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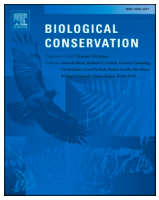

Effects of captivity and rewilding on amphibian skin microbiomes

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ABSTRACT

Captive breeding to safeguard against extirpation in the wild is a practice for many animal groups. Animals in captivity experience reduced contact with natural substrates and other animals, and consume atypical diets that may alter naturally occurring microbial associations. Amphibian skin microbiomes are vital for amphibian health, protecting them from pathogens and aiding in development, immune system training, and fecundity. Thus, understanding how changes associated with captivity influence microbial communities and the health of captive-reared amphibians is an important consideration in captive breeding and reintroduction programs. Overarching patterns of amphibian microbial diversity in captivity have not been previously explored. Therefore, we conducted a meta-analysis of skin microbes from captive-managed and wild individuals of 18 salamander and frog species from temperate and tropical biomes. We found that microbial composition of captive and wild amphibians differed for all species. However, while the overall captivity effect on amphibian skin richness was significant, the direction of the captivity effect on diversity metrics and antifungal function differed depending on the host species. One species exhibiting a large skin microbiome shift in captivity is the variable harlequin frog, *Atelopus varius*. A soft-release of *A. varius* to outdoor mesocosms "restored" the microbiome through time, and frogs also increased antifungal function of their skin microbiome with time in mesocosms. Rewilding the microbiome may influence resistance to diseases such as chytridiomycosis. Indeed, evaluating the outcome of individual species is necessary until we have a cohesive approach to mediate shifts of amphibian skin microbes that result from captivity.

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1. Introduction

The number of species listed as vulnerable, endangered, or critically endangered has increased dramatically in recent decades (González-del-Pliego et al., 2019), expanding the implementation of a wide range of conservation responses and intervention strategies. In some cases, the threats to species survival are sufficiently imminent and severe that animals are rapidly moved into ex situ captive programs to stave off extinction (Gratwicke et al., 2016). Commonly, captive programs are established with an initial goal of reintroducing the species back into their native range once the threat has abated (Lyles and May, 1987; Griffiths and Pavajeau, 2008). However, to date the outcomes of reintroduction efforts have been mixed, with some notable failures to establish viable populations in the wild (e.g., Seddon et al., 2007; Robert et al., 2015). Conservation biologists have attempted to use diverse approaches to increase the likelihood of success in reintroduction efforts, including strategies to mitigate or dampen the detrimental effects of captivity (van Wieren, 2012). While this has traditionally meant incorporating pre-release conditioning or a soft-release of captive animals (Seddon et al., 2007), the role of the microbiome of captive animals, and how it differs from that of their wild counterparts, has not figured prominently in reintroduction biology until relatively recently (Redford et al., 2012).

The conventional wisdom for captive breeding is that the microbiome of captive animals is depleted or disrupted relative to their wild counterparts (Ross et al., 2019). The disruption of the microbiome in captivity may be due to use of artificial substrates, frequent cleaning of housing spaces, provision of a non-native diet, decreased habitat complexity and stabilized climatic conditions, and/or reduced species interactions and behaviors which are constrained in captivity (McKenzie et al., 2017). A depleted microbiome can have a wide range of detrimental health effects (Redford et al., 2012). For example, conditions in captivity may prevent colonization by beneficial microbiota that could contribute to disease defenses, proper absorption of nutrients, and other biological functions that are critical for health (Sommer and Bäckhed, 2013; Cénit et al., 2014). Indeed, this paradigm is supported by data from diverse animals in captive populations (Kong et al., 2014; Alfano et al., 2015; Cheng et al., 2015). The studies included an in-depth analysis of mammalian gut microbiota in captivity compared to wild mammals showing consistent compositional differences between wild and captive counterparts but inconsistent responses of microbial richness, including decreased diversity, no change, or increased diversity (McKenzie et al., 2017).

One compelling example of threatened species that could benefit from investigations on the microbiome is that of amphibians (Bletz, 2013; Rebollar et al., 2016; Walke and Belden, 2016). The Conservation Needs Assessment, completed in 2019, recommended 577 amphibian species for ex situ captive breeding programs (www.conservationneeds.org). Currently, 180 at-risk species are subsisting in captive breeding programs, with a goal to reintroduce healthy individuals to depleted populations in the wild. For many of these amphibian species, emerging pathogens, including ranaviruses and *Batrachochytrium* fungi, such as, *Batrachochytrium dendrobatidis* (*Bd*) and *Batrachochytrium salamandrivorans* (*Bsal*), are severe and persistent threats, affecting hundreds of amphibian species globally (Spitzen-van der Sluijs et al., 2016; Scheele et al., 2019). As the risk of disease emergence and spread continues to threaten amphibian populations, the reliance on captive breeding programs will likely expand, potentially overtaxing the finite resources of captive breeding programs. Ultimately, amphibian conservation strategies will benefit from the development of innovative approaches to make reintroduction efforts successful.

The microbiome of amphibian skin has been a specific research focus due to the devastating impacts of the infectious disease chytridiomycosis, which is caused by lethal pathogens that colonize the epidermis (Voyles et al., 2009). The cutaneous microbiome can contribute directly to protection from invasive pathogens (Harris et al., 2009; Kueneman et al., 2016), and indirectly through priming immune defenses (Rollins-Smith et al., 2011). Recent studies have shown that the presence and, in some cases, the proportional abundance of bacteria that are known to inhibit *Bd*, can influence disease outcomes for individual amphibians and for populations of species across the landscape (Jani et al., 2017). Interactions between amphibian skin microbiota and amphibian pathogens may have consequences for amphibian communities because co-occurring amphibian species in the same habitats have distinct microbiomes (Kueneman et al., 2014), and therefore may have varying susceptibility to disease. In addition, because temperature can alter how the microbiome functions, recent research has focused on understanding how thermal conditions may impact the microbiome, thereby mediating amphibian defenses against *Bd* (Robak and Richards-Zawacki, 2018).

To date, our understanding of the effect of captive programs on the amphibian skin microbiome is limited. While several studies suggest that captive individuals have cutaneous microbiomes that retain lower microbial diversity compared to their wild counterparts (Becker et al., 2014; Antwis et al., 2014; Bates et al., 2019), other studies have reported no significant change (Flechas et al., 2017; Hernández-Gómez et al., 2017). Overall, in published studies on 18 amphibian species, 12 species showed decreased richness and altered bacterial composition in captive amphibians, while six species showed increases in bacterial richness or no change in microbial diversity (Supplemental Table 1). However, these studies used markedly different methods for experimental design, sample collection, and statistical analysis. These inconsistencies likely contributed to the mixed outcomes, making interpretation difficult. As such, there is a need for a reevaluation and synthesis of the available data on skin-associated microbiomes between captive and wild populations. In addition, we currently lack a deeper understanding of how the microbiome may shift – both in composition and function – as animals transition from captive conditions back into the wild during reintroduction efforts.

To advance our understanding of the amphibian microbiome in captive and wild conditions, we integrated two key approaches. First, we conducted a meta-analysis to examine the effects of captivity on amphibians using a standardized sampling protocol, a re-examination of published data, and a pipeline with identical measurement rubrics across amphibian species. We explored the consequences of captivity using a hypothesis-driven framework. Our main hypotheses were that captive conditions reduce amphibian skin microbiome diversity, antifungal function, and beta dispersion (a measure of the microbial community dissimilarity among a group of samples). In addition, we asked more specific questions concerning factors that might contribute to microbiome differences in captive populations, including (1) substrate type (semi-sterile or semi-natural), (2) hatching origin (captive or wild hatched), (3) amphibian order (Anura or Caudata), (4) and host bioregion (Temperate or Tropical species). Second, we conducted a soft-release reintroduction experiment to assess how captive amphibian skin microbiota changes over time (incorporating pre-release conditioning using outdoor mesocosms). We hypothesized that the skin microbiome – and the antifungal function of the microbiome – would shift to resemble the microbiome of wild frogs more closely. We consider the possibility that rewilding the microbiome (the process of restoring the wild-type microbiome of an animal) may improve reintroduction success by restoring beneficial microbiomes prior to release.

2. Methods

2.1. Summary of the meta-analysis

For this study, we sequenced bacterial community DNA collected on swabs gathered from 302 individual amphibians. We assembled the newly sequenced data with skin microbiome data from published studies to generate a dataset that includes samples collected from 578 individual post-metamorphic amphibians, representing 18 species and ten

amphibian families found in temperate and tropical regions. A summary of all amphibian samples included in the meta-analysis is provided in Table 1.

2.2. Skin sampling and bacterial community sequencing

We collected amphibian skin microbiota using sterile swabs and standardized sterile sampling techniques as descried in (Culp et al., 2007). We extracted DNA using Qiagen Power Soil kit and amplified the V4 region of the 16S rRNA gene with barcoded primers (515f–806r) (except for one published study which sequences V2) (Hernández-Gómez et al., 2017). We sequenced samples on Illumina MiSeq and Hiseq platforms (Table 1). We compiled the raw sequence data from published studies and data from manuscripts in preparation (Table 1).

Table 1
Shown are the amphibian species sampled, numbers of samples included by captive and wild conditions, the gene region sequenced, and the citation for the data included in this study. All data were collected on an Illumina MiSeq platform except for the two studies of captive and wild *Anaxyrus boreas* collected on an Illumina HiSeq.

Species	Status	Count	Rarefaction Depth	Reference
Ambystoma maculatum	Captive	17	2885	Barnhart, 2018
Ambystoma maculatum	Wild	13	2885	This study
Anaxyrus boreas	Captive	9	2885	Chen et al., 2022
Anaxyrus boreas	Wild	19	2885	Kueneman et al., 2016
Andrias japonicus	Captive	21	1254	Bletz et al., 2017
Andrias japonicus	Wild	13	1254	Bletz et al., 2017
Atelopus certus	Captive	5	2885	This study
Atelopus certus	Wild	8	2885	This study
Atelopus limosus	Captive	5	2885	This study
Atelopus limosus	Wild	28	2885	This study
Atelopus varius	Captive	10	2885	This study
Atelopus varius	Wild	28	2885	This study
Atelopus zeteki	Captive	10	2885	Becker et al., 2014
Atelopus zeteki	Wild	28	2885	Becker et al., 2014
Cryptobranchus alleganiensis	Captive	20	2885	Hernández-Gómez et al., 2019
Cryptobranchus alleganiensis	Wild	17	2885	Hernández-Gómez et al., 2019
Cynops pyrrhogaster	Captive	18	1432	Sabino-Pinto et al., 2016
Cynops pyrrhogaster	Wild	27	1432	Sabino-Pinto et al., 2016
Espadarana prosoblepon	Captive	4	2885	This study
Espadarana prosoblepon	Wild	48	2885	This study
Hylomantis lemur	Captive	17	2885	This study
Hylomantis lemur	Wild	5	2885	This study
Rana catesbeianus	Captive	13	2885	Chen et al., 2022 and this study
Rana catesbeianus	Wild	52	2885	Kueneman et al., 2019 and this sudy
Mantella aurantiaca	Captive	8	2885	This study
Mantella aurantiaca	Wild	6	2885	This study
Osteopilus septentrionalis	Captive	9	2885	Chen et al., 2022
Osteopilus septentrionalis	Wild	6	2885	Kueneman et al., 2019
Plethodon cinereus	Captive	18	2885	Loudon et al., 2014
Plethodon cinereus	Wild	20	2885	Loudon et al., 2014
Rana luteiventris	Captive	4	1234	Loudon et al., 2020
Rana luteiventris	Wild	10	1234	Loudon et al., 2020
Rana pretsiosa	Captive	31	2068	This study
Rana pretsiosa	Wild	17	2068	This study
Strabomantis bufoniformis	Captive	4	2885	This study
Strabomantis bufoniformis	Wild	10	2885	Rebollar et al., 2016

We quality filtered the sequences and further processed them using Quantitative Insights into Microbial Ecology 2 (QIIME2 (Bolyen et al., 2019)). Sub-Operational Taxonomic Units (sOTUs) were determined using deblur (Amir et al., 2017). Within this sOTU clustering, we trimmed all sequences to 90 bp to accommodate all studies. The final dataset comprised 15,816,777 reads with a mean frequency of 28,705 and a median frequency of 18,702 reads per sample.

2.3. Assessment of microbial diversity and composition

We subsequently rarefied most samples at 2885 reads per sample to allow for robust sample sizes across most species and to fully capture the microbial diversity (Supplemental Fig. 1). However, there were four species for which we rarefied at lower depths to allow for adequate sample size (Table 1). We used this approach to allow for the inclusion of amphibian species with lower sequencing depth while also providing a more in-depth analysis for species with higher sequence depth. We built a phylogenetic tree using fasttree2 using align-to-tree-mafft-fasttree, and taxonomy was assigned using sklearn and a pre-trained Greengenes database. We calculated alpha diversity [sOTU richness, phylogenetic diversity and evenness] and beta diversity (weighted and unweighted Unifrac; (Lozupone and Knight, 2005) in QIIME2. We used weighted Unifrac in our species by species (wild vs. captive) comparisons to capture the full compositional differences that exist (Fig. 1). We used unweighted Unifrac to capture the assimilation of rare taxa into the microbial community of amphibians undergoing a soft-release (Fig. 3). We subsequently analyzed and visualized the results in R (R Core Team, 2021).

2.4. Prediction of capacity to inhibit Batrachochytrium pathogens

We predicted *Batrachochytrium*-inhibitory function using a custom bash script (see Github: https://github.com/m-bletz/Amphibian-CaptiveWild-Metaanalysis) and a database containing 16S rRNA sequences from amphibian skin bacteria that have been tested for functional activity against the two *Batrachochytrium* pathogens, (*Bd*) and (*Bsal*). This database included 7382 total sequences, 1489 of which exhibited consistent inhibitory function (Woodhams et al., 2015). The bash script used vsearch to cluster sOTU sequences to potentially inhibitory sequences within the database at 99% similarity. We then calculated two response variables, antifungal function (the proportion of "inhibitory" reads with respect to the full, rarefied community) and antifungal richness (the number of inhibitory sOTUs).

2.5. Calculation of diversity and dispersion metrics

We calculated and pooled our effect sizes for three alpha diversity metrics, two functional prediction metrics, and community dispersion (specifically, the average dissimilarity from individual observation units to their group centroid in multivariate space) using the metafor package in R (Balduzzi et al., 2019; Viechtbauer, 2010). Briefly, we calculated arithmetic means, standard deviations, and sample sizes for wild and captive individuals from each species/dataset for each univariate metric. For community dispersion we calculated the average within group pairwise distances with the adegenet package in R (Jombart and Dray, 2008). We calculated bias-corrected standardized mean differences using Hedge's g (Hedges, 1981). We pooled and assessed these effect sizes using mixed effect models using the rma.mv() function with maximum likelihood estimation in R. We included a random term of study to account for non-independence of samples for two study species (*Plethodon cinereus* and *Ambystoma maculatum*). The rma.mv function includes a test for heterogeneity, using a generalized weighted least squares extension of Cochran's Q-test (Viechtbauer, 2010). Furthermore, we used subgroup or moderator analyses to examine a priori hypotheses about whether substrate type (semi-sterile or semi-natural), hatching origin (captive or wild hatched), amphibian order (Anura or

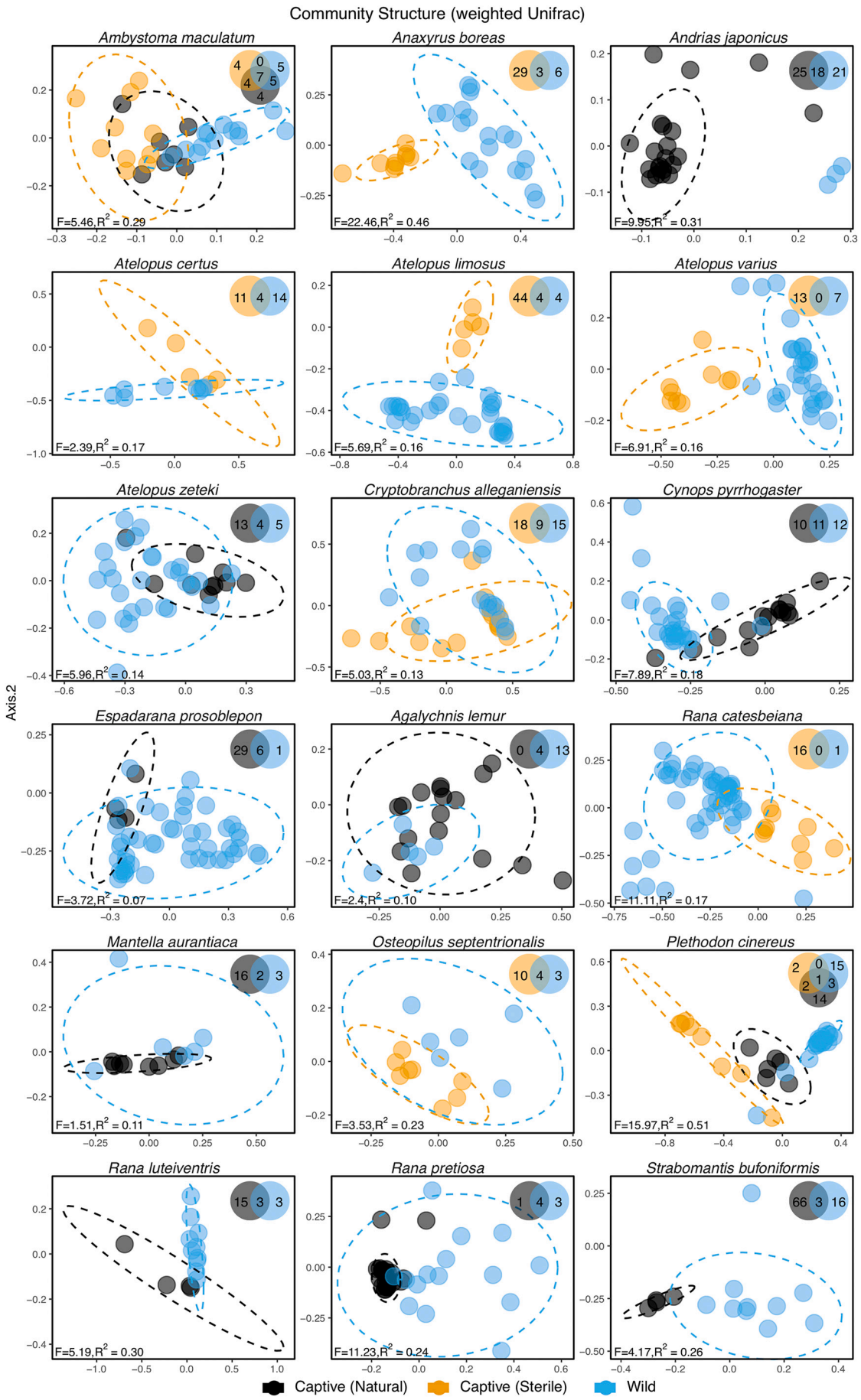

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Fig. 1. Captivity affects skin community structure measured as between-community (beta) diversity among samples using principal coordinates analysis (PCoA) of weighted unifrac distance. Black circles represent captive individuals housed in semi-natural environments (including soil and plant substrates), yellow circles represent captive individuals housed in semi-sterile conditions (no natural substrates), and blue circles represent wild individuals collected from their natural environments. Each species comparison of beta diversity between captive and wild individuals includes a Venn diagram showing overlapping core bacteria (bacteria found in >75% of samples by type). Adonis statistics are provided in the lower left of each plot; $p = 0.001$ for all, except *Atelopus certus* = 0.04, *Cryptobranchus alleganiensis* = 0.003, *Espadarana prosoblepon* = 0.007, *Agalychnis lemur* = 0.024, *Mantella aurantiaca* = 0.124, *Osteopilus septentrionalis* = 0.008, *Rana luteiventris* = 0.003, *Strabomantis bufoniformis* = 0.003.

Caudata), and host bioregion (Tropical or Temperate) explained the observed effect sizes or reduced heterogeneity in effect sizes. We define semi-sterile substrate conditions, as a housing tank that was near empty, except for treated water, or paper towels, and semi-natural substrate conditions, as a housing tank has soil and plants. We visualized effect sizes with ggplot2 in R (Wickham et al., 2019).

2.6. Analysis of beta diversity

We examined differences in beta diversity among captive and wild individuals using adonis() in the vegan package in R for both beta diversity metrics. We produced PCoA plots in R using the ape package (Paradis and Schliep, 2019) to compute axes and ggplot2 for visualization (Wickham et al., 2019). We identified differentially abundant sOTUs between captive and wild individuals for each species using linear decomposition models (LDM, (Hu and Satten, 2020)). We created heatmaps of identified differentially abundant sOTUs (q-value <0.01) using geom_tile() in ggplot2 (); see Github: https://github.com/m-bletz/Amphibian-CaptiveWild-Metaanalysis). In addition, we identified shared core sOTUs among captive and wild individuals within each species, as well as shared sOTUs among captive individuals across species, in R using ps_venn() in the MicrEco package (Liu et al., 2021). Core microbes are microbes that are common across individuals. We defined the core as the depth where we began to see shared taxa sOTUs for individuals of a given species. We chose 50% (core) for all species and 75% (core) for comparisons between wild and captive individuals of a given species (Fig. 1).

2.7. Rewilding of the microbiome with Atelopus varius

For the second part of this study, we used a species of conservation concern, the variable harlequin frog, *Atelopus varius*. This critically endangered species is bred in captivity with the aim of reintroducing and establishing viable wild populations (Lewis et al., 2019). However, like several other species, reintroduction efforts have been hampered by the persistent threat of *Bd*, which is still present and pathogenic in the native habitats of this species (Voyles et al., 2018; Linhoff et al., 2021).

Atelopus varius were captive reared from founders collected in the Donoso area of Panama. In captivity, they were maintained in same-sex groups of up to 10 individuals held in numbered glass tanks (size 25 × 53 × 38 cm) with automated misting systems lightly spraying the tank interiors with carbon-filtered water for 5 min every 2 h. Cages were initially sterilized with false bottoms installed (plastic egg crate covered in 0.5 mm screen mesh), keeping frogs out of contact with fecal pellets and any dirty water that may have pooled on the tank bottom. The false bottom was 20% covered with damp paper towel changed daily. Ultraviolet-emitting lights supplemented the 12-h overhead fluorescent lights for eight 45-min intervals per day. Each tank was furnished with one potted *Philodendron* plant. At the field site (8.91626°N 80.66267°W) located in the Donoso area, frogs were individually housed in 14 mesocosms (76 cm × 76 cm × 46 cm) built from a non-toxic, pliable, yet semi-rigid polyethylene mesh (0.6 mm) to prevent escape and exclude large predators but allow smaller invertebrates to enter the mesocosms. They were filled and kept with 2–4-in. depth of natural leaf-litter to maintain humidity and food for leaf-litter dwelling invertebrates and with a plant or piece of palm frond to allow animals to climb to elevated nocturnal sleeping positions. All the plant material used was obtained from the field site. During January 17–April 5, 2018, frogs inside the mesocosms were monitored weekly. A skin swab for microbiome analysis was obtained on day 0 ($n = 11$ pre-release), and again on either day 27 ($n = 5$) or day 79 ($n = 7$). Frogs were rinsed with 50 mL sterile water to eliminate transient bacteria and swabbed using a sterile rayon-tipped swab (MW113, Medical Wire & Equipment). Swabs were obtained by rubbing their skin for a total of 70 strokes, i.e., 10 times on the venter, 10 times on the dorsum, 10 times on each flank, 5 times on the ventral surface of each thigh and 5 times on each palmar and plantar. Skin swabs were kept in ice during fieldwork and stored at −20 °C in the laboratory. All amphibian sampling was conducted following IACUC approval and miAmbiente permitting.

3. Results

In our meta-analysis, we analyzed alpha diversity, predicted antifungal function and bacterial community composition from 20 published and unpublished studies representing 578 individual amphibians. We compared captive and wild samples for amphibian species collected from 10 families, 13 genera, and 18 species of frogs, toads, and salamanders across temperate and tropical localities (Table 1). In our field trial, we analyzed an additional 23 samples from *Atelopus varius* individuals to test for a rewilding response of skin microbiomes from captive individuals moved to outdoor enclosures.

3.1. Microbiome richness and predicted function

We calculated the effect sizes (Hedges g) for three alpha diversity metrics: richness, phylogenetic diversity and evenness (Fig. 2); two functional prediction metrics: antifungal richness, antifungal function (Fig. 2); and one beta diversity metric: community dispersion (Fig. 2). The overall effect of captivity (measured by Hedge's g) for richness and phylogenetic diversity were significant ($p = 0.02$ and $p = 0.04$, respectively) and predicted antifungal function approached significance ($p = 0.08$). We observed a lower richness and phylogenetic diversity in captive species for 13 out of 20 captive/wild comparisons, and a higher antifungal function in 12 out of 20 captive/wild comparisons. However, for evenness and antifungal richness, we did not observe a significant overarching effect of captivity (Fig. 2), and there was high heterogeneity among studies (Supplemental Table 2). We used subgroup analyses to explore the observed effect sizes and whether our four a priori hypotheses explained the observed variation (see methods). In all cases, the magnitude and direction of effect sizes did not support our hypotheses, and differences were non-significant, concerning substrate type (semi-sterile or semi-natural), hatching origin (captive versus wild hatching), amphibian order (Anura vs. Caudata), and host bioregion (Tropic vs. Temperate) (Supplemental Table 3).

3.2. Microbiome dispersion

In contrast, there was a significant overarching effect of captivity on community dispersion (SMD = −1.07, $p < 0.001$; Fig. 2). In nearly all cases (19/20), there was lower community dispersion for captive individuals compared to wild individuals. None of the moderators associated with our a priori hypotheses were significant ($p \geq 0.05$).

3.3. Microbiome composition

Except for one species (*Mantella aurantiaca*), we found significant

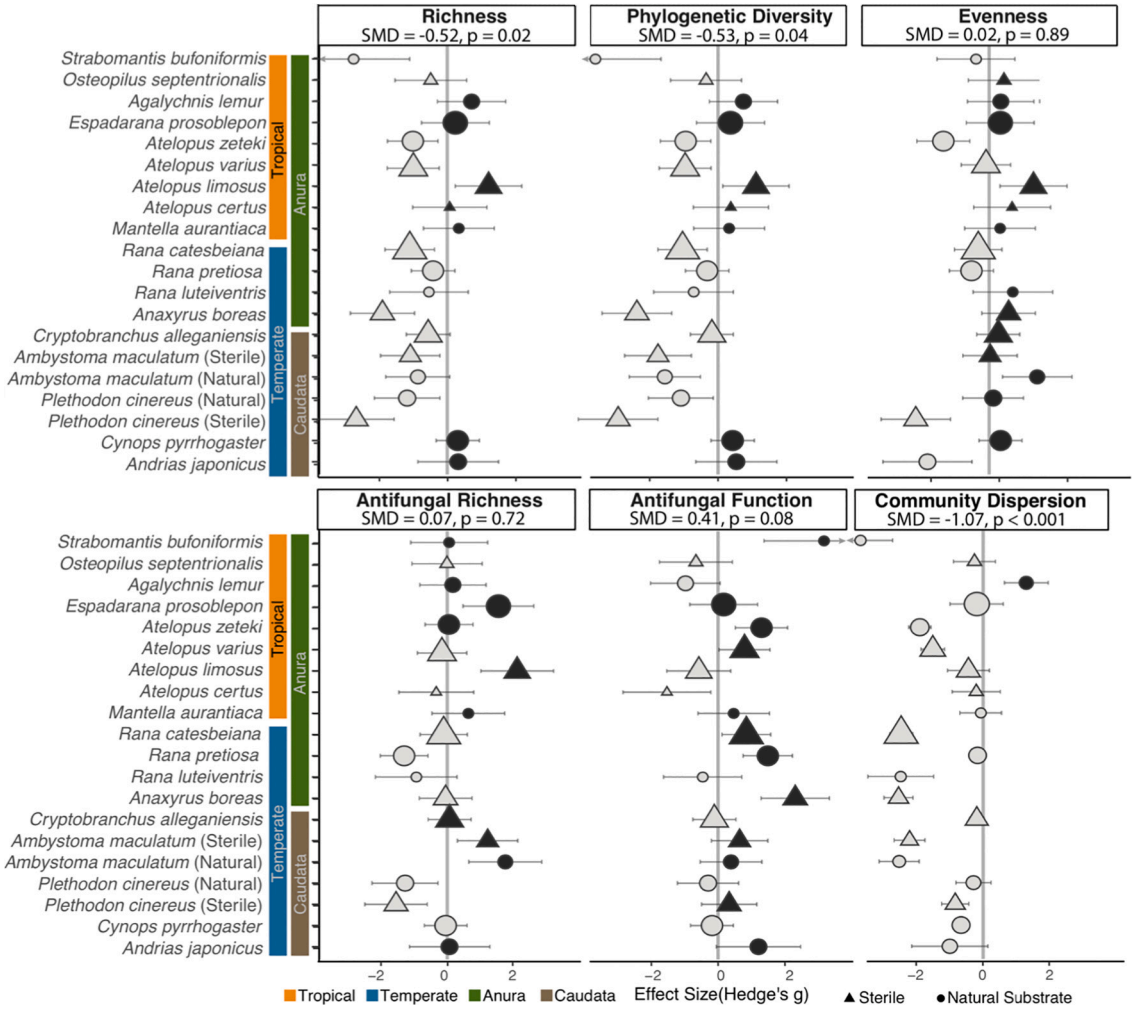

Fig. 2. Magnitude and direction of effect sizes vary across the studied species. Six panels show the mean effect sizes (Hedge's g, bias corrected standardized mean difference) across 18 studied species for alpha diversity (richness, phylogenetic diversity and evenness), beta diversity (dispersion) and antifungal prediction metrics (antifungal richness, antifungal function). Negative values indicate a reduction due to captivity, whereas positive values indicate an increase associated with captivity. Point size is scaled by sample size, and shape denotes substrate type (natural vs sterile) in captivity. Overall standard mean difference (SMD) and random effect model *p*-values are provided for each metric. Vertical color bars indicate amphibian order (Anura, Caudata) and bioregion (temperate, tropical).

differences in amphibian skin microbial community structure between captive and wild groups (Fig. 1). Adonis $p < 0.001$ for most species, and Adonis *p* values *Atelopus certus* = 0.04, *Cryptobranchus alleganiensis* = 0.003, *Espadarana prosoblepon* = 0.007, *Agalychnis lemur* = 0.024, *Osteopilus septentrionalis* = 0.008, *Rana luteiventris* = 0.003, *Strabomantis bufoniformis* = 0.003 (Fig. 1). Despite being significantly different in nearly all cases, the degree of community similarity between captive and wild individuals of the same species differed by species. Skin bacteria of captive individuals of *Anaxyrus boreas*, *Andrias japonicus, Atelopus limosus,* and *Plethodon cinereus* fell outside the community space of wild individuals (weighted Unifrac, visualized by the 95% confidence ellipses), whereas skin bacteria of captive individuals of *Mantella aurantiaca* and *Rana pretiosa* fell inside the community space of wild individuals. Indeed, the most common outcome was minimal to moderate overlap of community space between captive and wild individuals of the same species (Fig. 1).

To explore compositional differences of amphibian skin microbiomes in distinct habitat types (wild vs. captive) in more detail, we considered the conditions of captivity for each species. For instance, whether the individuals within a species were housed on semi natural substrates or semi-sterile substrates. Indeed, substrate type appeared to influence the differences between captive and wild individuals of an amphibian species. For the few species ($N = 2$) in which we have individuals housed in both semi-natural substrate conditions and semi-sterile substrate conditions (*Ambystoma maculatum, Plethodon cinereus*), we detected greater differences in bacterial community beta dispersion between captive and wild groups for individuals housed in semi-sterile conditions (Fig. 2).

3.4. Core microbiomes

We found no bacterial sOTUs that were shared across all wild amphibians, no taxa that were shared among all captive individuals on semi-sterile substrates, and only two bacterial sOTUs that were shared by captive individuals housed with semi-natural substrates. Two bacterial taxa were shared between wild and captive amphibians housed with semi-natural substates, and two bacterial taxa were shared between captive amphibians housed with semi-sterile substrates and semi-natural substrates. When we consider overlapping core bacteria between captive and wild individuals of the same species (bacteria found in >75% of samples by type), we found that all amphibians except *Atelopus varius* and *Rana catesbeiana* shared at least 1 core sOTU (Fig. 1, Supplemental Github; https://github.com/m-bletz/Amphibian-CaptiveWild-Metaanalysis).

3.5. Rewilding of the microbiome

One species within our study, *Atelopus varius*, was raised in captivity and then transferred to mesocosms and repeatedly sampled through time. This soft-release provided an opportunity to see how microbiomes shift when captive amphibians are returned to more semi-natural substrates. When amphibians were placed in mesocosms their skin microbiomes shifted to become more like the wild type over time. The PCOA unweighted Unifrac analysis revealed that individual samples at later time points in the mesocosms are more like wild sampled individuals (Adonis: Pseudo-F = 9.8521, $R^2 = 0.13905$, $p = 0.001$ and all pairwise comparison are significant $p = 0.01$, except mesocosms day 27, and day 79 where $p \geq 0.05$) (Fig. 3). We found that the percent of the bacterial community that matched to sequences with predicted antifungal function increased through time spent in the mesocosms (median value ± SE: mesocosm time point 0 = 0.39 ± 0.03, mesocosm time point 27 = 0.53 ± 0.08, and mesocosm time point 79 = 0.61 ± 0.09). Inferred antifungal function of the microbiome also increased through time spent in mesocosms (correlation tau = 0.43, z = 2.56, *p*-value = 0.01).

4. Discussion

The reliance on captive breeding programs has greatly increased in recent decades and amphibians have experienced the greatest numbers of species moved into captivity relative to any other class of vertebrates (Conservation Needs Assessment, 2019). We investigated the consequences of captivity on the skin microbiome of a diverse group of amphibians by synthesizing published data, generating additional data from new samples, and conducting a meta-analysis to probe specific questions concerning the microbiome in captive amphibians. We also conducted a soft-release reintroduction experiment to determine if the captive amphibian skin microbiota could shift over time to resemble more closely that of wild amphibians. Here, we provide a novel assessment that was only possible with assistance from amphibian conservation research communities, consistent methodological efforts, and standardized practices for animal handling, sampling, DNA extractions and sequencing (Thompson et al., 2017; Kueneman et al., 2019).

Although it is generally assumed that animals experience a loss of microbial diversity when they are moved into captivity (Kohl et al., 2014; Clayton et al., 2016; Borbón-García et al., 2017) and that amphibians lose protective skin microbiota, the first part of our study suggests that this assumption is not necessarily true. While our meta-analysis revealed an overarching responses of a decrease in amphibian skin microbial diversity in captivity, as measured by richness and phylogenetic diversity. We also observe an increase in amphibian skin microbial diversity for 35% (7/20) of the species in our study.

This finding may be explained by the fact that we detected high heterogeneity among studies, suggesting strong differences in the skin ecology of different amphibian species and/or that differences in experimental design that may account for inconsistencies in the direction of the shift in microbial diversity among amphibian species. The predicted antifungal function of the microbiome across all studies was only modestly higher in captive amphibians compared to their wild counterparts. While we did not directly test antifungal function (we made an inference from a database of microbial function), this finding of near significant ($P = 0.08$) increases in antifungal function in captivity is intriguing and worth further investigation. The presence of microbes with antifungal function may decrease infection risk and intensity (Kueneman et al., 2016; Chen et al., 2022). Irrespective of microbial function, our finding that some amphibians exhibited higher microbial richness when in captivity challenges the previously held notion that captivity necessarily leads to depletion of the microbiome (Bates et al., 2019; Chong et al., 2019).

We found that the community composition of amphibian skin microbiomes is consistently different between captive held individuals

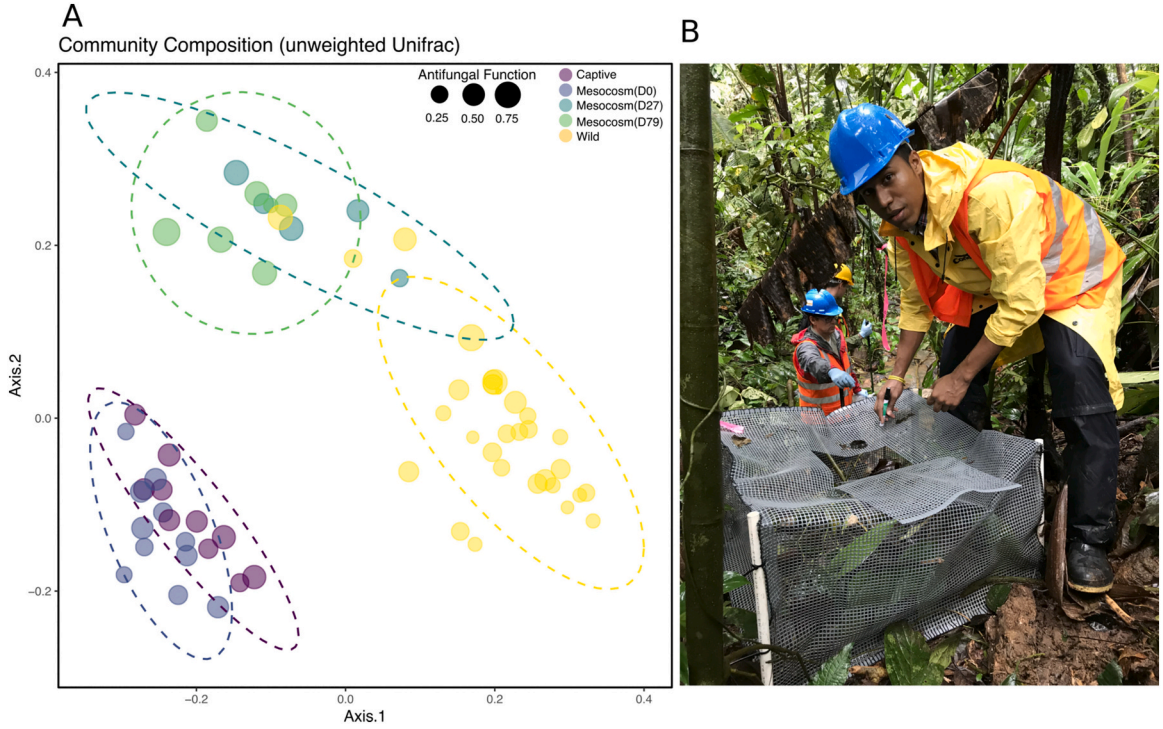

Fig. 3. Community composition *Atelopus varius*. (A) Community composition of *Atelopus varius* transitioning to wild habitats. Unweighted unifrac distances of juvenile *A. varius* born in captivity, transitioned to outdoor mesocosms (D0, D27, D79) and wild-caught field individuals. Sample groups are significantly distinct from one another Adonis: Pseudo-F = 9.8521, $R^2 = 0.13905$, p = 0.001 and all pairwise comparisons are significant $p = 0.01$, except mesocosms day 27, and day 79 where $p \geq 0.05$). Later mesocosm timepoints are closer to wild caught individuals from native habitats. Antifungal function (the proportion of the microbial community that matches known *Bd* inhibitory isolates) is depicted by the size of the spheres. (B) Image of *A. varius* in mesocosms.

and their wild counterparts (Species $N = 19/20$). These community differences could be a result of an increase or decrease of the abundance of certain microbial members, which could lead to differences in the overall composition of its microbiome. An additional component of skin microbial composition that could evoke differences between captive and wild group of samples is their dispersion. We measured this component of beta diversity to address our hypothesis that captive amphibians generally display greater microbiome homogeneity, while wild amphibians may exhibit greater microbiome dispersion. We found a significant effect of captivity on skin microbial homogeneity. Within amphibian species, captive animals had greater homogeneity, i.e., more similar microbiome composition to each other in comparison to wild animals that had more variability in the microbiomes. This result was expected, as amphibians in captivity are believed to be deprived of the same degree of microbial diversity that individuals of the same species are exposed to in the wild. Intriguingly, the one species (*Agalychnis lemur*) that had greater microbial community variation also had greater richness in captivity. This finding suggests that amphibian behavior or some other species-specific variable may play a role in decreasing skin microbial diversity in natural conditions. Indeed, a better understanding of the links between amphibian behavior and the microbial community may advance our understanding of the links between the microbiome and amphibian health in both captive and wild settings.

Using our meta-analysis we addressed additional specific questions concerning factors that might contribute to microbiome differences in captive populations, including the use of artificial housing substrates, captive hatching of the animals versus wild hatching, regional point of origin (e.g., temperate and tropical species), and amphibian order (e.g., Anura versus Caudata). To begin with, we detected greater, but non-significant, bacterial community differences between captive and wild groups for individuals housed with semi-sterile substrates compared to semi-natural substrates. As such, the substrate type that is used in captivity could potentially change the directionality of the richness response to captivity or further reinforce the captivity effect for a given species. Moreover, we compared the origins of amphibians in captivity by comparing captivity effects for amphibians raised in captivity to those that were brought into captivity and had been there for several months. We did not find a significant difference between these groups, suggesting that the effects of captivity on the skin-microbiome can occur rather rapidly, as demonstrated by Loudon et al. (2014). Additionally, we explored the hypotheses that host bioregion and host order may be important determinants of the effect of captivity on microbiome diversity and function. We did not find either factor (bioregion or host order) to have significant effects on the response of skin microbiome richness, community dispersion, or anti-fungal function to captivity. Instead, we found the strongest effects at the species level. Even within the same genera, amphibian species can behave uniquely with respect to their skin microbiome's response to captivity. Taken together, we report that captive conditions influence the composition of amphibian skin microbiome in predictable ways (altered composition, and decreased dispersion), but skin-microbial alpha diversity, antifungal richness and antifungal function have more variable responses than we had predicted.

In the second part of this study, we found that moving captive amphibians to experimental soft-release mesocosms (pre-release conditioning) resulted in rapid shifts in microbiome diversity, which we refer to as a rewilding the microbiome. While it is generally understood that the microbiome can shift over prolonged time scales (e.g., over the epizootic to enzootic transition (Jani et al., 2017), and over seasons (Longo et al., 2015; Tong et al., 2020; Le Sage et al., 2021), our results indicate that the microbiome shifted over a short amount of time for individuals that were moved out of a captive breeding program. Specifically, we found that a soft-release of captive-bred *A. varius* into outdoor mesocosms with natural substrates and food sources caused a shift in the skin microbiome toward a more wild-type microbial composition within 27 days. Furthermore, we estimated that the anti-*Batrachochyrium* function of the microbiome increased in mesocosms during this study. These findings provide further evidence that the microbiome is a naturally occurring mechanism helping to confer amphibian resistance to fungi such as *B. dendrobatidis* infection and they suggest that rewildling the microbiome can be an effective way to increase the success of reintroduction programs for some species.

4.1. Conservation implications

Conservation groups and agencies are often put into the position of either losing an amphibian species entirely or safeguarding the remaining depleted population by establishing ex situ programs and breeding them in captivity until conditions in the wild improve enough to reintroduce these threatened species (Linhoff et al., 2021). Safeguarding amphibians introduces substantial logistical challenges of how to care for a species and whether its new environment is sufficient for a species to develop and to reproduce. Supporting beneficial microbiomes in captivity is one tool conservation managers could use to improve the outcome of breeding and reintroduction programs. Comparing the skin microbiome from natural amphibian populations offers a vision of what the skin microbiomes of a captive-raised individual should re-wild into. Rewilding may be enhanced by incorporating wild individuals or natural substrates into the re-wilding protocol, thus facilitating acquisition of naturally occurring microbes. However, this approach should be weighed against the risk posed to the wild amphibians involved, and the risk of parasite transmission into captive colonies.

The composition and function of the microbiome may be one of several mechanisms that have facilitated disease resilience and allowed species to persist, and even recover from initial chytridiomycosis outbreaks (Voyles et al., 2018). Our results suggest that using prerelease conditioning, such as innovative soft-release approaches to "rewild the microbiome", may improve the likelihood of survival in reintroduction programs. In doing so, we found that *A. varius,* can be rehabilitated to a more natural wild-type skin microbiome with higher anti-*B. dendrobatidis* function through the course of our study, increasing through time spent in the outdoor mesocosms. Indeed, unpublished work also found that a soft-release improved survivorship of frogs, even though the odds of becoming infected also rose from the extra time exposed to *B. dendrobatidis* in the field (Estrada et al. in review). If native microbial defenses do not facilitate survival in landscapes with severe risk from skin pathogens, and rewilding is insufficient to support recovery of the species, more imaginative tools may be needed. Such tools could include the introduction of novel, altered, or even genetically modified microbiomes. In many cases the efficacy of such approaches may be short term, as the return of animals to wild-like conditions appears to facilitate the re-establishment of naturally occurring microbial associations. Given the severity of the amphibian decline crisis, more advanced strategies in conservation management are needed, but can also raise substantial ethical issues, or carry risks that should be fully considered.

The results of this metanalysis and soft-release for the goal of rewilding the microbiome can broadly inform strategies of captive breeding and reintroduction. The direction and the magnitude of the effect of captivity is not predetermined, and this study dispels conventional wisdom suggesting captivity always decreases microbial diversity, and protective components of the microbiome. Natural substrate conditions can help maintain wild-type microbial communities, and while microbial variability will likely be reduced in captivity, a soft-release can help recover beneficial groups of protective microbes if they are lost in captivity. Thus, we must continue to use science-based approaches to improve reintroduction efforts for the good of all species that require ex situ captive programs due to a variety of threats, and we must learn what we can from the successes and failures of all captive breeding programs.

Statement

We confirm that all the reported work is original, that this manuscript has not been submitted for publication elsewhere, and we have no conflicts of interest. Furthermore, all authors have seen and approved the final version submitted, all prevailing local, national and international regulations and conventions, and normal scientific ethical practices, have been respected.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocon.2022.109576.

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