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SPECIAL ISSUE: THE HOST-ASSOCIATED MICROBIOME: PATTERN, PROCESS AND FUNCTION

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Influence of immunogenetics, sex and body condition on the cutaneous microbial communities of two giant salamanders

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Abstract

The complex association between hosts and microbial symbionts requires the implementation of multiple approaches to evaluate variation in host physiology. Within amphibians, heterogeneity in immunogenetic traits and cutaneous microbiota is associated with variation in disease resistance. Ozark (*Cryptobranchus alleganiensis bishopi*) and eastern hellbenders (*C. a. alleganiensis*) provide a model system to assess variation in host traits and microbial communities. Ozark hellbenders have experienced declines throughout their range, are federally endangered and experience wound retardation that is absent in the eastern subspecies. Previous microbial investigations indicate differentiation in the composition of the skin microbiota of both hellbender subspecies, but it is not clear whether these patterns are concurrent with diversity in the major histocompatibility complex (MHC) genes. We characterized the MHC IIB and the skin microbiota of hellbenders in Missouri, where both subspecies co-occur though not sympatric. We compared the microbiota composition and MHC diversity between both subspecies and investigated whether individual-level MHC diversity, sex and body condition were associated with microbiota composition. Overall, MHC IIB diversity was lower in Ozark hellbenders compared to the eastern subspecies. Multivariate statistical comparisons identified microbiota differentiation between Ozark and eastern hellbenders. MHC IIB allele presence/absence, allele divergence, body composition and sex defined grouping of hellbender microbiotas within populations. Differentiation of the cutaneous microbiotas and MHC IIB genes between eastern and Ozark hellbenders suggests that differences exist in immunity between the two subspecies. This study demonstrates how simultaneous assessments of host genetic traits and microbiotas can inform patterns of microbial community structure in natural systems.

KEYWORDS
Cryptobranchus alleganiensis, hellbender, major histocompatibility complex, skin microbiota, trans-species polymorphism

1 | INTRODUCTION

Characterization of animal microbiomes has shed light on important associations between hosts and microbes. Recent work describes the role of microbial symbionts in the maintenance of important aspects of host physiology including metabolism and immunity (Costello, Stagaman, Dethlefsen, Bohannan, & Relman, 2012). Contributions provided by the microbiome often depend on the composition of the community (Costello et al., 2012; Round & Mazmanian, 2009). Variation in immunity/metabolism among hosts is associated with differences in symbiont community composition (Lam, Walke, Vredenburg, & Harris, 2010; Shafquat, Joice, Simmons, & Huttenhower,

2014). Composition of microbial symbiont communities is shaped by fundamental ecological processes (i.e., selection, competition, speciation, dispersal; Christian, Whitaker, & Clay, 2015). Habitat selection within the host environment (e.g., immune system response) can act as a strong influence in the assembly of host-associated microbial communities (Shafquat et al., 2014). In the case of immunological attack, functional overlap can exist between the host immune system and members of the microbiome. Thus, investigating host traits along with microbiome characterization is important to develop a full understanding of patterns of phenotypical variation among hosts.

Amphibians provide an opportunity to characterize the contributions of host traits and microbial community composition to host phenotypes. Composition of the skin microbial communities is attributed to host susceptibility among populations to cutaneous pathogens (Lam et al., 2010). Several amphibian skin bacteria produce antifungal metabolites that inhibit the growth of the lethal fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) in vitro and in vivo (Harris et al., 2009). As a result, previous research investigated how pressures from the host environment (e.g., temperature, land use, microbial reservoirs; Krynak, Burke, & Benard, 2016; Longo, Savage, Hewson, & Zamudio, 2015; Loudon et al., 2014) and host characteristics (e.g., body composition, immunity, ontogeny; Colombo, Scalvenzi, Benlamara, & Pollet, 2015; Kohl, Amaya, Passement, Dearing, & McCue, 2014; Kueneman et al., 2014; Longo et al., 2015) correlate with diversity and composition of amphibian cutaneous microbial communities. In addition to microbial contributions, other studies describe host factors such as adaptive immunity (Savage & Zamudio, 2011, 2016), innate immunity (Rollins-Smith et al., 2006; Woodhams, Voyles, Lips, Carey, & Rollins-Smith, 2006), body condition (Venesky et al., 2012) and neutral genetic diversity (Pearman & Garner, 2005) that correlate with observed heterogeneity in host immunity. Simultaneous influence of host filters on symbiont microbes and pathogen colonization suggests a complex system dominating immunity on the skin of amphibians. Therefore, it may be beneficial to investigate synergistic effects of host and skin microbial characteristics to patterns of disease resistance within natural populations.

Immunogenetic traits (e.g., major histocompatibility complex-MHC) are known to influence microbial community composition in other systems (Bolnick et al., 2014; Kubinak et al., 2015). The MHC class II genes encode for cell-surface glycoproteins that are expressed on antigen-presenting cells such as macrophages, dendritic and B cells (Cresswell, 1994). Function of the MHC class II involves presenting peptides derived from extracellular pathogens to components of the immune system (Kaufman, Flajnik, & Du Pasquier, 1985). MHC class II-expressing dendritic cells and Langerhans lymphocytes are present on the amphibian skin (Carrillofarga, Castell, Perez, & Rondan, 1990), indicating a possible link between MHC class II genes and cutaneous microbes. In fact, individual MHC class IIB genotype is associated with heterogeneity in resistance to the lethal amphibian skin fungus *Bd* (Savage & Zamudio, 2011, 2016). Despite the evidence for MHC expression on the skin of amphibians, no research has evaluated possible relationships between these loci and the skin symbiont community assembly of amphibians. To effectively manage for disease susceptibility among populations of amphibians, it is imperative to evaluate host characteristics (e.g., immunity or health status), skin microbiome and their interactions.

Hellbenders (*Cryptobranchus alleganiensis*) provide an ideal model to describe immune traits, host-symbiont communities and the interaction between these components. Currently, two hellbender subspecies are recognized: the Ozark (*C. a. bishopi*) and eastern hellbender (*C. a. alleganiensis*). Ozark hellbenders are endemic to south draining streams in the Ozark Plateau of southern Missouri and northern Arkansas (Nickerson & Mays, 1973). In contrast, eastern hellbenders inhabit rivers throughout the eastern United States, including a disjunct, isolated population in the north draining streams of the Missouri Ozark Plateau (Mayasich, Grandmaison, & Phillips, 2003). Genetic assessments using both mitochondrial (Sabatino & Routman, 2009) and microsatellite markers (Tonione, Johnson, & Routman, 2011) indicate that the two subspecies of hellbenders within Missouri are paraphyletic. In the past 30 years, hellbenders have faced population declines throughout their range (Wheeler, Prosen, Mathis, & Wilkinson, 2003). Declines in the Ozark subspecies resulted in federal listing under the Endangered Species Act in 2011 (Federal Register 2011). Along with population loss, most adult Ozark hellbenders experience chronic wounds that result in tissue necrosis on the head and limbs (Hernández-Gómez, Kimble, Briggler, & Williams, 2017b; Hiler, Wheeler, & Trauth, 2005; Nickerson et al., 2011; Wheeler, McCallum, & Trauth, 2002). While *Bd* and ranaviruses are not associated with the chronic wounds, opportunistic bacterial agents are suspected (Briggler et al., 2007; Hernández-Gómez et al., 2017b; Nickerson et al., 2011). In contrast, chronic skin lesions are not observed in eastern hellbenders within Missouri (J. Briggler, unpublished data). A previous investigation of the skin microbiota of hellbenders in Missouri suggests divergence in composition between subspecies; however, that study was limited in that it included individuals from only one population of each subspecies (Hernández-Gómez et al., 2017b). Moreover, it remains unclear whether the composition of the skin microbial communities is linked to diversity in the MHC class II genes.

We set out to characterize the MHC class IIB (MHC IIB) and the skin microbiota of Ozark and eastern hellbenders in Missouri. Our objectives were to (i) investigate differences in individual MHC IIB allele divergence (i.e., amino acid and nucleotide divergence) between the two subspecies, (ii) assess compositional differences of the cutaneous microbiota between the two subspecies of hellbenders and (iii) evaluate variation in the composition of the skin microbiota in relation to host traits (body condition, sex, presence/absence of MHC IIB alleles and amino acid divergence). We expected to observe differences in individual MHC IIB allele divergence between the two subspecies, with Ozark hellbenders possessing significantly lower individual amino acid divergence than eastern hellbenders. In addition, we predicted differentiation

in the skin microbiota of the subspecies, with the Ozark subspecies associated with potentially pathogenic lineages. Finally, we anticipated a negative correlation between individual MHC IIB diversity and richness of the skin microbiota and an effect of host traits on microbial community composition.

2 | MATERIALS AND METHODS

2.1 | Field methods

We sampled hellbenders between 24 August and 29 October 2015 within six rivers in Missouri (Ozark hellbenders: North Fork of the White, Eleven Point and Current River; eastern hellbenders: Niangua, Gasconade and Big Piney River; Table S1). We handled all hellbenders following an approved protocol by the Purdue University Animal Care and Use Committee (PACUC protocol # 1406001094). We captured hellbenders by hand after lifting any boulder-sized rocks within the stream as in Burgmeier et al. (2011) and sampled the skin microbiota from the dorsum of each individual following the protocol of Hernández-Gómez et al. (2017b). We measured each hellbender's total body length and mass using a portable CS-series 2,000 g scale (OHAUS Corp., Parsippany, NJ). We also collected 1–2 drops of blood using a 21 or 23-gage, 1.5-inch needle from the caudal vein of each hellbender and preserved each blood sample in lysis buffer (1 M Tris, 0.5 M EDTA pH 8.0, 5 M NaCl, 20% SDS). We returned all hellbenders to their original location of capture within the river after sampling was complete. After sampling was completed in each river, we collected 2 L of water 1 to 10 m upstream from where sampling began. We stored the river water in ice or in a −20°C freezer until filtering occurred in an aseptic environment using Whatman #1 11-μm filter paper (GE Healthcare, Chicago, IL). Water filters and skin swab samples were stored in liquid nitrogen until return to the laboratory.

2.2 | Laboratory methods

2.2.1 | MHC IIB amplification

We extracted genomic DNA from each individual's blood sample using a standard proteinase K/phenolchloroform/isoamyl alcohol procedure (Sambrook & Russell, 2001) and resuspended in 100 μl of TLE buffer (10 mM Tris–Cl, 0.1 mM EDTA). The MHC IIB of hellbenders had not been characterized before; therefore, we used a hellbender spleen transcriptome data set to characterize the MHC IIB of this species. Detailed information on our MHC IIB characterization protocol is located in MHC Characterization Methods, Supplemental Information. To amplify the MHC IIB gene, we performed 25 μl PCRs consisting 150 ng of template DNA, 1× MyTaq MasterMix (Bioline, Taunton, MA) and 0.4 μM of forward and reverse hellbender MHC IIB primers. PCR conditions consisted of 2 min at 94°C for 2 min, 35 cycles of 94°C for 30 s, 58.9°C for 30 s and 72°C for 30 s, followed by 72°C for 10 min. We cleaned the PCR products using a low-ethanol precipitation protocol (Sambrook & Russell, 2001).

2.2.2 | 16S rRNA amplification

We isolated DNA from river water samples using the PowerWater DNA isolation kit (MoBio Laboratories Inc., Carlsbad, CA) following manufacturer's instructions. We isolated DNA from hellbender skin swabs using the PowerSoil DNA Isolation Kit (MoBio Laboratories Inc.) following the protocol described in Hernández-Gómez et al. (2017b). In short, we amplified the bacterial 16S rRNA V2 region using primer pair 27F/338R (Fierer, Hamady, Lauber, & Knight, 2008) with the attachment of connector sequences (Hernández-Gómez et al., 2017b). We ran each sample in triplicate, and each reaction consisted of 5 μl of template DNA, 1× MyTaq Master Mix, 0.4 μM of forward and reverse primers, and 6.5 μl of PCR water (MoBio Laboratories Inc.) 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 16S rRNA amplicon triplicates and cleaned the products using the UltraClean PCR Clean-Up kit (MoBio Laboratories Inc.).

2.2.3 | Barcoding and sequencing

We performed a second PCR on 16S rRNA and MHC IIB amplicons to add-on dual-index barcodes connected to Illumina sequencing adaptors (Hernández-Gómez et al., 2017b) to the ends of amplicons. The PCR consisted of 5 μl of clean amplicons, 1× MyTaq Master Mix, 0.4 μM 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). We pooled MHC barcoded amplicons in equimolar amounts and purified on a 2% agarose gel. We isolated amplicons from the band corresponding to the expected amplified product size using a custom gel extraction protocol (Gel Extraction Protocol, Supplemental Information). We pooled 16S rRNA amplicons in equimolar amounts and cleaned using the UltraClean PCR Clean-Up kit. The 16S rRNA and MHC amplicons were sequenced separately on a MiSeq machine (Illumina Inc. San Diego, CA) using the Reagent Kit V2 to produce 250-bp paired end reads.

2.3 | Amplicon sequence analysis

We processed raw amplicon sequencing reads using Trimmomatic (Bolger, Lohse, & Usadel, 2014) 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 (Masella, Bartram, Truszkowski, Brown, & Neufeld, 2012). Only reads that paired successfully were employed in subsequent analysis.

2.3.1 | MHC genotyping

We performed our MHC IIB amplicon sequence analysis to differentiate between true allele reads from error variants in order to

determine each hellbender's allelic make-up. We wrote a custom PYTHON program to count sequencing depth for each variant within each individual sample (Python Code, Supplemental Information). We made a list of all unique variants and obtained the maximum per amplicon frequency depth (MPAF) for each variant among all samples following Radwan, Biedrzycka, and Babik (2010). To discern between true alleles and sequencing artefacts, we uploaded the unmultiplexed read file to AMPLISAS (Sebastian, Herdegen, Migalska, & Radwan, 2016). AMPLISAS is a web browser interface that performs read demultiplexing, variant clustering and putative allele filtering based on user-specified criteria. We implemented AMPLISAS's default settings to remove any samples with less than 100 read depth and set the max number of alleles per amplicon to ten. In addition, we directed AMPLISAS to filter out chimeric sequences and discard any variants within each individual if sequencing depth fell below the calculated MPAF (4.5%). This threshold excludes erroneous variants that do not cluster along with parental alleles (e.g., unremoved chimeras, contaminant reads, errors beyond the clustering algorithm). Because allele richness may be underrepresented in samples with low sequencing depth, we decided to test for a bias of sample coverage on individual allele richness through a correlation test (Stutz & Bolnick, 2014). We detected a nonsignificant and negligible correlation between these two variables; thus, we felt comfortable proceeding with allele analysis. We BLASTed all variants designated as true alleles against the NCBI GENBANK nucleotide database (Benson et al., 2013). We only retained variants that matched to the MHC class IIB of *Andrias davidianus* or other closely related amphibian species at an *E*-value <0.05.

2.3.2 | Microbiota sequence analysis

Our microbiota sequence analysis consisted of established read processing pipelines to filter erroneous reads, cluster reads into operational taxonomic units (OTUs) and generate abundance-based OTU tables. We used a custom PYTHON program to remove quality scores and rename reads with a name compatible with the chosen pipeline (Hernández-Gómez et al., 2017b). We processed the resulting read file using the QUANTITATIVE INSIGHTS INTO MICROBIAL ECOLOGY version 1.8.0 (QIIME) pipeline (Caporaso et al., 2010b). We clustered reads at the standard 97% similarity using the open-reference protocol (Rideout et al., 2004) and the GREENGENES 13_5 reference database (DeSantis et al., 2006). Reads that failed to cluster using the open-reference algorithm were clustered into de novo OTUs with UCLUST (Edgar, 2010). OTUs that clustered using the GREENGENES database retained the accorded taxonomy, while de novo OTUs were assigned taxonomy using the RDP Classifier (Wang, Garrity, Tiedje, & Cole, 2007) at 80% confidence. We aligned representative sequences to the pre-aligned GREENGENES reference using PYNAST (Caporaso et al., 2010a) and used the alignment to produce a phylogenetic tree through FASTTREE (Price, Dehal, & Arkin, 2010). 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, had matched taxonomy to chloroplasts, or were absent from more than one sample (Bokulich et al., 2013).

2.4 | Data analysis

To compare MHC IIB diversity between eastern and Ozark hellbenders, we first evaluated differences in population MHC IIB diversity and individual allele divergence between subspecies. We then compared the microbiota between the two subspecies by assessing differences in community richness/diversity, community structure and associations with bacterial families. Finally, we tested for a correlation between host traits and microbiota richness/composition of the skin microbiota of hellbenders. We performed all statistical analyses in R version 3.3.1 unless otherwise noted.

2.4.1 | MHC IIB divergence analysis

We uploaded the MHC IIB allele sequences to MEGA 6.0 and aligned the sequences using CLUSTALW (Larkin et al., 2007). To assess evidence of positive selection at the MHC IIB PBR in hellbenders, we calculated Tajima's D (Tajima, 1989). Tajima's D evaluates whether deviation from neutral processes exists in the MHC IIB. A positive Tajima's D value corresponds with positive/balancing selection or a population bottleneck, while a negative value suggests the presence of negative selection or a recent population expansion. We built an integer neighbour-joining haplotype network (French et al., 2013) through POPART (http://popart.otago.ac.nz) to visualize MHC IIB allele relationships across the populations sampled. We were unable to assign alleles to individual MHC IIB loci; therefore, we could not calculate individual-level heterozygosity. Instead, to assess differences in population-level MHC IIB genetic variation between the two subspecies, we calculated average per cent difference (APD) for each subspecies as described in Yuhki and Obrien (1990). APD is a measure of the average percentage of sequences that differ among individuals in each subspecies.

For each allele, we calculated Poisson corrected amino acid distances, nucleotide distances (Kimura 2-parameter model), and average rates of synonymous and nonsynonymous substitution using the Nei-Gojobori and Jukes Cantor correction for multiple substitutions. For all individuals, we derived average amino acid and nucleotide distances (e.g., divergence) among its alleles. We implemented a quasibinomial generalized linear model to assess differences in individual amino acid diversity and nucleotide diversity between both hellbender subspecies.

2.4.2 | Host species' microbiota comparisons

We compared alpha metrics of adult hellbender skin microbiota samples between the two hellbender subspecies. Before calculating alpha metrics, we rarefied the OTU table to 3,917 sequences per sample to standardize depth across all samples. We calculated

community richness (observed OTUs) and diversity (Shannon diversity) values on each sample in QIIME using the relative abundance-based OTU table. To evaluate whether OTU richness differs between the two subspecies, we implemented community richness as a dependent variable, subspecies identity as a fixed factor and river locality as a random variable in a Poisson regression model. To evaluate whether Shannon diversity differs between the two subspecies, we implemented individual subspecies identity as an independent variable, and river locality as a random variable and Shannon values as dependent variables in a generalized linear mixed model.

We transferred an OTU table containing only river water data and corresponding Newick phylogenetic tree to R. We implemented the packages GUNIFRAC (Chen et al., 2012) and VEGAN 2.2-1 (Oksanen, Kindt, Legendre, O'Hara, & Stevens, 2007) to build UniFrac distance (unweighted and weighted; Lozupone, Lladser, Knights, Stombaugh, & Knight, 2011) and Bray–Curtis dissimilarity matrices. For each matrix, we consolidated river water community variation using a principal component analysis. We retained the river scores of the first principal components for each distance matrix as variables in subsequent analyses. To reduce individual variance caused by rare skin OTUs specific to each population, we filtered out OTUs represented in less than 50% of individuals (nonubiquitous OTUs) from each population from the prerarified hellbender skin OTU table (removing 317 OTUs and ~12.7% of total depth). We rarified this OTU table to 3,419 sequences per sample to equalize depth across all samples and transferred the OTU table and corresponding Newick phylogenetic tree to R. We built UniFrac distance (unweighted/weighted) and Bray–Curtis dissimilarity matrices for all animal samples as well. We performed Adonis tests using the UniFrac/Bray–Curtis matrices to partition the variation between each subspecies. For each Adonis test, we also included river water community PC loadings from the corresponding distance matrix as grouping variable as well. We visualized differences in community structure among subspecies and populations using unweighted UniFrac distances through a principle co-ordinate analysis (PCoA) generated through the R package ADE4 (Dray & Dufour, 2007).

We implemented the LINEAR DISCRIMINANT ANALYSIS EFFECT SIZE (LEFSE) algorithm described in Segata et al. (2011) to test significant differences in OTU relative abundance between the two subspecies. The LEFSE algorithm identifies the OTUs whose abundance statistically differs between the subspecies through a nonparametric factorial Kruskal–Wallis rank-sum test ($\alpha < 0.05$). Subsequently, a pairwise Wilcoxon test is used to assess whether pairwise comparisons between rivers within each subspecies significantly agree with the subspecies-level trend. Finally, the algorithm generated 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 the assigned subspecies. OTU taxonomy data were retrieved from GREENGENES assignments performed in QIIME with an additional search of sequences in the Ribosomal Database Project (RDP) to confirm taxonomy or resolve unassigned sequences (Cole et al., 2014).

2.4.3 | Associations between host traits and the skin microbiota

To test associations between host traits and the skin microbiota, we generated an OTU table containing only samples for which we possessed sex information, MHC and microbiota data. Within each subspecies, we tested whether individual MHC IIB amino acid divergence had an association with OTU richness. We performed this analysis using a negative binomial generalized linear mixed model with river locality as a random variable.

High bacterial species turnover (e.g., replacements) has been described between individuals from separate sampling localities (Hernández-Gómez, Hoverman, & Williams, 2017a); thus, to limit variation from OTU replacements, we assessed individual-level effects on microbiota composition within each river separately. We recalculated UniFrac distance (unweighted/weighted) and Bray–Curtis dissimilarity matrices for individuals within each population (Big Piney/Gasconade River, Niangua River, North Fork of the White River, Current River and Eleven Point River). We combined samples from the Big Piney and Gasconade Rivers because we only obtained both MHC/microbiota data for a limited number of individuals from each population (Big Piney River: $n = 5$; Gasconade River: $n = 4$), and these two populations are genetically similar (Crowhurst et al., 2011). For each beta diversity matrix, we implemented distance-based redundancy analysis (dbRDA) to assess linear relationships between explanatory variables and grouping of samples (Legendre & Anderson, 1999). We tested for significant grouping of samples based on individual MHC IIB amino acid divergence, presence of common MHC IIB alleles (present in >3 individuals and <100% of individuals in the population), body condition (i.e., mass/total body length least squares regression residuals; Schulte-Hostedde, Zinner, Millar, & Hickling, 2005) and sex. We tested the significance of explanatory variables using a NPMANOVA at 999 permutations. For the Big Piney/Gasconade River analysis, we included river as a conditional variable to partition out variation created by OTU turnover. Within each dbRDA performed, we implemented stepwise elimination of nonsignificant variables and repeated the analysis until all variables in the model became significant at a $p < .10$.

3 | RESULTS

We collected a total of 25 eastern and 29 Ozark hellbender blood and microbiota samples (Table S1). Raw MHC IIB PBR amplicon sequencing data consisted of 1,113,312 raw reads with an average length of 130 base pairs. After quality filtering, we confidently characterized 25 true MHC IIB alleles of 192 bp length in 21 eastern and 28 Ozark hellbenders. However, we discarded one allele (CralDAB*17) because it did not BLAST to an MHC IIB domain in the NCBI database. 16S rRNA V2 amplicon sequencing resulted in 2,043,110 reads with an average length of 318 base pairs. After filtering out reads by base pair length, we processed the remaining reads through QIIME using the open-reference clustering method to return 1,422

OTUs for all skin and river water samples after OTU abundance filtration and rarefaction.

3.1 | MHC divergence analysis

Eastern hellbenders shared fewer MHC IIB alleles than Ozark hellbenders as evidenced by higher APD values (Ozark ADP ± *SE*: 75.31 ± 0.75; eastern ADP ± *SE*: 85.67 ± 1.10). Within both subspecies, we characterized between two to five alleles of the MHC IIB per individual, indicating the presence of at least three loci. Tajima's D value for the 24 alleles was 1.34, corresponding to a signal of positive selection. One allele (Cral-DAB*02) was recovered with varying frequency among populations of both subspecies, and this allele was more closely related to Ozark hellbender specific alleles than to those of the eastern subspecies (Figure S1). Within the Ozark subspecies, the North Fork of the White River population possessed the highest MHC allelic richness ($n = 8$) compared to the Eleven Point ($n = 7$) and Current River ($n = 4$) populations (Figure 1). In addition, alleles Cral-DAB*02 and Cral-DAB*03 were common within the Current and Eleven Point River populations. The Niangua River possessed the highest allele richness ($n = 8$) in the eastern subspecies compared to the Gasconade ($n = 4$) and Big Piney ($n = 4$) River populations (Figure 1). Alleles Cral-DAB*02 and Cral-DAB*04 were highly frequent in the Big Piney and Gasconade River populations. Ozark hellbenders possessed lower amino acid (Ozark mean ± *SE*: $0.17 \pm 1.4 \times 10^{-4}$; eastern mean ± *SE*: $0.26 \pm 3.4 \times 10^{-4}$; $F_{1,46} = 6.49$, $p = .014$) and nucleotide divergence (Ozark mean ± *SE*: $0.09 \pm 8.2 \times 10^{-5}$; eastern mean ± *SE*: $0.16 \pm 2.1 \times 10^{-4}$; $F_{1,46} = 9.01$, $p = .004$) than the eastern subspecies.

3.2 | Host subspecies microbiota comparisons

We characterized differentiation in the skin microbiota between eastern and Ozark hellbenders. OTU richness and evenness between the two hellbender subspecies were comparable (observed OTUs: LRT = 7.00×10^{-4}, $p = .977$; Shannon: LRT = 8.81×10^{-2}, $p = .767$), and both subspecies shared a considerable portion (~83.7% of total OTUs) of their microbiota with each other (Figure 2). All hellbenders shared a mean ± *SE* of 38.61 ± 0.020% of their OTUs with river water bacteria. We removed 317 OTUs after filtering nonubiquitous OTUs at each river locality (~12.7% of total read depth). The multivariate tests noted larger significant differences in the skin microbiota of eastern and Ozark hellbender using all beta diversity matrices (Adonis—unweighted UniFrac: $R = .12$, $p < .001$; weighted UniFrac: $R = .11$, $p < .001$; Bray–Curtis: $R = .12$, $p < .001$) compared to environmental microbiome variation (Adonis—unweighted UniFrac: $R = .06$, $p = .003$; weighted UniFrac: $R = .06$, $p = .014$; Bray–Curtis: $R = .06$, $p = .010$). The PCoA plots comparing both hellbender subspecies display distinct grouping by subspecies ID among coordinates derived from the unweighted UniFrac (Figure 3) and abundance-based metrics (Figure S2), indicating a pattern of strong OTU turnover among the subspecies. The LEFSE

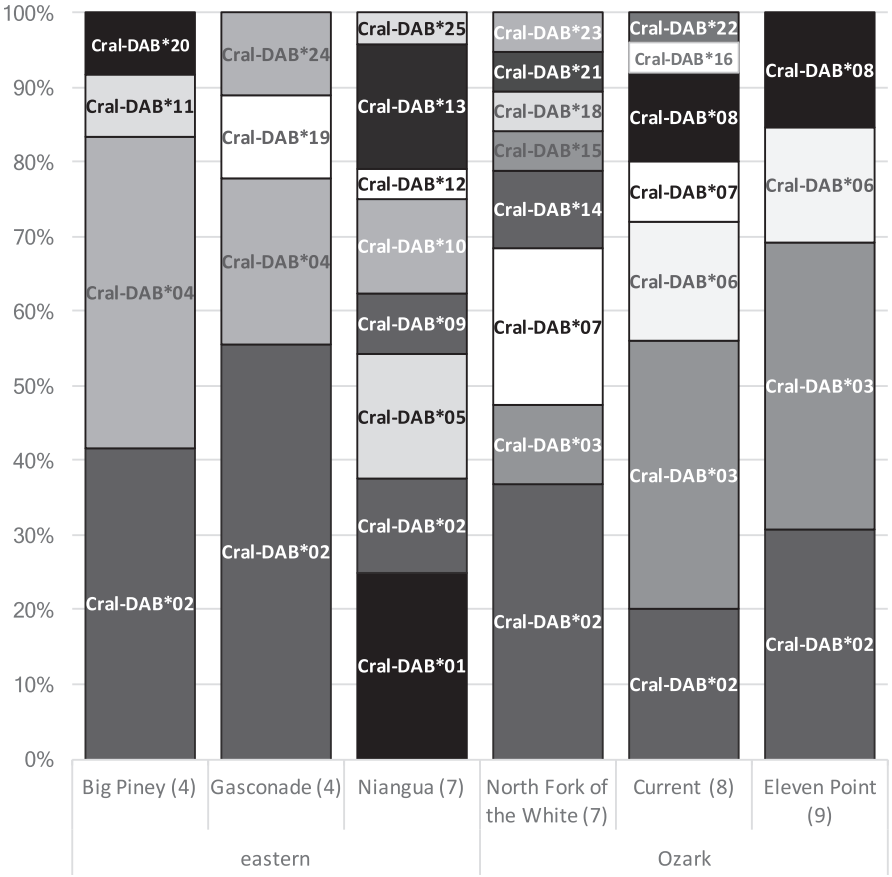

FIGURE 1 Hellbender major histocompatibility complex class IIB (MHC IIB) allele distribution. Allele frequency and distribution throughout six populations of eastern and Ozark hellbenders in Missouri. Sample sizes within each population are presented in parenthesis

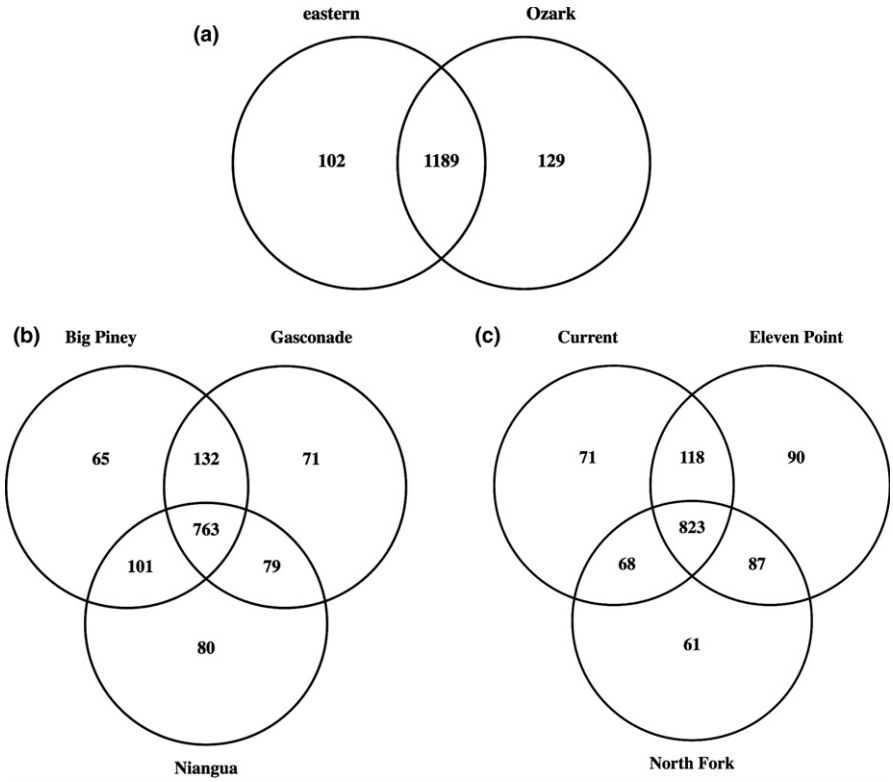

FIGURE 2 Venn diagram summarizing the overlap of hellbender skin microbiota OTUs. Diagrams portray overlap (a) between eastern (*Cryptobranchus alleganiensis alleganiensis*) and Ozark (*C. a. bishop*) hellbenders, (b) among populations of eastern hellbenders and (c) among populations of Ozark hellbenders

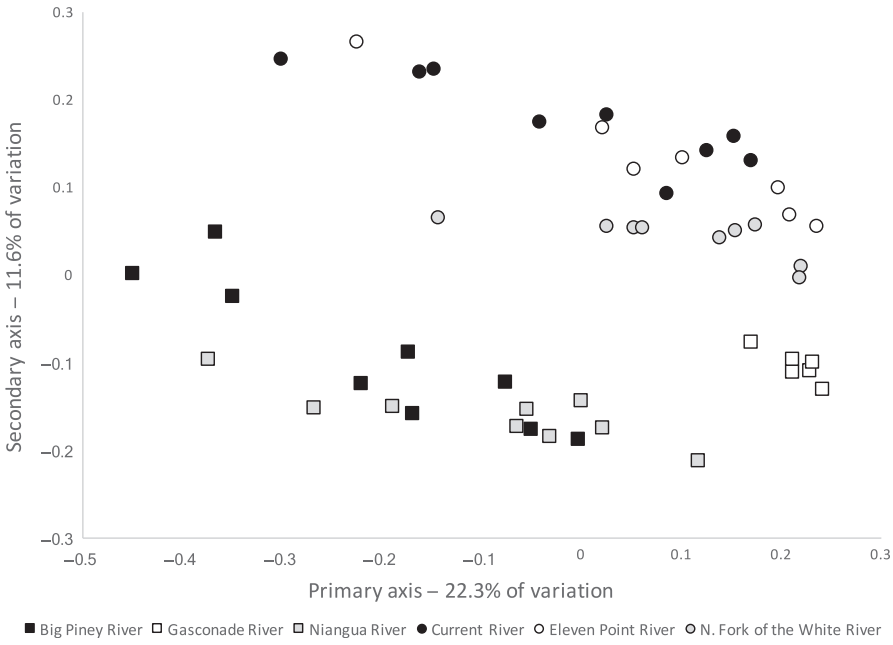

FIGURE 3 Principal co-ordinate analysis (PCoA) of unweighted UniFrac distances. Comparison between skin microbial communities of eastern (*Cryptobranchus a. alleganiensis*) and Ozark (*C. a. bishopi*) hellbenders across populations in each subspecies range. Total separation of individual points by subspecies identity (squares—eastern hellbenders or circles—Ozark hellbenders) is visible

algorithm identified 41 distinct bacterial OTUs between Ozark and eastern hellbenders (Table 1) at an LDA greater than 3.0. Twenty OTUs were associated with Ozark hellbenders, whereas 21 were associated with the eastern subspecies. The abundant OTUs (mean relative abundance >0.01) assigned to the family Comamonadaceae (OTU ID New.ReferenceOTU40) and order Bacteroidales (New.ReferenceOTU11) were associated with the skin of Ozark hellbenders. In addition, the skin of the Ozark subspecies was also associated with multiple rare OTUs assigned to the phyla Proteobacteria and Bacteroidetes, including the genera *Flavobacterium* (334370, 254696, New.ReferenceOTU530, and 114965), *Enhydrobacter* (New.CleanUp.ReferenceOTU69054, 574102 and New.CleanUp.ReferenceOTU28354), *Fluviicola* (702999), *Anaerospora* (New.CleanUp.ReferenceOTU59416) and *Acinetobacter* (4391687). In contrast, the only abundant OTU significantly assigned to the eastern subspecies was *Luteolibacter* sp (New.ReferenceOTU165). Members of the phyla

TABLE 1 Bacterial OTUs assigned to Ozark (*C. a. bishopi*) or eastern hellbenders (*C. a. alleganiensis*) at LDA > 3.0

			Eastern			Ozark			
Operational taxonomic unit	LDA	*p*-Value	BP	GA	NI	CU	EP	NF	Taxonomy
Eastern hellbenders									
New.ReferenceOTU165	4.88	<.001	2.6E-01	7.6E-02	1.6E-01	1.6E-03	2.2E-02	3.7E-02	Genus *Luteolibacter*
1054065	3.68	<.001	9.2E-03	2.1E-02	6.2E-04	0	0	0	Family Cerasicoccaceae
New.ReferenceOTU27	3.63	<.001	1.3E-02	1.9E-03	8.6E-03	0	0	0	Genus *Leptospira*
545460	3.44	.001	5.7E-03	1.3E-02	8.8E-04	0	1.2E-03	1.3E-04	Family ACK-M1
336935	3.39	.001	7.6E-03	8.4E-03	5.2E-04	0	1.0E-03	3.2E-04	Family ACK-M1
4426763	3.25	.001	2.7E-03	4.0E-03	5.7E-03	6.5E-04	3.8E-04	8.1E-04	Family Comamonadaceae
New.ReferenceOTU314	3.24	.014	3.0E-03	9.8E-03	2.8E-03	1.3E-04	1.9E-03	2.5E-03	Unassigned
4466646	3.24	<.001	6.4E-03	3.0E-03	4.2E-04	0	0	6.5E-05	Genus *Limnohabitans*
817953	3.24	.002	9.2E-03	5.1E-03	2.5E-03	2.3E-04	0	1.4E-02	Genus *Vogesella*
New.ReferenceOTU558	3.17	<.001	1.1E-03	3.6E-03	4.5E-03	0	0	3.2E-05	Phylum Chloroflexi
589360	3.12	.004	1.9E-04	9.7E-05	0	0	0	0	Genus *Crenothrix*
97094	3.11	.035	5.5E-04	0	0	0	0	0	Family Microbacteriaceae
New.ReferenceOTU74	3.10	<.001	4.2E-03	2.4E-03	0	0	0	0	Family Methylophilaceae
319691	3.09	<.001	4.5E-03	1.6E-03	8.8E-04	0	2.1E-04	1.6E-04	Family ACK-M1
New.CleanUp.ReferenceOTU47594	3.09	.033	1.9E-04	0	3.9E-04	0	0	1.3E-04	Family Methylophilaceae
New.CleanUp.ReferenceOTU60167	3.09	<.001	3.2E-03	3.0E-03	4.9E-04	0	0	0	Family Neisseriaceae
576960	3.08	<.001	3.5E-03	4.5E-03	2.9E-04	0	9.6E-04	0	Genus *Fluviicola*
New.ReferenceOTU444	3.06	.001	3.5E-03	2.7E-03	0	0	0	0	Genus *Flavobacterium*
729833	3.06	<.001	2.2E-03	4.0E-03	6.5E-04	0	0	0	family Armatimonadaceae
New.ReferenceOTU266	3.05	<.001	3.6E-04	0	5.5E-04	0	0	0	Genus *Flavobacterium*
New.CleanUp.ReferenceOTU52612	3.02	<.001	1.6E-04	6.7E-03	4.2E-04	0	0	0	Unassigned
Ozark hellbenders									
New.ReferenceOTU40	4.74	.024	2.1E-01	8.4E-02	2.4E-01	3.1E-01	2.5E-01	3.3E-01	Family Comamonadaceae
New.ReferenceOTU11	4.56	.005	9.1E-02	0	5.8E-02	1.7E-01	1.6E-01	8.4E-02	Order Bacteroidales
New.CleanUp.ReferenceOTU69054	3.68	.022	0	0	0	1.6E-04	4.2E-05	0	Genus *Enhydrobacter*
334370	3.64	<.001	7.5E-04	3.9E-04	2.9E-04	2.4E-02	3.3E-03	3.4E-03	Genus *Flavobacterium*
574102	3.53	.004	6.6E-03	1.4E-02	5.2E-04	9.6E-03	2.7E-02	8.0E-03	Genus *Enhydrobacter*
229011	3.43	.018	4.9E-03	3.4E-03	3.6E-04	9.0E-03	1.1E-02	3.4E-03	Family Cytophagaceae
254696	3.37	<.001	3.6E-04	8.3E-04	0	4.6E-03	5.6E-03	4.6E-03	Genus *Flavobacterium*
New.CleanUp.ReferenceOTU59416	3.35	.022	0	0	0	0	1.7E-04	1.3E-04	Genus *Anaerospora*
New.CleanUp.ReferenceOTU28354	3.27	.022	0	0	0	3.2E-05	2.1E-04	3.2E-05	Genus *Enhydrobacter*
New.ReferenceOTU104	3.26	<.001	0	0	1.6E-04	8.2E-03	1.5E-03	6.2E-04	Genus *Flavobacterium*
114965	3.21	<.001	0	0	0	5.2E-04	4.2E-04	8.5E-03	Genus *Flavobacterium*
New.CleanUp.ReferenceOTU47527	3.21	.043	0	0	0	3.2E-05	1.7E-04	0	Order Actinomycetales
702999	3.21	<.001	0	0	0	1.5E-03	6.2E-03	2.0E-03	Genus *Fluviicola*
New.CleanUp.ReferenceOTU38842	3.19	.043	0	0	0	0	4.2E-05	1.3E-04	Family Moraxellaceae
815062	3.16	<.001	2.9E-04	0	1.0E-03	1.1E-03	5.6E-03	2.7E-03	Genus *Leptolyngbya*
288283	3.12	.001	3.2E-03	4.1E-03	4.5E-04	6.0E-03	7.7E-03	3.4E-03	Family Caulobacteraceae
New.ReferenceOTU566	3.06	.003	0	4.9E-05	0	1.9E-04	1.7E-04	1.6E-04	Phylum Bacteroidetes
4391687	3.02	<.001	0	1.9E-04	0	4.6E-03	2.4E-03	4.2E-04	*Acinetobacter johnsonii*
444649	3.01	.003	0	0	0	2.9E-04	2.1E-04	0	Order Bacillales
358178	3.01	.001	0	7.1E-03	0	4.5E-03	1.2E-03	2.5E-03	Genus *Exiguobacterium*

A list of OTUs, LDA scores, mean relative abundance within each population (BP—Big Piney, GA—Gasconade, NI—Niangua, CU—Current, EP—Eleven Point, NF–North Fork of the White) and OTU taxonomy is displayed. Cell colour is assigned based on relative abundance value from 0 (white) to 0.33 (black).

TABLE 2 Host trait and hellbender (*Cryptobranchus alleganiensis*) skin microbial community turnover

Distance matrix	Factor	Pseudo-F	*p*-value	% variation
Ozark hellbenders				
Eleven Point River				
Unweighted UniFrac	Sex	3.63	.047	42.1
Current River				
Weighted UniFrac	Sex	3.49	.049	32.9
Bray–Curtis	Sex	1.89	.087	21.3
North Fork of the White River				
Unweighted UniFrac	Cral-DAB*02	1.83	.059	48.5
	Cral-DAB*07	2.88	.005	
Bray–Curtis	Cral-DAB*02	1.99	.021	42.8
	Cral-DAB*07	1.74	.054	
Eastern hellbenders				
Niangua River				
Unweighted UniFrac	AA	4.13	.001	37.1
Weighted UniFrac	AA	5.10	.002	71.9
	Cral-DAB*05	3.26	.026	
	Body condition	4.41	.017	
Bray–Curtis	Body condition	3.64	.065	50.4
	AA	2.45	.088	

Statistical values for distance-based redundancy analyses (dbRDA) of individual major histocompatibility complex (MHC) IIB amino acid divergence (AA), allele presence, body composition and sex on skin microbiota composition (unweighted/weighted UniFrac/Bray–Curtis distances) in Ozark and eastern hellbender populations. Only significant tests on variables ($p < 0.01$) through backwards selection are presented for each distance matrix.

Actinobacteria (family ACK-M1: 545460, 336935 and 319691; family Microbacteriaceae: 97094), Armatimonadetes (family Armatimonadaceae: 729833), Bacteroidetes (genus *Fluviicola*: 576960; genus *Flavobacterium*: New.ReferenceOTU2666 and New.ReferenceOTU444), Proteobacteria (family Comamonadaceae: 4426763; genus *Limnohabitans*: 4466646; family Methylophilaceae: New.ReferenceOTU74 and New.CleanUp.ReferenceOTU47594; family Neisseriaceae: New.CleanUp.ReferenceOTU60167; genus Vogesella: 817953; genus Crenothrix: 589360), Spirochetes (genus Leptospira: New.ReferenceOTU27) and Verrucomicrobia (family Cerasicoccaceae: 1054065) were among the rare OTUs associated with the eastern subspecies skin.

3.3 | Associations between host traits and the skin microbiota

We noted no significant associations between individual MHC IIB amino acid divergence and skin community richness in the Ozark subspecies (LTR = 0.32, $p = .569$). When testing for correlations between Ozark hellbender skin microbiota samples and host traits, we detected significant grouping of samples defined by sex within the Eleven Point and Current River populations and the presence of alleles Cral-DAB*02/Cral-DAB*07 within the North Fork of the White River population (Table 2). Within eastern hellbenders, we observed a significant positive relationship between individual MHC IIB amino acid divergence and microbial community richness (LTR = 3.04, $p = .081$; estimate = 1.54 ± 0.77; $z = 1.99$; $p = .047$). In regards to correlations between host traits and the skin microbiota of eastern hellbenders, we did not detect significant grouping of samples defined by these variables in the Big Piney/Gasconade River populations (Table 2). However, in the Niangua River population, we identified significant grouping of samples by amino acid divergence using unweighted UniFrac distances/Bray–Curtis dissimilarities (Table 2). Amino acid divergence, the presence/absence of allele Cral-DAB*05 and body condition dictated grouping of samples within the Niangua River population based on weighted UniFrac distances (Table 2). Allele Cral-DAB*05 was a relatively common allele within the Niangua population (Figure 1). In addition, Cral-DAB*05 was highly divergent (Figure S1) compared to other alleles present in Niangua individuals with an average of 0.240 amino acid substitutions per site.

4 | DISCUSSION

We implemented culture-independent microbiota characterization and MHC IIB sequencing to describe two known components of amphibian immunity for the two subspecies of hellbenders. We identified low MHC IIB allele richness between populations of each subspecies, and one allele (Cral-DAB*02) present across all populations of hellbenders sampled. In addition, Ozark hellbenders possessed lower MHC IIB allele divergence than eastern hellbenders. Our

exploration of the cutaneous microbiota of hellbenders revealed bacterial OTUs that significantly associate with one subspecies over the other. We also observed correlations between the skin microbiota composition and host MHC IIB amino acid divergence, common allele presence and sex. These results support the role of subspecies identity as an important determinant of skin bacterial communities in hellbenders, and the importance of MHC IIB genotype as a selective force on bacteria on the skin of amphibians.

4.1 | MHC IIB variation between Ozark and eastern hellbenders

We identified between one and five alleles per individual in this study, suggesting that hellbenders possess at least three MHC IIB loci. This estimate differs from gene copy numbers observed in other Cryptobranchids ($n = 2$, Zhu et al., 2014), urodeles ($n = 1$, Babik et al., 2009; $n = 1$, Bos & DeWoody, 2005) and anurans ($n = 2$, Kiemnec-Tyburczy, Richmond, Savage, & Zamudio, 2010). However, our methodology did not allow us to define copy number of these genes and accurately genotype every individual. Given the evidence of disproportionate levels of long terminal repeat retrotransposons contributing to genome expansions in hellbenders (Sun & Mueller, 2014), duplication of the MHC is a possibility. In addition, primer bias and cross-contamination may result in preferential amplification of specific alleles leading to an underrepresentation of allelic richness. By simply considering allele presence, we may be overestimating individual amino acid divergence. Still, we can make conservative inferences regarding the distribution of MHC IIB alleles between both subspecies of hellbenders, and potential associations between individual-level allele divergence and microbiota composition. Numerous other investigations have resorted to implementing similar methods when handling multigene families as the MHC in nonmodel organisms (Miller, Allendorf, & Daugherty, 2010; Whittaker, Dapper, Peterson, Atwell, & Ketterson, 2012; Yuhki & Obrien, 1990).

The presence of allele Cral-DAB*02 in populations of both hellbender subspecies corresponds with patterns of trans-species polymorphism (TSP) in the MHC (Figure 1). TSP refers to the retention of identical or similar alleles in closely related species (Klein, Sato, Nagl, & O'hUigín, 1998). Patterns of TSP have been heavily documented in the MHC of multiple taxa due to balancing selection maintaining beneficial alleles within populations (Těšický & Vinkler, 2015). Within amphibians, cases of TSP in the MHC have been described in *Lithobates* spp. (Kiemnec-Tyburczy et al., 2010), *Ambystoma* spp. (Bos & DeWoody, 2005) and *Xenopus* spp. (Bos & Waldman, 2006). In hellbenders, allele Cral-DAB*02 likely predates the divergence of the two hellbender subspecies. Mitochondrial genome analysis has been used to describe the family *Cryptobranchidae* as a monophyletic group, with divergence of the genus *Andrias* (Asian giant salamanders) and *Cryptobranchus* (North American giant salamanders) around 70 MYA (Zhang & Wake, 2009). Furthermore, mitochondrial and microsatellite markers describe the root of the *Cryptobranchus* genus lying within populations of the Current/Eleven Point rivers (currently Ozark hellbender inhabited) and the New/Tennessee Rivers in the southern Appalachia (currently eastern hellbenders; Sabatino & Routman, 2009). Ozark and eastern hellbenders expanded their range following glacial meltdown 11,000 years ago (Sabatino & Routman, 2009). We did not find Cral-DAB*02 within the list of alleles that Zhu et al. (2014) characterized in the hellbender's sister species, *Andrias davidianus*. Therefore, it is likely that Cral-DAB*02 originated after divergence of *Andrias* and *Cryptobranchus* 70 MYA and has been maintained in North American *Cryptobranchus* populations through balancing selection (e.g., heterozygote advantage, negative frequency dependent selection). The presence of this allele through the divergence of eastern and Ozark hellbenders suggests it may offer a degree of selective advantage for disease resistance.

Lower MHC IIB allele divergence in the Ozark subspecies compared to the eastern hellbender is among the first concrete explanations for the unique presence of chronic wounds in Ozark hellbenders. Infectious disease resistance has been positively associated with MHC allele divergence in other systems as well (Lenz, Wells, Pfeiffer, & Sommer, 2009), suggesting an advantage of divergent allele genotypes in pathogen detection (Wakeland et al., 1990). Thus, differences in MHC IIB allele divergence may contribute to immunity differences between the two subspecies of hellbenders. Decreased MHC divergence corresponds with risk of population extirpations due to future disease challenges (Radwan et al., 2010). As a result, current conservation management programs should consider incorporating approaches to preserve MHC diversity among both subspecies of hellbenders. Captive breeding and rearing of both hellbender subspecies is employed by the Missouri Department of Conservation and the St. Louis Zoo-Ron Goellner Center for Hellbender Conservation (Ettling et al., 2013). Data from neutral genetic makers are currently considered prior to arrangement of captive mating pairs and wild population supplementation (Ettling et al., 2013). This program provides the opportunity to also pair breeding to preserve MHC allele diversity. While overall genetic diversity should continue to be a priority, attention to MHC diversity in conservation management can bolster immunocompetence in declining species (Madsen, Shine, Olsson, & Wittzell, 1999; Madsen, Ujvari, & Olsson, 2004; Ujvari & Belov, 2011).

4.2 | Microbial community structure between Ozark and eastern hellbenders

Subspecies identity was a primary driver of Ozark and eastern hellbender skin microbial community composition, with variation in environmental microbiota as a secondary factor. Differences in the cutaneous microbiota of Ozark and eastern hellbenders suggest that subspecies-specific factors are important in the assembly processes of microbial communities. While we only characterized bacteria in the water and not substrate/biofilm communities, our results indicate that variation in environmental bacterial reservoirs can influence community assembly among populations of hellbender hosts. Our results parallel conclusions drawn from previous

assessments of the skin microbiota in both subspecies (Hernández-Gómez et al., 2017b), but expand that work to multiple populations of each subspecies. The current study confirms a pattern of skin symbiont divergence among the two subspecies by including multiple populations within the range of both subspecies. Factors specific to each subspecies' physiology (e.g., ontogeny, innate/adaptive immunity, metabolism) could contribute to the success of different microbes in colonizing skin microbial communities (Costello et al., 2012; Longo et al., 2015; Phillips et al., 2012; Walke et al., 2014). Species identity as a predictor of cutaneous bacterial community composition is an established pattern among other amphibian systems as well. McKenzie, Bowers, Fierer, Knight, and Lauber (2012) characterized strong differences in the microbiota throughout four sympatric amphibian species. Furthermore, Kueneman et al. (2014) identified host species as a predictor of skin microbiota diversity from samples collected across geographical distances. Therefore, divergence in the microbial make-up of the two hellbender subspecies indicates a mechanism through which host selection processeson skin symbionts change over evolutionary time (Zilber-Rosenberg & Rosenberg, 2008).

Given the compositional differences between both hellbender subspecies, differences in skin OTU associations could correlate with the presence of wounds in the Ozark hellbender. A previous microbiota study on Ozark hellbenders identified abundant OTUs on wound tissues compared to the healthy skin (Hernández-Gómez et al., 2017b). These OTUs included members of the families Comamonadaceae, Moraxellaceae and Flavobacteriaceae and were assumed to be opportunistic pathogens of wound tissue. In the present study, the abundance of OTUs identified to the family Comamonadaceae, genus *Flavobacterium* (family Flavobacteriaceae) and genus *Acinetobacter* (family Moraxellaceae) on the skin of Ozark hellbenders was significantly higher compared to the skin from the eastern subspecies. These taxa are described as common environmental microbes; however, all three also contain numerous opportunistic pathogenic species that affect humans, animals and plants (Teixeira & Merquior, 2014; Willems, 2014). Within amphibians, members of these families are commonly described as part of the skin microbiota (Jiménez & Sommer, 2017). However, *Batrachochytrium dendrobatidis* infection can induce a positive response in the abundance of *Comamonadaceae*, *Flavobacteriaceae* and *Moraxellaceae* (Federici et al., 2015; Jani & Briggs, 2014; Walke et al., 2015), and studies recording the effects of captivity on the skin of amphibians have recorded positive responses in members of these families as well (Becker et al. 2014). We cannot confidently say these families play a role in the development of wounds in the Ozark hellbender without further investigation; however, increased abundance could result from decreased immunity within this hellbender subspecies.

4.3 | Host traits and skin microbiota associations

We provide the first report linking MHC IIB, body condition and sex on composition of the skin microbiota in an amphibian, the hellbender. A limited number of studies have also drawn links between the MHC IIB and the microbiota, but these have evaluated the effect of the MHC on the gut of mammals and fish. In laboratory experiments, Kubinak et al. (2015) described an influence of individual MHC polymorphism on the composition of the mouse gut microbiota. Bolnick et al. (2014) took this methodology to the field and observed a relationship between stickleback (*Gasterosteus aculeatus*) individual MHC IIB allele divergence and composition of the gut microbiota. Our study expands on previous work by incorporating numerous populations of two wild amphibians to quantify the role of immunogenetics and host characteristics on processes shaping skin microbial community assembly.

We noted several correlations between the MHC IIB genes and the skin microbiota in hellbenders; however, our small sample size restricted our ability to accurately assess an influence of MHC IIB on specific OTUs. Admittedly, another limitation of our study is that we did not measure other aspects of immunity (e.g., Toll receptors, skin peptides) that may contribute to selective pressures on skin symbionts (Hopkins & DuRant, 2011; Woodhams et al., 2014). Still, we identified correlations between MHC IIB amino acid divergence/allele presence and skin community richness/composition of hellbenders. Within the eastern subspecies, we characterized a positive correlation between amino acid divergence and OTU richness. This observation is contrary to what previous microbiota/MHC association studies have described (Bolnick et al., 2014). However, eastern hellbenders with high allele divergence were also more likely to possess the common alleles such as the TSP allele Cral-DAB*02 and allele Cral-DAB*01. This pattern might explain the lack of a significant association between MHC IIB amino acid divergence and microbiota richness among Ozark hellbenders. The Ozark subspecies' MHC IIB alleles were not as divergent as the eastern hellbender's alleles from the common alleles (Figure S1). In fact, amino acid divergence and/or the presence/absence of common alleles consistently had an impact on compositional variation in the skin microbial communities of Ozark and eastern hellbenders. Pressure from parasites may induce a selective disadvantage to individuals possessing common MHC alleles, as parasites evolve ways to deflect presentation by these alleles (Bernatchez & Landry, 2003). Thus, it is possible that common alleles induce different selective pressures on the microbiota compared to the rare variants. To better understand how the MHC influences microbial composition in amphibians, future studies should continue to evaluate the influence of host traits on the composition of the skin microbiome of amphibians in controlled environments.

We only observed an association between body condition and compositional turnover in the skin microbiota of eastern hellbenders from the Niangua River population. The absence of an effect of body condition among the other populations likely results from our reduced sample size within each river. Given that body condition has been associated with resistance to integumentary pathogens (Searle et al., 2011), more research needs to evaluate the effect of body condition on associations with microbial symbionts. We did note significant grouping of skin communities in response to individual sex among populations of the Ozark subspecies. This pattern is contrary to what has been previously documented in other salamander

systems where individuals from both sexes shared similar microbial communities (Prado-Irwin, Bird, Zink, & Vredenburg, 2017). Sex-related differences in skin microbiota composition may result from physiological variation between male and female hellbenders. Some of our hellbender populations were sampled around the breeding season (eastern hellbenders: mid-/late August to mid-September; Ozark hellbenders: mid-/late September to mid-October; Nickerson & Mays, 1973; Humphries & Pauley, 2005). During the mating season, male hellbenders guard nests and can engage in intraspecific aggression (Miller & Miller, 2005), suggesting a spike in androgenic hormones and subsequent altered immunity (Eikenaar, Husak, Escallon, & Moore, 2012; Klein, 2000). As such, variation in hormone/metabolism between males and females may alter associations between hellbenders and their microbial communities. To further evaluate the effect of sex and seasonality on the skin microbiome of hellbenders, future studies should consider serial microbial surveys on the skin of this salamander.

5 | CONCLUSIONS

Our investigation of Missouri hellbender immunogenetics and cutaneous microbiota provided several insights related to the possible link between host traits and the cutaneous microbiome within amphibians. Disentangling the complex association between hosts, parasites and the microbiome is important to understanding the role of skin symbionts on pathogen defence among this class (Jiménez & Sommer, 2017). Our results support the role of individual host characteristics on the assembly processes of the cutaneous microbiome of amphibians. These findings suggest the urgent need to continue to monitor/manage the immunogenetics of captive and wild populations of hellbenders in North America. In the end, assessing the influence of host traits in the formation of microbial symbiont communities can provide insight into microbiome structure and phenotype heterogeneity within natural systems.

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DATA ACCESSIBILITY

Individual subspecies identity, population and morphological data are uploaded as online supporting information (Table S1). Python scripts and an OTU table containing both environmental and animal samples are also available as supplemental information. Because this research involves an endangered species, no geographical information was recorded.

Raw sequence data for spleen transcriptome, skin microbiome and MHC IIB amplicon reads can be accessed via the BioProject Accession Number PRJNA382978 in the NCBI BioProject database (https://www.ncbi.nlm.nih.gov/bioproject/). The assembled spleen transcriptome can be accessed in the Dryad Digital Repository via the DOI 10.5061/dryad.7bj0f.

MHC allele sequences were deposited in GenBank (Accession Numbers KY947277–KY947300).

AUTHOR CONTRIBUTIONS

O.H.G., J.T.B. and R.N.W. contributed in the research design. O.H.G. and J.T.B. performed sample collection, using J.T.B.'s sampling permit. Laboratory and bioinformatics work was performed by O.H.G. The manuscript was written by O.H.G. with input from J.T.B. and R.N.W.

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REFERENCES

Babik, W., Pabijan, M., Arntzen, J. W., Cogalniceanu, D., Durka, W., & Radwan, J. (2009). Long-term survival of a urodele amphibian despite depleted major histocompatibility complex variation. *Molecular Ecology*, *18*(5), 769–781. https://doi.org/10.1111/j.1365-294X.2008.04057.x

Becker, M. H., Richards-Zawacki, C. L., Gratwicke, B., Belden, L. K. (2014). The effect of captivity on the cutanous bacterial community of the critically endangered Panamanian golden frog (Atelopus zeteki). *Biological Conservation*, *176*, 199–206. https://doi.org/10.1016/j.biocon.2014.05.029

Benson, D. A., Cavanaugh, M., Clark, K., Karsch-Mizrachi, I., Lipman, D. J., Ostell, J., & Sayers, E. W. (2013). GenBank. *Nucleic Acids Research*, *41*, D36–D42.

Bernatchez, L., & Landry, C. (2003). MHC studies in nonmodel vertebrates: What have we learned about natural selection in 15 years? *Journal of Evolutionary Biology*, *16*(3), 363–377. https://doi.org/10.1046/j.1420-9101.2003.00531.x

Bokulich, N. A., Subramanian, S., Faith, J. J., Gevers, D., Gordon, J. I., Knight, R., . . . Caporaso, J. G. (2013). Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nature Methods*, *10*(1), 57–59. https://doi.org/10.1038/nmeth.2276

Bolger, D., Lohse, M., & Usadel, B. (2014). Trimmomatic: A flexible trimmer for illumina sequence data. *Bioinformatics*, *30*, 2114–2120. https://doi.org/10.1093/bioinformatics/btu170

Bolnick, D. I., Snowberg, L. K., Caporaso, J. G., Lauber, C., Knight, R., & Stutz, W. E. (2014). Major histocompatibility complex class IIb polymorphism influences gut microbiota composition and diversity. *Molecular Ecology*, *23*(19), 4831–4845. https://doi.org/10.1111/mec.12846

Bos, D. H., & DeWoody, J. A. (2005). Molecular characterization of major histocompatibility complex class II alleles in wild tiger salamanders (*Ambystoma tigrinum*). *Immunogenetics*, *57*(10), 775–781. https://doi.org/10.1007/s00251-005-0038-5

Bos, D. H., & Waldman, B. (2006). Evolution by recombination and trans-species polymorphism in the MHC class I gene of *Xenopus laevis*. *Molecular Biology and Evolution*, *23*(1), 137–143. https://doi.org/10.1093/molbev/msj016

Briggler, J., Utrup, J., Davidson, C., Humphries, J., Groves, J., Johnson, T., … Byers, O. (2007). *Hellbender population and habitat viability assessment: Final report*. Apple Valley, MN: IUCN/SSC Conservation Breeding Specialist Group.

Burgmeier, N. G., Unger, S. D., Sutton, T. M., Williams, R. N. (2011). Populations status of the eastern hellbender (Cryptobranchus alleganiensis alleganiensis) in Indiana. *Journal of Herpetology*, *45*(2), 195–201. https://doi.org/10.1670/10-094.1

Caporaso, J. G., Bittinger, K., Bushman, F. D., DeSantis, T. Z., Andersen, G. L., & Knight, R. (2010a). PyNAST: A flexible tool for aligning sequences to a template alignment. *Bioinformatics*, *26*(2), 266–267. https://doi.org/10.1093/bioinformatics/btp636

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., … Knight, R. (2010b). QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, *7*(5), 335–336. https://doi.org/10.1038/nmeth.f.303

Carrillofarga, J., Castell, A., Perez, A., & Rondan, A. (1990). Langerhans-like cells in amphibian epidermis. *Journal of Anatomy*, *172*, 39–45.

Chen, J., Bittinger, K., Charlson, E. S., Hoffmann, C., Lewis, J., Wu, G. D., … Li, H. Z. (2012). Associating microbiome composition with environmental covariates using generalized UniFrac distances. *Bioinformatics*, *28*(16), 2106–2113. https://doi.org/10.1093/bioinformatics/bts342

Christian, N., Whitaker, B. K., & Clay, K. (2015). Microbiomes: Unifying animal and plant systems through the lens of community ecology theory. *Frontiers in Microbiology*, *6*, 869.

Cole, J. R., Wang, Q., Fish, J. A., Chai, B., McGarrell, D. M., Sun, Y., … Tiedje, J. M. (2014). Ribosomal database project: Data and tools for high throughput rRNA analysis. *Nucleic Acids Research*, *42*, D633–D642. https://doi.org/10.1093/nar/gkt1244

Colombo, B. M., Scalvenzi, T., Benlamara, S., & Pollet, N. (2015). Microbiota and mucosal immunity in amphibians. *Frontiers in Immunology*, *6*, 111.

Costello, E. K., Stagaman, K., Dethlefsen, L., Bohannan, B. J. M., & Relman, D. A. (2012). The application of ecological theory toward an understanding of the human microbiome. *Science*, *336*(6086), 1255–1262. https://doi.org/10.1126/science.1224203

Cresswell, P. (1994). Assembly, transport, and function of MHC class II molecules. *Annual Review of Immunology*, *12*(1), 259–291. https://doi.org/10.1146/annurev.iy.12.040194.001355

Crowhurst, R. S., Faries, K. M., Collantes, J., Briggler, J. T., Koppelman, J. B., & Eggert, L. S. (2011). Genetic relationships of hellbenders in the Ozark highlands of Missouri and conservation implications for the Ozark subspecies (*Cryptobranchus alleganiensis bishopi*). *Conservation Genetics*, *12*(3), 637–646. https://doi.org/10.1007/s10592-010-0170-0

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., … Andersen, G. L. (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Applied and Environmental Microbiology*, *72*(7), 5069–5072. https://doi.org/10.1128/AEM.03006-05

Dray, S., & Dufour, A. B. (2007). The ade4 package: Implementing the duality diagram for ecologists. *Journal of Statistical Software*, *22*(4), 1–20.

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*, *26*(19), 2460–2461. https://doi.org/10.1093/bioinformatics/btq461

Eikenaar, C., Husak, J., Escallon, C., & Moore, I. T. (2012). Variation in testosterone and corticosterone in amphibians and reptiles: Relationships with latitude, elevation, and breeding season length. *The American Naturalist*, *180*(5), 642–654. https://doi.org/10.1086/667891

Ettling, J. A., Wanner, M. D., Schuette, C. D., Armstrong, S. L., Pedigo, A. S., & Briggler, J. T. (2013). Captive reproduction and husbandry of adult Ozark hellbenders, *Cryptobranchus alleganiensis bishopi*. *Herpetoculture*, *44*(4), 605–610.

Federal Register. (2011). Endangered and threatened wildlife and plants; endangered status for the Ozark hellbender salamander. *Federal Communications Commission*, *76*, 61956–61978.

Federici, E., Rossi, R., Fidati, L., Paracucchi, R., Scargetta, S., Montalbani, E., … Di Rosa, I. (2015). Characterization of the skin microbiota in Italian stream frogs (*Rana italica*) infected and uninfected by a cutaneous parasitic disease. *Microbes and Environments*, *30*(3), 262–269. https://doi.org/10.1264/jsme2.ME15041

Fierer, N., Hamady, M., Lauber, C. L., & Knight, R. (2008). The influence of sex, handedness, and washing on the diversity of hand surface bacteria. *Proceedings of the National Academy of Sciences of the United States of America*, *105*, 17994–17999. https://doi.org/10.1073/pnas.0807920105

French, N., Yu, S., Biggs, P., Holland, B., Fearnhead, P., Binney, B., … Carter, P. (2013). Evolution of Campylobacter species in New Zealand. In S. Sheppard, & G. Méric (Eds.), *Campylobacter ecology and evolution* (pp. 221–240). Norfolk, VA: Horizon Scientific Press.

Harris, R. N., Brucker, R. M., Walke, J. B., Becker, M. H., Schwantes, C. R., Flaherty, D. C., … Minbiole, K. P. C. (2009). Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. *The ISME Journal*, *3*(7), 818–824. https://doi.org/10.1038/ismej.2009.27

Hernández-Gómez, O., Hoverman, J. T., & Williams, R. N. (2017a). Cutaneous microbial community variation across populations of eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*). *Frontiers in Microbiology*, https://doi.org/10.3389/fmicb.2017.01379

Hernández-Gómez, O., Kimble, S. J. A., Briggler, J. T., & Williams, R. N. (2017b). Characterization of the cutaneous bacterial communities of two giant salamander subspecies. *Microbial Ecology*, *73*(2), 445–454. https://doi.org/10.1007/s00248-016-0859-9

Hiler, W. R., Wheeler, B. A., & Trauth, S. E. (2005). Abnormalities in the Ozark hellbender (*Cryptobranchus alleganiensis bishopi*) in Arkansas: A comparison between two rivers with a historical perspective. *Journal of the Arkansas Academy of Science*, *59*, 88–94.

Hopkins, W. A., & DuRant, S. E. (2011). Innate immunity and stress physiology of eastern hellbenders (*Cryptobranchus alleganiensis*) from two stream reaches with differing habitat quality. *General and Comparative Endocrinology*, *174*(2), 107–115. https://doi.org/10.1016/j.ygcen.2011.08.006

Humphries, W. J., & Pauley, T. K. (2005). Life history of the hellbender, *Cryptobranchus alleganiensis*, in a West Virginia stream. *American Midland Naturalist*, *154*, 135–142. https://doi.org/10.1674/0003-0031(2005)154[0135:LHOTHC]2.0.CO;2

Jani, A. J., & Briggs, C. J. (2014). The pathogen *Batrachochytrium dendrobatidis* disturbs the frog skin microbiome during a natural epidemic and experimental infection. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(47), E5049–E5058. https://doi.org/10.1073/pnas.1412752111

Jiménez, R. R., & Sommer, S. (2017). The amphibian microbiome: Natural range of variation, pathogenic dysbiosis, and role in conservation. *Biodiversity Conservation*, *26*(4), 763–786. https://doi.org/10.1007/s10531-016-1272-x

Kaufman, J. F., Flajnik, M. F., & Du Pasquier, L. (1985). *Xenopus* MHC class II molecules. II. Polymorphism as determined by two-dimensional gel electrophoresis. *Journal of Immunology*, *134*(5), 3258–3264.

Kiemnec-Tyburczy, K. M., Richmond, J. Q., Savage, A. E., & Zamudio, K. R. (2010). Selection, trans-species polymorphism, and locus identification of major histocompatibility complex class IIβ alleles of New World ranid frogs. *Immunogenetics*, *62*(11–12), 741–751. https://doi.org/10.1007/s00251-010-0476-6

Klein, S. L. (2000). Hormones and mating system affect sex and species differences in immune function among vertebrates. *Behavioural Processes*, *51*, 149–166. https://doi.org/10.1016/S0376-6357(00)00125-X

Klein, J., Sato, A., Nagl, S., & O'hUigín, C. (1998). Molecular trans-species polymorphism. *Annual Review of Ecology and Systematics*, *29*(1), 1–21. https://doi.org/10.1146/annurev.ecolsys.29.1.1

Kohl, K. D., Amaya, J., Passement, C. A., Dearing, M. D., & McCue, M. D. (2014). Unique and shared responses of the gut microbiota to prolonged fasting: A comparative study across five classes of vertebrate hosts. *FEMS Microbiology Ecology*, *90*(3), 883–894. https://doi.org/10.1111/1574-6941.12442

Krynak, K. L., Burke, D. J., & Benard, M. F. (2016). Landscape and water characteristics correlate with immune defense traits across Blanchard's cricket frog (*Acris blanchardi*) populations. *Biological Conservation*, *193*, 153–167. https://doi.org/10.1016/j.biocon.2015.11.019

Kubinak, J. L., Stephens, W. Z., Soto, R., Petersen, C., Chiaro, T., Gogokhia, L., … Round, J. L. (2015). MHC variation sculpts individualized microbial communities that control susceptibility to enteric infection. *Nature Communications*, *6*, 8642. https://doi.org/10.1038/ncomms9642

Kueneman, J. G., Parfrey, L. W., Woodhams, D. C., Archer, H. M., Knight, R., & McKenzie, V. J. (2014). The amphibian skin-associated microbiome across species, space and life history stages. *Molecular Ecology*, *23*(6), 1238–1250. https://doi.org/10.1111/mec.12510

Lam, B. A., Walke, J. B., Vredenburg, V. T., & Harris, R. N. (2010). Proportion of individuals with anti-*Batrachochytrium dendrobatidis* skin bacteria is associated with population persistence in the frog *Rana muscosa*. *Biological Conservation*, *143*(2), 529–531. https://doi.org/10.1016/j.biocon.2009.11.015

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., … Higgins, D. G. (2007). ClustalW and ClustalX version 2. *Bioinformatics*, *23*(21), 2947–2948. https://doi.org/10.1093/bioinformatics/btm404

Legendre, P., & Anderson, M. J. (1999). Distance-based redundancy analysis: Testing multispecies responses in multifactorial ecological experiments. *Ecological Monographs*, *69*(1), 1–24. https://doi.org/10.1890/0012-9615(1999)069[0001:DBRATM]2.0.CO;2

Lenz, T. L., Wells, K., Pfeiffer, M., & Sommer, S. (2009). Diverse MHC IIB allele repertoire increases parasite resistance and body condition in the long-tailed rat (*Leopoldamys sabanus*). *BMC Evolutionary Biology*, *9*, 269. https://doi.org/10.1186/1471-2148-9-269

Longo, A. V., Savage, A. E., Hewson, I., & Zamudio, K. R. (2015). Seasonal and ontogenetic variation of skin microbial communities and relationships to natural disease dynamics in declining amphibians. *The Royal Society Open Science*, *2*(7), 140377. https://doi.org/10.1098/rsos.140377

Loudon, A. H., Woodhams, D. C., Parfrey, L. W., Archer, H., Knight, R., McKenzie, V., & Harris, R. N. (2014). Microbial community dynamics and effect of environmental microbial reservoirs on red-backed salamanders (*Plethodon cinereus*). *The ISME Journal*, *8*(4), 830–840. https://doi.org/10.1038/ismej.2013.200

Lozupone, C., Lladser, M. E., Knights, D., Stombaugh, J., & Knight, R. (2011). UniFrac: An effective distance metric for microbial community comparison. *The ISME Journal*, *5*(2), 169. https://doi.org/10.1038/ismej.2010.133

Madsen, T., Shine, R., Olsson, M., & Wittzell, H. (1999). Conservation biology: Restoration of an inbred adder population. *Nature*, *402*(6757), 34–35. https://doi.org/10.1038/46941

Madsen, T., Ujvari, B., & Olsson, M. (2004). Novel genes continue to enhance population growth in adders (*Vipera berus*). *Biological Conservation*, *120*(1), 145–147. https://doi.org/10.1016/j.biocon.2004.01.022

Masella, A. P., Bartram, A. K., Truszkowski, J. M., Brown, D. G., & Neufeld, J. D. (2012). PANDAseq: Paired-end assembler for illumina sequences. *BMC Bioinformatics*, *13*, 31. https://doi.org/10.1186/1471-2105-13-31

Mayasich, J., Grandmaison, D., & Phillips, C. (2003). *Eastern hellbender status assessment report*. *Final report*. Fort Snelling, MN: U. S. Fish and Wildlife Service, Region 3.

McKenzie, V., Bowers, R. M., Fierer, N., Knight, R., & Lauber, C. L. (2012). Co-habiting amphibian species harbor unique skin bacterial communities in wild populations. *The ISME Journal*, *6*, 558–596.

Miller, H. C., Allendorf, F., & Daugherty, C. H. (2010). Genetic diversity and differentiation at MHC genes in island populations of tuatara (*Sphenodon* spp.). *Molecular Ecology*, *19*(18), 3894–3908. https://doi.org/10.1111/j.1365-294X.2010.04771.x

Miller, B. T., & Miller, J. L. (2005). Prevalence of physical abnormalities in eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*) populations of middle Tennessee. *Southeastern Naturalist*, *4*(3), 513–520. https://doi.org/10.1656/1528-7092(2005)004[0513:POPAIE]2.0.CO;2

Nickerson, M. A., & Mays, C. E. (1973). *The hellbenders: North American 'giant salamanders'*. Milwaukee, WI: Milwaukee Public Museum.

Nickerson, C. A., Ott, C. M., Castro, S. L., Garcia, V. M., Molina, T. C., Briggler, J. T., … Nickerson, M. A. (2011). Evaluation of microorganisms cultured from injured and repressed tissue regeneration sites in endangered giant aquatic Ozark Hellbender Salamanders. *PLoS ONE*, *6*(12), e28906. https://doi.org/10.1371/journal.pone.0028906

Oksanen, J., Kindt, R., Legendre, P., O'Hara, B., & Stevens, M. H. H. (2007). vegan. Community ecology package R package version 2.4-1.

Pearman, P. B., & Garner, T. W. J. (2005). Susceptibility of Italian agile frog populations to an emerging strain of *Ranavirus* parallels population genetic diversity. *Ecology Letters*, *8*(4), 401–408. https://doi.org/10.1111/j.1461-0248.2005.00735.x

Phillips, C. D., Phelan, G., Dowd, S. E., McDonough, M. M., Ferguson, A. W., Hanson, J. D., … Baker, R. J. (2012). Microbiome analysis among bats describes influences of host phylogeny, life history, physiology and geography. *Molecular Ecology*, *21*(11), 2617–2627. https://doi.org/10.1111/j.1365-294X.2012.05568.x

Prado-Irwin, S. R., Bird, A. K., Zink, A. G., & Vredenburg, V. T. (2017). Intraspecific variation in the skin-associated microbiome of a terrestrial salamander. *Microbial Ecology*, *74*, 745–756. https://doi.org/10.1007/s00248-017-0986-y

Price, M. N., Dehal, P. S., & Arkin, A. P. (2010). FastTree 2 – approximately maximum-likelihood trees for large alignments. *PLoS ONE*, *5*(3), e9490. https://doi.org/10.1371/journal.pone.0009490

Radwan, J., Biedrzycka, A., & Babik, W. L. (2010). Does reduced MHC diversity decrease viability of vertebrate populations? *Biological Conservation*, *143*(3), 537–544. https://doi.org/10.1016/j.biocon.2009.07.026

Rideout, J. R., He, Y., Navas-Molina, J. A., Walters, W. A., Ursell, L. K., Gibbons, S. M., & Caporaso, J. G. (2004). Subsampled open reference clustering creates consistent, comprehensive OTU definitions and scales to billions of sequences. *PeerJ*, *2*, e545.

Rollins-Smith, L. A., Woodhams, D. C., Reinert, L. K., Vredenburg, V. T., Briggs, C. J., Nielsen, P. F., & Conlon, J. M. (2006). Antimicrobial peptide defenses of the mountain yellow-legged frog (*Rana muscosa*). *Developmental and Comparative Immunology*, *30*(9), 831–842. https://doi.org/10.1016/j.dci.2005.10.005

Round, J. L., & Mazmanian, S. K. (2009). The gut microbiome shapes intestinal immune responses during health and disease. *Nature Reviews Immunology*, *9*(5), 313–323. https://doi.org/10.1038/nri2515

Sabatino, S. J., & Routman, E. J. (2009). Phylogeography and conservation genetics of the hellbender salamander (*Cryptobranchus alleganiensis*). *Conservation Genetics*, *10*(5), 1235–1246. https://doi.org/10.1007/s10592-008-9655-5

Sambrook, J., & Russell, D. (2001). *Molecular cloning: A laboratory manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Savage, A. E., & Zamudio, K. R. (2011). MHC genotypes associate with resistance to a frog-killing fungus. *Proceedings of the National Academy of Sciences*, *108*(40), 16705–16710. https://doi.org/10.1073/pnas.1106893108

Savage, A. E., & Zamudio, K. R. (2016). Adaptive tolerance to a pathogenic fungus drives major histocompatibility complex evolution in natural amphibian populations. *Proceedings of the Royal Society B-Biological Sciences*, *283*, 20153115. https://doi.org/10.1098/rspb.2015.3115

Schulte-Hostedde, A. I., Zinner, B., Millar, J. S., & Hickling, G. J. (2005). Restitution of mass-size residuals: Validating body condition indices. *Ecology*, *86*(1), 155–163. https://doi.org/10.1890/04-0232

Searle, C. L., Gervasi, S. S., Hua, J., Hammond, J. I., Relyea, R. A., & Blaustein, A. R. (2011). Differential host susceptibility to *Batrachochytrium dendrobatidis*, an emerging amphibian pathogen. *Conservation Biology*, *25*(5), 965–974. https://doi.org/10.1111/j.1523-1739.2011.01708.x

Sebastian, A., Herdegen, M., Migalska, M., & Radwan, J. (2016). amplisas: A web server for multilocus genotyping using next-generation amplicon sequencing data. *Molecular Ecology Resources*, *16*(2), 498–510. https://doi.org/10.1111/1755-0998.12453

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., & Huttenhower, C. (2011). Metagenomic biomarker discovery and explanation. *Genome Biology*, *12*(6), R60. https://doi.org/10.1186/gb-2011-12-6-r60

Shafquat, A., Joice, R., Simmons, S. L., & Huttenhower, C. (2014). Functional and phylogenetic assembly of microbial communities in the human microbiome. *Trends in Microbiology*, *22*(5), 261–266. https://doi.org/10.1016/j.tim.2014.01.011

Stutz, W. E., & Bolnick, D. I. (2014). Stepwise threshold clustering: A new method for genotyping MHC loci using next-generation sequencing technology. *PLoS ONE*, *9*(7), e100587. https://doi.org/10.1371/journal.pone.0100587

Sun, C., & Mueller, R. L. (2014). Hellbender genome sequences shed light on genomic expansion at the base of crown salamanders. *Genome Biology and Evolution*, *6*(7), 1818–1829. https://doi.org/10.1093/gbe/evu143

Tajima, F. (1989). Statistical-method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics*, *123*(3), 585–595.

Teixeira, L. M., & Merquior, V. L. C. (2014). The Family Moraxellaceae. In E. Rosenberg, E. F. DeLong, S. Lory, E. Stackebrandt, & F. Thompson (Eds.), *The Prokaryotes* (pp. 443–476). New York, NY: Springer.

Těšický, M., & Vinkler, M. (2015). Trans-species polymorphism in immune genes: General pattern or MHC-restricted phenomenon? *Journal of Immunology Research*, *2015*, https://doi.org/10.1155/2015/838035

Tonione, M., Johnson, J. R., & Routman, E. J. (2011). Microsatellite analysis supports mitochondrial phylogeography of the hellbender (*Cryptobranchus alleganiensis*). *Genetica*, *139*(2), 209–219. https://doi.org/10.1007/s10709-010-9538-9

Ujvari, B., & Belov, K. (2011). Major Histocompatibility Complex (MHC) markers in conservation biology. *International Journal of Molecular Sciences*, *12*(8), 5168–5186. https://doi.org/10.3390/ijms12085168

Venesky, M. D., Wilcoxen, T. E., Rensel, M. A., Rollins-Smith, L., Kerby, J. L., & Parris, M. J. (2012). Dietary protein restriction impairs growth, immunity, and disease resistance in southern leopard frog tadpoles. *Oecologia*, *169*(1), 23–31. https://doi.org/10.1007/s00442-011-2171-1

Wakeland, E. K., Boehme, S., She, J. X., Lu, C. C., McIndoe, R. A., Cheng, I., … Potts, W. K. (1990). Ancestral polymorphisms of MHC class II genes: Divergent allele advantage. *Immunologic Research*, *9*(2), 115–122. https://doi.org/10.1007/BF02918202

Walke, J. B., Becker, M. H., Loftus, S. C., House, L. L., Cormier, G., Jensen, R. V., & Belden, L. K. (2014). Amphibian skin may select for rare environmental microbes. *The ISME Journal*, *8*(11), 2207–2217. https://doi.org/10.1038/ismej.2014.77

Walke, J. B., Becker, M. H., Loftus, S. C., House, L. L., Teotonio, T. L., Minbiole, K. P. C., & Belden, L. K. (2015). Community structure and function of amphibian skin microbes: An experiment with bullfrogs exposed to a chytrid fungus. *PLoS ONE*, *10*(10), e0139848. https://doi.org/10.1371/journal.pone.0139848

Wang, Q., Garrity, G. M., Tiedje, J. M., & Cole, J. R. (2007). Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology*, *73*(16), 5261–5267. https://doi.org/10.1128/AEM.00062-07

Wheeler, B. A., McCallum, M. L., & Trauth, S. E. (2002). Abnormalities in the Ozark Hellbender, *Cryptobranchus alleganiensis bishopi*. *Journal of the Arkansas Academy of Science*, *56*, 250–252.

Wheeler, B. A., Prosen, E., Mathis, A., & Wilkinson, R. F. (2003). Population declines of a long-lived salamander: A 20+-year study of hellbenders, *Cryptobranchus alleganiensis*. *Biological Conservation*, *109*(1), 151–156. https://doi.org/10.1016/S0006-3207(02)00136-2

Whittaker, D. J., Dapper, A. L., Peterson, M. P., Atwell, J. W., & Ketterson, E. D. (2012). Maintenance of MHC Class IIB diversity in a recently established songbird population. *Journal of Avian Biology*, *43*(2), 109–118. https://doi.org/10.1111/j.1600-048X.2012.05504.x

Willems, A. (2014). The family comamonadaceae. In E. Rosenberg, E. F. DeLong, S. Lory, E. Stackebrandt, & F. Thompson (Eds.), *The Prokaryotes* (pp. 777–851). New York, NY: Springer. https://doi.org/10.1007/978-3-642-30197-1

Woodhams, D. C., Brandt, H., Baumgartner, S., Kielgast, J., Küpfer, E., Tobler, U., … McKenzie, V. (2014). Interacting symbionts and immunity in the amphibian skin mucosome predict disease risk and probiotic effectiveness. *PLoS ONE*, *9*(4), e96375. https://doi.org/10.1371/journal.pone.0096375

Woodhams, D. C., Voyles, J., Lips, K. R., Carey, C., & Rollins-Smith, L. A. (2006). Predicted disease susceptibility in a panamanian amphibian assemblage based on skin peptide defenses. *Journal of Wildlife Diseases*, *42*(2), 207–218. https://doi.org/10.7589/0090-3558-42.2.207

Yuhki, N., & Obrien, S. J. (1990). DNA variation of the mammalian major histocompatibility complex reflects genomic diversity and population history. *Proceedings of the National Academy of Sciences of the United States of America*, *87*(2), 836–840. https://doi.org/10.1073/pnas.87.2.836

Zhang, P., & Wake, D. B. (2009). Higher-level salamander relationships and divergence dates inferred from complete mitochondrial genomes. *Molecular Phylogenetics and Evolution*, *53*(2), 492–508. https://doi.org/10.1016/j.ympev.2009.07.010

Zhu, R., Chen, Z.-Y., Wang, J., Yuan, J.-D., Liao, X.-Y., Gui, J.-F., & Zhang, Q.-Y. (2014). Extensive diversification of MHC in Chinese giant salamanders *Andrias davidianus* (Anda-MHC) reveals novel splice variants. *Developmental and Comparative Immunology*, *42*(2), 311–322. https://doi.org/10.1016/j.dci.2013.10.001

Zilber-Rosenberg, I., & Rosenberg, E. (2008). Role of microorganisms in the evolution of animals and plants: The hologenome theory of evolution. *FEMS Microbiology Reviews*, *32*(5), 723–735. https://doi.org/10.1111/j.1574-6976.2008.00123.x

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