Dorsal Skin Microbial Assemblages in Free-ranging Hellbenders, *Cryptobranchus Alleganiensis*, Associated With Subspecies and Chronic Toe Lesions, but Not With Chytrid Fungal Infection.

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Research Article

Keywords: Hellbender, amphibian microbiome, salamander, wildlife disease, cutaneous lesions, chytrid, Bd

Posted Date: August 30th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-827234/v1>

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Abstract

Background

Skin microbiomes are important components of skin health and have been shown to contribute to immunity in amphibians, especially against the chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*). Hellbenders (*Cryptobranchus alleganiensis*) are large aquatic amphibians (Order Caudata) native to the eastern United States that have experienced population declines of both the Ozark and eastern subspecies, *C. a. bishopi* and *C. a. alleganiensis*, respectively. In addition, ulcerative non-healing toe lesions have become increasingly prevalent in *C. a. bishopi*, in Arkansas (AR) where populations are now reduced to a single river. To evaluate the potential impacts of both chronic toe lesions and *Bd* on hellbender health, we compared dorsal skin microbial assemblages based on 16S rRNA gene amplicons between a declining Ozark hellbender population in Arkansas (AR) presenting lesions and a reference, recruiting, lesion-free, population of eastern hellbenders in eastern Tennessee (ETN). We further evaluated effects of mass and life stage across both subspecies, as well as toe lesion severity and *Bd* infection status within AR, to better understand the associations between microbiomes and disease in a wild salamander.

Results

We found skin of ETN hellbenders to have greater bacterial alpha diversity compared to AR, with this disparity decreasing as Hill number order increased. Conversely, within AR, animals with more severe lesions had decreased alpha diversity than those with mild lesions, which became more pronounced with increasing Hill number. Further, the average microbial assemblage structure differed between ETN and AR. Specifically, AR communities displayed increased beta diversity compared to those from ETN, which appeared to be linked to toe lesion severity. Neither size class (mass) nor *Bd* infection status had a significant effect on alpha or beta diversity. Taxonomic analysis revealed ETN to have greater OTU abundance of phylum Cyanobacteria (24.3%) compared to AR (5.9%); whereas AR had increased abundance of Proteobacteria (48.5%), Firmicutes (9.1%), and Synergistetes (1.5%), in comparison to ETN (31.5%, 2.6%, 0.2%, respectively).

Conclusions

Results demonstrate that eastern hellbenders of ETN have richer and less dispersed dorsal skin bacterial assemblages compared to Ozark hellbenders of AR. Furthermore, we suggest that increased severity of toe lesions may be linked to systemic changes resulting in skin microbial dysbiosis, independent of *Bd* infection. Although lesions remain to have an unknown etiology, this study is another step towards understanding skin bacterial microbiomes in hellbenders, and their potential associations with chronic disease.

Background

The skin is the first line of defense against invading pathogens and despite a highly developed immune system, thousands of bacterial species are resident on all vertebrate skin surfaces (Grice and Serge 2011). Increasing evidence reveals the critical role these microbial ecosystems (microbiomes) play in skin maintenance (Rodriguez Hoffman et al. 2015) and host immunity and health (de Vos and de Vos 2012; Shreiner et al. 2015; Arnolds and Lozupone 2016). Further, alterations in microbiomes are associated with important diseases in humans (Ilseung and Blaser 2012) and companion animals (Rodriguez Hoffman et al. 2015). As we continue to learn more about this complex ecosystem on host surfaces, we are discovering its importance as both an indicator of host health (Shreiner et al. 2015), and medical treatment tool (Kang et al. 2017).

Evaluation of altered skin microbiomes may be especially important in amphibians. They rely on their skin for osmotic regulation, which is subsequently more permeable than other vertebrate hosts (Shoemaker and Nagy 1977). In addition, microbial community shifts may be major factors in amphibian host susceptibility to pathogens such as the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*), which is a fungal dermal pathogen responsible for the extinction of approximately 90 amphibian species worldwide (Scheele et al. 2019).

Intrinsic factors related to the amphibian host are important for colonization and maintenance of these skin bacterial communities (Jiménez and Sommer 2017). In apparently healthy amphibians, bacterial skin communities associate to species across different isolated ponds (McKenzie et al. 2011). Amphibian skin communities can also resist colonization from repeatedly applied bacterial skin washes (Küng et al. 2014). However, other studies reveal amphibian skin bacteria can in fact be shaped by the surrounding environment. Amphibian skin microbial community metrics have been linked to climate and biosphere (Kueneman et al. 2019; Jani 2019), environmental substrate (Fitzpatrick and Allison 2014), and microhabitat type (Bletz et al. 2017a). This dynamic relationship between amphibian host and environment on skin microbiome composition is an important consideration during an assessment of wild amphibian health (Jani and Briggs 2018; Bernardo-Cravo et al. 2020). Associations between environmental degradation and altered host-associated microbial communities are documented in other wildlife species such as black howler monkeys (Amato et al. 2013) and corals (Krediet et al. 2013). In corals particularly, these host-associated microbial community changes have been further linked to decreased coral immunity (Glasl et al. 2016).

Hellbenders, *Cryptobranchus alleganiensis*, are fully aquatic riverine amphibians (Order Caudata) of the eastern United States and one of only three species of giant salamanders (family: Cryptobranchidae). Populations of both subspecies, the Ozark hellbender, *C. a. bishopi*, and eastern hellbender, *C. a. alleganiensis*, have experienced considerable declines since the early 1990's resulting in listing of the Ozark hellbender as endangered in 2011 (USFWS 2011). Declines in both subspecies are linked to habitat degradation and sedimentation from eroding stream banks (Humphries and Pauley 2005; Hopkins and DuRant 2011; USFWS 2011, Pugh et al. 2015), but disease is also hypothesized to play a role (Hardman et al. 2020a,b; Nickerson et al. 2011). In Ozark hellbenders of Arkansas (AR), over 93% of individuals had progressive distal limb lesions that ranged from toe swelling to ulceration and digital necrosis (Hardman et al. 2020a). Toe lesions are documented to occur and worsen over several years. There is no identified etiology but disease is likely manifesting from a multifactorial process (Hardman et al. 2020a). These lesions may be linked to environmental factors, although, host and infectious factors are hypothesized to contribute (Hardman et al. 2020a; Nickerson et al. 2011). For example, Ozark hellbenders have been identified to have decreased allelic diversity of genes important for adaptive immunity (Hernández-Gómez et al. 2018). Changes in the skin microbiome may help identify important aspects of disease pathophysiology. In humans, skin microbial community alterations can be associated with chronic skin wounds (Grice and Segre 2012; Scales and Huffnagle 2012; Pang et al. 2019) and chronic skin diseases such as psoriasis (Thio 2018). However, it is still speculative as to how changes in skin immunity may be associated with chronic toe lesions observed in wild hellbender populations.

Although there has been extensive research on how skin microbiomes may modulate susceptibility of amphibian hosts to *Bd* infection (Becker and Harris 2010; Bletz et al. 2017a; Jani and Briggs 2018; Bernardo-Cravo et al. 2020), there is a paucity of research on how these surface communities may be related to wound healing. Only one study has evaluated foot lesions in a small sample of Ozark hellbenders and found bacterial assemblages on toe lesions were richer and had differential abundance of core taxa when compared to the dorsal skin (Hernández-Gómez et al. 2017b). It will be important to also understand how lesion presence and severity may be associated with overall changes in the skin microbiome to better mitigate this disease. Furthermore, hellbenders are long-lived animals (> 20

years) and present an opportunity for us to understand the long-term effect of chronic disease in a wild amphibian species, particularly related to the association between chronic non-healing wounds and skin microbiomes.

Therefore, we evaluated dorsal skin bacterial assemblages of hellbender populations from two rivers of different conservation status: a stable population of eastern hellbenders in eastern Tennessee (ETN) and a declining and diseased population of Ozark hellbenders in Arkansas (AR). We further evaluated whether variation within the unhealthy AR population could be explained by toe lesion severity or *Bd* infection status. Lastly we evaluated how life stage and mass could help explain any observed variation.

Results

We sampled 63 individuals, 37 from AR and 26 from ETN (Fig. 1). We recovered 2,804,796 sequences assigned to 911 phylotypes after sequence processing in MOTHUR. Sequence counts per sample ranged from 3 to 379,352. Subsampling and quality control resulted in subsequent removal of 25 samples with less than 4000 sequences, leaving 40 samples remaining for analysis (15 AR and 25 ETN). Within the 15 AR samples, two were from individuals with mild toe lesions, seven with moderate lesions, and six with severe lesions. The 15 AR samples were also screened for the presence of *Bd*, using quantitative PCR (qPCR). This screening process identified six individuals that were *Bd* positive within the AR samples. We encountered and sampled juveniles and adults in ETN (nine and 16, respectively), but only adults were sampled from AR. Mass and subsequent size class data were available for all individuals sampled for both subspecies with eight small and eight medium adults in ETN and six medium and nine large adults in AR. Mass ranged from 3g – 807g with a median of 320g.

Subspecies, size, and life stage

Eastern hellbenders of ETN had greater relativized richness (Hill number $q = 0$) than Ozark hellbenders of AR ($p < 0.001$; Fig. 2, Table 1). Hill numbers for exponential Shannon Diversity ($q = 1$) and inverse Simpson ($q = 2$) were also greater for ETN compared to AR hellbender skin but were not significant ($p = 0.24$ and 0.53 , respectively; Fig. 2, Table 1). Average assemblages significantly differed between subspecies via both Sorenson Index ($F_{1,39} = 9.7$, $p < 0.001$, $R^2 = 0.21$) and Morisita-Horn ($F_{1,39} = 12.5$, $p < 0.001$, $R^2 = 0.25$). Non-metric Multidimensional Scaling (NMDS) ordination plots highlighted distinct grouping between subspecies and displayed increased beta diversity of AR hellbender skin assemblages compared to ETN (Fig. 4). This was confirmed via beta dispersion analysis (betadisper, Morisita-Horn, $p = 0.01$; Sorenson Index, $p = 0.002$). Mass was not predictive of alpha diversity for hellbender subspecies (Hill $q = 0$: $p = 0.48$, 1: $p = 0.90$, 2: $p = 0.83$; Table 1). Similarly, adult size class was not predictive of average assembly structure of hellbender subspecies (Sorenson Index: $p = 0.81$ Morisita-Horn: $p = 0.95$). Within ETN, no effect of life stage was seen for alpha diversity (Hill $q = 0$: $p = 0.19$, 1: $p = 0.19$, 2: $p = 0.77$; Table 1). Average assemblage structure of juvenile skin differed from adults using the Sorensen Index ($F_{1,24} = 1.8$, $p = 0.03$, $R^2 = 0.07$, Fig. 4), but not Morisita-Horn ($F_{1,24} = 0.4$, $p = 0.92$, $R^2 = 0.02$, Fig. 4). To remove potential effects of juvenile sampling on subspecies comparisons, we reran all subspecies analyses with only adults from ETN ($n = 16$) and found that the previously mentioned relationships remained unchanged (see Table 1, Fig. 2,4).

Table 1

Average Hill numbers (orders $q = 0, 1, 2$) for each factor comparison of skin microbial assemblage alpha diversity from the dorsum of eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*) of eastern Tennessee (ETN) and Ozark hellbenders (*Cryptobranchus alleganiensis bishopi*) of Arkansas (AR). Comparison groups were as follows: subspecies (ETN, AR), life stage within ETN only (juvenile, adult), toe lesion severity (mild, moderate, severe) within AR only, and *Batrachochytrium dendrobatidis* (*Bd*) infection status within AR only (*Bd*+, *Bd*-).

Factor	n	Hill Number (avg +/- SD)					
		q = 0		q = 1		q = 2	
Subspecies							
ETN	25	140	+/- 17.8	45.4	+/- 13.51	19.0	+/- 8.92
AR	15	111.4	+/- 23.9	40.3	+/- 12.3	20.1	+/- 8.49
Life Stage (ETN Only)							
Juvenile	9	133.0	+/- 24.3	44.3	+/- 18.0	19.8	+/- 12.5
Adult	16	144.0	+/- 12.1	46.0	+/- 10.9	18.6	+/- 6.6
Lesion Severity (AR Only)							
Mild	2	132.4	+/- 0.9	60.4	+/- 3.5	36.0	+/- 0.2
Moderate	7	104.3	+/- 23.7	34.8	+/- 5.4	16.5	+/- 3.7
Severe	6	112.7	+/- 26.1	39.9	+/- 13.5	19.0	+/- 8.0
<i>Bd</i> Infection Status (AR Only)							
<i>Bd</i> +	6	111.6	+/- 19.2	39.0	+/- 10.6	18.4	+/- 9.1
<i>Bd</i> -	9	111.2	+/- 27.8	41.1	+/- 13.9	21.2	+/- 8.4

Lesion severity and *Bd* infection within AR populations

Within AR, disparity between mild and moderate/severe lesion groups increased with increasing q order. Dorsal skin assemblages from animals with mild toe lesions had greater relativized richness compared to those with both moderate ($p = 0.18$), and severe ($p = 0.31$), and had significantly greater exponential Shannon Diversity ($q = 1$; $p = 0.009$ and 0.024 , respectively) and inverse Simpson ($q = 2$); $p = 0.010$ and 0.017 , respectively) (Fig. 3, Table 1). Average assemblage structure did not differ between lesion severity groups especially when relative abundance was not considered (Sorenson Index, $F_{2,14} = 0.85$, $p = 0.59$, $R^2 = 0.13$; Morisita-Horn, $F_{2,14} = 1.86$, $p = 0.08$, $R^2 = 0.24$; Fig. 5). Beta dispersion increased with increasing lesion severity for both Morisita-Horn and Sorenson Indices but was not significant ($p = 0.09$ and 0.12 , respectively; Fig. 5).

There was no effect of *Bd* infection status on measures of alpha diversity (Table 1, Fig. 3) or average assemblage structure (Sorenson Index, $F_{1,14} = 0.73$, $p = 0.68$, $R^2 = 0.05$; Morisita Horn, $F_{1,14} = 0.70$, $p = 0.56$, $R^2 = 0.05$; Fig. 5). There was no difference in beta dispersion between *Bd* infection-status groups of AR individuals for both Morisita-Horn and Sorenson Indices ($p = 0.80$ and 0.42 , respectively; Fig. 4)

Bacterial Taxonomic Analysis

We identified 468 phylotypes represented by 28 bacterial phyla during sequence processing. Ozark AR and eastern ETN hellbenders shared 312 out of 468 phylotypes, with 121 unique to ETN and 35 unique to AR. ETN had on average greater phylotype abundance of phylum Cyanobacteria (24.3%), Acidobacteria (2.7%), and Armatimonadetes (1.0%) compared to AR (5.9%, 1.1%, and 0.3%, respectively). Hellbenders of AR had a greater average relative abundance of Proteobacteria (48.5%), Firmicutes (9.1%), and Synergistetes (1.5%), in comparison to ETN with (31.5%, 2.6%, 0.2%, respectively) (Fig. 6, Table 2). For indicator species analysis, 65 phylotypes were considered indicative of ETN dorsal skin compared to 34 for AR (Table 3). There were three top indicators (IndVal Index stat > 0.9) for ETN which belonged to genus *Ferruginibacter*, an unclassified order of Cyanobacteria (incertae sedis), and an unclassified group of Verrucomicrobia (subdivision 3). Only one top phylotype (genus *Limnohabitans*) was identified for AR (Table 3).

Table 2

Average % phylum abundance of the 28 bacterial phyla identified from 1000 sequence subsamples of 16S sequencing from dorsal skin swabs of eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*) of eastern Tennessee (ETN) and Ozark hellbenders (*Cryptobranchus alleganiensis bishopi*) of Arkansas (AR). AR hellbenders are further grouped by toe lesion severity (mild, moderate serve) and *Batrachochytrium dendrobatidis* (*Bd*) infection status (*Bd*+, *Bd*). ETN hellbenders are further grouped by life stage (juvenile, adult).

Phylum	ETN Eastern Hellbender			AR Ozark Hellbender					
	All	Juvenile	Adult ETN	All	Mild	Moderate	Severe	<i>Bd</i> -	<i>Bd</i> +
Bacteria unclassified	7.54	6.93	7.88	5.91	7.65	7.51	3.47	5.37	6.73
Proteobacteria	31.49	31.51	31.48	48.48	41.00	56.81	41.25	48.59	48.32
Cyanobacteria	24.34	24.49	24.25	5.89	8.25	4.31	6.95	6.58	4.87
Planctomycetes	4.47	3.41	5.07	2.65	5.45	2.96	1.35	1.63	4.17
Bacteroidetes	11.96	13.14	11.29	11.91	16.65	7.79	15.15	10.76	13.65
Verrucomicrobia	4.12	3.11	4.69	2.17	2.75	2.51	4.02	3.52	2.58
Deinococcus-Thermus	1.63	2.56	1.11	3.15	0.80	0.30	0.10	0.27	0.32
OD1	0.72	0.90	0.62	0.29	0.55	1.37	0.67	1.20	0.65
TM7	0.98	0.93	1.01	0.98	6.35	5.24	3.67	4.58	5.03
Actinobacteria	4.54	3.63	5.04	4.76	2.15	1.06	0.85	1.09	1.17
Acidobacteria	2.69	2.12	3.01	1.12	5.20	1.76	1.65	1.80	2.73
Armatimonadetes	1.04	1.07	1.03	0.26	0.50	0.17	0.28	0.24	0.28
Firmicutes	2.58	3.59	2.01	9.13	0.85	5.01	16.70	10.61	6.92
Chloroflexi	0.60	0.18	0.17	0.39	0.40	0.44	0.32	0.24	0.60
WS3	0.17	0.58	0.61	0.05	0.05	0.00	0.10	0.02	0.08
Gemmatimonadetes	0.14	0.06	0.19	0.08	0.30	0.06	0.03	0.09	0.07
Nitrospira	0.13	0.14	0.13	0.07	0.15	0.06	0.07	0.10	0.03
Fusobacteria	0.14	0.20	0.11	0.48	0.05	0.10	1.07	0.67	0.20
Synergistetes	0.16	0.46	0.00	1.49	0.00	1.77	1.67	1.76	1.10
Chlamydiae	0.22	0.22	0.21	0.13	0.50	0.09	0.05	0.10	0.17
Spirochaetes	0.14	0.40	0.00	0.04	0.10	0.06	0.00	0.06	0.02
BRC1	0.03	0.29	0.05	0.01	0.05	0.00	0.00	0.01	0.00
Lentisphaerae	0.14	0.04	0.03	0.36	0.00	0.39	0.45	0.54	0.08
SR1	0.02	0.01	0.02	0.04	0.00	0.09	0.00	0.00	0.10
Chlorobi	0.00	0.00	0.01	0.08	0.25	0.06	0.05	0.06	0.12

Phylum	ETN Eastern Hellbender			AR Ozark Hellbender					
Deferribacteres	0.00	0.00	0.00	0.04	0.00	0.06	0.03	0.06	0.02
Elusimicrobia	0.00	0.00	0.00	0.04	0.00	0.03	0.07	0.07	0.00
Fibrobacteres	0.01	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table 3

List of indicator taxa based on indicator species analysis significantly associated with dorsal skin of hellbenders within each comparison group based on subspecies (eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*) of eastern Tennessee (ETN) and Ozark hellbenders (*Cryptobranchus alleganiensis bishopi*) of Arkansas (AR)), life stage within ETN (juvenile vs adult), lesion severity within AR (mild vs moderate vs severe) and *Batrachochytrium dendrobatidis* (Bd) infection status (Bd+ vs Bd-). No taxa were identified as significantly associated with animals with severe lesions. Taxa with a stat > 0.9 were considered top indicator taxa and are noted in bold.

Factor	Group	stat	p value	Phylum	Genus
Subspecies					
	ETN	0.954	0.001	Bacteroidetes	Ferruginibacter
	ETN	0.945	0.001	Cyanobacteria Chloroplast	Cyanobacteria order incertae sedis unclassified
	ETN	0.915	0.001	Verrucomicrobia	3 genus incertae sedis
	ETN	0.882	0.001	Cyanobacteria Chloroplast	<i>Bacillariophyta</i>
	ETN	0.879	0.001	Verrucomicrobia	<i>Opitutus</i>
	ETN	0.874	0.001	Acidobacteria	<i>Gp3</i>
	ETN	0.869	0.001	Armatimonadetes	<i>Armatimonas Armatimonadetes gp1</i>
	ETN	0.867	0.001	Actinobacteria	<i>Actinobacteria unclassified</i>
	ETN	0.864	0.001	Bacteroidetes	<i>Sphingobacteriales unclassified</i>
	ETN	0.862	0.001	Cyanobacteria Chloroplast	<i>GpI</i>
	ETN	0.855	0.001	Proteobacteria	<i>Myxococcales unclassified</i>
	ETN	0.854	0.001	Bacteroidetes	<i>Ohtaekwangia</i>
	ETN	0.846	0.001	OD1	<i>OD1 genus incertae sedis</i>
	ETN	0.841	0.001	Proteobacteria	<i>Betaproteobacteria unclassified</i>
	ETN	0.838	0.001	Cyanobacteria Chloroplast	<i>GpVI</i>
	ETN	0.836	0.001	Proteobacteria	<i>Proteobacteria unclassified</i>
	ETN	0.833	0.001	Actinobacteria	<i>Acidimicrobiales unclassified</i>
	ETN	0.830	0.002	Proteobacteria	<i>Alphaproteobacteria unclassified</i>
	ETN	0.826	0.001	Proteobacteria	<i>Hyphomicrobium</i>
	ETN	0.826	0.001	Bacteroidetes	<i>Flammeovirgaceae unclassified</i>
	ETN	0.825	0.001	Chloroflexi	<i>Ktedonobacterales unclassified</i>
	ETN	0.824	0.001	Proteobacteria	<i>Cellvibrio</i>
	ETN	0.819	0.001	Proteobacteria	<i>Rhodospirillales unclassified</i>

Factor	Group	stat	p value	Phylum	Genus
	ETN	0.807	0.002	Deinococcus-Thermus	<i>Deinobacterium</i>
	ETN	0.803	0.007	Verrucomicrobia	<i>Spartobacteria genera incertae sedis</i>
	ETN	0.802	0.015	Planctomycetes	<i>Planctomycetaceae unclassified</i>
	ETN	0.802	0.001	Proteobacteria	<i>Phaselicystis</i>
	ETN	0.800	0.001	Proteobacteria	<i>Bradyrhizobiaceae unclassified</i>
	ETN	0.790	0.01	Proteobacteria	<i>Gammaproteobacteria unclassified</i>
	ETN	0.784	0.02	Acidobacteria	<i>Gp16</i>
	ETN	0.781	0.033	Planctomycetes	<i>Planctomyces</i>
	ETN	0.781	0.021	Acidobacteria	<i>Gp4</i>
	ETN	0.777	0.01	Verrucomicrobia	<i>Verrucomicrobia unclassified</i>
	ETN	0.775	0.003	Deinococcus-Thermus	<i>Deinococcaceae unclassified</i>
	ETN	0.765	0.038	Planctomycetes	<i>Gemmata</i>
	ETN	0.762	0.002	Armatimonadetes	<i>Chthonomonas Armatimonadetes gp3</i>
	ETN	0.753	0.005	WS3	<i>WS3 genus incertae sedis</i>
	ETN	0.751	0.011	Proteobacteria	<i>Bradyrhizobium</i>
	ETN	0.748	0.041	Proteobacteria	<i>Deltaproteobacteria unclassified</i>
	ETN	0.748	0.001	Proteobacteria	<i>Rhizobacter</i>
	ETN	0.741	0.001	Proteobacteria	<i>Acetobacteraceae unclassified</i>
	ETN	0.721	0.005	Bacteroidetes	<i>Arcicella</i>
	ETN	0.721	0.001	Actinobacteria	<i>Conexibacter</i>
	ETN	0.701	0.005	Actinobacteria	<i>Pseudonocardia</i>
	ETN	0.693	0.003	Proteobacteria	<i>Undibacterium</i>
	ETN	0.693	0.002	Acidobacteria	<i>Gp1</i>
	ETN	0.683	0.014	Actinobacteria	<i>Solirubrobacterales unclassified</i>
	ETN	0.670	0.02	Proteobacteria	<i>Kofleria</i>
	ETN	0.662	0.037	Proteobacteria	<i>Anaeromyxobacter</i>
	ETN	0.658	0.009	Proteobacteria	<i>Polyangiaceae unclassified</i>
	ETN	0.646	0.021	Actinobacteria	<i>Nocardioideaceae unclassified</i>

Factor	Group	stat	p value	Phylum	Genus
	ETN	0.645	0.018	Cyanobacteria Chloroplast	<i>Cyanobacteria Chloroplast unclassified</i>
	ETN	0.638	0.014	Bacteroidetes	<i>Rudanella</i>
	ETN	0.632	0.013	Bacteroidetes	<i>Fibrella</i>
	ETN	0.632	0.012	Acidobacteria	<i>Gp2</i>
	ETN	0.632	0.007	Acidobacteria	<i>Gp5</i>
	ETN	0.631	0.02	Actinobacteria	<i>Marmoricola</i>
	ETN	0.620	0.026	Acidobacteria	<i>Geothrix</i>
	ETN	0.609	0.033	Proteobacteria	<i>Byssovorax</i>
	ETN	0.600	0.05	Planctomycetes	<i>Schlesneria</i>
	ETN	0.600	0.021	Chloroflexi	<i>Ktedonobacter</i>
	ETN	0.566	0.029	Acidobacteria	<i>Holophagaceae unclassified</i>
	ETN	0.566	0.025	Proteobacteria	<i>Rhodoferrax</i>
	ETN	0.562	0.048	Actinobacteria	<i>Sporichthya</i>
	ETN	0.529	0.048	Actinobacteria	<i>Phycococcus</i>
	AR	0.925	0.001	Proteobacteria	Limnohabitans
	AR	0.894	0.002	Proteobacteria	<i>Acinetobacter</i>
	AR	0.874	0.001	Bacteroidetes	<i>Cytophagaceae unclassified</i>
	AR	0.845	0.006	Proteobacteria	<i>Burkholderiales unclassified</i>
	AR	0.840	0.015	Proteobacteria	<i>Aeromonas</i>
	AR	0.836	0.008	Deinococcus-Thermus	<i>Deinococcus</i>
	AR	0.834	0.002	Proteobacteria	<i>Rhodobacteraceae unclassified</i>
	AR	0.832	0.002	Bacteroidetes	<i>Flavobacterium</i>
	AR	0.831	0.01	Proteobacteria	<i>Sphingomonas</i>
	AR	0.825	0.004	Verrucomicrobia	<i>Luteolibacter</i>
	AR	0.806	0.006	Actinobacteria	<i>Microbacteriaceae unclassified</i>
	AR	0.800	0.001	Proteobacteria	<i>Vogesella</i>
	AR	0.773	0.001	Proteobacteria	<i>Roseomonas</i>
	AR	0.760	0.001	Bacteroidetes	<i>Cryomorphaceae unclassified</i>
	AR	0.758	0.037	Verrucomicrobia	<i>Verrucomicrobiaceae unclassified</i>

Factor	Group	stat	p value	Phylum	Genus
	AR	0.740	0.041	Proteobacteria	<i>Xanthomonadaceae unclassified</i>
	AR	0.728	0.003	Bacteroidetes	<i>Chryseobacterium</i>
	AR	0.727	0.044	Bacteroidetes	<i>Flavobacteriaceae unclassified</i>
	AR	0.720	0.003	Actinobacteria	<i>Coriobacteriaceae unclassified</i>
	AR	0.699	0.002	Proteobacteria	<i>Methylobacter</i>
	AR	0.696	0.016	Proteobacteria	<i>Haliea</i>
	AR	0.664	0.005	Proteobacteria	<i>Pseudomonadaceae unclassified</i>
	AR	0.657	0.003	Proteobacteria	<i>Polynucleobacter</i>
	AR	0.642	0.015	Firmicutes	<i>Exiguobacterium</i>
	AR	0.627	0.02	Proteobacteria	<i>Dechloromonas</i>
	AR	0.626	0.005	Verrucomicrobia	<i>Opitutae unclassified</i>
	AR	0.608	0.037	Firmicutes	<i>Planococcaceae unclassified</i>
	AR	0.600	0.035	Firmicutes	<i>Clostridium XI</i>
	AR	0.598	0.028	Firmicutes	<i>Clostridiales unclassified</i>
	AR	0.563	0.019	Chlorobi	<i>Ignavibacterium</i>
	AR	0.516	0.016	Proteobacteria	<i>Methylosoma</i>
	AR	0.516	0.015	Proteobacteria	<i>Silanimonas</i>
	AR	0.500	0.036	Cyanobacteria Chloroplast	<i>Cryptomonadaceae</i>
	AR	0.488	0.039	Proteobacteria	<i>Phyllobacteriaceae unclassified</i>
Life Stage (ETN Only)					
	Adult	0.824	0.004	Planctomycetes	<i>Planctomyces</i>
	Adult	0.811	0.033	Verrucomicrobia	<i>3 genus incertae sedis</i>
	Adult	0.796	0.049	Actinobacteria	<i>Actinomycetales unclassified</i>
	Adult	0.792	0.028	Gemmatimonadetes	<i>Gemmatimonas</i>
	Adult	0.784	0.039	Planctomycetes	<i>Zavarzinella</i>
	Adult	0.777	0.019	Actinobacteria	<i>Solirubrobacterales unclassified</i>
	Adult	0.775	0.04	Proteobacteria	<i>Betaproteobacteria unclassified</i>
	Adult	0.744	0.029	Proteobacteria	<i>Undibacterium</i>

Factor	Group	stat	p value	Phylum	Genus
	Adult	0.707	0.026	Proteobacteria	<i>Haliea</i>
	Adult	0.707	0.024	Proteobacteria	<i>Inhella</i>
	Juvenile	0.867	0.002	Deinococcus-Thermus	<i>Deinococcus</i>
	Juvenile	0.833	0.013	Proteobacteria	<i>Acinetobacter</i>
	Juvenile	0.820	0.026	Proteobacteria	<i>Sphingomonas</i>
	Juvenile	0.809	0.022	Proteobacteria	<i>Oxalobacteraceae unclassified</i>
	Juvenile	0.785	0.04	Proteobacteria	<i>Methylobacterium</i>
	Juvenile	0.760	0.007	Proteobacteria	<i>Delftia</i>
	Juvenile	0.680	0.041	Cyanobacteria Chloroplast	<i>Chlorophyta</i>
	Juvenile	0.624	0.039	Proteobacteria	<i>Caulobacter</i>
	Juvenile	0.577	0.036	Bacteroidetes	<i>Chitinophaga</i>
	Juvenile	0.577	0.035	Bacteroidetes	<i>Cytophaga</i>
Lesion Severity (AR Only)					
	Mild	0.953	0.009	Verrucomicrobia	Opitutus
	Mild	0.947	0.011	Bacteroidetes	Emticicia
	Mild	0.920	0.021	Bacteroidetes	Ferruginibacter
	Mild	0.906	0.036	Proteobacteria	Myxococcales unclassified
	Mild	0.901	0.008	Planctomycetes	Zavarzinella
	Mild	0.892	0.026	Bacteroidetes	<i>Fluviicola</i>
	Mild	0.877	0.01	Gemmatimonadetes	<i>Gemmatimonas</i>
	Mild	0.874	0.046	Chlamydiae	<i>Parachlamydiaceae unclassified</i>
	Mild	0.873	0.025	Verrucomicrobia	<i>Verrucomicrobia unclassified</i>
	Mild	0.862	0.04	Proteobacteria	<i>Inhella</i>
	Mild	0.856	0.008	Bacteroidetes	<i>Sphingobacteriales unclassified</i>
	Mild	0.849	0.023	Proteobacteria	<i>Steroidobacter</i>
	Mild	0.846	0.041	Acidobacteria	<i>Gp7</i>
	Mild	0.846	0.036	Proteobacteria	<i>Desulfocapsa</i>
	Mild	0.846	0.029	Proteobacteria	<i>Hyphomicrobiaceae unclassified</i>
	Mild	0.846	0.022	Proteobacteria	<i>Haliea</i>

Factor	Group	stat	p value	Phylum	Genus
	Mild	0.837	0.047	Chlorobi	<i>Ignavibacterium</i>
	Mild	0.833	0.034	Proteobacteria	<i>Comamonadaceae unclassified</i>
	Mild	0.832	0.023	Verrucomicrobia	<i>Verrucomicrobium</i>
	Mild	0.828	0.018	Proteobacteria	<i>Xanthomonadaceae unclassified</i>
	Mild	0.826	0.021	Proteobacteria	<i>Rhodocyclaceae unclassified</i>
	Mild	0.817	0.024	Verrucomicrobia	<i>3 genus incertae sedis</i>
	Mild	0.816	0.034	OD1	<i>OD1 genus incertae sedis</i>
	Mild	0.804	0.022	Proteobacteria	<i>Alphaproteobacteria unclassified</i>
	Mild	0.796	0.04	Bacteroidetes	<i>Chitinophagaceae unclassified</i>
	Mild	0.794	0.02	Proteobacteria	<i>Rhodobacter</i>
	Mild	0.751	0.05	Proteobacteria	<i>Gammaproteobacteria unclassified</i>
	Moderate	0.761	0.027	Proteobacteria	<i>Burkholderiales unclassified</i>
<i>Bd</i> Infection Status (AR Only)					
	<i>Bd</i> -	0.888	0.05	Proteobacteria	<i>Aquabacterium</i>
	<i>Bd</i> +	0.902	0.029	Proteobacteria	Novosphingobium
	<i>Bd</i> +	0.881	0.035	Bacteroidetes	<i>Sediminibacterium</i>
	<i>Bd</i> +	0.878	0.032	Proteobacteria	<i>Sphingomonadaceae unclassified</i>
	<i>Bd</i> +	0.816	0.012	Chloroflexi	<i>Chloroflexi unclassified</i>
	<i>Bd</i> +	0.707	0.048	Proteobacteria	<i>Neisseriaceae unclassified</i>

A total of 136 phylotypes were shared between lesion severity groups within AR, with 17 unique to animals with mild lesions, 50 to animals with moderate lesions, and 48 to animals with severe lesions. Increased abundance in Firmicutes and Synergistetes was correlated with moderate and severe lesion groups for AR hellbenders (Fig. 7, Table 2). Indicator species analysis identified 27 phylotypes representative of individuals with mild toe lesions, only one for those with moderate lesions, and none for those with severe lesions (Table 3). Top indicator phylotypes (IndVal Index stat > 0.9) for mild lesions were species in the genera *Opitutus*, *Emticicia*, *Ferruginibacter*, *Zavarzinella*, and an unclassified of order Myxococcales. Five of the 27 indicator phylotypes for mild lesions within AR were also indicators for ETN, including three out of the five top mild lesion indicator taxa *Opitutus*, *Ferruginibacter*, and an unclassified order Myxococcales. The only indicator species for animals with moderate lesions was an unclassified phylotype in the order Burkholderiales.

Although bacterial alpha diversity and assemblage structure was not significant between *Bd* positive and negative individuals, indicator species analysis identified one phylotype (*Aquabacterium*) indicative of *Bd* negative skin

assemblages and five for *Bd* positive skin (Table 3). For life stage analysis within ETN, we identified a total of 10 phylotypes as indicative of adult skin, and 10 of juvenile skin, none with a IndVal Index stat > 0.9 (Table 3)

Discussion

Our results showed differences in dorsal skin bacterial assemblage structure and diversity between two subspecies of hellbenders from populations not previously sampled. Furthermore, we showed that variation in these communities could be explained by subspecies, severity of chronic lesions in the feet, and partially by life stage. *Bd* infection status and adult size class (mass) were not correlated with dorsal skin bacterial assemblages. Several factors including both environment and host health may be contributing to these differences. Host populations are separate subspecies from different ecoregions. Furthermore, the ETN eastern hellbender population we sampled is considered healthy and stable and surrounded by forested land (USFWS 2018; Da Silva Neto et al. 2019) whereas the AR Ozark population is in danger of extirpation with little surrounding forest buffer (USFWS 2018).

Distinction between ETN and AR dorsal skin microbial assemblage was greatest via Sorenson Index for average assemblage structure and via lower Hill numbers for alpha diversity. This highlights that subspecies differences were driven by presence of relatively rarer phylotypes. Similar to other studies, we determined that skin microbial assemblage structure of hellbenders differed by subspecies (Hernández-Gómez et al. 2017b, 2018). However, in contrast to those previous studies, we showed eastern hellbenders to have greater richness. We sampled Ozark hellbenders from AR and eastern hellbenders from ETN, whereas previous work focused on populations of both subspecies within Missouri (MO). This is an important distinction for eastern hellbender sampling. While Ozark hellbenders have a small contiguous range within the Ozark highlands of AR and MO only, eastern hellbenders span a large geographic range with eastern MO populations disjunct from all other eastern hellbenders and phylogenetically distinct from ETN (Unger et al. 2013; Hime 2017). Disparate results are further confounded by differential population health within the eastern hellbender subspecies. Eastern hellbenders of MO have been classified as an endangered population alongside the Ozark subspecies whereas the rest of the eastern hellbenders remain unlisted (USFWS 2018).

Interestingly, we found a trend of decreased alpha diversity with increased lesion severity with the apparently unhealthy Ozark hellbender population of AR. This, along with subspecies differences, may suggest that skin bacterial diversity is negatively associated with hellbender skin health. This is in contrast to Hernández-Gómez et al. (2017b) who found toe lesions to have richer assemblages than apparently healthy dorsal skin within a given individual. However, our study differed in that we compared dorsum between individuals of varying toe lesion severity, as opposed to direct comparison between lesioned and lesion-free skin within an individual. Furthermore, along with decreased diversity, we showed skin microbiomes of AR hellbenders, particularly those with severe lesions, had increased beta dispersion. This suggests severe lesions may be associated with uneven, random, and potentially dysbiotic, skin bacterial communities. In contrast to subspecies comparisons, dorsal skin bacterial assemblage differences between AR individuals of varying toe lesion severity were driven by relative abundance of more common phylotypes. A similar relationship was observed between dorsum and toe lesions in Ozark hellbenders of MO in that differences were driven by changes in relative abundance of shared OTUs (Hernández-Gómez et al. 2017b).

These distal limb lesions are progressive and can take several years to increase in severity (Hardman et al. 2020a), and the same factors affecting toe lesions may also be affecting dorsal skin communities. Changes we observed between AR hellbenders of varying toe lesion severity could represent a decreased ability to maintain an optimal microbial community as disease progresses in an individual. Another explanation could be that local microbial changes due to chronic lesions can eventually generate changes over the entire skin surface as has been seen around

chronic ulceration due to leishmaniasis in humans (Gimblet et al. 2017). Alternatively, shifts in microbial communities could be the main mechanism by which lesions manifest, and lesion severity represent a sequela of this dysbiosis that manifests specifically in an area of increased use (e.g. toes). The mechanism by which these patterns may arise is still speculative and it is possible these trends are not causal or are potentially spurious from limited sample size within AR ($n = 15$). More intensive within-river surveys alongside more detailed measurements of health (i.e., blood and immunological parameters, body condition score, and detailed lesion scoring) will be needed to better understand within watershed variation and drivers from host health and environment on changes in skin bacterial community parameters.

Unexpectedly, infection status of *Bd* had no effect on beta or alpha diversity. This was an unexpected result based on other studies of *Bd* infection being associated with skin microbial community shifts in amphibians (Jani and Briggs 2014; Muletz-Wolz et al. 2019). Wild hellbenders appear to be tolerant to *Bd* infection and there are no confirmed reports of chytridiomycosis in a wild hellbender despite the impressive amount of *Bd* surveillance studies with often 20–30% infection prevalence (Gonynor et al. 2011; Souza et al. 2012; Bales et al. 2015; Seeley et al. 2016; Hardman et al. 2020b). However, *Bd* may be an important secondary invader during instances of decreased skin immunity as chytridiomycosis is reported in captive and stressful conditions (Dean et al. 2016; Dusick et al. 2017), and *Bd* infection rates are positively associated with higher lesion scores in wild AR populations (Hardman et al. 2020a). Still, data from this study suggest that even if *Bd* affects AR hellbender skin health, it is not in association with changes in the skin bacterial community.

Life stage also had minimal effect on the bacterial skin community, at least within the ETN population for which we were able to sample juveniles. Similar to subspecies differences, community membership but not relative abundance, was predictive of hellbender life stage. Our study was limited in the fact that only two juveniles were still gilled larvae (pre-metamorphosis). Evaluation of larval and post-metamorphic juvenile substages within a single site may reveal significant microbiome shifts after metamorphosis we were unable to evaluate.

We found commonalities in indicator taxa that overlapped with those found in previous hellbender studies. The top indicator phylotypes for AR (genera *Limnohabitans* and *Acinetobacter*) belonged to families Comamonadaceae and Morallaceae, respectively. These taxa were previously identified with increased abundance in toe lesions compared to dorsal skin in Ozark hellbenders of MO (Hernández-Gómez et al. 2017b), and further characterized as significantly more abundant in Ozark vs eastern hellbender skin of MO (Hernández-Gómez et al. 2018). The genus *Acinetobacter* is of particular interest as an indicator taxon because this genus has been previously cultured from Ozark hellbender toe lesions (Nickerson et al. 2011) and is also highly correlated with differential amphibian host responses to *Bd* infection in both laboratory and field experiments (Bates et al. 2018). Similarly, the only indicator taxon for moderate lesions was an unclassified Burkholderiales, and an OTU in the Burkholderiales was recently shown to increase in abundance on skin of Sierra Nevada yellow-legged frogs, *Rana sierrae*, experimentally infected with *Bd* (Ellison et al. 2019).

Some phylotypes may associate with healthy hellbender skin. Several of the indicator taxa that distinguished dorsal skin assemblages of AR animals with mild versus moderate or severe toe lesions were the same that distinguished ETN from AR. One particular (genus *Ferruginibacter*) has also been identified as a core taxon on alpine newt (*Ichthyosaura alpestris*) skin (Bletz et al. 2017b).

No indicator phylotype for ETN eastern hellbenders from this study overlapped with taxa previously identified as significantly associated with eastern hellbender skin. As mentioned above, our study evaluated eastern hellbenders from ETN whereas the previous studies comparing hellbender subspecies evaluated those from MO. Populations

from ETN and MO vary greatly in phylogeny, ecoregion, and population health. Conversely, AR Ozark hellbenders are phylogenetically very similar to MO Ozark hellbenders and individuals sampled from our study were from one of the same watersheds sampled in these previous studies. It is no surprise then that our indicator species analysis revealed similar results for Ozark hellbenders yet disparate results for eastern hellbenders. The importance of specific taxa of bacteria and their function within a community context remains an understudied area in amphibian microbiome research. This study contributes to our understanding of the microbial assemblages of hellbenders and their correlation with skin health.

Conclusions

Our study demonstrated that bacterial assemblages on hellbender dorsal skin are not only associated with subspecies but could be shaped by skin health. Regardless of the immediate mechanism driving hellbender skin microbial communities, continued evaluation of their structure and diversity, when added to traditional amphibian disease surveillance, may provide a more comprehensive assessment of wild population health. We found a correlation between decreased dorsal skin bacterial assemblage similarity and richness within Ozark hellbenders compared to eastern hellbenders. This trend was further explained by severity of toe lesions in Ozark hellbenders. This pattern was not affected by presence of *Bd* on the skin and was likely related to other factors linked with chronic changes in the host and/or aquatic environment. These salamanders are long lived, river-dependent organisms with documented population declines associated with habitat changes, especially sedimentation. The skin environment is constantly exposed overtime to both this altered lotic environment as well as its secondary effects on host skin maintenance. Intensive sampling will be required in both healthy and unhealthy populations of both subspecies to disentangle factors affecting these important skin microbial communities, and to ultimately inform management to maintain and promote future healthy wild hellbenders.

Methods

We sampled hellbenders from 2014–2015 from mid-July to mid-August in two rivers, one from the Blue Ridge ecoregion of eastern Tennessee (ETN), the other in the Ozark Highlands of Arkansas (AR) (Fig. 1). We sampled in ETN for the eastern hellbender (*C. a. alleganiensis*) and in AR for the Ozark hellbender (*C. a. bishopi*). All sampling in Tennessee with approval from Tennessee Wildlife Resources Agency (TWRA) (Permit # 1877 (RHH)) and in AR under observation of the USFWS permit TE66039A-0 issued to KJI and Arkansas Game and Fish Commission. In TN, and shallower water in AR, we used standard snorkeling techniques to locate individuals. In AR, we also sampled hellbenders via artificial nest boxes (Briggler and Ackerson 2012) and sampled deep water habitats (up to 4 meters) using a hookah dive system (i.e. gasoline powered air compressor with tethered air supply lines to dive regulators). We captured any animals encountered under cover objects and nest boxes and placed them in a clean, soft cotton or mesh bag. We kept bags submerged in the river before and after animals were processed. (University of Tennessee IACUC protocol # 2481 – 0916). We changed dive gloves between animal captures to reduce pathogen transfer and contamination of samples. Skin microbiome samples were collected from a subset of hellbenders surveyed for disease and amphibian pathogen prevalence (see Hardman et al. 2020 a,b). We placed each individual in a new, cleaned plastic tub and rinsed the dorsum with approximately 200 mL sterile distilled water. We immediately rubbed a sterile cotton swab (Fisherbrand product# 23400111) over the dorsal skin surface for 30 seconds and placed it in a sterile 1.5 mL tube on dry ice for transport to a -80°C freezer. For individuals in AR only, we also obtained a swab for *Bd* load analysis for a concurrent pathogen prevalence study (see Hardman et al. 2020b), as well as, lesion severity score (scored from 0–7) for toe lesions of unknown etiology (Hardman et al. 2020a). All individuals sampled from AR for the microbiome analysis had toe lesions, but because of low sample size within AR, and subsequent inability to

compare across all 8 lesion scores, we binned scores of 0–7 into three biologically relevant severity levels of mild (score 0–2; toe swelling only), moderate (3–4; toe swelling and some shortened) and severe (5–7; most toes shortened and/or missing).

Amplicon Library Sequence Preparation

We extracted DNA from skin swabs using ZR Fungal/ Bacterial DNA Miniprep kits (Zymo Research Corporation, Irvine, CA, USA) and sent extracted DNA to Hudson Alpha (Huntsville, AL) for 16S rDNA amplicon sequencing on the Illumina MiSeq platform (v2 2 x 250) of the V3-V4 region of 350 bp length. Bioinformatic preparation of the resulting sequence data was performed in the program MOTHUR, following the MiSeq protocol (Schloss et al. 2009) with minor modifications including the creation of an oligo file of primer sequences to recognize the target V3-V4 region from primer trimming after making contigs. We used a phylotype based approach to assign phylotypes to the rank of genus based on the Silva v128 reference database (Quast et al. 2013; Yilmaz et al. 2014).

Statistical Analysis

We performed all subsequent cleanup and analyses using RStudio v1.2.5019 (R Studio Team 2016). We removed rare phylotypes with less than five total sequence reads or occurring in less than three samples. We also removed samples with less than 4000 sequence reads based on rarefaction curves performed in package Vegan (Oksanen et al. 2015). To compare alpha diversity among samples we used packages Vegetarian (Charney and Record 2012) and Vegan to calculate an averaged 1000 iteration bootstrapped value generated from 1000 sequence subsamples for the following Hill numbers: relativized richness ($q = 0$), exponential of Shannon's index ($q = 1$) and the inverse Simpson's index ($q = 2$). We used Hill numbers as they provide better evaluation of changes in both richness and evenness (Chao et al. 2014). We performed the same bootstrap technique to calculate beta diversity distance matrices of the Sorenson Index and Morisita-Horn to evaluate structural changes based on phylotype presence/absence and relative abundance, respectively. We performed all statistical comparisons of both beta and alpha diversity in package Vegan. For alpha diversity analysis we normalized each response variable (either natural log or cubed transformation) and performed an analysis of variance (ANOVA) across all samples to test for variation in alpha diversity by subspecies and mass. We performed a second ANOVA from AR samples only to test for variation in alpha diversity by *Bd* infection status and lesion severity within this population. A final ANOVA was performed to evaluate variation in alpha diversity that correlated with life stage effects (juvenile vs adult) within ETN only where both adults and juveniles were encountered. We used PERMANOVA (adonis – 999 permutations) to test for differences in average assemblage structure using Sorenson Index and Morisita-Horn distances and previously mentioned categorical predictors mentioned for the alpha diversity analysis. The only exception was that mass was binned into natural breaks of adult size class of small (150–299g), medium (300–449), and large (450+) adults. We also assessed beta diversity as a multivariate dispersion (community consistency among samples) by calculating the multidimensional area of minimum convex polygons fit to clusters using the 'betadisp' function within vegan based on distances created from principal coordinates analysis (PCoA) from previously calculated Sorenson and Morisita-Horn indices. For visualization of alpha diversity differences, we created boxplots for each Hill number for each parameter using package ggplot2 (Wickham 2016). For visualization of beta diversity between these same groups we created nonmetric multidimensional scaling (NMDS) plots based on distance matrices via packages Vegan and ggplot2.

Bacterial Taxonomic Analysis

We calculated and graphed phylotype abundance by bacterial phylum for a single subsample of 1000 sequences for each sample in package phyloseq (McMurdie and Holmes 2013). Finally, we identified significant indicator phylotypes for each comparison group via the indicspecies package (De Cáceres and Legendre 2009) using the

'multipatt' function with 'IndVal' setting, considering top indicator phylotypes those with a p value < 0.05 and IndVal Index stat > 0.9.

Abbreviations

AR

ETN

OTU

MO

ANOVA

NMDS

Declarations

Ethics approval and consent to participate

This study was approved by the University of Tennessee IACUC Committee. Protocol #2481

Consent for publication

Not applicable

Availability of data and materials

The datasets supporting the conclusions of this article are available in the NCBI SRA repository, [PRJNA755662, <http://www.ncbi.nlm.nih.gov/bioproject/755662>] and included within the article additional files (for sequence and SRA metadata and MOTHUR and R code, respectively).

Competing interests

The authors declare that they have no competing interests

Funding

Funding was provided by the Arkansas Game and Fish Commission

Authors' contributions

RHH contributed to project inception, field and laboratory work, data analysis and manuscript writing. GL contributed to project inception, sample preparation, data analysis, and manuscript editing. BF contributed to data analysis, and manuscript editing. KJI contributed to project inception, acquisition of funds and field collection. WBS, JGD, and MJF contributed to field collection, manuscript editing, and sample transport. DMW contributed to data analysis. DLM contributed to project inception, acquisition of funds, project oversight, and manuscript editing.

Acknowledgements

We would like to thank the staff of the Arkansas Game and Fish Commission and Arkansas office of the USFWS, especially Chris Davidson and Erin Knoll for help with Arkansas field work. Thank you to Jessica Braunstein and Ruth Freeman for help with Tennessee field work. Thanks to Davis Carter for help with laboratory preparation.

Authors' information

Endnotes

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Figures

Figure 1

Map of United States displaying historical range of hellbenders (*Cryptobranchus alleganiensis*) with cross hatching and zoomed insert displaying relative location of field sampling denoted by hellbender icons in Arkansas (AR) and eastern Tennessee (ETN). Ozark hellbenders (*C. a. bishop*) were sampled in AR along with collection *Batrachochytrium dendrobatidis* (Bd) skin swabs and observations of toe lesions. Eastern hellbenders (*C. a. alleganiensis*) were sampled in ETN where both adult and juveniles were encountered.

Figure 2

Alpha diversity comparison of bacterial 16S assemblages from dorsal skin swabs of hellbenders (*Cryptobranchus alleganiensis*) using Hill numbers from order $q=0$ (relativized richness), $q=1$ (exponential of Shannon Index) and $q=2$ (inverse Simpson's Index). Comparison between eastern hellbenders (*C. a. alleganiensis*) of eastern Tennessee (ETN) and Ozark hellbenders (*C. a. bishopi*) of Arkansas (AR). ETN hellbenders are further grouped into juveniles and adults.

Figure 3

Alpha diversity comparison of bacterial 16S assemblages from dorsal skin swabs of Ozark hellbenders (*Cryptobranchus alleganiensis bishopi*) using Hill numbers from order $q=0$ (relativized richness), $q=1$ (exponential of Shannon Index) and $q=2$ (inverse Simpson's Index). Comparisons between individuals with varying toe lesion severity (mild, moderate, severe) in top row, and individuals with differing *Batrachochytrium dendrobatidis* (Bd) infection status (Bd + vs Bd -) in bottom row.

Figure 4

NMDS ordination plots of hellbender skin bacterial assemblages based on Sorenson and Morisita-Horn Indices. Each point represents a single skin bacterial assemblage from dorsal skin swabs. Groupings by subspecies of eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*) of eastern Tennessee (ETN) (blue) and Ozark hellbenders (*Cryptobranchus alleganiensis bishopi*) of Arkansas (red) with associated ellipses base on standard error. ETN hellbenders are further grouped by life stage of juvenile (gray-blue) and adult (dark blue).

Figure 5

NMDS ordination plots of hellbender skin bacterial assemblages based on Sorenson and Morisita-Horn Indices. Each point represents a single skin bacterial assemblage from dorsal skin swabs of Ozark hellbenders (*Cryptobranchus alleganiensis bishopi*) of Arkansas (AR). Top plots grouped by toe lesion severity of mild (yellow), moderate (orange), or severe (red). Bottom plots groups by *Batrachochytrium dendrobatidis* (Bd) infection status of Bd + (green) or Bd - (gray).

Figure 6

Phylum abundance of the 28 bacterial phyla identified from 1000 sequence subsamples of 16S sequences obtained from dorsal skin swabs from eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*) captured in eastern Tennessee (ETN) and Ozark hellbenders (*Cryptobranchus alleganiensis bishopi*) in Arkansas (AR). ETN hellbenders are further grouped by life stage of juvenile (ETN-Juv) and adult (ETN-AR). Only adults were encountered for AR (AR-Ad).

Figure 7

Phylum abundance of the 28 bacterial phyla identified from 1000 sequence subsamples of 16S sequences obtained from dorsal skin swabs from Ozark hellbenders (*Cryptobranchus alleganiensis bishopi*) captured in Arkansas (AR) grouped by toe lesion severity of mild, moderate, and severe.

Supplementary Files

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