Microbial Ecology
https://doi.org/10.1007/s00248-023-02229-3

HOST MICROBE INTERACTIONS

# Captivity, Reintroductions, and the Rewilding of Amphibian-associated Bacterial Communities

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Received: 20 September 2022 / Accepted: 23 April 2023


## Abstract

Many studies have noted differences in microbes associated with animals reared in captivity compared to their wild counterparts, but few studies have examined how microbes change when animals are reintroduced to the wild after captive rearing. As captive assurance populations and reintroduction programs increase, a better understanding of how microbial symbionts respond during animal translocations is critical. We examined changes in microbes associated with boreal toads (*Anaxyrus boreas*), a threatened amphibian, after reintroduction to the wild following captive rearing. Previous studies demonstrate that developmental life stage is an important factor in amphibian microbiomes. We collected 16S marker-gene sequencing datasets to investigate: (i) comparisons of the skin, mouth, and fecal bacteria of boreal toads across four developmental life stages in captivity and the wild, (ii) tadpole skin bacteria before and after reintroduction to the wild, and (iii) adult skin bacteria during reintroduction to the wild. We demonstrated that differences occur across skin, fecal, and mouth bacterial communities in captive versus wild boreal toads, and that the degree of difference depends on developmental stage. Skin bacterial communities from captive tadpoles were more similar to their wild counterparts than captive post-metamorphic individuals were to their wild counterparts. When captive-reared tadpoles were introduced to a wild site, their skin bacteria changed rapidly to resemble wild tadpoles. Similarly, the skin bacterial communities of reintroduced adult boreal toads also shifted to resemble those of wild toads. Our results indicate that a clear microbial signature of captivity in amphibians does not persist after release into natural habitat.

**Keywords** Captivity · Reintroduction · Microbiome · Amphibian

## Introduction

The communities of microbes that live in and on larger hosts play a significant role in the functioning of most organisms [1]. Investigating the factors that influence host-associated microbial communities has been a major focus of research for the past decade [2, 3]. Understanding microbial communities and how they may shift is particularly relevant to endangered wildlife that are moved from the wild into captivity as well as those reared in captivity for reintroduction to the wild. Animals experience an enormous shift between captivity and the wild, including changes in diet, veterinary treatments, substrate, and exposure to other species. These changes make the microbial environment extremely different in captive vs. wild settings, which can produce major changes in the microbial communities associated with animals. We do not fully understand what these microbial shifts mean in terms of their effects on host health, but in a few cases there is a potential link between captivity and poor health due to microbial dysbiosis [4–6].

Altered host-associated microbial communities are just one source of potential health issues in captive populations, but conservation biologists are often dependent on captive assurance populations and reintroductions (moving individuals from captivity to the wild) to prevent extinctions (e.g., [7]). Consequently, conservation-related reintroductions are projected to increase in the coming decades [8]. Given the necessity of captive environments in conservation and their

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Published online: 24 May 2023

potential impacts on vital microbial communities, microbiologists have started advocating for the incorporation of microbiome studies in conservation decisions [9–11]. Relatively few studies have examined how animal translocations from captivity to the wild affect host microbial communities and whether any signature of captivity remains in the host-associated microbes. One study in white-footed mice found that a captivity signature may persist upon reintroduction to the wild in the gut microbiome and that its persistence depended on the diet in captivity [12]. Conversely, wild-type microbes persisted in the gut of woodrats upon translocation from the wild to a captive setting [13]. If shifts in the microbiome impact animal health, understanding how microbes respond to a change in habitat is critical for ex situ conservation strategies that use assurance colonies and reintroductions.

Amphibians are a particularly important group for investigating shifts in the microbiome in captivity and during reintroductions to the wild. Amphibians are declining globally at an alarming pace, with nearly 40% of species threatened [14], leaving many species dependent on captive breeding and reintroduction efforts. In many cases, the small size, short generation time, and large clutch sizes make amphibians particularly amenable to captive breeding [15]. Finally, there is evidence that the skin microbial communities of amphibians play a role in their ability to overcome one of the major threats identified in driving global declines: the recently emerged fungal skin pathogen *Batrachochytrium dendrobatidis* (*Bd*) [16, 17].

Previous work on the effect of captivity of the microbial communities of amphibians has focused exclusively on the skin bacterial communities of adult amphibians. The general trend is toward altered community structure and reduced alpha diversity of bacterial skin communities on captive amphibians relative to wild counterparts [16, 18–23], but see [24]. In some systems, the captivity-induced changes were quite rapid [21]. In one study that examined multiple species, the effect of captivity was clear in all of them, and they maintained species-specific skin bacterial communities generations into their captivity [18], underscoring that captivity does not erase intrinsic, biotic factors that influence skin microbial communities. A recent meta-analysis involving 18 amphibian species failed to find a consistent impact of captivity on multiple microbial diversity and function metrics [25]. Data from our system, boreal toads (*Anaxyrus boreas boreas*), show that there are large differences in skin bacterial communities between captive and wild post-metamorphic animals [16]. Recent work also shows that there is a significant reorganization of skin microbial communities around metamorphosis, and that communities are comparatively stable both before and after metamorphosis in this species [26]. To date, few studies have investigated the impact of amphibian reintroductions on their microbial communities.

Boreal toads (*Anaxyrus boreas boreas*) are long-lived, high elevation amphibians that have declined precipitously in Colorado over the last few decades, mainly due to their susceptibility to the fungal pathogen *Bd* [27–29]. The boreal toad is listed as a state endangered species in Colorado and efforts to restore it to its former range have been ongoing for over 20 years [30, 31]. Much of this work has involved captive rearing/breeding efforts at Colorado's Native Aquatic Species Restoration Facility (NASRF), which has maintained assurance populations from various regions and raises tadpoles for field reintroduction trials. This is an excellent system to assess the impacts of captivity and reintroduction on host-associated microbial communities, and can also inform decision-making for active ex situ conservation efforts.

Our goals for this study were two-fold: (1) understand how boreal toad developmental life stage and body location influence the effect of captivity on host-associated microbial communities and (2) evaluate the persistence of a microbial signature during reintroduction to a natural environment in pre- and post-metamorphic individuals. We hypothesized that there would be a large impact of captivity across all life stages and body sites given the massive departure from a natural environment that captivity represents, and that the microbial impact of captivity would persist throughout the duration of our sampling efforts upon reintroduction to the wild. Given the impacts of the microbiome on host health, this has practical implications for decision-making regarding the developmental timing of reintroduction efforts.

## Methods

### Sample Collection

Boreal toads (*Anaxyrus boreas boreas*) were sampled over four summers in Colorado (2015, 2017, 2018, 2019). This sampling was conducted with approved IACUC protocols 1505.04 and 2629 from the University of Colorado Boulder and Colorado Parks and Wildlife research permit number HP0998. Samples from Rocky Mountain National Park were collected by park staff and United States Geological Survey staff under permit number 2013-09_RENEWAL_C. All sampling from captive individuals occurred at NASRF in Alamosa, Colorado, the Colorado Parks, and Wildlife facility responsible for the captive breeding and rearing efforts for the boreal toad. Housing for toads includes plastic raceways with run-through aquifer-sourced water, and artificial light exposure. Toads in this facility are fed commercially available pellet food as tadpoles, and cultured feed-insects post-metamorphosis. They do not have contact with soil or

natural pond water. Some wild toad samples from a previously published study were included in the analysis [26].

We conducted three different sampling efforts concurrently throughout the summers of 2017–2019. First, we compared the skin, fecal, and mouth bacterial communities of boreal toads at four distinct life stages in captivity (NASRF) and the wild (Sup. Table 1). The four life stages were tadpoles (Gosner 35–41), metamorphs (young of the year shortly after metamorphosis), juveniles (1–2 year old, non-reproductive individuals), and adults (3 years and older, reproductive individuals). Wild toads were sampled from multiple high elevation wetland sites (typically beaver ponds surrounded by willow-dominated meadows). These sites, as well the reintroduction sites, were located within a 120-km radius in central and northern Colorado, ranging from 7 to 210 km away from each other. The reintroduction sites ranged from 7 to 18 km to the nearest sampled wild site. All toads were captured by hand while wearing clean nitrile gloves and placed in clean, plastic bags until sampling. To collect samples of the skin bacterial community, individual toads were handled with sterile nitrile gloves, rinsed briefly with sterile water, and rubbed gently with a sterile rayon-tipped swab for 30 s. To collect samples of the oral microbial communities, sterilized tweezers were used to open toads' mouths and a sterile rayon-tipped swab was rubbed on the internal surface of the oral cavity for 10 s. For fecal microbial communities, individuals were placed in plastic containers with sterile water for up to 1 h, during which a proportion of the toads defecated. The feces were then rubbed/pressed with a sterile rayon-tipped swab. All samples were stored on dry ice at the point of collection and then at −20°C until DNA extraction.

Second, we sampled the skin bacterial communities of two sets of tadpoles that were reared at NASRF and released into suitable, but unoccupied habitats, wetlands in Brittle Silver Basin (elevation 3495 m) in Gunnison County, Colorado, and near Brown's Creek in Chaffee County, Colorado (elevation 2977 m). As all potential reintroduction sites are extensively monitored by Colorado Parks and Wildlife, we can be confident that they were unoccupied by toads for many years prior to our sampling, and all sampled individuals were from the reintroduction effort. The tadpoles originated from eggs that were produced in the wild at other sites in each county, and then taken into captivity shortly after fertilization. The tadpoles were reared at NASRF in large tanks in aquifer-sourced water. Late-stage tadpoles (mainly Gosner stage 38–41) were transported near to the wetland in a water tank, then hiked to the wetland and released. Both sites consist of beaver ponds and associated wetlands. Tadpoles (and recently metamorphosed individuals during the final timepoints) at the Brittle Silver site were sampled five times, once prior to release and four times after recapture over the subsequent 20 days. Tadpoles at Brown's Creek were sampled five times, once prior to release and four times after recapture over the subsequent 9 days.

Third, we sampled the skin bacterial communities of four adult boreal toads that were reared at NASRF and introduced to a suitable, but unoccupied wild site, near Boulder Brook in Rocky Mountain National Park. The site consists of an old beaver pond and associated upstream wetlands. These adult toads were part of a sentinel effort to assist in determining the *Bd* status prior to a planned release of captive-reared tadpoles. The four adult boreal toads released included one female and three males. Individuals were fitted with radio telemetry belts for monitoring, and released then recaptured up to 11 times over the subsequent 57 days. Skin swabs for skin bacterial community work were collected each time a toad was recaptured.

### DNA Extraction, Amplification, Sequencing, Analyses, and Data Accessibility

Genomic DNA was extracted from skin swabs using the DNeasy Blood and Tissue 96-Well plate kit (Qiagen, Germany) following the manufacturers instruction with the exception of the volume of buffer AE in the elution step, which we adjusted to 50 µl from 200 µl. Amplification of the V4 region of the 16S rRNA gene was performed using a barcoded 515f/806r primer set [32]. Barcoded amplicons were pooled in equimolar concentrations and sequenced on an Illumina MiSeq sequencing platform in rapid run mode (V2 chemistry 150 bp paired-end sequencing protocol) at the University of Colorado Boulder. Filtering, dereplication, denoising, chimera identification and removal, and end read merging were performed on the raw sequences using functions in the DADA2 package in R [33]. Taxonomy was assigned to each exact sequence variant (ESV) to the genus level using the SILVA database [34] version 138.1 as the reference database. Sequences corresponding to eukaryotes, mitochondria, or chloroplasts were removed and samples were rarefied to 2000 sequences per sample to control for uneven sequencing depth. Samples with insufficient sequences were removed prior to downstream analysis. Data visualizations and statistical analyses were generated with the vegan and mctoolsr packages in R [35, 36]. See the supplementary methods for further detail on extraction, sequence processing, and analyses. The bacterial sequence data and meta-data are accessible through NCBI SRA with BioProject ID PRJNA854967.

## Results

We processed 851 samples, and 817 were sequenced at sufficient depth (2000 sequences per sample) to be included in our analysis. The 12,879,243 sequences were assigned to 42,381 unique exact sequence variants (ESVs).

## Captive vs. Wild Comparisons

The skin microbial communities of both captive and wild tadpoles were dominated by a single ESV, an unidentified member of the Burkholderiales order. This single taxon represented, on average, 48.9% (S.E. = 0.32%) of sequences and 46.6% (S.E = 0.68%) in wild individuals. Despite this dominance, captive and wild microbial communities were significantly different based on a PERMANOVA ($F = 49.852$, df = (1, 224), $p < 0.001$). They were, however, similar in alpha diversity (wild mean Shannon diversity: 1.75, $n = 184$; captive mean Shannon diversity: 1.63, $n = 40$; ANOVA and Tukey HSD: $F = 0.589$, df = (1, 224), $p = 0.444$). There were a few taxa that were clear markers of captivity in tadpole skin communities, which were abundant in captive samples and very rare in wild samples. One of these was the *Aeromonas* genus (mainly ESV_10), (captive mean relative abundance: 0.104 vs. 0.004 wild; Mann-Whitney Bonferroni corrected $p = 4.26E-26$), members of which are common in aquaculture settings. We also identified the *Cetobacterium* genus (mainly ESVs 19 and 32), (captive mean relative abundance: 0.127 vs. 0.001 wild; Mann-Whitney Bonferroni corrected $p = 5.52E-37$). Taxa that were significantly more abundant in wild tadpoles include the genera *Flavobacterium* (captive mean relative abundance: 0.005 vs. 0.186 wild; Mann-Whitney Bonferroni corrected $p = 1.53E-14$) and *Sphingorhabdus* (captive mean relative abundance: 0.006 vs. 0.073 wild; Mann-Whitney Bonferroni corrected $p = 3.45E-8$).

In adults, juveniles, and metamorphs, we found that captive and wild skin microbial communities were different (PERMANOVA $F = 13.989$; df = (1, 207), $p < 0.001$) (Fig. 1A). The skin communities of captive adult toads had lower alpha diversity than their wild counterparts (Shannon; Wild = 3.44, captive = 2.14, ANOVA and Tukey HSD: $F = 16.49$, df = (1, 68), $p = 0.0001$) (Sup. Fig 7). There were no significant differences in metamorphs or yearling alpha diversity. Unlike in the larval life stage, there were no extremely clear markers of captivity in adults. The most significant differentiators between wild and captive toads post-metamorphosis were the genera *Perlucidbaca* (captive mean relative abundance: 0.052 vs. <0.001 wild; Mann-Whitney Bonferroni corrected $p = 5.98E-33$), *Antricoccus* (captive mean relative abundance: 0.064 vs. 0.006 wild; Mann-Whitney Bonferroni corrected $p = 0.003$), and *Chryseobacterium* (captive mean relative abundance: 0.122 vs. 0.076 wild; Mann-Whitney Bonferroni corrected $p = 0.043$).

The fecal and oral microbial communities of captive post-metamorphic toads were both different from their wild counterparts (fecal PERMANOVA $F = 6.667$, df = (1, 21), $p < 0.001$) (oral PERMANOVA $F = 10.123$, df = (1, 41), $p < 0.001$) (Fig. 1C). Captive toad mouths had significantly higher proportions of the genera *Marmoricola*, (captive mean relative abundance: 0.043 vs. <0.001 wild; Mann-Whitney Bonferroni corrected $p = 1.080E-7$), *Antricoccus*, (captive mean relative abundance: 0.264 vs. 0.004 wild; Mann-Whitney Bonferroni corrected $p = 1.017E-6$), and *Akkermansia*, (captive mean relative abundance: 0.042 vs. <0.001 wild; Mann-Whitney Bonferroni corrected $p = 1.024E-6$). The genus *Mycoplasma* was not found in the captive samples and very abundant in the mouths of wild toads (captive mean relative abundance: 0.000 vs. 0.381 wild; Mann-Whitney Bonferroni corrected $p = 4.513E-4$). The fecal taxa that were differentially abundant between the captive and wild samples were not identified to the genus level by our pipeline, but include members of the Bacteroidales, Enterobacterales, and Verrucomicrobiales orders.

The signature of captivity, when measured by the median Bray-Curtis dissimilarity between captive-wild pairs of samples, was different across life stage (Kruskal-Wallis rank sum test; df = (3, 1978), chi-square = 10,265, $p < 2.2E-16$) (Fig. 1B). The median dissimilarity of the tadpole captive-wild pairs (0.82) was lower than the metamorph (0.96), juvenile (0.94), or adult (0.92) pairs. Within adults and juvenile groups, the signature of captivity was larger in the fecal samples (median dissimilarity of captive-wild pairs = 0.96), than the oral (0.93) or skin samples (0.92). These differences between body sites were smaller than between life stages, but were still statistically significant (Kruskal-Wallis rank sum test; chi-square = 10.524, df = (2, 1979) $p = 0.005$) (Fig. 1D).

## Tadpole Reintroduction

The two ESVs most strongly correlated with the skin microbial community in captive animals, a *Cetobacterium* and an *Aeromonas*, decreased dramatically in relative abundance upon release into the wild. Pre-release, the *Cetobacterium* represented, on average, 14.2% of the sequences, and the *Aeromonas* represented, on average, 7.8% of the sequences. A few days post-release in a natural habitat (still pre-metamorphosis; 2 days at the Brittle Silver site and 4 days at the Brown's Creek site), both of these taxa were almost absent (less than 1% of sequences combined). The taxa that is highly dominant in both wild and captive tadpoles, the undescribed Burkholderiales, increased in relative abundance in the skin community at the Brown's Creek site over the same time period (45.3 to 91.1%) and the Brittle Silver site (49.7 to 59.6%) (Fig. 2B, C).

After metamorphosis, the translocated individuals at Brittle Silver were still different from the toads that completed metamorphosis in the wild (PERMANOVA $F = 10.482$, df = (2, 223), $p < 0.001$) (Fig. 2A), but the most common taxa in skin microbial community of wild metamorphs were largely present (see Sup. Fig. 1). Of the 10 most common genera in wild metamorphs, 9 were found on the reintroduced Brittle

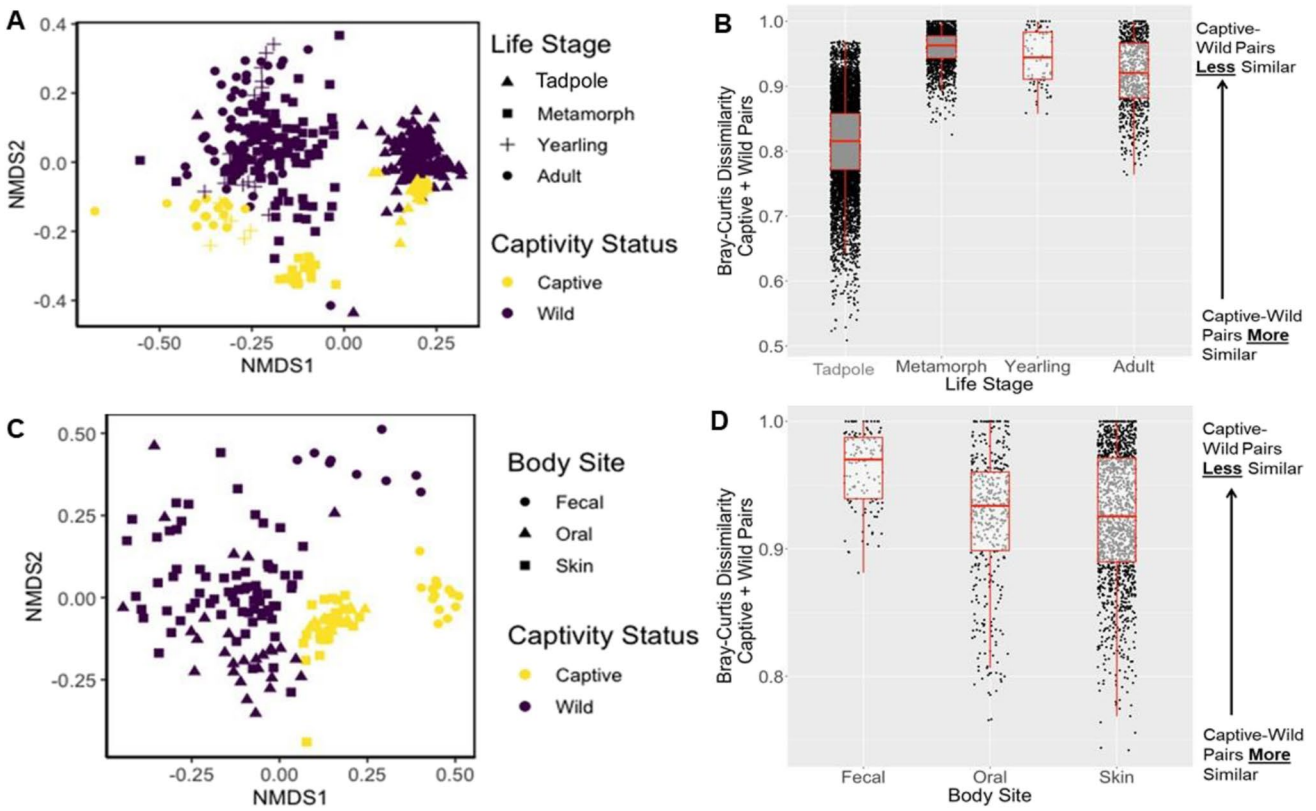


**Fig. 1** **A** The differences in captive and wild boreal toad associated bacterial communities. NMDS plot of boreal toad skin bacterial communities labeled by life stage and captive/wild status. Tadpole means larval individuals that are nearing metamorphosis, roughly equivalent to Gosner stages 36–42. **B** Plot of pairwise Bray-Curtis dissimilarities of skin bacterial communities from boreal toads of different life stages. Each dot represents the Bray-Curtis dissimilarity between one combination of a captive and a wild toad of the same life stage (e.g., captive adult skin and wild adult skin). The boxes represent the median and 25–75% range for the pairwise combinations. **C** NMDS plot of boreal toad bacterial communities labeled by body site and captive/wild status. **D** Plot of pairwise Bray-Curtis dissimilarities of skin bacterial communities from different body sites of adult boreal toads. Each dot represents the Bray-Curtis dissimilarity between one combination of a captive and a wild toad of the same body site (e.g., captive adult fecal and wild adult fecal). The boxes represent the median and 25–75% range for the pairwise combinations

Silver metamorphs just three weeks after they were released as tadpoles. The one exception was an unidentified genus in the Dermabacteraceae, home to two of the most common ESVs in the dataset.

### Adult Reintroduction

The skin microbial community of the adult toads that were translocated from a captive to a wild environment experienced multiple transitions in their skin microbial community. Generally, these transitions were similarly timed and involved increases in proportional abundance of the same microbial taxa in each of the four individual adult toads tracked during the experiment. The first transition, occurring immediately after release into the wild site, involved a large increase in the relative abundance of an *Acinetobacter* ESV, with concurrent decreases in the relative abundance of a Dermabacteraceae ESV. The next transition occurred a few weeks later, when the skin of all four toads showed an increase in a *Chryseobacterium* ESV (Fig. 3A). There were 556 ESVs shared between reintroduced and fully wild toads but not captive adults, and 87 ESVs shared between captive and reintroduced adults but not found in fully wild adults (Sup. Fig. 5). The similarity between the reintroduced individuals is also visible in the similar trajectories of the four individuals in multivariate space through time (Fig. 3B). The mean Bray-Curtis dissimilarity from captive adults only increased slightly from the first post-release recapture (0.71) to the final recapture timepoint 50 days later (0.79) (Sup. Fig. 5).

## Discussion

Our study expands upon the growing body of work that seeks to understand how captivity influences the microbiome of animals. First, we add a new dimension to comparisons of

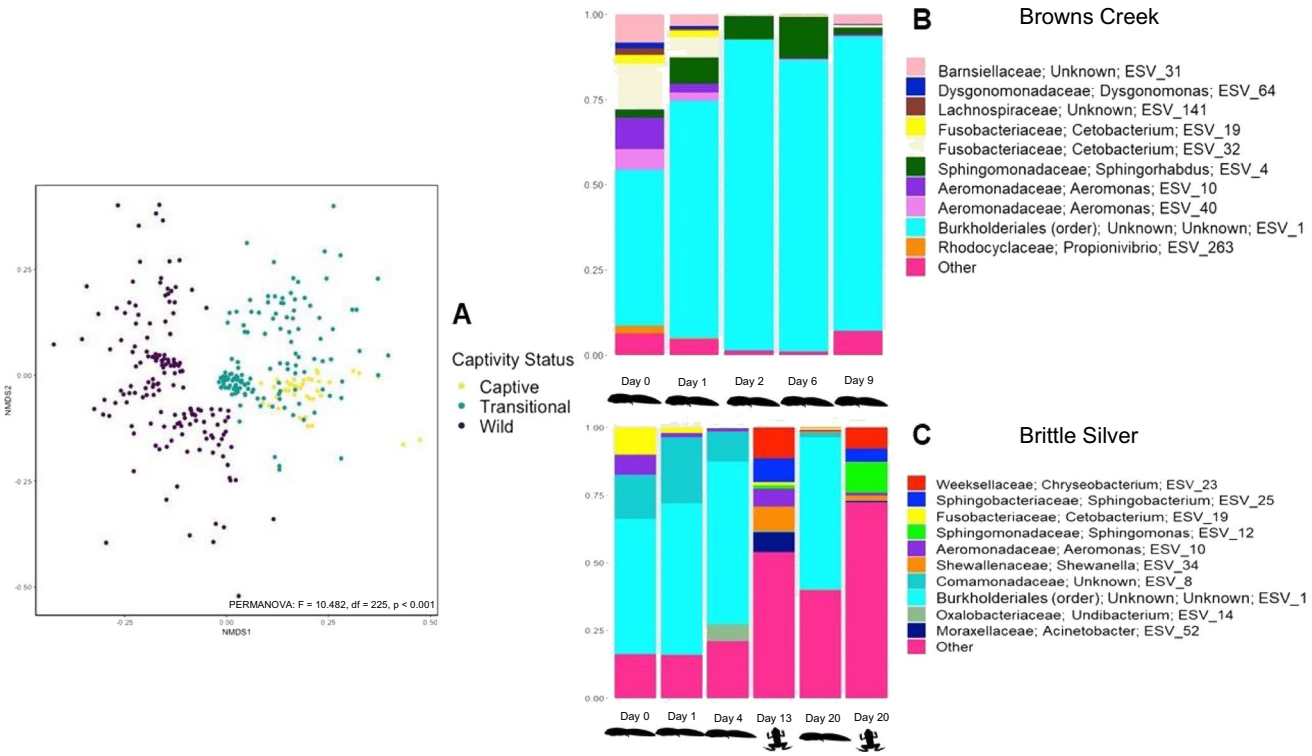


**Fig. 2** The transition of skin bacterial communities of boreal toads released into the wild from captivity as tadpoles. **A** NMDS plot of the skin bacterial communities of larval boreal toads. Each point represents a single tadpole that was either captive, fully wild, or released into the wild from captivity. **B** Stacked bar charts displaying the ten most common ESVs found in larval (and metamorphic) boreal toad skin bacterial communities sampled during captive to wild reintroduction effort at Brown's Creek (*N* per timepoint = 10, 10, 10, 10, 30). The initial timepoint represents the pre-release (captive) state. The columns represent the mean proportional abundances from individuals sampled at that timepoint. The illustrations below represent the life stage of the toads at that timepoint. **C** Stacked bar charts displaying the ten most common ESVs found in larval (and metamorphic) boreal toad skin bacterial communities sampled during captive to wild reintroduction effort at Brittle Silver (*N* per timepoint = 30, 30, 27, 30, 30, 15). The initial timepoint represents the pre-release (captive) state. The columns represent the mean proportional abundances from individuals sampled at that timepoint. The illustrations below represent the life stage that the toads were at that timepoint. The final timepoint included sampling from members of the cohort that were both pre- and post-metamorphosis, and those groups are displayed separately (metamorphosis does not happen simultaneously in all members of the cohort)

captive versus wild amphibians by studying different body sites (skin, oral, and fecal). Second, we examine how the effect of captivity differs across pre- and post-metamorphic developmental life stages of amphibians. And lastly, we examine how translocations of amphibians from captivity to the wild affect bacterial communities in both pre- and post-metamorphic life stages.

## Effects of Captivity on Different Body Sites and Developmental Life Stages

Unsurprisingly, the microbial communities associated with captive boreal toads were different than those associated with wild toads. The strength of the impact of captivity varied across body sites and developmental stage. For example, the skin bacterial communities of captive and wild tadpoles did not differ significantly in alpha diversity, but there were still multiple bacterial taxa that were differentially abundant and identified a sample as wild or captive. Sequence variants from the *Aeromonas* and *Cetobacterium* genera were common in captive samples, but nearly absent from wild samples (Fig 2B/C). *Aeromonas* species are ubiquitous in fresh water and many are associated with diseases in aquaculture facilities [37]. Members of the *Cetobacterium* genus are also associated with freshwater fish and aquaculture [38], so its presence on tadpoles from NASRF (primarily a fish hatchery) is not surprising. Even with these captivity-associated ESVs, the effect of captivity on tadpole skin bacteria is smaller than in the other life stages and body sites (Fig. 1B). This trend was largely driven by the fact that in both wild and captive tadpoles, a large fraction of the sequences from skin swabs came from a single ESV, an unidentified member of the Burkholderiales order. The dominance of this single sequence variant on both captive and wild skin indicates that it may be an important and durable larval symbiont. It is not known how this bacterium is acquired by boreal toads; it

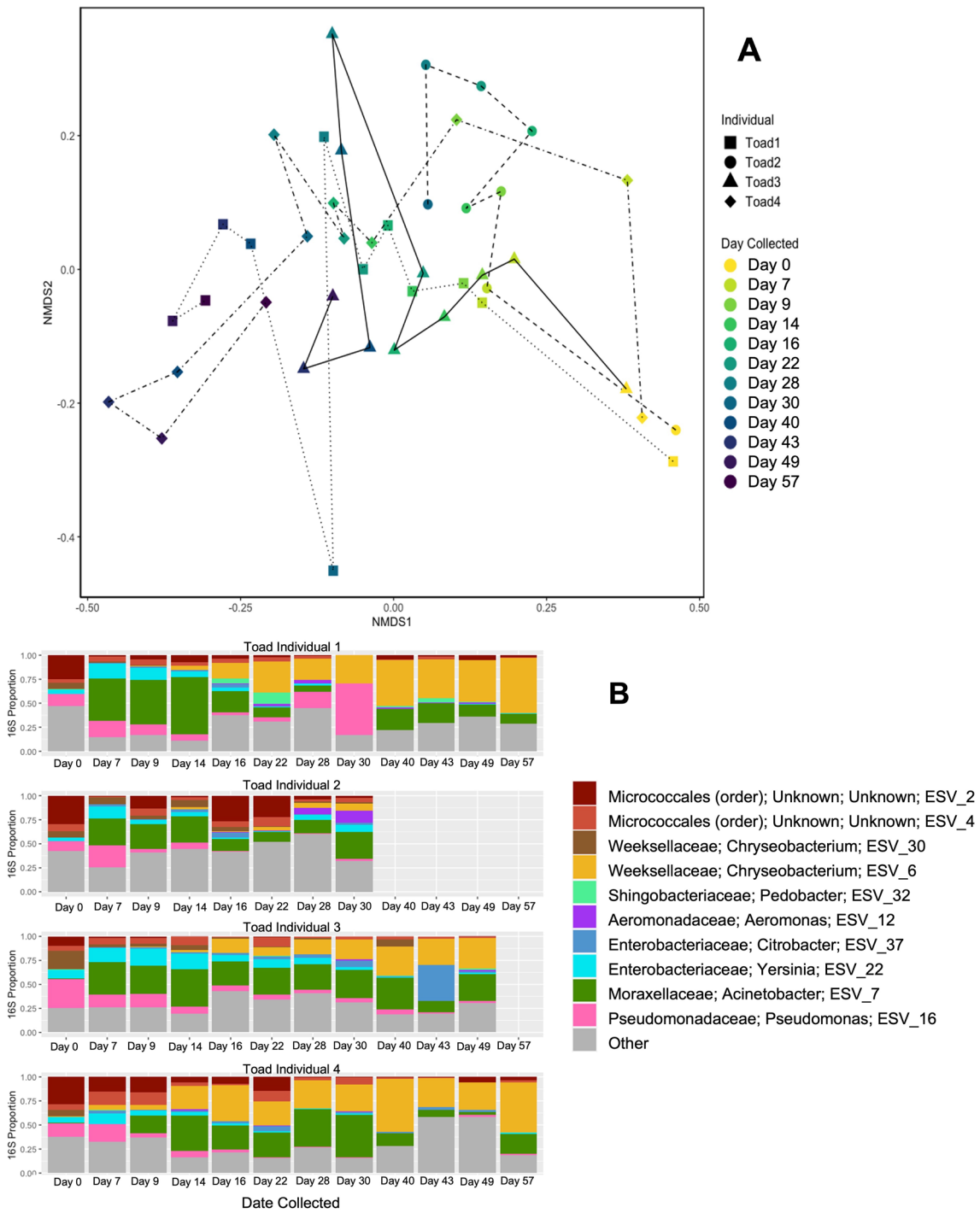


**Fig. 3** The transition of skin bacterial communities of boreal toads released into the wild from captivity as adults. **A** Stacked bar charts displaying the relative abundance of the ten most common sequence variants (and all others combined) of the skin bacterial communities of captive adult boreal toads released into the wild. Each row represents a single individual toad. The most common ESVs were determined across the whole dataset, including all four toads and all time points. **B** NMDS plot showing the movement in multi-dimensional space over time of the skin bacterial communities of captive adult boreal toads released into the wild

may be selected for by the host from the environment or it may be vertically transmitted. Swabs from egg masses show this ESV is present shortly after fertilization, though at lower proportional abundances (see Sup. Fig 2).

Post-metamorphosis, the effect of captivity is stronger. Bacterial Shannon diversity is lower in captive adult skin relative to wild toads, and each life stage has a number of microbial taxa that are differentially abundant across captivity states (Sup. Table 2). This generally matches what other studies that compared the skin bacterial communities of captive and wild individuals have found [16, 23]. A multispecies meta-analysis recently found no consistent impact of captivity on the skin bacterial communities of adult amphibians, but that dataset did include some boreal toad samples which did indicate a large impact of captivity on microbial richness and phylogenetic diversity [25]. The lower alpha diversity in captive toad skin is intuitive, since their environment has significantly less source inoculum than wild individuals are likely exposed to. Boreal toad rearing conditions at NASRF involve plastic raceways without soil or pond water which would provide a rich diversity of environmentally abundant bacteria that are often found in the skin communities of wild boreal toads (also see [16]). The lack of this pattern pre-metamorphosis suggests that the host control over the skin bacterial community may be stronger pre-metamorphosis than post-metamorphosis.

The effect of captivity across body sites was also variable. Captive skin was less diverse than wild skin, the oral communities were similarly diverse, and fecal communities were much more diverse than wild feces (Sup. Fig 3,7). All three body sites had multiple taxa that were significantly differentially abundant in wild and captive samples. The impacts of captivity on the gut microbiome in wildlife are well documented [39], and the large impact in our system could easily be attributed to dietary shifts. Interestingly, captive oral and skin microbial communities were more similar to each other in captivity than they were to the same body site in the wild (Fig. 1C).

## Tracking the Effects of Translocations from Captivity to the Wild

The small effect of captivity that we observed in pre-metamorphic toads (tadpoles) decreased rapidly after release into wild habitats, and disappeared nearly completely after metamorphosis. This transition happened over the course of just 3 weeks. The dominance of the undescribed Burkholderiales ESV on larval skin, along with the previously documented reorganization of skin microbial communities around metamorphosis [30] probably contributed to this result. The Burkholderiales was already dominant on the skin of captive larval toads, and upon release it increased to over 50% of the sequences within a few days in each of the larval reintroductions we sampled. Some of these differences are difficult to disentangle from the developmental changes we would expect even in the absence of a captive/wild transition. The skin bacterial communities of reintroduced individuals still cluster away from fully wild larval individuals (Fig. 2A), but this is explained by the fact that the reintroductions took place at different sites from the sampling of fully wild individuals and site has an impact on amphibian skin microbial communities [40]. Post-metamorphosis, the released toads resembled their wild counterparts much more than their fellow cohort members in the captive breeding facility (Sup. Fig. 4).

When sampling adult toads released from captivity into the wild, a shift to more wild-type skin bacterial communities began at the first recapture timepoints (1-week post-release). Notably, even though the four adult toads moved independently following release, their skin bacteria changed in a strikingly similar fashion. Members of the Micrococcales and Pseudomonadales orders decreased in relative abundance in the first weeks after release, followed by increases in the relative abundance of the genera *Acinetobacter*, and subsequently, *Chryseobacterium*. It did appear that the changes in the skin microbial community slowed dramatically after about one month in the wild, possibly indicating a "wild-type" equilibrium had been reached on the skin (Sup. Fig. 5, 6). This rapid initial shift suggests that post-metamorphosis, skin bacterial communities are mutable, and that a change in environmental inoculum will produce a change in skin microbial communities in a somewhat predictable fashion given the consistency across individual toads. This is in relative contrast to the pre-metamorphic individuals, where it appears that deterministic host factors play a larger role in bacterial community assembly, as evidenced by the larger overlap in communities across captivity status. There is a growing body of literature that demonstrates the large impact of the environment (and thus the potential source inoculum) on amphibian skin bacterial communities, but these studies are largely conducted in post-metamorphic individuals [41–43]. It is conceivable that, absent the major reorganization of skin microbial communities at metamorphosis, a change in environment would not produce significant immediate changes in the skin bacterial communities of toads because of priority effects, but our data contradict this hypothesis. It is possible that the microbes that established on adult toad skin in captivity were not particularly well adapted to the niches available, but were able to establish there in the relative absence of better competitors in the depauperate source inoculum of captivity. Additionally, the taxa that were markers of captivity may have been absent in the source inoculum at the reintroduction sites.

## Implications for Conservation and Amphibian Translocations

In the few studies that examine host-associated microbial communities across the transition from captivity to the wild, results are mixed with regards to the persistence of

the microbial signature of captivity. In Tasmanian devils reintroduced to the wild, gut microbial communities began to resemble wild counterparts in as little as 3 weeks [44]. White-footed mice fed a special diet in captivity returned to a gut microbiome more similar to their wild counterparts than a standard captive style diet [12]. Translocations in deer mice (both captive to wild and wild to captive) produced gut microbial communities that resembled their cohabitants without a signature of the environment in which they were reared [45]. In the only other study to look at amphibian reintroductions, adult *Atelopus varius* transitioned from captivity to outdoor mesocosms acquired skin bacterial communities that more closely resembled those of wild individuals than those from captivity [25]. On the other end of the spectrum, in giant pandas released into the wild, authors concluded that 2–3 months may not be long enough to establish a fully wild-type gut microbial community in captive born individuals [46].

Our results with the skin communities of an amphibian more closely resemble those results from mice, Tasmanian devils, and *Atelopus* that reported limited persistence of the signature of captivity. Amphibian skin microbes play a large role in host health [47], and our results indicating a quick "rewilding" of skin bacterial communities upon release into the wild may be positive news. It is not yet clear whether rearing amphibians with a captive-type microbiome has longer term health effects that are not observed in a short-term study such as this one. However, the ability of these toad bacterial communities to resume a wild-type state suggest that long-term microbial impacts on amphibians reared in captivity and returned to the wild are likely negligible. Many reintroductions/translocations use the amphibian larval stage for efficiency/logistical reasons [15], and knowing that there is not a lasting impact on the toads (at least from a microbial perspective) supports the usefulness of these conservation strategies. This data also suggests that natural conditions during captive rearing may not be critical for the microbial health of amphibians in reintroduction efforts. Because the impacts of captivity on microbial communities are not consistent across species and habitats, our results for boreal toads may not be applicable to all amphibians. In the future, similar studies should be conducted in other threatened species for which captive rearing and reintroductions represent a significant component of the management strategy.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s00248-023-02229-3.

**Acknowledgments** The authors would like to thank the staff of the J. W. Mumma Native Aquatic Species Restoration Facility for their assistance with sample collection. This manuscript is contribution #853 of the USGS Amphibian Research and Monitoring Initiative. Any use of trade, firm, and product names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

**Author Contribution** All authors contributed to the study conception and design. Data collection was performed by Timothy Korpita, Mary Kay Watry and Erin Muths. The first draft of the manuscript was written by Timothy Korpita and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

**Funding** The Morris Animal Foundation (award D19ZO-044 to Valerie McKenzie) provided salary support for Timothy Korpita and the sequencing work in this study. An NSF grant (award #2123583 to VJM) also supported the completion of analyses and publication.

**Data Availability** The bacterial sequence data and meta-data are accessible through NCBI SRA with BioProject ID PRJNA854967.

## Declarations

**Ethics Approval** Sampling for this study was conducted with approved IACUC protocols 1505.04 and 2629 from the University of Colorado Boulder.

**Competing Interests** The authors declare no competing interests.

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