family as a product of OTU abundance, and generates a metagenome table (i.e., trait counts per sample). We collapsed the predicted metagenomes to the Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology group level 3, removed rare genes that were represented by fewer than 0.005% of the total trait count, and standardized total gene copies by rarifying the metagenome table to 555,616 gene copies per sample. To assess the accuracy of PICRUSt's predictions, we calculated weighted-Nearest Sequenced Taxon Index (weighted-NSTI) scores to estimate the extent that microorganisms in each sample are related to sequenced genomes in the database [45]. Low weighted-NSTI reflects a closer match between abundant microorganisms in each sample and reference genomes.

Statistical Analysis

We compared the microbiota between wild and captive individuals from both hellbender subspecies separately by assessing differences in community richness/diversity through linear models and community structure using multivariate statistical tests. We also used multivariate statistical tests to evaluate differences in metabolite composition among wild and captive hellbenders, and assessed for associations of metabolites and captive or wild status at KEGG level 3. In all Ozark hellbender wild versus captive analyses, we did not differentiate between captive individuals sampled from display tanks or aquaria. We performed all statistical analyses in R version 3.3.1 unless otherwise noted.

Microbiota Comparisons

We compared alpha metrics of skin microbiota samples between wild and captive hellbenders of each subspecies. We calculated community richness (observed OTUs) and diversity (phylogenetic diversity) values on each sample in QIIME using the relative abundance-based OTU table. To evaluate whether OTU richness differs between wild and captive individuals, we implemented community richness as a dependent variable and captivity status as an independent variable in negative binomial linear models. To evaluate whether community diversity differs between wild and captive individuals of each subspecies, we implemented diversity as a dependent variable and captivity status as an independent variable in a linear model.

We used the package GUniFrac [46] in R to build UniFrac distance matrices (unweighted) [47]. We performed Adonis tests using the unweighted UniFrac distance matrices within the R package vegan 2.4-4 [48] to partition the variation between wild and captive individuals within each subspecies. We visualized differences in community structure between all hellbenders using unweighted UniFrac distances through a principal coordinate analysis (PCoA) using the R package ade4 [49]. We implemented the linear discriminant analysis effect size (LEfSe) algorithm described in Segata et al. [50] to test significant differences in OTU relative abundance between wild and captive hellbenders in each subspecies. The LEfSe algorithm identifies the OTUs whose abundance statistically differs between the wild and captive individuals through a nonparametric factorial Kruskal-Wallis sum rank test ($\alpha < 0.05$). Subsequently, the algorithm generates effect sizes for divergent OTUs through a linear discriminant analysis (LDA). The effect sizes represent the magnitude of the association of each relevant OTU. To maximize the stringency of our analysis and simplify the list of divergent OTUs, we excluded all singleton OTUs from the analysis and only retained divergent OTUs with an LDA effect size greater than 3.0. Finally, we calculated the core microbiota (OTUs present across 80% of individuals) for each category within each subspecies. To visualize the number of shared core OTUs among wild and captive hellbenders in each subspecies, we produced Venn diagrams using the program Venny 2.1.0 [51].

Predicted Metagenome Comparisons

We built Bray-Curtis distance matrices from each subspecies predicted metagenome table using vegan, and performed Adonis tests to partition the variation between wild and captive individuals. We visualized differences in metagenome composition of all hellbenders using Bray-Curtis distances through a PCoA generated with ade4. We also used each subspecies' predicted metagenome table to perform a species indicator analysis using the package labdsv in R [52]. The indicator species analysis allowed us to identify the most prominent pathways found on wild or captive hellbenders.

Results

At the time of sampling, all captive hellbenders appeared in good health and free of any relevant skin conditions. We noted cutaneous wounds in the extremities of one wild eastern hellbender and three wild Ozark hellbenders. These wounds are consistent with previous observations in wild individuals of hellbenders [33, 53, 54], and likely result from territorial bouts. From 19 Ozark (nine wild individuals, ten captive individuals) and 18 eastern (eight wild individuals, ten captive individuals) hellbender microbiota samples, 16S rRNA V2

amplicon sequencing resulted in 926,882 reads with an average length of 318 base pairs. We processed the remaining reads through QIIME using the open-reference clustering method to return 1596 OTUs for all skin samples. Our metagenome prediction consisted of 2858 functional genes. We deposited sequencing data into the NCBI Sequence Read Archive (project accession number PRJNA382978).

Microbiota Comparisons

We characterized differences in the skin microbiota between wild and captive individuals of both subspecies of hellbenders. Wild Ozark hellbenders possessed richer but not more diverse communities than those in captivity (observed OTUs $F_{1,17} = 5.64$, $p = 0.030$; phylogenetic diversity $F_{1,17} = 2.18$, $p = 0.158$; Fig. 1a, b). Conversely, captive eastern hellbenders possessed richer and more diverse communities than wild individuals (observed OTUs $F_{1,16} = 7.13$, $p = 0.017$; phylogenetic diversity $F_{1,16} = 10.16$, $p = 0.006$; Fig. 1a, b). Multivariate tests (i.e., Adonis) noted significant differences between the skin microbiota of wild and captive hellbenders (Ozark pseudo $F = 5.67$, $R = 0.25$, $p < 0.001$; eastern pseudo $F = 8.80$, $R = 0.35$, $p < 0.001$). We visualized stronger segregation of microbial communities between wild and captive hellbenders than between the two subspecies on the PCoA plot (Fig. 1c).

The LEfSe analyses identified 118 divergent OTUs between wild and captive eastern hellbenders (34 OTUs were markers for wild and 92 for captivity) and 68 divergent OTUs between wild and captive Ozark hellbenders (40 for wild and 28 for captivity; Table S1). Captive hellbenders from both subspecies had significant associations with multiple OTUs identified to the family Oxalobacteraceae, family Comamonadaceae, genus *Paucibacter*, order Bacillales, genus *Nevskia*, genus *Flavobacterium*, genus *Nitrospira*, order Burkholderiales, and order Rhizobiales. Within wild hellbenders of both subspecies, we saw associations with the same OTUs identified to the family Comamonadaceae, phylum Cyanobacteria, family Cytophagaceae, genus *Fluviicola*, order Bacteroidales, family Ruminococcaceae, class Chloroflexia, genus *Deinococcus*, and genus *Streptococcus*. All hellbenders in this study shared five OTUs in their core microbiota identified to the family Bradyrhizobiaceae, the family Comamonadaceae (× 2), genus *Propionibacterium*, and order Burkholderiales. In addition, we observed overlap in core OTUs between wild and captive hellbenders of both subspecies, among captive hellbenders of both subspecies, and among wild hellbenders of both subspecies (Fig. 2). There was a noticeable shift in the core microbiota between wild and captive eastern hellbenders with the wild eastern hellbender communities dominated by Verrucomicrobia (28.0%) and Proteobacteria (25.0%) and the captive eastern hellbender communities dominated by Proteobacteria (41.8%), Bacteroidetes (12.3%), Actinobacteria (7.5%), Firmicutes

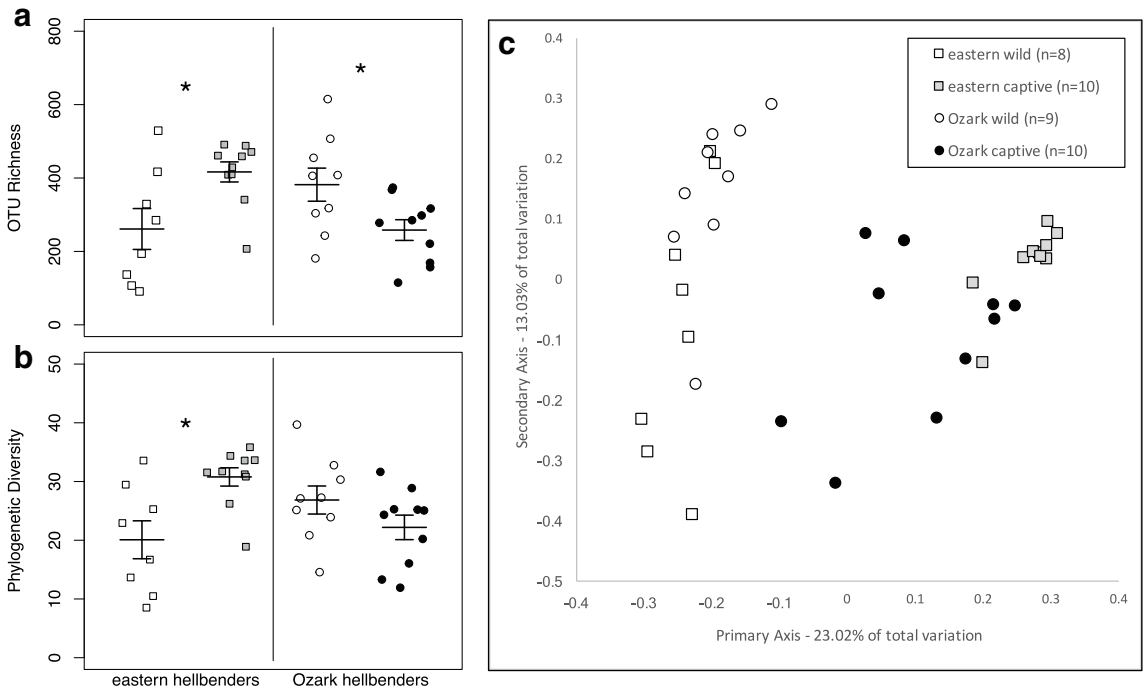

Fig. 1 Alpha and beta diversity comparisons of wild/captive eastern and hellbender skin microbiotas. **a** Dot plot of OTU richness values and **b** dot plot of phylogenetic diversity values are presented for both subspecies. Group means and standard error bars are present in dot plots and significant differences are marked with an asterisk. **c** Principal coordinate analysis using unweighted UniFrac distances is pictured. Each point represents the skin bacterial community of an individual hellbender. Clustering by subspecies identity and captivity status is visible

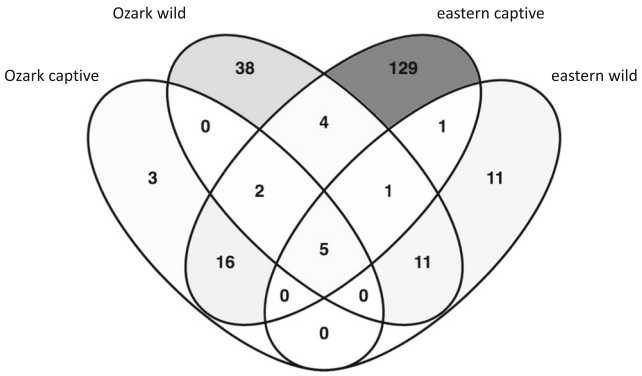

Fig. 2 Venn diagram displaying overlap in core microbiota of captive/wild Ozark and eastern hellbenders. Core microbiota represents OTUs present in 80% of individuals within each group. Shading corresponds to magnitude of OTUs in each category. Ubiquitous OTU taxonomies are as follows: 165,421 *Propionibacterium* sp.; 574,721 Comamonadaceae; 750,411 Burkolderiales; 826,276 Bradyrhizobiaceae; and 1,108,726 Comamonadaceae

(3.8%), and Nitrospirae (2.7%; Fig. S1). A similar pattern was observed among Ozark hellbender samples, with the wild hellbender core microbiota dominated by the phyla Proteobacteria (30.3%), unassigned OTUs (21.0%), Bacteroidetes (4.3%), and Verrucomicrobia (3.1%); and the captive Ozark hellbender skin core microbiota dominated by the phyla Proteobacteria (8.1%) and Bacteroidetes (5.1%; Fig. S1).

Metagenome Prediction Analysis

Our metagenome predictions had a low weighted-NSTI value (mean ± SE 0.088 ± 0.0029), corresponding with accurate relationship with sequenced genome representatives. Using the predicted metagenomes, we also identified significant differences in putative function composition and abundance between wild and captive hellbenders. Multivariate tests (i.e., Adonis) on Bray-Curtis distances noted significant grouping of samples based on wild or captive status (Ozark pseudo $F = 9.28$, $R = 0.35$, $p < 0.001$; eastern pseudo $F = 2.90$, $R = 0.15$, $p = 0.023$). In addition, we visualized marginal clustering between the putative metagenomes of captive and wild hellbenders in the PCoA (Fig. 3a). At KEGG Orthology hierarchy level 3, we identified significant associations between wild and captive individuals and specific functional pathways across both subspecies of hellbenders (Fig. 3b). A majority of the predicted differences observed in the abundance of functional pathways between wild and captive hellbenders were in genes contributing to cell metabolism. Pathways related to protein breakdown (e.g., amino acid metabolism), metabolism of terpenoids/polyketides, biosynthesis of secondary metabolites, and glycan biosynthesis/metabolism were consistently predicted to be abundant among wild hellbenders of both subspecies (Fig. 3c). On the contrary, captive hellbenders possessed a higher number of putative pathways related to xenobiotic biodegradation and metabolism (Fig. 3c).

Discussion

Characterizing host-associated microbiotas throughout distinct habitats is imperative to predict changes in microbial community and functionality that parallel environmental change. In our system, we observed significant differences in the microbiota of wild and captive hellbenders in Missouri. Among Ozark hellbenders, we observed higher diversity of bacteria on the skin of wild individuals compared to captive. However, we detected an opposite pattern among eastern hellbenders with captive individuals possessing higher diversity than wild ones. Moreover, we noted significant divergence in microbial community composition and predicted metagenomes between wild and captive hellbenders, and observed consistent shifts in the abundance of bacteria in the phyla Proteobacteria, Bacteroidetes, and Verrucomicrobia between wild and captive hellbender populations. These results show that raising hellbenders in captivity results in disparate microbial communities compared to wild cohorts. In addition, wild hellbenders possessed a greater number of metabolism-related genes than captive individuals in their skin microbiota, suggesting that the skin microbial communities of wild hellbenders possess higher metabolic plasticity. Therefore, our results imply that the captive environment induces divergent assemblies of the skin microbiota of hellbenders that could influence the success of reintroduction efforts.

We expected that captive hellbenders would possess reduced cutaneous microbial community richness and diversity; however, this pattern was only observed in Ozark hellbenders. Interestingly, captive eastern hellbenders possessed richer and more phylogenetically diverse communities than their wild cohorts. Contradicting patterns in the influence of captivity on the richness/diversity of the amphibian skin microbiota are reported in the literature. A number of studies have described richer or more diverse cutaneous communities in wild ranids and plethodontids compared to captive ones [14, 16, 20, 22, 55]. However, Becker et al. [21] characterized the skin microbiota of wild and captive Panamanian golden frogs (*Atelopus zeteki*) and observed that captive frogs possessed richer and more diverse communities than wild individuals. Inconsistency in richness patterns between eastern and Ozark hellbenders may result from variation in age and time spent in captivity between the two captive cohorts. In our study, the captive Ozark hellbenders hatched in 2010, whereas the eastern hellbender cohort hatched in 2013. Previous studies have observed a correlation between time in captivity and the loss of natural and functionally important bacteria from host-associated amphibian microbiotas [22]. Thus, it is likely that captive Ozark hellbenders have lost a greater number of their

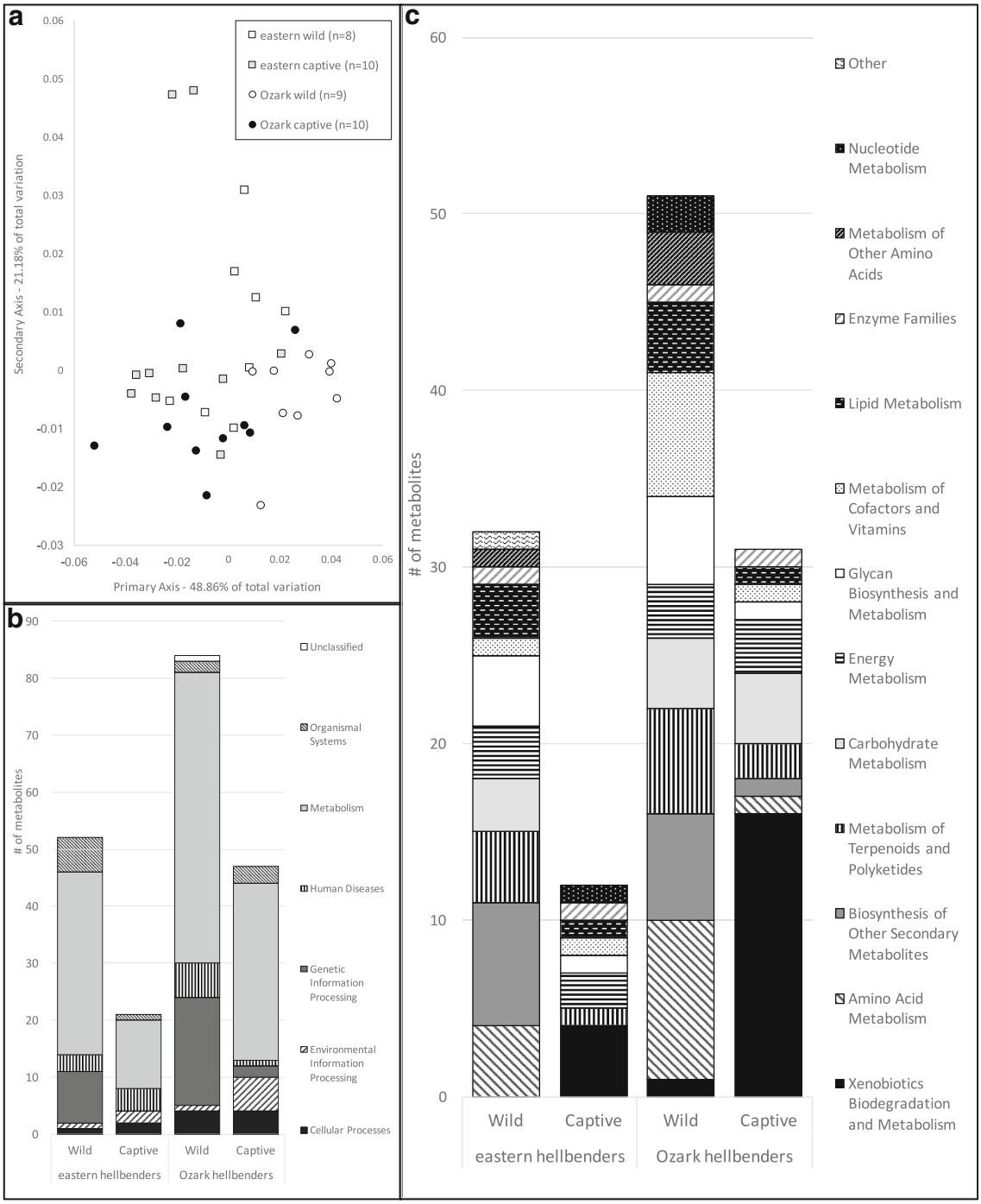

Fig. 3 **a** Principal coordinate analysis of Bray-Curtis dissimilarity matrices from wild/captive Ozark and eastern hellbender skin bacterial community predicted metabolites. Each point represents the skin bacterial community predicted metabolites of an individual hellbender. Marginal clustering by captivity status is visible. **b** Frequency of metabolites significantly associated with the skin microbial communities of wild/captive eastern or Ozark hellbenders. **c** Frequency of metabolites contributing to cell metabolism which are significantly associated with cutaneous microbial communities of wild/captive eastern or Ozark hellbenders

skin microbes due to their prolonged exposure to captive conditions.

Our study differs from previous investigations in that we simultaneously characterized the effect of captivity on the skin microbiota of two closely related amphibians. Previous studies on the skin microbiota of wild hellbenders describe differences between the two subspecies [33, 56]. In the current study, we noted marginal differences between the two subspecies and a much stronger pattern of microbial community structure between wild and captive. Even within captive

Ozark hellbenders, we noted variation in the microbial communities that was associated with the split of samples between the CHH and RGCHC. The effect of captivity on the microbiota of hellbenders is consistent with previous studies evaluating the effect of environment on the microbiota of amphibians [14, 20, 21, 57]. However, our data illustrates how the captive environment can erode natural differences between the skin microbial communities of wild amphibian species. These observations suggest that captive conditions may disturb natural variation in the skin microbiota of amphibian species. In addition, even small changes to habitat conditions, such as the differences between CHH and RGCHC, can result in disparate microbial community structure.

Our hellbender skin microbiotas were dominated by the phyla Proteobacteria, Bacteroidetes, Firmicutes, Verrucomicrobia and Actinobacteria. These phyla have been universally characterized as common members of wild amphibian skin microbiotas [8, 33, 58]. However, previous studies have recorded abundance shifts of these phyla on the skin microbial communities between wild and captive amphibians [16, 20, 21]. In our study, we observed higher abundances of Bacteroidetes and Proteobacteria among captive hellbenders of both subspecies. In addition, we observed associations between captivity status and specific OTUs belonging to the phyla Bacteroidetes, Proteobacteria, and Firmicutes. Commonalities in OTU associations between captive and wild hellbenders could result from the unique presence of OTUs in each environment, as most of the bacteria associated with either group of hellbenders is commonly found in environmental habitats [59, 60]. However, it is also likely that captivity provides a favorable environment for certain bacteria to successfully grow or outcompete against other skin microbes. In addition, we recorded less bacteria of the phylum Verrucomicrobia in captive eastern hellbenders (mainly a single OTU assigned to the genus *Luteolibacter* which was identified in captive eastern hellbenders, but significantly enriched in wild individuals). Verrucomicrobia are common microbes of aquatic and soil habitats [61], and the genus *Luteolibacter* has been described in high abundance in wild eastern hellbenders outside of Missouri [9]. As such, it is likely that *Luteolibacter* is common in wild eastern hellbender habitats, transmitted vertically between generations, or unable to survive in the captive environment. Therefore, current captive-rearing conditions may alter the assembly of hellbender cutaneous microbiotas by restricting exposure to common environmental microbes, limiting the inheritance/permanence of microbes, and selecting for divergent microbial structure.

It is important to acknowledge that the captive hellbenders sampled in our study have remained in captivity since first brought into the zoo as eggs. Thus, a strong effect of the environment throughout development can explain the significant patterns of microbial community structure observed. As such, more research needs to evaluate how hellbenders, and other amphibians, develop associations with natural microbes throughout development in the wild. Amphibians can acquire microbes by direct contact between individuals (i.e., both horizontal and vertical transfer), or through colonization from environmental reservoirs [62–64]. Vertical transmission of microbial symbionts is a probable mechanism through which microbial symbiont uptake occurs in hellbenders. Male hellbenders exhibit parental care of eggs and larvae [23], suggesting the presence of microbial symbiont inheritance [64, 65]. Horizontal transmission is also probable as adult hellbenders interact regularly throughout their lifetime, either through territorial bouts [66, 67], breeding [23], or cohabitation of shelter rocks [68, 69]. In addition to inter-host transmission mechanisms, previous research on wild hellbenders has found overlap in the skin microbiota and environmental material (e.g., river water) suggesting that environmental reservoirs also play a role in the assembly of hellbender skin microbiotas [9]. As such, more research needs to investigate the assembly mechanisms, chronological variation, and reservoirs of the skin microbiota of hellbenders and other amphibians.

In our case, differences in community composition translated into divergence of predicted metabolites. One caveat, however, is the fact that our predicted metabolite analysis only applies to a set of OTUs that have been previously cultured and sequenced. Therefore, we were unable to characterize functionality of novel bacteria such as the common *Luteiobacter* sp. In addition, our methodology (i.e., metabarcoding) restricted our ability to evaluate whether the functional genes identified are expressed by members of the community or present in dormant microbes. Therefore, our results are conservative in that they may not include a full functional profile of the active microbial community. Still, we were able to observe divergence in potential functional profiles between wild and captive hellbenders for both hellbender subspecies based on the subset of OTUs analyzed. Captive hellbenders of both subspecies possessed a higher number of xenobiotic degrading genes, indicating that captive amphibians may experience increased exposure to xenobiotics. In the case of hellbenders, possible sources of xenobiotics are not limited to, but could include antibiotics used to treat infectious diseases or biphenols released from plastic tubing [29, 70]. Diverse metabolism-related genes were highly associated with wild versus captive individuals among both subspecies, indicating a higher degree of metabolic plasticity in the microbiota of wild hellbenders. In freshwater bacterioplankton communities, the contribution of metabolic plasticity to colonization resistance and functional redundancy in response to environmental shifts has deemed it an evolutionary important trait [71]. As a result, a high degree of metabolic plasticity in the microbiota of wild hellbenders can result in higher resistance, resilience, or functional redundancy in response to environmental disturbances (e.g., translocations, pollution, temperature variations, pathogen invasion) [72].

Conservation Implications and Future Directions

Our exploration of the cutaneous microbiota of wild and captive hellbenders provides important considerations for the ex situ management of this species. We noted strong structure in the skin microbial community composition between wild and captive hellbenders. Microbiota structure translated into differences in the predicted metagenome of wild and captive individuals as well. As such, we can expect captive hellbenders to experience alternative microbial structure and function upon reintroduction into the wild. In Missouri, previous reintroduction attempts using hellbenders raised at the St. Louis Zoo have been moderately successful with annual survival rates of up to 75% [73], suggesting that captive-raised individuals fare well in the wild despite possessing alternative microbiotas. Still, reintroduction programs throughout the range of the eastern subspecies (e.g., Indiana, New York) are smaller than Missouri's, release far fewer hellbenders, and have noted far lower survival rates (0–53%) which are attributed to predation, flooding, and disease [29, 30, 74]. Given the increased risk of disease associated with depauperate microbial communities in amphibians [75], microbiota management in captivity merits consideration in hellbender captive-rearing programs as a mean to increase survival rates in states where risks of reintroduction failures are higher.

Incorporating environmental reservoirs (i.e., river water or substrate) into captive hellbender enclosures has been previously suggested as a way to assimilate microbiotas to that of wild counterparts [9]. Managers should be aware that microbial supplementations may introduce harmful pathogens into captive stock, and care should be taken to monitor the health of supplemented individuals and provide treatment in the event of an infectious disease outbreak. However, the benefits of microbial supplementations might outweigh potential risks, as previous studies have shown that environmental reservoirs can be successful in preserving wild-type community composition and/or supplementing depauperate microbial communities in the skin of captive plethodontid salamanders [16, 63]. In addition, given that vertical transmission of microbes among generations of hellbenders is likely due to the presence of parental care in this species, the skin microbiota of adult individuals could also serve as a source of microbial symbionts for captive-reared hellbenders. Microbial transplants using skin washes have been previously implemented to transfer anti-pathogenic bacteria from one amphibian species to another [76]. However, transplant trials showed little to no success in the incorporation of foreign microbes into the recipients' skin, likely due to host-specific immune function [76]. As such, there is a need to evaluate whether a similar pattern is evident in intraspecies microbial transplants, where host species-specific defenses may not be an issue.

Continuing to explore the assembly, maintenance, and function of amphibian microbiomes is crucial for developing successful implementations for the ex situ management of hellbenders and other amphibians. Given the role of the gut microbiome in organismal health in other vertebrates [6], there is a need to expand microbial community surveys to the gut of captive and wild amphibians in order to determine which factors are more important in shaping the gut microbiota (e.g., captive diets, environmental reservoirs) [77]. In addition, future studies should evaluate how captivity-associated microbiotas impact the health and survivorship of captive-reared individuals after release into the wild. If microbial supplementation is to be implemented in captivity, it is essential to evaluate the effects that exposure to novel microbes may bestow in captive stock, characterize differences in microbiota composition between control and treatment individuals, and assess the microbial communities of treatment and control groups following reintroductions. In addition, more work needs to characterize temporal variation, role of microbial inheritance, and contribution of different environmental reservoirs to the assembly of the amphibian cutaneous microbiota.

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Compliance with Ethical Standards

We handled hellbenders following an approved protocol by the Purdue University Animal Care and Use Committee (PACUC protocol no. 14060011094).

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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