

Understanding the temporal variability of skin-associated bacterial communities for the
conservation of threatened amphibian species

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ACADEMIC ABSTRACT

Amphibians harbor beneficial skin bacteria that can contribute to host defense against chytridiomycosis, an infectious disease caused by the lethal fungal pathogen *Batrachochytrium dendrobatidis* (Bd). However, while skin-associated microbial communities may alter host infection risk, the structure of these complex microbial communities can be impacted by both biotic and abiotic factors. In a series of three studies, I investigated the natural temporal and spatial variation in bacterial communities on the skin of wild and captive-born amphibians using 16S rRNA gene amplicon sequencing to characterize bacterial community diversity. First, in a study examining the skin bacterial communities of two sympatric treefrog species (*Agalychnis callidryas* and *Dendropsophus ebraccatus*) at a single pond over multiple seasons and years, I found that annual, seasonal, and even daily fluctuations in temperature and rainfall changed the skin bacterial communities on these species. Second, I further investigated the impact of seasonality and rainfall on amphibian skin bacterial communities with a study of the bacterial communities on *Craugastor fitzingeri*, a common terrestrial species, along a rainfall gradient, and five co-occurring amphibian species at a single site. The strong wet and dry seasonality in the tropical lowland forest impacted the bacterial communities of multiple stream-dwelling co-occurring species, but the nature of the changes differed among the frog species. For *C. fitzingeri* sampled along the rainfall gradient, I found there was variation in bacterial community structure among sites, although this was not correlated with the latitudinal rainfall gradient. Finally, I investigated the challenges faced by captive-reared *Atelopus limosus*, an endangered amphibian species, after soft-release into natural habitat with the use of mesocosms. I found that the skin bacterial communities reverted to wild-type fairly quickly, body condition decreased to come closer to wild conspecifics, and 15% of the frogs became infected with Bd during the 27 day trial in mesocosms. Overall, I found that skin bacterial communities of lowland amphibians change across time and space, that variation sometimes correlates with environmental conditions at the time and the site of sampling, and that skin bacterial communities on captive-born frogs revert to wild-frog's state soon after soft-release to natural habitat.

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GENERAL AUDIENCE ABSTRACT

Beneficial bacteria found on amphibian skin can provide protection against an infectious disease caused by the lethal amphibian chytrid fungus (*Batrachochytrium dendrobatidis*), that has been linked with the decline and extinction of amphibian species worldwide. However, while skin bacterial communities may play a key role in determining disease outcome, these complex microbial communities can be impacted by biological and environmental factors. In a series of three studies, I investigated the natural variation in skin bacterial communities on wild and captive-born amphibians through time and space using modern DNA sequencing technologies to characterize bacterial community diversity. First, in a study examining the skin bacterial communities of two treefrog species at a single pond over multiple years and seasons, I found that annual, seasonal, and even daily fluctuations in temperature and rainfall changed the skin bacterial communities on these species. Second, I further investigated the impact of seasonality and rainfall with a study sampling the skin of one common frog species along a rainfall gradient, and five amphibian species at a single site across seasons. The strong wet and dry seasonality in the tropical lowland forest impacted the bacterial communities of multiple species found near streams, but the nature of the changes differed among the different frogs. For the common species sampled along the rainfall gradient, I found there was variation in bacterial community structure among sites, although this was not correlated with the rainfall gradient. Finally, I investigated the challenges faced by captive-reared *Atelopus limosus*, an endangered amphibian species from Panama, after release into field enclosures in the natural habitat. I found that the skin bacterial communities reverted to wild-type fairly quickly, body mass decreased to come closer to wild frogs of the same species, and 15% of the frogs became infected with the chytrid fungus during the 27 day trial in the field enclosures. Overall, I found that skin bacterial communities of lowland amphibians change across time and space, that variation is sometimes linked with environmental conditions at the time and site of sampling, and that captive-born frogs revert to wild states soon after release to natural habitat.

Dedication

To my family.

To those who have always been there and those that are now gone.

(Para mi familia.

Para los que siempre han estado y los que ya no estan)

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Attributions

Chapter 2. Skin bacterial communities of neotropical treefrogs vary with local environmental conditions at the time of sampling

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The published article included six additional authors and below is a list of their contributions and affiliations:

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Chapter 1: Introduction

The crisis: disease-driven biodiversity loss

Human activities are the largest drivers of global change and from that, the Anthropocene is branded with the greatest loss of biodiversity in history. The loss of Earth's biodiversity is attributed mainly to habitat alteration and loss, overexploitation, introduction of invasive species, disease, and climate change (Collins 2010). As wild habitat is converted to service human activities, less is available for biodiversity and the ecosystem functions biodiversity provides (McLellan *et al.*, 2014). Moreover, when habitat loss is compounded by the added pressure of emergent infectious disease, the impact on species can be devastating. In recent decades, increasing infectious diseases have caused mass mortalities in corals, amphibians, bats, bees, and snakes, just to mention a few, and will probably have far-reaching ecological consequences (Blehert *et al.*, 2009; Allender *et al.*, 2011; Cameron *et al.*, 2011; Fisher *et al.*, 2012). For amphibians, one-third of species are threatened with extinction and 43% are experiencing dramatic declines (Wake 1991; Stuart *et al.*, 2004; Wake and Vrederbug, 2008; Collins 2013). Woefully, diversity loss caused by disease outbreaks might even go beyond known species. Crawford *et al.* (2010) estimated that disease epidemics in montane forest in Panama resulted in the loss of 41% of the total amphibian lineages in the community, including five undescribed species. Moreover, population declines across the Neotropics have led to the reduction of beta diversity in local amphibian communities, resulting in regional homogenization in terms of species composition, phylogenetic diversity, and species life history (Smith *et al.*, 2009; Nowakowski *et al.*, 2018).

For amphibians, an aquatic chytrid fungus, *Batrachochytrium dendrobatidis* (Bd) is a main cause of catastrophic population declines and species extinctions first observed in Central America and Australia (Berger and Speare 1998; Lips 1999; Pounds *et al.*, 2006; Skerratt *et al.*, 2007); what followed is still known as the “greatest disease-driven loss of biodiversity ever documented” (Fisher *et al.*, 2012). More recently, a second chytrid fungus, *B. salamandrivorans* (Bsal) has been associated with salamander declines in Europe (Martel *et al.*, 2014). Bd is present on every continent inhabited by amphibians, and it continues to spread, with a highly heterogeneous distribution, as it encounters naïve populations (Olson *et al.*, 2013). That being said, not all amphibians are susceptible to Bd or succumb to declines. Some species are resistant and/or tolerant to high levels of infection, without high mortality (Alford and Richards, 1999; Lips 1999; Stuart *et al.*, 2004; Woodhams *et al.*, 2007), while others seem to find protection from Bd in climatic refugia (Puschenforf *et al.*, 2005; 2009; Zumbado-Ulate *et al.*, 2014). Understanding the environmental conditions and host traits that favor resilience has been the target of researchers and conservationists in the aftermath of the epidemics, particularly in the tropics, where diversity is high.

The trends: disease across species, habitat and seasons

Despite minimal annual temperature variation in the tropics, chytridiomycosis has had stronger impacts in the tropics than in temperate regions in terms of species loss and population declines (Skerratt *et al.*, 2007; Whitfield *et al.*, 2012; Becker *et al.*, 2016; Lips *et al.*, 2016). In general, Bd-associated amphibian declines have occurred more often in riparian species at higher

elevations (Lips *et al.*, 2006), in cooler months (Berger *et al.*, 2005; Retallick *et al.*, 2004; Kriger and Hero, 2006) and in warmer years (Berger *et al.*, 2005; Pounds *et al.*, 2006; Rohr and Raffel, 2010; Rohr *et al.*, 2011; Raffel *et al.*, 2012; Venesky *et al.*, 2013). These patterns hold true especially for endemic species with small distributions (i.e., the genus *Atelopus*); in these species, chytridiomycosis has had catastrophic results (La Marca *et al.*, 2005). Different hypotheses have attempted to explain the declines of *Atelopus* species in Central and South America in relation to the limited environmental pockets of mountain forest that they occupy. While in other tropical regions, climatic extremes did not explain population declines (Murray *et al.*, 2009), climate-linked disease hypotheses suggested that *Atelopus* declines in the Neotropics were linked with temperature fluctuations at small- (Pounds *et al.*, 2006) and large spatio-temporal scales (Rohr and Raffel, 2010). More recently, work conducted in the Atlantic Brazilian forest has found strong correlations with disease status, amphibian declines, and water availability (Ruggeri *et al.*, 2015).

While amphibians in the mountains of Panama underwent severe population declines (Lips *et al.*, 2006), lowland species persisted despite Bd occurrence (Woodhams *et al.*, 2008; Kilburn *et al.*, 2010; Rebollar *et al.*, 2014). In the lowlands, fewer individuals in the population were estimated to be infected with Bd (i.e., low prevalence) and those that tested positive harbored lower pathogen loads (i.e., low infection intensity (Kilburn *et al.*, 2010; Whitfield *et al.*, 2012; Rebollar *et al.*, 2014; Gray *et al.*, 2017). This pattern of disease status is common in other lowland sites across Central America (Puschendorf *et al.*, 2009; Flechas *et al.*, 2012; Whitfield *et al.*, 2012), and there is little evidence for lowland population declines (Kilburn *et al.*, 2010). Hence, many lowland sites are believed to be climatic refugia for some amphibian species, where dry and hot environments might limit the growth and spread of Bd (Puschendorf *et al.*, 2005; 2009; Zumbado-Ulate *et al.*, 2014). Optimal Bd growth, observed both in field and lab studies, occurs at intermediate temperatures (17° to 25°C) and high humidity (Woodhams *et al.*, 2008). Bd persists at lower temperatures (4°C), but dies at 30°C (Longcore *et al.*, 1999).

Seasonality is expected to have a direct effect on disease dynamics (pathogen prevalence and infection load) because natural changes in temperature and humidity can affect fungal growth and host behavior, abundance, susceptibility, and immune response (Berger *et al.*, 2005; Woodhams and Alford, 2005; Richards-Zawacki 2010; Phillott *et al.*, 2013; Lenker *et al.*, 2014). However, patterns associated with strong seasonality vary among study sites. In tropical forests with very little seasonal temperature variation compared to the temperate zone (Bosch *et al.*, 2001; Berger *et al.*, 2005; Skerratt *et al.*, 2007), previous studies indicate strong seasonal variation in the prevalence and infection of Bd (Longo *et al.*, 2010; Whitfield *et al.*, 2012). For example, in Puerto Rico, higher Bd prevalence and lower infection intensity occurs in the wet-warm months, with disease outbreaks strongly correlated with rainfall (Lips and Burrowes, 2005; Longo *et al.*, 2010). However, in Costa Rica, Whitfield *et al.* (2012) conducted a field survey where temperature was the main predictor of disease prevalence and infection intensity among terrestrial amphibian species.

Overall, strong seasonality in lowland tropical forests could be essential for amphibian resilience, as infections do not seem to reach lethal levels (Kilburn *et al.*, 2010; Woodhams *et al.*, 2008). Ultimately, how lowland amphibians are able to survive with enzootic Bd infections remains unclear, though many studies suggest that a combination of sub-optimal environmental

conditions for Bd and host traits such as behavioral modifications, production of antimicrobial peptides (AMPs) or metabolites produced by symbiotic bacteria could explain differences between declining-highland and persisting-lowland amphibian species (Woodhams *et al.*, 2011; Rebollar *et al.*, 2014). Understanding these mechanisms will improve our ability to forecast disease outbreaks across changing environments, identify species that will require limited conservation interventions, improve the selection of reintroduction sites, and develop management strategies for those animals currently maintained in captivity.

One mechanism: Amphibian skin-microbial communities

Amphibians have different strategies to defend themselves against infections, including both innate and acquired immune responses (Voyles *et al.*, 2011). Although amphibians have acquired immune responses (Berger *et al.*, 2005; Lips *et al.*, 2006; Kriger and Hero, 2006; Woodhams *et al.*, 2008; Bishop *et al.*, 2009), there are only a few studies that suggest some amphibian species can acquire immunological resistance to Bd (McMahon *et al.*, 2014). Most studies have found inconsistent results or fail to provide strong support for an important role of acquired immunity in response to Bd (review by both Woodhams *et al.*, 2008; Venesky *et al.*, 2014). However, the innate immune system, which includes antimicrobial peptides (AMPs) and the community of microbes associated with amphibian skin, may play an important role in amphibian host defense to Bd (Harris *et al.*, 2006; Belden and Harris, 2007; Bletz *et al.*, 2013). Many AMPs effectively inhibit Bd growth and prevent higher infection loads in laboratory assays. For example, a study examining 40 different types of peptides from 30 different amphibian species found that 83% of these peptides were effective at killing Bd (Rollins-Smith 2009; Voyles *et al.*, 2011). But AMPs are a genetically-based defense, and hence may not effectively save species currently fighting Bd infections in nature (Bletz *et al.*, 2013). Recently, studies are looking at the interactions between AMPs, skin microbes, and environmental conditions that might be beneficial in frog defense (Daskin *et al.*, 2011; K  ng *et al.*, 2014).

Conservation interventions

The case of probiotic treatments

There is a clear link between host-associated microbes and host health in animals. In amphibians, numerous anti-fungal bacteria have been cultured from amphibian skin (Lauer *et al.*, 2007; Woodhams *et al.*, 2015), and their presence can be associated with disease resistance (Becker and Harris, 2010). One promising mitigation strategy against Bd is bio-augmentation of beneficial bacteria on amphibian skin to increase disease resistance (Harris *et al.*, 2006; Daskin *et al.*, 2011; Becker *et al.*, 2011; Flechas *et al.*, 2012; Bletz *et al.*, 2013; Loudon *et al.*, 2014). Cutaneous bacteria can directly produce metabolites (Harris *et al.*, 2009) or indirectly regulate the production of AMPs that inhibit Bd (Woodhams *et al.*, 2007). One of the most well-known bacteria that has been tested under lab and field conditions is *Janthinobacterium lividum* (Jliv). The augmentation of Jliv on *Rana muscosa* resulted in recovery from Bd infection without infection reaching the lethal threshold (Vredenburg *et al.*, 2011).

There are interesting patterns that have surfaced from research on beneficial bacteria. For example, microbes can be transmitted vertically, horizontally, and environmentally (reviewed in

Bletz *et al.*, 2013). Although how amphibians obtain their skin microbiota remains unclear, recent studies in glass frogs that exhibit parental care (e.g. *Hyalinobatrachium colymbiphyllum*, Walke *et al.*, 2011) and plethodontid salamanders (e.g. Muletz *et al.*, 2012; Loudon *et al.*, 2014) provide evidence for vertical and environmental transmission of skin bacteria. Bletz *et al.*, (2013) suggested that horizontal transmission could occur during mating and congregation in winter hibernacula. In the tropics, amphibians congregate at specific times of the year to meet their moisture requirements and to breed, and the timing of both events mostly depends on species life history traits and habitat (Savage 2002; Ibáñez *et al.*, 1999). Specifically, in the lowland tropics, terrestrial amphibians generally congregate along streams or permanent pools during the dry season and disperse to the forest floor after the first rains of the wet season (Ibáñez *et al.*, 1999; Ibáñez *et al.*, 2002). If horizontal transmission of skin microbes occurs at high rates when amphibians are more abundant at one site, then microbial communities on amphibian skin will be affected by seasonality in the lowlands. Understanding when horizontal transmission occurs at higher rates can inform conservation. If probiotics should be applied during times of congregation, such as the dry season for terrestrial frogs and stream-associated amphibians, fewer individuals will require treatment because treated individuals could transfer the probiotic to untreated individuals as adults would to offspring.

Interventions with beneficial bacteria have enormous potential; however, natural variations in host-associated microbial communities are driven by biotic and abiotic conditions that can ultimately impact their functionality (Jimenez and Sommer, 2017). Despite strong support for the anti-Bd properties of some bacteria (Harris *et al.*, 2009; Becker *et al.*, 2009; 2011), few of the observations have been reported in vivo under natural environmental conditions (Bletz *et al.*, 2013). Although experiments under laboratory conditions allow for a better understanding of the mechanisms behind the effect of environmental conditions on microbiota, those results might not translate to natural conditions (Lokmer *et al.*, 2016). Hence, baseline knowledge of natural microbiome variation under different environmental conditions is crucial for the development of therapies involving beneficial bacteria.

Captive breeding programs and reintroductions

The unprecedented rate of amphibian biodiversity loss and the lack of effective treatments to manage chytridiomycosis in wild populations prompted the creation of ex-situ breeding programs as part of a global ‘Amphibian Ark’ effort (Mendelson *et al.*, 2006; Zippel *et al.*, 2011). More recently, adaptive management frameworks advocate for in-situ maintenance of threatened species in order to avoid negative consequences of captivity (Scheele *et al.*, 2014; Garner *et al.*, 2016). For example, captivity affects the microbial communities associated with the skin and gut of amphibians, and generally, captive microbiomes are less diverse and differ in structure from wild microbiomes (Loudon *et al.*, 2014; Bataille *et al.*, 2015; Sabino-Pinto *et al.*, 2016; Kueneman *et al.*, 2016; Flechas *et al.*, 2017). Less diverse bacterial communities in captivity are attributed to sterile environments with an absence of natural bacterial reservoirs (Loudon *et al.*, 2014), less diverse diets (Antwis *et al.*, 2014), and the extensive use of antibiotics and antifungals (reviewed by West *et al.* 2019). Although research conducted in boreal toads, *Anaxyrus boreas*, showed that beneficial bacteria decrease over time in captivity, captive frogs from the genus *Atelopus* maintained a high proportion of Bd-inhibitory bacteria taxa soon after being brought into captivity (Flechas *et al.*, 2017) and even after several years under captive

conditions (Becker *et al.*, 2014). These results suggest that despite changes in bacterial community diversity and structure the protective function of the skin microbiome might be maintained under captive conditions in some tropical species (i.e., *Atelopus*). However, empirical evidence supporting the protective function of the natural skin microbial communities in *Atelopus* is lacking, and wild frogs remain highly susceptible to Bd infections. The effect of captivity on host-associated microbial communities has been described for a wide range of vertebrate species (McKenzie *et al.*, 2017), yet bacterial community shift after release has been less explored.

Spatio-temporal variation of skin-microbes

Spatio-temporal studies of microbiome variation are challenging, hence lacking, especially for extremely diverse amphibian communities in the tropics. Longitudinal studies on amphibian microbiomes have been mostly limited to single species (Fitzpatrick and Allison, 2014; Longo *et al.*, 2015) and temperate zone species with strong seasonal temperature fluctuations (Kueneman *et al.*, 2014; Longo *et al.*, 2015; Bletz *et al.*, 2017). Several of these studies have focused on examining temporal changes across developmental stages (e.g. through amphibian metamorphosis), or during breeding events. Interpretations of spatial variation in host-associated microbes depend on the sampling resolution and on environmental conditions. However, spatial sampling across known environmental gradients can disentangle geographical influences from interactions with synergistic variables, such as life stage, breeding, or diet. Undoubtedly, spatio-temporal variation in microbiomes is coupled with fluctuations, or gradual differences, in environmental conditions across temporal and spatial scales. Tropical lowland amphibians offer a good opportunity to examine factors and processes shaping microbiome dynamics and the potential impacts of the microbiome on Bd resilience.

The natural variation in microbial communities associated with amphibian skin and gut has been thoroughly reviewed by Jimenez and Sommer (2017). In general, the amphibian microbiome varies across temporal and spatial scales, and is influenced by internal (i.e., host phylogeny, age) and external factors (i.e., microbiota reservoirs, diet, environmental conditions). Assessing spatio-temporal dynamics of host-associated microbial communities is crucial for understanding and forecasting their response to disturbances and changing environmental conditions. Ultimately, for species of conservation concern, it is important to study not just the spatio-temporal variation of their skin microbiome, but whether fluctuations across time and space are also reflected in their protective function. In the case of biphasic newts in temperate zones (Bletz *et al.*, 2017) and endemic harlequin frogs in the tropics (Flechas *et al.*, 2017), temporal variation of their skin-associated microbes are not linked to changes in the proportion of Bd-inhibitory bacteria, suggesting that their protective function might be maintained through time. Moreover, understanding the microbiome's response to disturbance (i.e., pathogens, captivity, fasting) and environmental variables (i.e., temperature and rainfall) across time can also assist in making predictions about the changes that the host microbiome may undergo after conservation interventions (i.e., probiotic therapies and reintroductions).

Dissertation research overview

In my dissertation, I aim to increase our current understanding of spatio-temporal variation of skin-associated bacterial communities on amphibians to inform the development of conservation interventions, including beneficial bacteria treatment and captive-to-wild release methods. Both of these interventions have had mixed results thus far. The development and efficacy of probiotic treatments requires a deep understanding of the ecology of microbial communities associated with amphibian hosts and their environments. Thus, in Chapter 2, I present the results of a study examining the effect of temperature and rainfall on the natural variation of bacterial communities associated with the skin of two treefrog species, *Agalychnis callidryas* and *Dendropsophus ebraccatus*, at different temporal scales (Estrada *et al.*, 2019). Studies on temporal variation of skin-associated microbes have been almost exclusively conducted for single species or on those inhabiting temperate regions. To determine the natural variation in the skin bacterial communities across multiple host species and along spatial environmental gradients, in Chapter 3, I present a study assessing skin bacterial community diversity of *Craugastor fitzingeri*, a common species along the Panama Canal rainfall gradient, and the seasonal variation in skin bacterial communities of co-occurring amphibian species in a highly diverse host community. Lastly, since most probiotic and Bd infection trials have been conducted under laboratory conditions, I studied the natural variation of bacterial communities through field surveys and under natural conditions using field enclosures. The results of this study are presented in Chapter 4. Utilizing field enclosures allowed me to monitor not only changes in skin bacteria, but body condition and disease infection status of captive-born *Atelopus limosus*, a threatened Panamanian endemic, after soft-release into a site where the species used to thrive. Longitudinal, individual-based approaches with repeated measurements are necessary to identify potential mechanisms that could allow amphibians to survive at sites where Bd is now endemic. I finalize my dissertation in Chapter 5 with a synthesis of my findings and suggestions for future research aiming to better understand links between amphibian skin microbiomes and host health, and the implications for amphibian conservation in the tropics.

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Chapter 2. Skin bacterial communities of neotropical treefrogs vary with local environmental conditions at the time of sampling

Abstract

The amphibian skin microbiome has been the focus of recent studies aiming to better understand the role of these microbial symbionts in host defense against disease. However, host-associated microbial communities are complex and dynamic, and changes in their composition and structure can influence their function. Understanding temporal variation of bacterial communities on amphibian skin is critical for establishing baselines from which to improve the development of mitigation techniques based on probiotic therapy and provides long-term host protection in a changing environment. Here, we investigated whether microbial communities on amphibian skin change over time at a single site. To examine this, we collected skin swabs from two pond-breeding species of treefrogs, *Agalychnis callidryas* and *Dendropsophus ebraccatus*, over four years at a single lowland tropical pond in Panamá. Relative abundance of OTUs based on 16S rRNA gene amplicon sequencing was used to determine bacterial community diversity on the skin of both treefrog species. We found significant variation in bacterial community structure across long and short-term time scales. Skin bacterial communities differed across years on both species and between seasons and sampling days only in *D. ebraccatus*. Importantly, bacterial community structures across days were as variable as year level comparisons. The differences in bacterial community were driven primarily by differences in relative abundance of key OTUs and explained by rainfall at the time of sampling. These findings suggest that skin-associated microbiomes are highly variable across time, and that for tropical lowland sites, rainfall is a good predictor of variability. However, more research is necessary to elucidate the significance of temporal variation in bacterial skin communities and their maintenance for amphibian conservation efforts.

Introduction

Host-associated microbial communities promote health and fitness for animal and plant hosts alike. Although a healthy microbiome is yet to be defined, assessing the stability of microbial communities, their impact on host fitness and their response to disturbances at different spatio-temporal scales have become fundamental issues in microbial ecology (Antwis *et al.*, 2017). However, measuring natural temporal variation in the microbiome can be daunting, despite emergent technologies for DNA sequencing and advanced computational tools (Arnold *et al.*, 2016). Challenges include determining the necessary frequency of sampling, detecting, measuring and gauging the impact of ecosystem disturbance and tackling difficulties in the analysis of large time-series data (Gerber, 2014). Additionally, the environmental scales over which community change occurs still need to be determined (Shade *et al.*, 2013). The latter represents one of the greatest challenges in the study of symbionts associated with free-living wildlife hosts. Depending on the host species and habitat type, survey efforts and physical monitoring may lack the temporal resolution to capture rapid community changes (Shade *et al.*, 2013) and to disentangle the effect of environmental variables on the microbiome.

The relevance of temporal scale on variation of host-associated microbial communities has been mostly studied in human and model organisms (reviewed by Robinson *et al.*, 2010). In free-living wildlife, studies are limited, but, for example, recent field surveys combined with transplant experiments in wild Pacific oysters revealed temporal stability of their microbiome maintained across months (Lokmer *et al.*, 2016). Amphibian studies generally suggest that longitudinal changes in skin microbiome occur during life stage transitions, especially from larvae to adults (Kueneman *et al.*, 2014; Longo *et al.*, 2015; Sabino-Pinto *et al.*, 2017) and in adults, with transitions from aquatic to terrestrial environments (Bletz *et al.*, 2017). These transitions in microbial community composition are likely correlated with marked shifts in environmental factors associated with the life stages or microhabitats. As in other ectotherms (e.g.: lizards, Bestion *et al.*, 2017), seasonal shifts in temperature are one of the strongest drivers known to impact amphibian-associated microbial communities, having been observed in both tadpoles (Kohl and Yahn, 2016) and adults (Bletz *et al.*, 2017; Longo and Zamudio, 2017). Moreover, amphibians tend to host skin communities that are unique to each host species, even when living in the same habitat and experiencing the same environmental conditions (McKenzie *et al.*, 2012; Kueneman *et al.*, 2014; Walke *et al.*, 2014; Belden *et al.*, 2015; Vences *et al.*, 2016; Sabino-Pinto *et al.*, 2017). Our understanding of the relative importance of fluctuation in other environmental variables, such as moisture, on skin-associated microbial communities remains poorly understood, especially for terrestrial ectotherms (Bird *et al.*, 2018, but see Varela *et al.*, 2018).

We sampled the skin of red-eye tree frog (*Agalychnis callidryas*) and hourglass frog (*Dendropsophus ebraccatus*), two of the most abundant, common and sympatric pond-breeding amphibian species in the Neotropics (Donnelly and Guyer, 1994) to examine how time scale and environmental conditions, specifically rainfall and temperature, affect their skin-associated bacterial communities. In this study, we: 1) described amphibian skin bacterial communities at three different time scales: years, seasons and days, 2) identified the bacterial taxa driving variation at different time scales, and 3) addressed potential links between temporal changes on skin bacterial communities and rainfall events or temperature shifts at the lowland tropical pond where samples were collected. By temporally assessing variation in host-associated microbial communities and identifying the environmental variables linked with such shifts, we can better understand factors that impact the structure of skin bacterial communities.

Materials and methods

Study site and field survey

We collected skin samples of adult *Agalychnis callidryas* and *Dendropsophus ebraccatus* at a single pond to examine long-term (annual and seasonal) and short-term (sampling day) variation in the skin bacterial communities. The study site, Ocelot Pond at Parque Nacional Soberanía, Panamá (50 m elevation), is a lowland, permanent, rain-fed pond. Ocelot Pond lies in seasonal moist forest characterized by a pronounced dry season from mid-December to mid-April/early May, and an extended wet season (Condit *et al.*, 2001, STRI BCI Meteorological Report 2016). The region receives approximately 2,700 mm of rain per year. On average, only about 300 mm

of rain falls during the dry season. Temperatures vary relatively little throughout the year (average annual temperature = 26.3 ± 0.54 °C). We obtained daily average precipitation and air temperature measurements from the Barro Colorado Station at the Smithsonian Tropical Research Institute Physical Monitoring Program (STRI).

We took one sample of skin bacterial communities from each individual of red-eyed treefrogs, *A. callidryas*, (N = 48) and hourglass treefrogs, *D. ebraccatus*, (N = 49) using sterile skin swabs as described in detail by Walke et al. (2015) and Belden et al. (2015). Swabs were placed individually in 1.5 ml sterile microcentrifuge tubes and were then stored at -80 °C until processing. Most animals were released on site immediately after sampling, except for individuals captured in 2013 (Table 1), which were used later in an experiment. Animal use was approved by the Institutional Animal Care and Use Committees of Virginia Tech (11-105- BIOL and 13-097-BIOL) and the Smithsonian Tropical Research Institute (2011-1110-2014 and 2013-0401-2016-A3), and was completed with permission from the Ministerio de Ambiente in Panamá (SE/A-47-12, SEX/A-65-12, SEX/A-77-12, SEX/A-89-12 and SEX/A-113-13).

DNA extraction and sequencing

DNA was extracted from swabs using the Qiagen DNeasy blood and tissue kit (Valencia, CA), with an initial incubation step with Lysozyme of 1 h at 37 °C. Relative abundance of operational taxonomic units (OTUs, which approximate bacterial species) based on 16S rRNA gene amplicon sequencing was used to assess bacterial community structure. For the community characterization, the V4 region of the 16S rRNA gene was amplified using the primers 515F and individually barcoded 806R (Caporaso et al. 2012). PCRs were run in triplicate, pooled and visualized on a 1% agarose gel, and quantified using a Qubit 2.0 fluorometer (Invitrogen, Carlsbad, California). These samples were included in seven different sequencing runs. For each run, PCR products (200 ng/sample) were pooled to make a composite sample and cleaned with the QIAquick PCR Purification Kit (Qiagen, Valencia, CA). Final pooled samples were sent for sequencing on an Illumina MiSeq platform using the 250bp paired-end (2013 samples) or single-end (2012, 2014 and 2015 samples) strategy at the Dana-Farber Cancer Institute of Harvard University.

Raw forward sequences from the Illumina 16S rRNA gene amplicon sequencing from the seven sequencing runs were processed and quality-filtered using the default parameters of the Quantitative Insights Into Microbial Ecology pipeline (QIIME, v. 1.9.1; Caporaso *et al.*, 2010a), with the exception that we allowed for no errors in the barcodes, the minimum Phred quality score (q) was 20, we increased the number of minimum consecutive low-quality base calls allowed before truncating a read (r) to 10, and decreased the fraction of the minimum number of consecutive high-quality base calls to include a read (p) to 0.5. Sequences were clustered into OTUs based on a 97% similarity threshold using the UCLUST method (Edgar, 2010), and representative OTUs were the most abundant sequence within the cluster. Representative sequences were aligned to the Greengenes 13_8 reference database (DeSantis et al. 2006) using PyNAST (Caporaso *et al.*, 2010b) and assigned taxonomy using the RDP classifier (Wang *et al.*, 2007). Prior to statistical analyses, we removed all OTUs with less than 0.01% of the total number of sequences (Bokulich *et al.*, 2013), as well as all Archaea, chloroplast, and

mitochondrial sequences. Then the dataset was rarefied at a depth of 11,500 reads/sample. The final dataset after rarefaction included 97 samples, with a range of 128 to 344 OTUs/sample.

Data analysis and statistical methods

Overview

Temporal variation in bacterial community diversity (alpha diversity) and community structure (beta diversity) in *A. callidryas* and *D. ebraccatus* were evaluated at three time scales. Larger temporal scales were assessed with annual and seasonal variation; one shorter temporal scale was assessed across sampling days. Annual variation was assessed using samples of *A. callidryas* and *D. ebraccatus* collected in June or July from 2012 to 2015 (N = 39 individuals per species across the four years). Seasonal variation was assessed by comparing *A. callidryas* (N = 19) and *D. ebraccatus* (N = 19) sampled between the dry and wet seasons (*A. callidryas*: December 2014 and July 2014; *D. ebraccatus*: March 2015 and July 2015). Daily temporal variation was assessed with a subset of samples from 2014 and 2015 using individuals that were sampled within days of one another. If analyses indicated differences among years, we subsequently identified the OTUs driving those differences. We used indicator species analysis to identify the OTUs exclusively associated with a specific time point as well as those found across all frogs from each species at every sampling year (core microbiome). Finally, we examined associations between temporal variation in the skin microbiome and environmental variables (temperature and rainfall) using climate data obtained from the Smithsonian Tropical Research Institute Physical Monitoring Program (STRI). Amplicon sequences from the 2012 survey were deposited in the NCBI Sequence Read Archive (SRA, study accession number: SRP062596) as part of Belden et al. (2015), and sequences from 2013 were deposited in PRJNA504463. The remainder of the sequences was submitted as part of the present study in PRJNA504466. All statistical analyses were conducted in R v. 3.3.3 (R Core Team, 2017) using the vegan package (v. 2.4-5; Oksanen *et al.*, 2017) unless noted otherwise.

Temporal variation in alpha and beta diversity on different time scales

Differences in alpha diversity across years, seasons and days were individually tested using OTU richness (estimated as the total number of OTUs per individual frog), Faith's phylogenetic diversity and the Shannon index, which accounts for species abundance and evenness. These metrics were computed with QIIME 1.9 (Caporaso *et al.*, 2010a) and were fitted to generalized linear models (GLMs) for each species with year, season and sampling day as fixed effects. GLMs were run separately for each temporal scale. OTU richness analyses were performed with a negative binomial error distribution (function `glm.nb`, package MASS, Venables and Ripley, 2002), while Faith's phylogenetic diversity and the Shannon index were performed with a gamma error distribution (function `glm`, package lme4, Bates *et al.*, 2015). The reported test statistic was Chi-Square (X^2). Lastly, post-hoc multiple comparisons of years and sampling days were conducted with Tukey tests (function `glht`, package multcomp, Hothorn *et al.*, 2008).

Permutational multivariate analyses of variance (PERMANOVA, function `adonis`) and homogeneity of multivariate dispersion (PERMUTEST, function `betadisper`) were performed to determine if differences in bacterial community structure (location) and variability (dispersion)

were explained by temporal variation. Pairwise comparisons using Tukey's honest significance difference method were conducted on the betadisper distances. Bray-Curtis dissimilarities, based on OTU relative abundance data, were visualized with principal coordinate analysis (PCoA). Results from the Jaccard dissimilarity distance matrix, which is based on OTU presence/absence, showed a similar pattern.

When PERMANOVA results suggested significant differences between skin-associated bacterial communities on frogs through time, indicator species analyses (function `IndVal`, package `labdsv`, Roberts, 2016) were conducted to identify bacterial taxa that showed exclusivity or fidelity to a year. Indicator OTUs were defined as a $P < 0.05$ and an indicator value of more than 0.7 (as in Becker *et al.*, 2015). We controlled for the false discovery rate (FDR) of multiple comparisons using the Benjamini & Hochberg (1995) method. Additionally, the core microbiome for each species, defined here as OTUs present on 100% of all the frogs across years, was calculated with QIIME 1.9.1 and was visualized with a heatmap (function `heatmap.2`, package `gplots`; Warnes *et al.*, 2016).

Links between environmental variables and community composition

For our environmental variables of rainfall and temperature, we obtained daily average precipitation and air temperature measurements from the Barro Colorado Station at the Smithsonian Tropical Research Institute Physical Monitoring Program. For rainfall, we initially calculated the monthly and weekly averages and sums. To assess daily variation, additional rainfall metrics were calculated: the average and sum from two days prior to and on the day of sampling (as in Bletz *et al.*, 2017); and the sum of day before, day of and day after collection. Daily rainfall is manually recorded the following morning; therefore, "day after" measurements were included to consider rainfall events during the night of sampling. Average temperatures were also calculated at the monthly, weekly and both daily scales. We were unable to include temperature calculations for two sampling days (27 and 28 of July 2014) due to instrument malfunctions. To use the most complete temperature data set, we tested for differences between average temperature for months, weeks and days. High collinearity between explanatory variables was anticipated (Zuur *et al.*, 2009); therefore, to identify which specific environmental variables for rainfall had the highest explanatory power, we conducted a variable model selection based on the lowest Akaike's information criterion (AIC) in canonical (constrained) correspondence analysis or CCA (functions `add1` and `cca`). Models that included variables with higher AIC were dropped until the best-fit model was selected (Gardener, 2014). The best-fit model resulted in us using the summation of day before, day of and day after sampling and the average monthly temperature as explanatory variables in subsequent models examining impacts on the skin bacterial communities.

To examine how rainfall and temperature affected community structure at each sampling day, we separately analyzed the skin bacterial communities of each treefrog species using a CCA, performed by the function `cca` (Ter Braak, 1986). We ran permutation tests on the overall CCA model (function `anova`) and on each variable (`anova`, `by = "terms"`) to test the significance of each environmental variable as a predictor of community composition. Only explanatory variables that explained variation in beta diversity are represented in the final results (function `autoplot`, package `ggvegan`, Simpson, 2017).

To further explore impacts of temporal variation on associations between bacterial community and environmental variables, we conducted a Mantel test (function `mantel`) using 1,000 permutations per each of the distance matrix pairs. Euclidean distance matrices were used for rainfall and temperature, and Bray-Curtis distances were used for bacterial community structure. Finally, we performed variation partitioning (Borcard *et al.*, 1992; Legendre, 2008) to assess how much variation in community structure could be attributed to rainfall and temperature independently and combined.

Results

Bacterial diversity and community structure varied at different time scales

Bacterial community diversity (alpha diversity) on the skin of *Agalychnis callidryas* and *Dendropsophus ebraccatus* varied through time at different scales. At large time scales, we found significant differences in OTU richness (ANOVA, *A. callidryas*: $X^2 = 13.31$; *D. ebraccatus*: $X^2 = 30.37$, both $df = 3$, $P < 0.01$; Fig. 1A) and Faith's phylogenetic diversity in both treefrog species across years (ANOVA, *A. callidryas*: $X^2 = 13.94$; *D. ebraccatus*: $X^2 = 58.87$, both $df = 3$, $P < 0.01$; Fig. S1A). Community evenness (Shannon index) was not significantly different across years in *A. callidryas* (ANOVA, $X^2 = 6.25$, $df = 3$, $P = 0.10$; Fig. S2A), but was significantly different in *D. ebraccatus* (ANOVA, $X^2 = 17.67$, $df = 3$, $P < 0.001$; Fig. S2A). Generally, there was an overall increase in OTU richness and phylogenetic diversity from 2012 to 2015 in the skin microbiome of both frog species with the exception of 2013.

When examining seasonality in alpha diversity of bacterial communities we found differences between the dry and wet season for *D. ebraccatus*, but not for *A. callidryas*. Community diversity and evenness did not differ across seasons in *A. callidryas* (ANOVA, richness: $X^2 = 0.06$, $df = 1$, $P = 0.81$, Fig. 1B; Faith's phylogenetic diversity: $X^2 = 0.01$, $df = 1$, $P = 0.98$, Fig. S1B; Shannon index: $X^2 = 0.57$, $df = 1$, $P = 0.45$, Fig. S2B), but did in *D. ebraccatus* (ANOVA, richness: $X^2 = 61.13$, $df = 1$, $P < 0.001$, Fig. 1B; Faith's phylogenetic diversity: $X^2 = 24.18$, $df = 1$, $P < 0.001$, Fig. S1B; Shannon index: $X^2 = 3.56$, $df = 1$, $P = 0.05$, Fig. S2B).

At short time scales, comparisons of OTU richness across sampling days were significant for *D. ebraccatus* (ANOVA, $X^2 = 10.75$, $df = 2$, $P = 0.005$), but not for *A. callidryas* (ANOVA, $X^2 = 1.47$, $df = 1$, $P = 0.22$, Fig. 1C). We found no significant differences in Faith's phylogenetic diversity and Shannon index across sampling days in either treefrog species (ANOVA, *A. callidryas*: $X^2 = 0.67$ and 0.17 , both $df = 1$ and $P > 0.05$; *D. ebraccatus*: $X^2 = 3.66$ and 0.02 , both $df = 2$, $P > 0.05$; Fig. S1C and S2C).

We found temporal changes in bacterial community structure (beta diversity) based on Bray-Curtis dissimilarity distances at all time scales. Community structure differed across years (PERMANOVA for *A. callidryas*: Pseudo-F = 14.016, $R^2 = 0.53$ and *D. ebraccatus*: Pseudo-F = 9.28, $R^2 = 0.44$; both $P < 0.001$, Fig. S3A and S3B), seasons (PERMANOVA for *A. callidryas*: Pseudo-F = 2.19, $R^2 = 0.12$, $P = 0.05$ and; *D. ebraccatus*: Pseudo-F = 5.48, $R^2 = 0.24$, $P = 0.0026$; Fig. S3C and S3D) and sampling days (PERMANOVA, *A. callidryas*: Pseudo-F = 3.58,

$R^2 = 0.31$, $P=0.01$; Fig. 2A and *D. ebraccatus*: Pseudo-F = 4.23, $R^2 = 0.55$, $P < 0.01$; Fig 2B) in both treefrog species. Dispersion of individuals from the centroid was significantly different across years, with frogs in 2013 having communities more similar to one another (Permutest, *A. callidryas*: $F = 26.696$, $P < 0.001$ and *D. ebraccatus*: $F = 27.402$, $P = 0.001$). Dispersion between seasons was significantly different for *A. callidryas* (Permutest, $F = 13.45$, $P = 0.005$), but not for *D. ebraccatus* (Permutest, $F = 0.3047$, $P = 0.58$). Lastly, dispersion between sampling days was significant in *D. ebraccatus* (Permutest, $F = 21.84$, $P = 0.001$) but not in *A. callidryas* (Permutest, $F = 4.51$, $P = 0.07$).

OTUs driving temporal variation

We identified 540 OTUs in the skin of treefrogs and three phyla contributed 96% of the total relative abundance: Proteobacteria (63%), Actinobacteria (29%) and Firmicutes (4%). However, indicator species analysis identified a distinct set of OTUs associated with *A. callidryas* and *D. ebraccatus* across years with few OTUs shared between species (supplementary Table S1). By far, the highest number of indicator OTUs was recorded for samples from 2013 in both species: *A. callidryas* (N = 88) and *D. ebraccatus* (N = 75). Most indicator OTUs shared between treefrog species were in the phylum Proteobacteria (68%), including 3 OTUs associated with individuals of both frog species sampled in different seasons. We also found higher numbers of unique OTUs in both species in the dry season when comparing across seasons (*A. callidryas*: dry season N = 26/wet season N = 10; *D. ebraccatus* dry season N = 93/wet season N = 9).

The core bacterial communities (OTUs present in 100% of the samples) for *A. callidryas* and *D. ebraccatus* were comprised of only 10 and 6 OTUs, respectively. Five of these OTUs were shared between the two treefrog species. The vast majority of OTUs in the core community belonged to the phylum Proteobacteria (*A. callidryas* = 70% and *D. ebraccatus* = 67%). Moreover, all Proteobacteria belonged to the class Gammaproteobacteria, including: Pseudomonadaceae, Xantomonadaceae, Commamonadaceae and Enterobacteriaceae. The relative abundance of the core OTUs varied considerably across individuals sampled at the same time point. Interestingly, individuals collected in 2013 had more similar abundances in their core communities, but strikingly differed from other years in both *A. callidryas* (Fig. 3) and *D. ebraccatus* (Fig. 4). Relative abundance of two core OTUs varied notably in 2013 compared to the other years in *A. callidryas*: Pseudomonadaceae X589596 (relative abundance, mean $\pm$ s.d. in 2012 = $50 \pm 9\%$; 2013 = $0.5 \pm 0.4\%$; 2014 = $34 \pm 15\%$; 2015 = $37 \pm 20\%$) and Cellumonadaceae X624310 (relative abundance, mean $\pm$ s.d. in 2012 = $6 \pm 6\%$; 2013 = $89 \pm 3\%$; 2014 = $16 \pm 11\%$; 2015 = $3 \pm 4\%$). Similar shifts in relative abundance across years were observed in *D. ebraccatus* for Pseudomonadaceae X9744121 (relative abundance, mean $\pm$ s.d. in 2012 = $14 \pm 21\%$; 2013 = $46 \pm 3\%$; 2014 = $22 \pm 20\%$; 2015 = $11 \pm 10\%$). No core OTUs were identified by indicator species analysis.

Rainfall and temperature are linked to temporal variation in skin-associated bacterial communities

At short time scales, environmental variables (rainfall and temperature) were linked with bacterial community structure. In both treefrog species, we found significant correlations between the rainfall and temperature around the day of sampling and bacterial community

structure (Mantel test, *A. callidryas*: $R = 0.5496$; *D. ebraccatus*: $R = 0.5097$, both $P < 0.001$). Variance partitioning analysis showed that rainfall, calculated as the sum of day before, day of and day after sampling, explained most of the variation in community structure in both species. However, the proportion of variance explained for rainfall was larger in *A. callidryas* (21%) than *D. ebraccatus* (9%). Temperature explained a smaller proportion of variation in *A. callidryas* (3%) and a similar proportion in *D. ebraccatus* (8%). Finally, most of the variance, 72% and 80%, respectively, was unexplained by the environmental variables measured in this study (Fig. 5).

Discussion

We found clear temporal differences in skin bacterial communities on the skin of *Agalychnis callidryas* and *Dendropsophus ebraccatus*. Time of sampling accounted for significant proportions of variation (years = 44% to 53%, seasons = 12% to 24% and days = 31% to 55%) observed in bacterial community diversity on both treefrog species. This, together with strong correlations found between community structure and rainfall and temperature on the day of sampling, emphasizes the role that rapidly changing environmental conditions may play on microbial community assembly and dynamics. Differences in response to shifts in environmental conditions among individuals and between species, despite cohabiting the same ponds, suggests that temporal dynamics observed in skin-associated bacterial communities could also be influenced by host-related factors and by biotic interactions among the bacteria.

The importance of temporal variability and time scale has been well established in human gut microbiome studies. Age is known to greatly alter microbial communities in the gut, where variation has been detected at time scales of months and years; however, changes in adult diets can result in parallel changes in the gut microbiome in just a few days (Gerber, 2014). Our study suggests that temporal instability of the skin bacterial communities through time might be largely regulated by external factors: abiotic conditions preceding sampling and the effects of these conditions on free-living microbial communities in the surrounding environment. This supports previous findings that amphibians, unlike humans (Costello *et al.*, 2009; Oh *et al.*, 2016), might continuously reacquire new microbes for their skin from the surrounding environments (Loudon *et al.*, 2014). Although our study did not allow for repeated samples of individuals and did not have a large sample size at finer temporal scales, our results highlight variation in both alpha diversity metrics and community structure across sampling years, seasons and days.

In our study, the differences observed in bacterial communities through time were mainly associated with shifts in relative abundance of core taxa and indicator OTUs, associated with each sampling point. Both core and indicator OTUs were dominated by the phyla Proteobacteria, Actinobacteria, Firmicutes, Bacteroides and Verrucomicrobia. These major phyla also contribute key OTUs in the skin bacterial communities of other tropical amphibians (Longo *et al.*, 2015; Hughey *et al.*, 2017), including amphibians from the same region in eastern Panama (Belden *et al.*, 2015; Rebollar *et al.*, 2016). Other recent studies are consistent with this finding that changes in the relative abundance of key bacterial OTUs are mainly responsible for the temporal patterns observed in amphibian skin bacterial communities (Bletz *et al.*, 2017; Sabino-Pinto *et al.*, 2017). However, most studies on temporal variation of amphibian skin bacterial communities have

focused on describing community shifts between different life stages (Kueneman *et al.*, 2014; Longo *et al.*, 2015; Bletz *et al.*, 2017; Sabino-Pinto *et al.*, 2017; Muletz-Wolz *et al.*, 2018). Consequently, the microbes associated with changes in community composition are most likely acquired from environmental sources corresponding to various life stages (Prest *et al.*, 2018). In addition, differences in the skin itself across life stages likely selects for different bacterial communities. For our study, only terrestrial adult stages were sampled, and variation in skin communities through time were not expected to be explained solely by differences in age nor environmental sources of bacteria (Walke *et al.*, 2014; Rebollar *et al.*, 2016; Varela *et al.*, 2018).

We found that rainfall and temperature immediately preceding the day of sampling were strongly correlated with bacterial community structure on both treefrog species. In fact, rainfall alone explained as much as 21% of the temporal variation observed on *A. callidryas*. Although temperature can have a strong effect on community diversity and composition of host-associated microbiomes (Carrier and Reitzel, 2017), including amphibians (Kohl and Yahn, 2016), and free-living microbial communities (Pettersson and Bååth, 2003), we found that rainfall best explained observed shifts in the skin bacterial community of our study species. Our study site, Ocelot Pond, like other tropical lowland ecosystems, is characterized by little intra-annual variation in temperature (Whitfield *et al.*, 2012) and high seasonality of precipitation (Condit *et al.*, 2001). Moreover, different microhabitats around pond edges reduce ambient temperature variability in the canopy of lowland tropical forest (Scheffers *et al.*, 2014); thus, arboreal amphibian hosts and their associated bacteria might be less exposed to temperature fluctuations. Rainfall events, however, are infrequent during the dry season, but short-term fluctuations in rainfall are common during the rainy season (Condit *et al.*, 2001). Bacterial community diversity and evenness in both treefrogs increased across years from samples collected in 2012 to 2015. Likewise, rainfall generally decreased through time, with 2015 having the lowest rainfall recorded. Changes in environmental conditions in amphibian microhabitat have been previously linked to variation in diversity and composition of skin bacterial communities in temperate amphibians (Bletz *et al.*, 2017; Kueneman *et al.*, 2014; Longo *et al.*, 2015). It has been proposed that changing environmental conditions and bacterial reservoirs can induce temporal shifts in amphibian skin bacterial communities (Belden and Harris, 2007; Loudon *et al.*, 2014; Loudon *et al.*, 2016). For example, cold temperatures increased OTU richness in adult *Lithobates yavapaiensis* during the winter months (Longo *et al.*, 2015), and fluctuations in OTU relative abundances on newt skin were linked to water temperature (Bletz *et al.*, 2017). However, in these studies water temperatures have extreme seasonal fluctuations that may exceed the physiological tolerance levels for some symbionts (Erwin *et al.*, 2015), and therefore, the conditions are quite different than in the tropical system we studied.

Interestingly, the dynamics of the amphibian fungal pathogen, *Batrachochytrium dendrobatidis*, have also been associated with patterns of rainfall and temperature in the Neotropics (James *et al.*, 2015). Panamanian amphibian communities at higher elevations have been particularly hard hit by the spread of Bd, with some communities experiencing loss of > 50% of species (Lips 1999; Lips *et al.*, 2003; Lips *et al.*, 2006). In our study, we examined amphibian species that are not highly susceptible to Bd, so that we could focus on non-pathogen induced changes in the skin bacterial communities. However, it could be important to ultimately examine links between these rapid changes we observed in OTU relative abundances and Bd infection dynamics in more susceptible host species. Overall, little is known about the functional significance of varying

OTU relative abundances on amphibian skin bacterial communities. We have found that different bacterial communities can produce secondary metabolite profiles that are quite similar, which suggests that function may not be closely linked to structure in at least some skin bacterial communities (Belden *et al.*, 2015). Our present data do not allow us to determine the functional outcome of changing OTU relative abundances through time, but we do provide evidence that small fluctuations in environmental conditions at time of sampling can have an important and rapid effect on OTU relative abundances, and these could potentially impact the dynamics of Bd.

Our observations revealed two specific patterns that might suggest the broader importance of environmental conditions on bacterial communities. First, there were significant seasonal effects in *D. ebraccatus*, and not in *A. callidryas*. This pattern suggests that different processes influenced the skin bacterial communities of each species. Specifically, community diversity and evenness were significantly higher in the wet season of 2015 in *D. ebraccatus*. Interestingly, the 2015 wet season was characterized by extended dry periods typical of the El Niño-Southern Oscillation (ENSO) cycle in Panamá. Since there was little rainfall for both dry and wet seasons of 2015, we would expect to see similar temporal microbiome responses. However, some individuals of *D. ebraccatus*, but none of *A. callidryas*, were found closer to or partially submerged in the water during the wet season of 2015 (A. Estrada, pers. obs.) potentially allowing bacterial colonization from a different environmental source. While *A. callidryas* and *D. ebraccatus* adults live in the forest canopy (Ibáñez, 1999; Duellman, 2001), *D. ebraccatus* can change their reproductive mode based on microhabitat conditions, and lay eggs in either aquatic or terrestrial substrates (Touchon and Warkentin, 2009), while *A. callidryas* lays only on vegetation. Environmental conditions that impact variation in behavior and microhabitat use between these two sympatric species might also drive differences in skin bacterial communities. Second, samples from 2013 displayed less variation among individual frogs and greater difference in alpha and beta community diversity in comparison to other years. Interestingly, environmental conditions around the day of sampling in 2013 had two of the most extreme values for rainfall (high) and temperature (low) reported for the duration of our study. Frogs sampled in 2013 were likely exposed to an intense short-term rain event, which could have washed-in or altered (Elmir *et al.*, 2007) the bacterial communities of both species. Taken together, these results emphasize the role that abiotic factors play in natural variation of bacterial communities associated with amphibian skin.

Although shifts in amphibian skin bacterial diversity and relative abundance have recently been linked to extreme weather events (Familiar-López *et al.*, 2017), the effect of large-scale climate fluctuations on bacterial symbionts is not well understood. The potential for microbes to influence amphibians' fitness could change our current understanding of how both aquatic and terrestrial organisms adapt to global climate change. Overall, our results suggest that short-term amphibian microbiome studies may not be sufficient for finding clear patterns and making predictions about the community's response to such large-scale events. Thus, an assessment of the effects of large-scale climate variability on microbiome stability and function will require long-term surveys and high-resolution environmental variables. Research on marine organisms also proposes that the host-microbe relationship is altered by fluctuations in environmental conditions (reviewed by Apprill, 2017). Most notably, the increase of ocean temperatures may alter the temporal stability of microbial communities of marine taxa. Coral bleaching, the most visible consequence of microbiome dysbiosis, occurs after long-term, yet small, increases in

seawater temperature (Brown, 1997). Although long-term monitoring of microclimatic conditions will be challenging, future work targeting long-term microbiome studies and their temporal fluctuations are necessary. Moreover, manipulative experiments involving precipitation changes (Beier *et al.*, 2012) will provide us with a better understanding of the direct effects of rainfall and temperature on structural and functional stability of the amphibian skin-associated microbial communities under changing environmental conditions.

Conclusions

Our study aimed to examine natural temporal dynamics of skin-associated bacterial communities of two common Neotropical treefrog species. By exploring the effect of large- and small-temporal scales and accounting for short-term environmental heterogeneity, we observed that variation detected daily, seasonally and annually was mainly explained by short-term rainfall events. Our findings suggest some caution should be taken in interpreting results from one sampling period, since these results cannot capture variation based on time scale and variation in local short-term environmental conditions. Considering natural variation in the microbiome could be important for studies of host-microbiome interactions.

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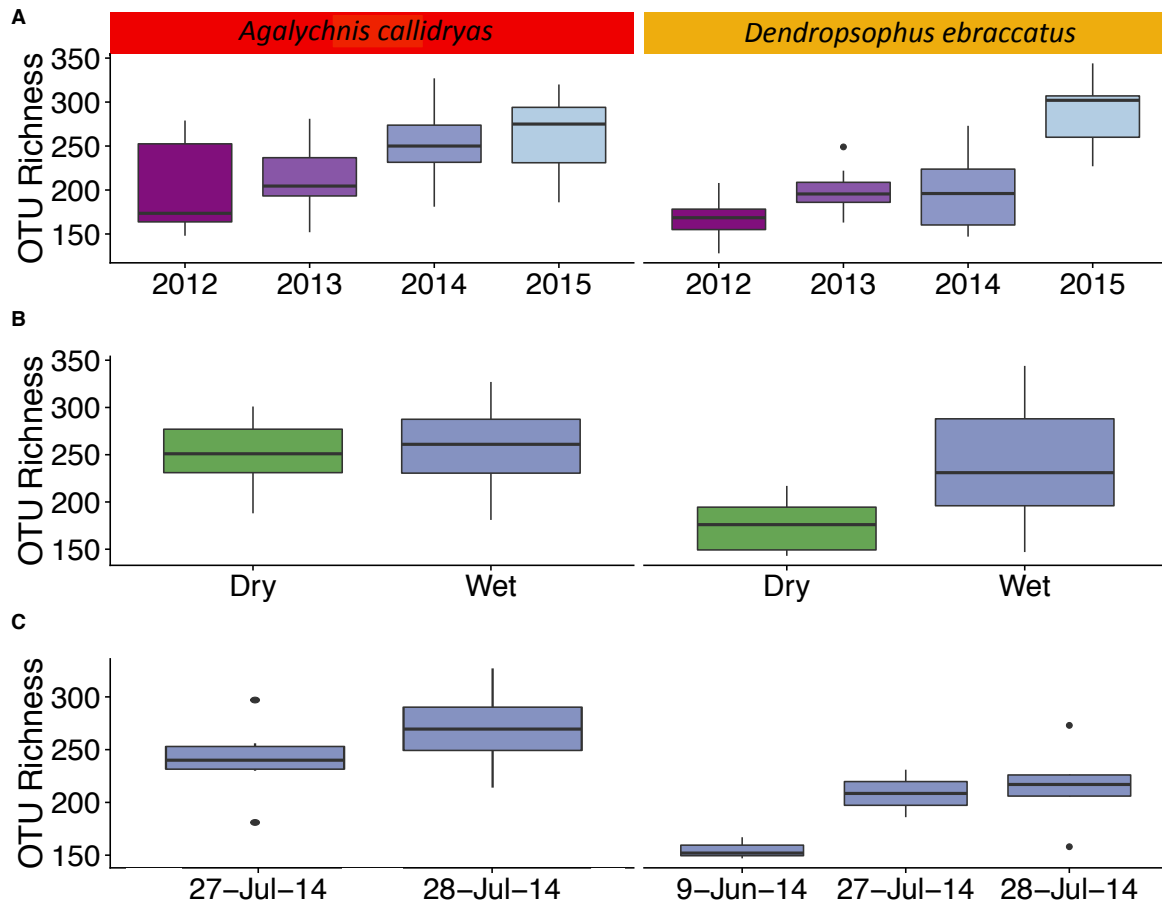
Tables and Figures

Table 1. Sample sizes for field surveys assessing temporal differences in amphibian skin-associated bacterial communities on *Agalychnis callidryas* and *Dendropsophus ebraccatus*.

Sampling Year	Sampling Season	Sampling Day	Time Scale Analysis	<i>Agalychnis callidryas</i>	<i>Dendropsophus ebraccatus</i>
2012	Wet	29-Aug-12	y	10	10
2013	Wet	14-Jun-13	y	10	10
2014	Wet	9-Jun-14	y/s/d		3
2014	Wet	27-Jul-14	y/s/d	6	2
2014	Wet	28-Jul-14	y/s/d	4	5
2014	Dry	21-Dec-14	s	3	
2014	Dry	22-Dec-14	s	4	
2014	Dry	30-Dec-14	s	2	
2015	Dry	11-Mar-15	s		4
2015	Dry	15-Mar-15	s		6
2015	Wet	28-Jun-15	y/s/d		9
2015	Wet	25-Jul-15	y/s/d	9	

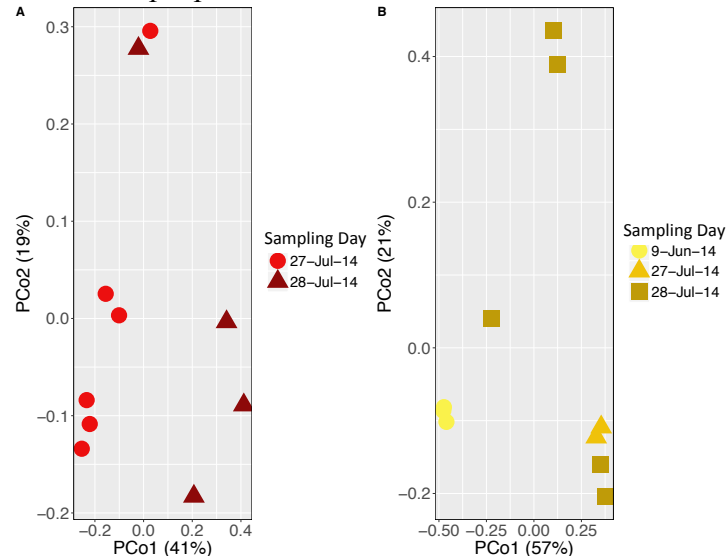
Time scale analysis indicates when samples were used in analysis of variation in sampling years (y), seasons (s) or days (d) for each treefrog species.

Figure 1. Alpha diversity (OTU Richness) of skin microbiome of *Agalychnis callidryas* and *Dendropsophus ebraccatus* at different temporal scales.



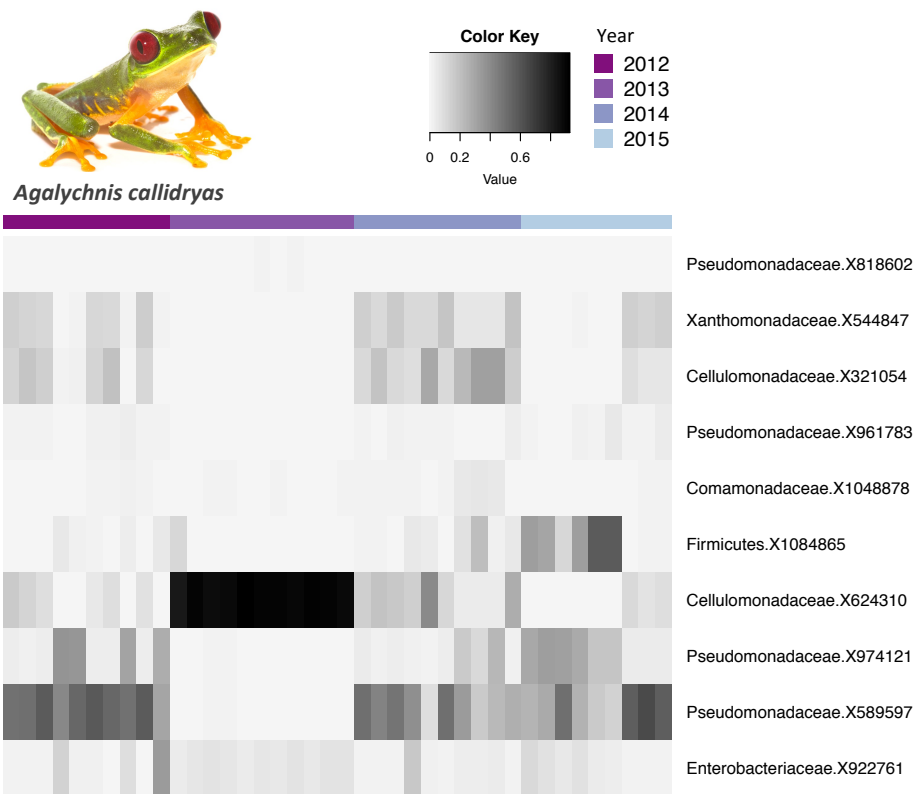
Alpha diversity (OTU Richness) of skin microbiome of *Agalychnis callidryas* and *Dendropsophus ebraccatus* across sampling years (A), seasons (B) and days (C). Annual values for OTU Richness include only the wet seasons of four consecutive years. Seasonal values include dry and wet seasons for 2014 and 2015. Daily values include only multiple sampling days in the wet season of 2014. Additional alpha diversity metrics are in supplementary materials.

Figure 2. Daily variation of bacterial community structure in the skin of *Agalychnis callidryas* and *Dendropsophus ebraccatus*.



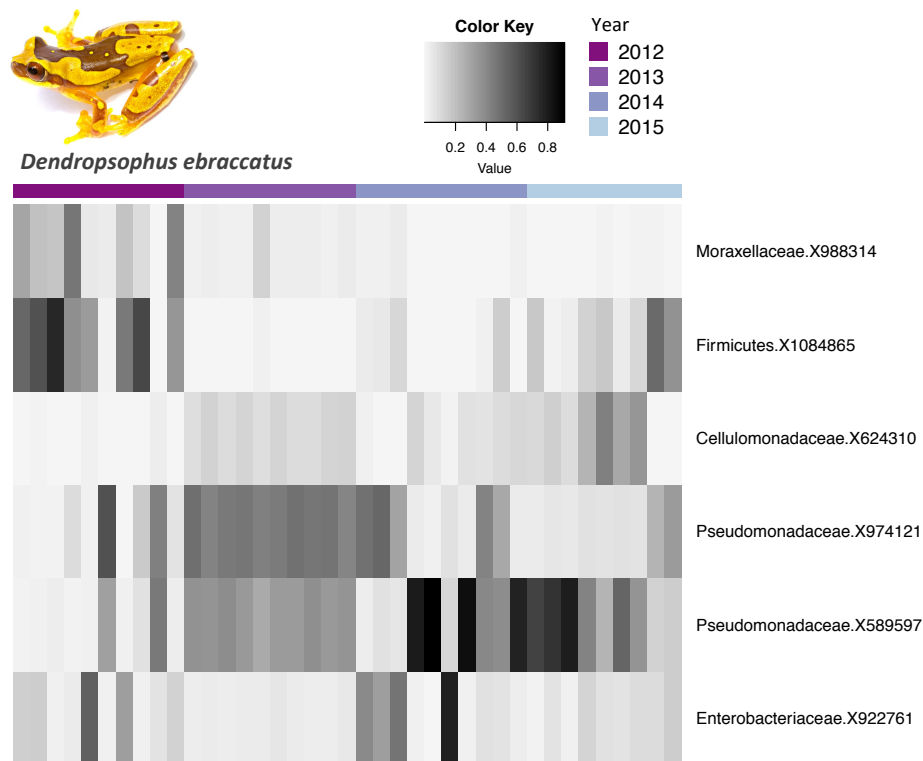
Beta diversity of bacterial communities based on Bray-Curtis dissimilarity of *A. callidryas* (A) and *D. ebraccatus* (B) grouped by sampling day. Each point represents the microbiome on a single individual.

Figure 3. Relative abundance of core microbiome in *Agalychnis callidryas* across years.



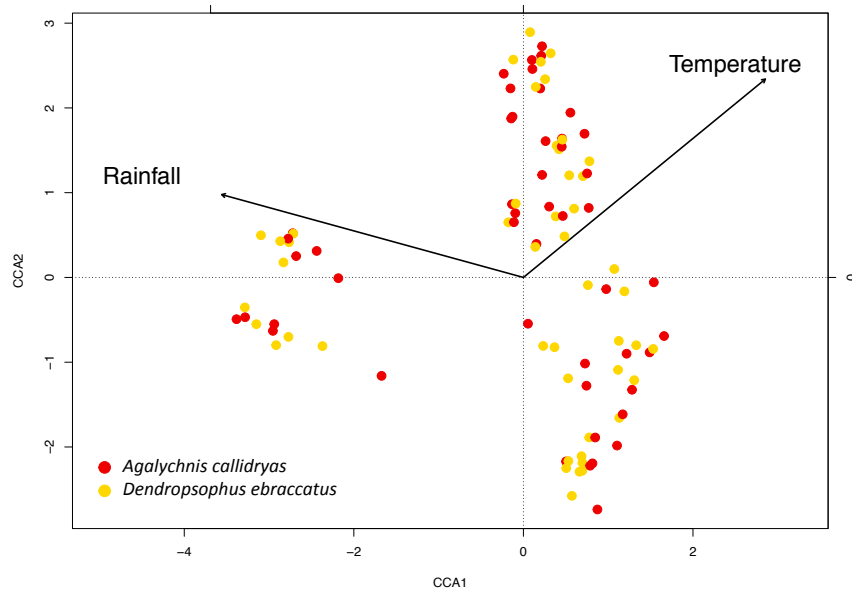
Relative abundance of core microbiome (OTUs found in 100% of the samples) in *Agalychnis callidryas* across years. Darker shades indicate higher relative abundances and lighter shades indicate lower relative abundances. Photo credit: Brian Gratwicke.

Figure 4. Relative abundance of core microbiome in *Dendropsophus ebraccatus* across years.



Relative abundance of core microbiome (OTUs found in 100% of the samples) in *Dendropsophus ebraccatus* across years. Darker shades indicate higher relative abundances and lighter shades indicate lower relative abundances. Photo credit: Brian Gratwicke.

Figure 5. Canonical correspondence analysis of the variation in bacterial community composition on the skin of the two treefrog species.



Canonical (constrained) correspondence analysis shows that environmental variables (rainfall and temperature) are associated with temporal variation in community composition of skin-associated bacteria on *Agalychnis callidryas* (red) and *Dendropsophus ebraccatus* (yellow).

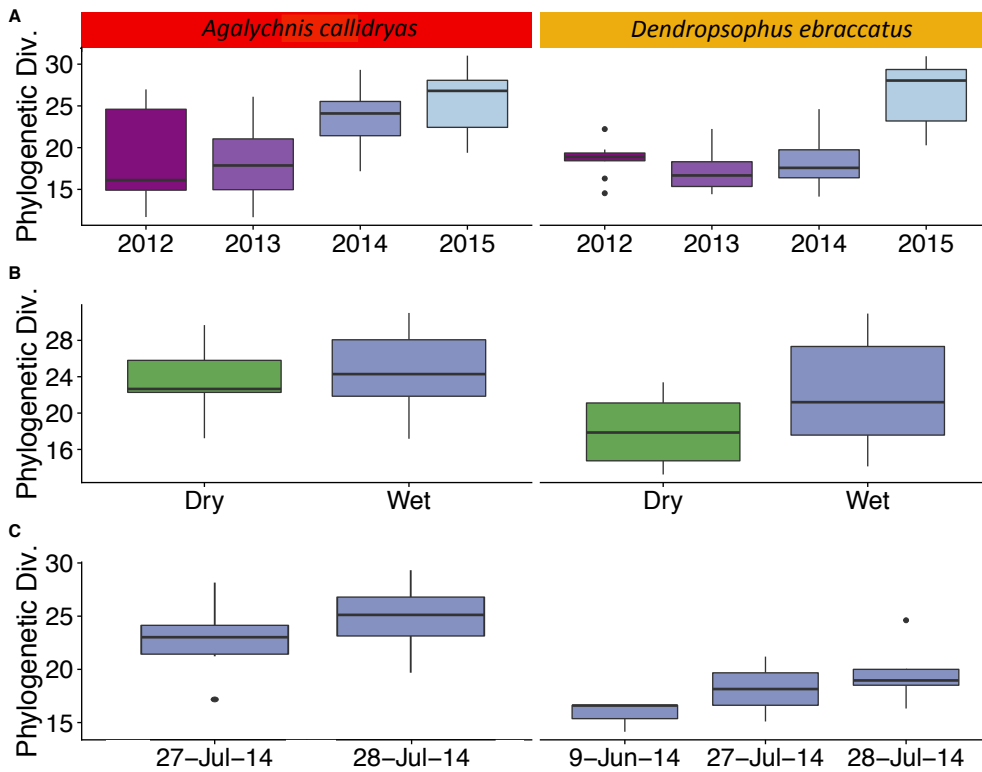
Supplementary Material

Table S1. Indicator OTUs associated with different years shared in *Agalychnis callidryas* and *Dendropsophus ebraccatus*.

Sampling year	OTU ID	Phylum	Class	Order	Family	Genus	<i>Agalychnis callidryas</i> Indicator value index P-value		<i>Dendropsophus ebraccatus</i> Indicator value index P-value	
2012	denovo70491	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae		0.66	0.001	0.882	0.01
2012	X960463	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	0.756	0.031	0.735	0.012
2013	X4470206	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	1	0.001	0.837	0.001
2013	X4434190	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	1	0.001	0.837	0.001
2013	X1834768	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Stenotrophomonas	0.959	0.001	1	0.001
2013	X1109607	Actinobacteria	Actinobacteria	Actinomycetales	Cellulomonadaceae	Cellulomonas	0.945	0.001	0.984	0.001
2013	X1083508	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Stenotrophomonas	0.999	0.001	0.964	0.001
2013	X1060517	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Janthinobacterium	0.728	0.003	0.699	0.01
2013	X969805	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Agrobacterium	0.937	0.001	0.996	0.001
2013	X915948	Actinobacteria	Actinobacteria	Actinomycetales	Nocardiaceae	Rhodococcus	1	0.001	0.956	0.001
2013	X895220	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae		0.692	0.005	0.992	0.001
2013	X750016	Bacteroidetes	Flavobacteriia	Flavobacteriales	[Weeksellaceae]	Chryseobacterium	0.993	0.001	0.993	0.001
2013	X646549	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	0.97	0.001	0.963	0.001
2013	X592636	Proteobacteria	Alphaproteobacteria	Rhizobiales	Brucellaceae	Ochrobactrum	1	0.001	1	0.001
2013	X591708	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Stenotrophomonas	0.604	0.014	0.996	0.001
2013	X578701	Actinobacteria	Actinobacteria	Actinomycetales	Nocardiaceae	Rhodococcus	0.979	0.001	0.834	0.001
2013	X573976	Actinobacteria	Actinobacteria	Actinomycetales	Nocardiaceae	Rhodococcus	0.998	0.001	0.975	0.001
2013	X570350	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingobium	0.739	0.001	0.775	0.001
2013	X566578	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae		0.931	0.001	0.949	0.001
2013	X558264	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Achromobacter	1	0.001	0.994	0.001
2013	X544313	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	0.984	0.001	0.881	0.001
2013	X536008	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae		0.954	0.001	0.975	0.001
2013	X528421	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Citrobacter	0.749	0.001	0.989	0.001
2013	X451696	Proteobacteria	Alphaproteobacteria	Rhizobiales	Brucellaceae	Ochrobactrum	1	0.001	1	0.001
2013	X429838	Actinobacteria	Actinobacteria	Actinomycetales	Cellulomonadaceae	Cellulomonas	1	0.001	1	0.001
2013	X323068	Actinobacteria	Actinobacteria	Actinomycetales	Cellulomonadaceae	Cellulomonas	0.953	0.001	1	0.001
2013	X271876	Proteobacteria	Alphaproteobacteria	Rhizobiales	Brucellaceae	Ochrobactrum	1	0.001	0.995	0.001
2013	X269781	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae		0.982	0.001	0.99	0.001
2013	X193536	Actinobacteria	Actinobacteria	Actinomycetales	Cellulomonadaceae	Cellulomonas	0.842	0.001	0.991	0.001
2013	X130455	Actinobacteria	Actinobacteria	Actinomycetales	Nocardiaceae	Rhodococcus	0.994	0.001	0.679	0.002
2014	denovo42701	Bacteroidetes	Flavobacteriia	Flavobacteriales	[Weeksellaceae]	Chryseobacterium	0.775	0.001	0.774	0.001
2014	denovo288776	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Alcanivoracaceae	Alcanivorax dieselolei	0.992	0.001	0.981	0.001
2014	X793461	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Alcanivoracaceae	Alcanivorax dieselolei	0.968	0.001	0.986	0.001
2014	X254890	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Stenotrophomonas	0.515	0.038	0.917	0.001
2015	X1042671	Bacteroidetes	[Saprospirae]	[Saprospirales]	Chitinophagaceae		0.79	0.009	0.726	0.003
2015	X786876	Bacteroidetes	Flavobacteriia	Flavobacteriales	[Weeksellaceae]	Elizabethkingia meningoseptica	0.941	0.001	0.748	0.001
2015	X767063	Bacteroidetes	[Saprospirae]	[Saprospirales]	Chitinophagaceae	Chitinophaga	0.789	0.002	0.816	0.001
2015	X527033	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae		0.662	0.011	0.648	0.004
2015	X377546	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Mycoplana	0.759	0.033	0.694	0.008

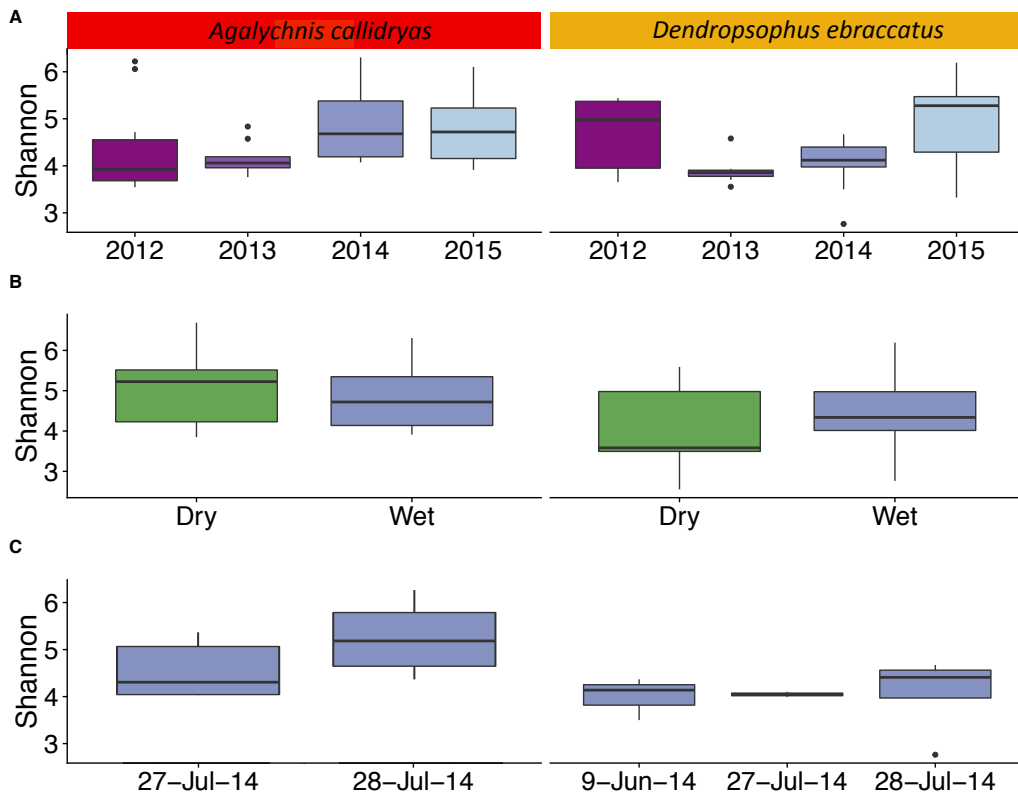
The indicator value index represents a measure of the association between an OTU and a year, and range from 0 to 1, where a value close to 1 implies a relatively stronger association.

Figure S1: Alpha diversity (Faith's phylogenetic diversity) of skin microbiome of *Agalychnis callidryas* and *Dendropsophus ebraccatus* at different temporal scales.



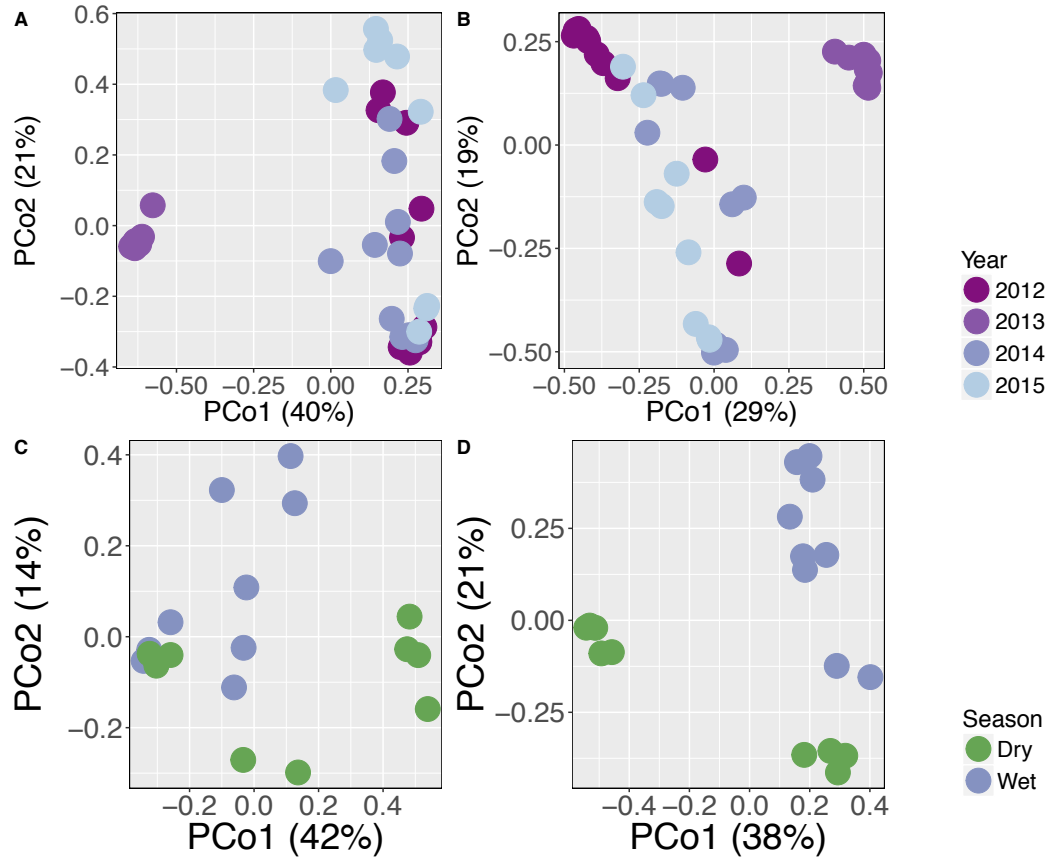
Alpha diversity (Faith's phylogenetic diversity) of skin microbiome of *Agalychnis callidryas* and *Dendropsophus ebraccatus* across sampling years (A), seasons (B) and days (C). Annual values for phylogenetic diversity include only the wet seasons of four consecutive years. Seasonal values include dry and wet seasons for 2014 and 2015. Daily values include only multiple sampling days in the wet season of 2014.

Figure S2: Community evenness (Shannon) of skin microbiome of *Agalychnis callidryas* and *Dendropsophus ebraccatus* at different temporal scales.



Community evenness (Shannon) of skin microbiome of *Agalychnis callidryas* and *Dendropsophus ebraccatus* across sampling years (A), seasons (B) and days (C). Annual values for evenness include only the wet seasons of four consecutive years. Seasonal values include dry and wet seasons for 2014 and 2015. Daily values include only multiple sampling days in the wet season of 2014.

Figure S3: Annual and seasonal variation of bacterial community structure in the skin of *Agalychnis callidryas* and *Dendropsophus ebraccatus*.



Beta diversity of bacterial communities based on Bray-Curtis dissimilarity of *A. callidryas* (left column) and *D. ebraccatus* (right column) grouped by year (AB) and season (CD). Each point represents the microbiome on a single individual.

Chapter 3. Variation of bacterial communities associated with the skin of lowland amphibians in the Panama Canal Watershed

Introduction

One critical function of microbial symbionts of animals (the microbiome) is in host defense against disease. In amphibians, interest in the skin microbiome has been spurred by the emerging wildlife disease, chytridiomycosis, a lethal infectious disease caused by the aquatic fungi, *Batrachochytrium dendrobatidis* (Bd) and *B. salamandrivorans* (Bsal). Numerous bacteria that produce anti-fungal metabolites have now been cultured from amphibian skin (Lauer *et al.*, 2007, Woodhams *et al.*, 2008), and their presence can be associated with Bd resistance (Belden and Harris 2007, Woodhams *et al.*, 2008, Becker and Harris 2010, Becker *et al.*, 2011, Bletz *et al.*, 2013). However, biotic and abiotic factors regulate the normal variation in amphibian-associated microbial communities, and this variation could potentially alter the protective function of these bacteria (Jiménez and Sommer, 2017).

The response of host-associated microbial communities to abiotic factors can vary among different host species and across diverse environmental gradients. For example, microbial communities associated with marine sponges show strong seasonal and interannual stability despite drastic changes in water temperature (Erwin *et al.*, 2015). Conversely, skin-bacterial communities in six fish species seem to correlate with temperature fluctuations in marine environments (Larsen *et al.*, 2013). These studies highlight temporal responses not only among host species, but also across host communities. Temporal variation in amphibian skin bacterial community composition has primarily been studied in single species (Longo *et al.*, 2015) and species inhabiting temperate regions with drastic seasonal changes in environmental conditions throughout the year (Kueneman *et al.*, 2014; Longo *et al.*, 2015). Together, these studies suggest direct and indirect effects of environmental variables on temporal variation of host-associated bacteria. However, two recent studies in tropical species suggest direct connections between temporal variation of skin microbiome and fluctuation in environmental variables at different temporal and spatial scales (Varela *et al.*, 2018; Estrada *et al.*, 2019). Varela *et al.*, (2017) found that the microbiome on three species of dendrobatid frogs varied across soil pH and rainfall gradients in Panama (Varela *et al.*, 2017). In the same region, Estrada *et al.*, (2019) found strong correlations between rainfall and temperature at the time of sampling on skin bacterial communities of treefrogs. Overall, these studies highlight that the site environmental conditions influence the skin microbiome of co-occurring species differently, suggesting species-specific microbiome response.

The Panama Canal is surrounded by protected tropical moist forest characterized by a marked rainfall gradient and strong seasonality, with the northern, or Atlantic slope, of the canal being wetter with a shorter dry season (Condit *et al.*, 2001). Also, in the north, tree, bird, and amphibian communities are more diverse than in the drier southern end on the Pacific slope (Condit *et al.*, 2001). Although there is some evidence supporting a relationship between host intraspecific diversity and host-associated microbiomes (Kueneman *et al.*, 2019), the region provides the opportunity to further investigate this idea. With hosts experiencing temporal and spatial changes in environmental conditions, one might expect that their associated bacterial

communities will also vary along physical gradients and between seasons. Environmental reservoirs of bacteria can also change across spatio-temporal scales (Ortmann and Ortell, 2014), influencing bacterial community composition in host skin. We hypothesize that bacterial communities associated with common amphibian species will vary among sites along the Panama Canal rainfall gradient. Since amphibian host plays an active role selecting which bacteria colonize their skin, we also expect that different amphibian species will show different seasonal responses in bacterial community diversity and structure.

Here, 16S rRNA gene amplicon sequencing was used to characterize the bacterial communities associated with the skin of lowland amphibian species in the Panama Canal Watershed. Specifically, our goal was to determine: (1) if skin bacterial communities vary across sites along a latitudinal rainfall gradient; (2) if seasonal changes in skin bacterial communities are consistent across diverse host species; (3) if a diverse amphibian assemblage share bacteria associated with the skin of co-occurring host species; and (4) if Bd status varies between different amphibian species in the watershed. To address our first objective, we sampled the skin of a common leaf litter species, Fitzinger's robber frog (*Craugastor fitzingeri*), across five sites along the rainfall gradient of the Panama Canal Watershed (Ibáñez *et al.*, 1999). This species is mostly active during the night, though it can be observed during the day on the forest floor or along streams in dry periods. It shows direct development and lays eggs in the leaf litter, and the eggs are cared by the females until hatching. The skin of *C. fitzingeri* has been extensively characterized, in terms of bacterial community diversity and structure (Belden *et al.*, 2015; Rebollar *et al.*, 2016; Abarca *et al.*, 2018), skin symbiotic community function (Rebollar *et al.*, 2019) and disease susceptibility (Rebollar *et al.*, 2014). Finally, their populations are stable and the species is little impacted by habitat disturbance, hence it is of low conservation concern (Gratwicke *et al.*, 2016). To assess our second objective, we sampled the skin of five co-occurring lowland species (*C. fitzingeri*, *Engystomops pustulosus*, *Hypsiboas rosenbergi*, *Rhinella alata* and *Silverstoneia flotator*), which were selected based on the likelihood of finding enough individuals in both seasons. The skin microbial community of *S. flotator* has been previously described in relations to differences across elevations and metabolite production (Medina *et al.*, 2017), and seasonal variation (Varela *et al.*, 2018). To address our third objective, our study is the first to characterize the skin bacterial communities of *E. pustulosus*, *H. rosenbergi*, *R. alata* and other lowland species. Lastly, for our fourth objective, we determine Bd prevalence in all lowland amphibian species encountered and Bd infection intensity in a subset of all sampled.

Methods

Field sampling and sample processing

We sampled frogs within 2 m of the riparian edge on each side of 200 m stream transects at five lowland sites (< 200 m.a.s.l) in the wet and dry seasons of 2015 and 2016. The five sites fall along a latitudinal rainfall gradient characteristic of the Panama Canal Watershed (Fig. 1), from the driest site Campo Scout (CS) in the southern end of the gradient, followed by Camino de Plantación (PL) and Camino de Oleoducto (OL) near Gatun Lake, to the wettest sites in the north at Sherman (SH) and San Lorenzo (SL).

Encountered frogs (total N = 742; total species = 20) were caught by hand with a new pair of gloves and placed individually in sterile whirl pack bags until processing (<1 hour from time of capture). Skin swabs were collected with a non-invasive technique, as previously described by Estrada *et al.*, (2019). All animals were released on-site immediately after swabbing, and swabs were stored at -80 °C until DNA extraction.

Samples were collected under a research permit granted by the Panamanian authority of environment (Ministerio de Ambiente, Permit No. SE/A-47-12). Animal research was approved by corresponding animal care protocols from the Animal Care Committees at the Smithsonian Tropical Research Institute (2013-0401-2016-A3) and Virginia Tech (11-105-BIOL).

DNA extraction and sequencing protocol

For both the analysis of the skin bacterial communities and assessment of *Bd* infection, DNA was extracted from the skin swabs using the DNeasy Blood and Tissue kit (Qiagen, Valencia CA, USA) following the manufacturer's protocol and with an initial 1 h lysozyme incubation at 37 °C.

Skin bacterial communities were assessed in four sequencing runs by amplifying and sequencing the V4 region of the 16S rRNA gene with primers 515F and barcoded 806R (Caporaso *et al.*, 2012), using PCR protocols as described in Medina *et al.*, (2017). For each run, the barcoded PCR products were pooled to make a composite sample and were cleaned with the QIAquick PCR Purification Kit (Qiagen, Valencia, CA). The four resulting pooled samples were sent to the Dana Farber Cancer Institute at Harvard University for single-end 250bp sequencing on an Illumina MiSeq instrument (Caporaso *et al.*, 2012).

Forward reads from the four sequencing runs were processed using the QIIME2 pipeline (Bolyen *et al.*, 2018). Following import, the sequences were demultiplexed. DADA2 (Callahan *et al.*, 2016) was used to quality filter the sequences, denoise them, remove chimeras and remove any remaining PhiX sequence (used to increase base diversity in the sequencing run). Default parameters were used for DADA2 filtering, except that q (phred quality score for truncation) was set at 11, per the recommendation in the DADA2 “big data” workflow. After filtering, the four resulting amplicon sequence variant (ASV) tables were merged into a single feature table, which contained 267 samples, 120,947 ASVs and 24,308,497 total reads, with a range of 26085-170923 reads/sample. To remove potential contaminants and other exceptionally low abundance taxa, we filtered the ASV file to remove ASVs that composed less than 0.01% of the total read abundance. This reduced the number of ASVs to 1032. Taxonomy was assigned to these sequences using sklearn (Bokulich *et al.*, 2018), with the SILVA_132_99 pre-classifier (Quast *et al.*, 2012). After assigning taxonomy, we filtered out taxa assigned as mitochondria or chloroplast, which brought the number of ASVs down to 987. Following examination of alpha rarefaction plots, we rarefied all samples at 13,000 reads, which resulted in the loss of a single sample. The final table, therefore, contained 263 samples and 983 ASVs.

An aliquot of the extracted DNA was tested for the presence of *Bd* in all samples using a conventional PCR method (Annis *et al.*, 2004). Due to low *Bd* prevalence (11%), infection intensity was only evaluated in a subset of randomized *Bd* samples from all species and sites (N

= 40 out of 71 positive samples) using Taqman real-time PCR (Boyle *et al.*, 2004). Bd strain JEL 310, isolated from *Smilisca phaeota* in Panama, was used as a positive control for regular PCR and to create standards for the qPCR reaction. No differences were detected in skin bacterial communities between infected and uninfected *C. fitzingeri* in a previous study (Belden *et al.*, 2015); however, we still chose to use only Bd negative samples to examine variation in bacterial communities across sites along the rainfall gradient.

Overview of statistical analysis

To determine variation in skin bacterial communities along a rainfall gradient, we characterized the skin communities of a widely-distributed species, *C. fitzingeri* (N = 103), across five sites along the Panama Canal Watershed (hereafter, gradient dataset). To characterize seasonal differences in skin-associated bacteria of diverse amphibian hosts, we studied five co-occurring lowland species (*C. fitzingeri*, *E. pustulosus*, *H. rosenbergi*, *R. alata* and *S. flotator*) at Camino de Plantacion between the wet season of 2015 (N = 63) and the dry season of 2016 (N = 48) (hereafter, seasonal dataset). Table 1 summarizes species' natural history, sample sizes and previous research on their skin microbiomes. We also described bacterial communities associated with the skin of 11 lowland amphibian species that have not yet been characterized (hereafter, community dataset). Lastly, we reported Bd status in the watershed including all five sites and 20 species surveyed in this study. For the seasonal and community dataset, we determined richness or the total number of bacterial taxa at each taxonomic level for each species, the dominant bacterial taxa at all taxonomic levels for each species, and the proportion of unique and shared ASVs between co-occurring host species. Dominant bacterial phyla, class, order, family and genus were defined as those with the highest proportion (%) of bacteria belonging to each taxon. Dominant ASVs were defined as those with the highest mean relative abundance for each host species.

Alpha diversity metrics, including the total number of different bacterial taxa (ASV richness) and the Shannon index (transformed to Hill numbers), were calculated for all sites, seasons and species. Generalized mixed models (GLMMs) were used to determine changes in bacterial community diversity and evenness across sites, seasons, and species. We used negative binomial error for ASV richness and gamma error distributions for Shannon index hill numbers. GLMMs included random effects of "season" for the gradient dataset set and "collection day" for the seasonal dataset. Additionally, "individual" was used as a random effect in both analyses. Chi square and likelihood ratio tests were used to estimate P-values of fixed factors, and multiple comparisons with Tukey tests were conducted to determine differences between pairs of groups. Additionally, generalized linear models or GLMs were used to examine the relationship between alpha diversity and latitude along the gradient. Latitude (UTM) was used to represent the rainfall gradient. GLMs were performed as described above with negative binomial and gamma error distributions for ASV richness and Shannon index hill numbers, respectively.

Variation in bacterial community structure across sites along the latitudinal rainfall gradient and between seasons across co-occurring species were visualized with non-metric multi-dimensional scaling plots (NMDS) based on the Bray-Curtis (relative abundance based) and Jaccard (presence/absence based) distance matrices. Permutational multivariate analysis of variance (PERMANOVA) was used to test for differences in bacterial beta diversity in *C. fitzingeri* across

sites along the rainfall gradient, between seasons within co-occurring host species in Camino de Plantacion, and among different lowland amphibian species. We controlled for seasonal effects (gradient dataset analysis) and collection day effects (seasonal dataset analysis) by including these variables as strata. Strata do not correspond directly to a random factor, but they control for repeated measures within seasons or collection days. Analysis of multivariate homogeneity of group dispersions (betadisper) was used to determine whether the effect of seasonality on skin bacterial communities varied between and within host species. Lastly, Indicator Species Analysis (ISA; Dufrene and Legendre, 1997) was used to identify ASVs that most likely explained differences across sites along the gradient.

Results

Bacterial community stability along a rainfall gradient

In *C. fitzingeri*, alpha diversity metrics did not differ across sites along the rainfall gradient of the Panama Canal Watershed (ASVs richness: Chisq = 0.9368, P-value = 0.9192, Fig. 2A and Shannon Hill: Chisq = 2.6342, P-value = 0.6208, Fig. 2B). The average frog had 222 ASVs, with a range of 93 - 366 ASVs. We also found no relationship between latitude (as UTM coordinates) and ASV richness (slope = -4.068e-06, SE = 2.696e-06, P-value = 0.1313, Fig. 2C) or Shannon index Hill numbers (slope = 1.241e-07, SE = 1.098e-07, P-value = 0.3810, Fig. 2D).

The spatial stability of the ASV richness and evenness on *C. fitzingeri* skin contrasts with analysis of community structure based on the Bray-Curtis distance matrix, where site explained 15% of the variation observed among bacterial communities ($R^2 = 0.1535$, F-model = 2.1815, P-value = 0.0071, Fig 3A). The effect of site was observed after considering that frogs were sampled in different seasons and days across all the sites (strata = site and day, both P-values > 0.05, Table S1). NMDS plots graphically showed overlap between most sites, excluding apparent clustering among the wettest sites (Sherman and San Lorenzo), suggesting that the variation is not solely driven by rainfall gradient. Betadisper analysis demonstrated no differences in dispersion among sites (Bray-Curtis F-stat = 2.0863, P-value = 0.0986). The analyses of bacterial community structure and dispersion with the Jaccard dissimilarity matrix were similar, indicating differences not only in the relative abundance, but also in the presence/absence of ASVs in these communities ($R^2 = 0.1289$ F-model = 1.7312, P-value = 0.0060 Fig. 3B)

Changes in community structure extended to indicator species, with respect to both variation in relative abundance and presence/absence. Analysis determined that of the 983 ASVs, there were 140 ASVs indicator species on *C. fitzingeri* between and within sites along the rainfall gradient (Table S2). Proportionally to the total number of bacteria, indicator ASVs belonged to the classes: Gammaproteobacteria (32%), Alphaproteobacteria (28%), Actinobacteria (22%), Bacteroidia (11%), Bacilli (5%), Deltaproteobacteria (< 1%) and Negativicutes (< 1%). The family *Pseudomonadaceae* (Gammaproteobacteria) consistently dominated all bacterial communities, and their relative abundance differed across sites, with the highest values in Camino de Oleoducto (OL = 63%, SL = 53%, SH = 40%, CS and PL = 36%). At the other end of the gradient, the wettest, most Northern sites (SH and SL) were additionally characterized by families such as *Micrococcaceae* (Actinobacteria) and *Moraxellaceae* (Gammaproteobacteria),

and there too, relative abundances of the dominant ASVs changed between sites. In total, 22 ASVs were associated with CS, 2 with OL, 26 with PL, 21 with SH, and 12 with SL.

Seasonal changes in skin microbiome among co-occurring species

Seasonal changes in skin bacterial community in a lowland amphibian community

Amphibians from Camino de Plantacion varied in the total number of different ASVs that characterized their skin. For example, *R. alata* (904 ASVs) had the highest number of recovered ASVs, followed by *E. pustulosus* (879 ASVs), *C. fitzingeri* (841 ASVs), *S. flotator* (813 ASVs) and *H. rosenbergi* (785 ASVs). The bacterial communities of the five selected amphibian hosts varied in terms of both ASV richness (Deviance = 1268.2, Chisq = 40.55 P-value < 0.001, Fig. S1A) and evenness (Shannon index Hill numbers, Chisq = 31.58 P-value < 0.001, Fig. S1B). Considering all species together, no differences were detected between seasons (ASV richness Deviance = 1282.5, Chisq = 0.614, P-value = 0.4333; Shannon index Hill numbers, Chisq = 2.7026, P-value = 0.1002). *R. alata* had the most diverse bacterial communities, in terms of ASV richness (P < 0.001), followed by *E. pustulosus*. In terms of Shannon index hill numbers these two species were not significantly different (z = -1.790 P-value = 0.36). *C. fitzingeri*, *H. rosenbergi* and *S. flotator* had similar but less diverse and less even communities (P-value > 0.05). However, both richness and evenness varied across seasons for four of the species, although not in a consistent direction (ASV richness; Chisq = 24.0860 P-value = 0.0042, Fig. 4A; Shannon index transformed into hill numbers; Chisq = 142.74 P-value < 0.0001, Fig. 4B). For example, ASV richness on *R. alata* was higher in the wet season, while communities on other co-occurring species (*E. pustulosus*, *H. rosenbergi*) were higher in the dry season. Season had no effect on ASV richness or evenness on *S. flotator*.

Skin bacterial community structure changed between seasons in the five most abundant amphibian hosts, though season explains a very small proportion of the variation observed (PERMANOVA, $R^2 = 0.06568$, F-model = 8.9526, P-value < 0.001, Fig. 5A). Yet, when considering species as strata, which is represented by an interaction factor in the analysis, both season and species best explain differences in bacterial community structure (PERMANOVA, $R^2 = 0.19336$, F-model = 3.2946, P-value < 0.001, Fig. 5B). However, within-host seasonal responses are not consistent (betadisper, F = 0.5858 P-value = 0.4457). For example, in *H. rosenbergi* and *S. flotator*, the average distance from centroid in the dry season was shorter than in the wet season, meaning that communities were more similar to each other in the dry season (betadisper, both P-value < 0.05). Whereas, *C. fitzingeri*, *E. pustulosus* and *R. alata* showed no differences in dispersion between dry and wet season, suggesting that communities responded similarly in both seasons (betadisper, all P-value > 0.05).

Assessment of bacterial communities associated with the skin of lowland amphibians

For the entire host community at Camino de Plantacion (N = 11 species), amphibian species differed in the total number of phyla (5-16), classes (8-26), orders (27-71), families (44-136), genera (50-256) and ASVs (87-904) that characterized their skin communities. In general, we recovered less bacterial diversity from the skin of amphibians with a smaller number of surveyed individuals, such as *Rhinella marina* and *Hyalinobatrachium fleischmanni*, with 137 and 87 ASVs, respectively.

Across all amphibian species, most bacteria belonged to three phyla: Proteobacteria (62% of the relative abundance), Actinobacteria (21%) and Bacteroidia (9%). Yet at lower taxonomic levels, skin communities were enriched by different taxa. For example, at the genus level, the skin of *Dendrobates auratus* (10%), *Diasporus diastema* (7%) and *H. fleschmanni* (17%) were enriched by *Pseudomonas*. However, the skin of more common amphibian species *C. fitzingeri*, *E. pustulosus* and *H. rosenbergi* (all 4%) were enriched by *Sphingomonas*. Several genera in the Rhizobiaceae family were more diverse in *Leptodactylus savagei*. The dominant ASV found on the skin of each species generally did not belong to the dominant genus, for example: *H. rosenbergi* skin was enriched by the genus *Sphingomonas* (4%), but the single ASV with the highest relative abundance belonged to the genus *Acinetobacter* (0.07). See Tables S3 for a complete list of dominant AVSs or those with the highest relative abundance, and Table S4 for dominant bacterial taxa by host species.

Of the ASVs recovered, most were shared by some host species (93%), and a large proportion was shared by the five most abundant amphibian species (68.27%). While a small percent (5.3%) were shared across all host species, few were unique to one amphibian species (5.2%) (Fig. 6). The percent of unique ASVs varied between species: *H. fleschmanni* (27.2%), *R. marina* (24.5%), *L. savagei* (4.2%), *R. alata* (0.7%), *E. pustulosus* (0.2%), *S. flotator* (0.2%), *C. fitzingeri* (0.04%). No unique ASVs were found for *H. rosenbergi*. These unique ASVs were frequently found in low relative abundances, except for species of the Micrococcales order (0.24) found on *R. marina* and one species of the Betaproteobacteriales order (0.24) on *H. fleschmanni*. For other amphibians in the community, the ASVs with the highest relative abundance were also found in the skin of other amphibian species. *Pseudomonas spp.* were more abundant in the skin of *D. auratus*, *C. fitzingeri*, *H. rosenbergi* and *S. flotator*.

Bd infection status in lowland amphibians in the Panama Canal Watershed

During our field surveys in five sites along the Panama Canal Watershed, we surveyed 742 individuals from 20 different amphibian species and found 71 individuals from 9 species infected with Bd (11% prevalence) (Table 2). From a subset of samples (N = 40), Bd infection loads averaged 9156.24 zoospores equivalents (0.89 to 172,464.77 zoospores equivalents). Prevalence was overall low, except for *P. gaigei* (50%) and especially *L. warszewitschii* where 3 out of 5 frogs surveyed tested positive for Bd (60% prevalence), one of which had the highest infection load recorded in this study (172,464.77 zoospores equivalents). See Table S5 for disease prevalence and infection load of a subset of infected amphibian species.

Discussion

Based on the marked rainfall gradient and strong seasonality in the narrowest stretch of land connecting the Atlantic and Pacific Oceans, the Panama Canal watershed offers a unique opportunity to understand how factors that structure host-associated microbial communities act on spatial and temporal scales within diverse host communities. We hypothesized that the diversity of bacterial communities associated with amphibian skin would increase with latitude as the more northern sites are significantly wetter. Although there are marked differences of

environmental variables along the gradient, we did not detect differences in bacterial community richness and evenness across sites, but site was the strongest predictor of differences in community structure based on relative abundance and presence/absence associated with the skin of *C. fitzingeri*. We also hypothesized that strong seasonality patterns in the region would have similar effects on skin-associated bacterial communities across amphibian hosts. However, seasonal patterns of bacterial community diversity and structure were host-specific. Together, these findings suggest strong selection by the hosts for symbiotic bacteria, despite spatial and temporal environmental gradients.

Skin-associated bacterial communities on *C. fitzingeri* showed a large amount of variation within individuals at each site. In terms of bacterial community richness and evenness, less variation was observed across sites, despite the marked spatial differences in abiotic and biotic factors that characterized the Panama Canal Watershed. However, the structure of the skin bacterial communities, both in terms of relative abundances and presence/absence, did vary among sites. Our results are consistent with the only additional study conducted in the watershed evaluating skin-associated microbial communities, where soil pH and rainfall explained differences in skin communities of another terrestrial amphibian, *D. auratus* (Varela *et al.*, 2018). For other amphibian host species, environmental gradients created by changes in land use (Hughey *et al.*, 2017) and latitude (Bletz *et al.*, 2017) explain variation in community composition. However, patterns across environmental gradients are context-dependent (Medina *et al.*, 2017). For example, while no difference was observed in skin bacterial community composition of *S. flotator*, bacterial metabolite profiles varied across elevations. Indeed, amphibians at higher latitudes have suffered severe disease-related population declines, and Medina *et al.*, (2017) suggested that the same bacteria produce different metabolites under different conditions. In the Panama Canal, Varela *et al.*, (2018) found correlations between soil pH and variation in bacterial functional groups associated with the skin of *D. auratus*; at soils of pH 5.4, there were less diverse bacterial communities, with more predicted Bd-inhibitory bacteria and fewer predicted Bd-enhancing bacteria.

While no systematic disease surveillance have been conducted along the rainfall gradient of the Panama Canal, several publications have reported overall low Bd prevalence and infection intensities in the region (Woodhams *et al.*, 2008; Kilburn *et al.*, 2010; Rebollar *et al.*, 2014). We too detected only low levels of Bd prevalence during our study (11%). Most amphibian population declines associated with Bd in Central America have been documented at higher elevations, whereas ours was a lowland study. Although the mechanisms that allow lowland amphibians to survive despite Bd presence are unclear, suboptimal environmental conditions for Bd growth and host characteristics, such as the presence of beneficial bacteria, have been suggested as potential explanations (Rebollar *et al.*, 2014). Thus, amphibians found in the lowlands along the Panama Canal might harbor beneficial bacteria that allow them to survive with low levels of infection.

For *C. fitzingeri*, only 4% of individuals were infected with Bd, and though we did not include disease status in our analysis of bacterial communities, a previous study found no differences in the bacterial composition or metabolite profiles between Bd-infected and -uninfected individuals (Belden *et al.*, 2015). We identified several *Pseudomonas* sp. ASVs that largely dominated the skin bacterial communities of *C. fitzingeri*. Other amphibian species harbor *Pseudomonas* in

their skin (Bletz *et al.*, 2017), some of which are uniquely found in tropical species (Belden *et al.*, 2015), and others of which harbor Bd-inhibiting properties (Muletz-Wolz *et al.*, 2017). Ultimately, *C. fitzingeri* is not susceptible to Bd infections and populations are stable (IUCN, 2008), hence their skin bacterial communities might be characteristic of non-susceptible frogs that are presently dealing with low endemic Bd infection dynamics (Rebollar *et al.*, 2014, Belden *et al.*, 2015, Rebollar *et al.*, 2016). Alternatively, the same environmental conditions that limit pathogen growth (i.e. temperature and humidity) might affect the bacteria associated with amphibian skin and their antifungal capabilities (Muletz-Wolz *et al.*, 2017). In that case, species distributed along the Panama Canal watershed could vary in susceptibility to pathogen infections and in skin bacterial community composition (Rebollar *et al.*, 2016). Disease surveillance efforts and the characterization of host microbiomes should prioritize susceptible species at low and mid elevation in northern sites where disease-related amphibian declines might still occur (Kilburn *et al.*, 2010).

We found that seasonality appears to be an important driver of variation of bacterial community richness, evenness, and structure within host species, providing support for species-specific temporal variation in skin microbiomes. In Camino de Plantacion, differences in natural history, microhabitat use or extreme climate patterns affecting the area could partially explain seasonal differences in skin bacterial communities among species. For example, though all species were surveyed along the riparian zone and most breed in the wet season, species such as *R. alata* are mostly terrestrial and were never found in direct contact with water at any sampling season. A previous study on *S. flotator* found that skin-bacteria community diversity was higher during dry conditions and structure differed between seasons (Varela *et al.*, 2018). Though we did not detect seasonal changes in community diversity in *S. flotator*, this could be due to inter-annual differences in seasonality in central Panama. Our surveys were conducted during 2015-16 when the Panama Canal watershed experienced the lowest totals recorded for monthly and weekly rainfall, soil humidity, and stream discharge corresponding to an intense El Niño Southern Oscillation (STRI physical monitoring program). Hence, opposing patterns in bacterial diversity and structure across studies conducted in different years might be explained by the effect of long- and short-term fluctuation in environmental conditions on skin bacterial communities (Estrada *et al.*, 2019).

Predictably, we described clear differences in skin bacterial communities of co-occurring amphibian host species (Walke *et al.*, 2014; Kueneman *et al.*, 2014; Belden *et al.*, 2015; Rebollar *et al.*, 2016). Former studies acknowledged host identity as the main predictor of variation in skin bacterial communities, but few have clearly described variation in the proportion of dominant bacterial taxa and those shared among other host species in the community (Rebollar *et al.*, 2016). Although dominant taxa persisted across seasons, the relative abundance of specific bacteria, including key ASVs, changed across species within seasons, as shown in ordinations based on relative-abundance analysis. Although our study is not the first to look at skin bacterial communities within highly diverse tropical amphibian communities (Rebollar *et al.*, 2016; Bletz *et al.*, 2017; Abarca *et al.*, 2018), we are among the first to describe host-specific seasonal responses in skin-associated bacterial communities. The relevance of these studies relies on the link between seasonal disease dynamics in lowland tropical regions and the potential disruption of the protective function of the amphibian skin microbiome (Jimenez and Sommer, 2015).

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Figure 1. Study sites along the Panama Canal Watershed

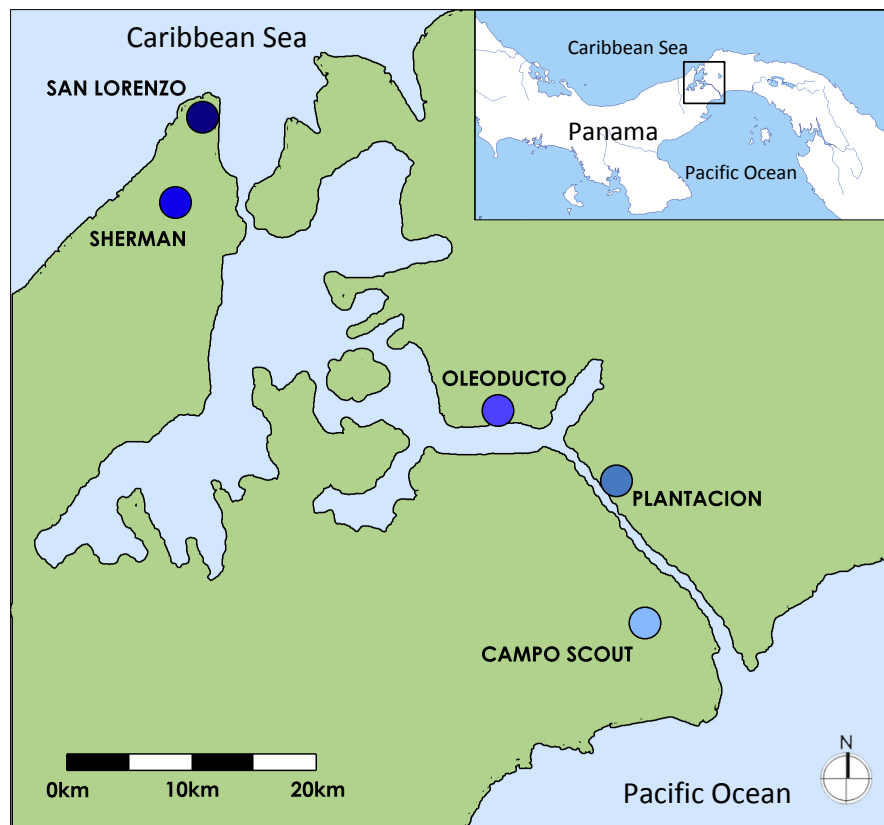
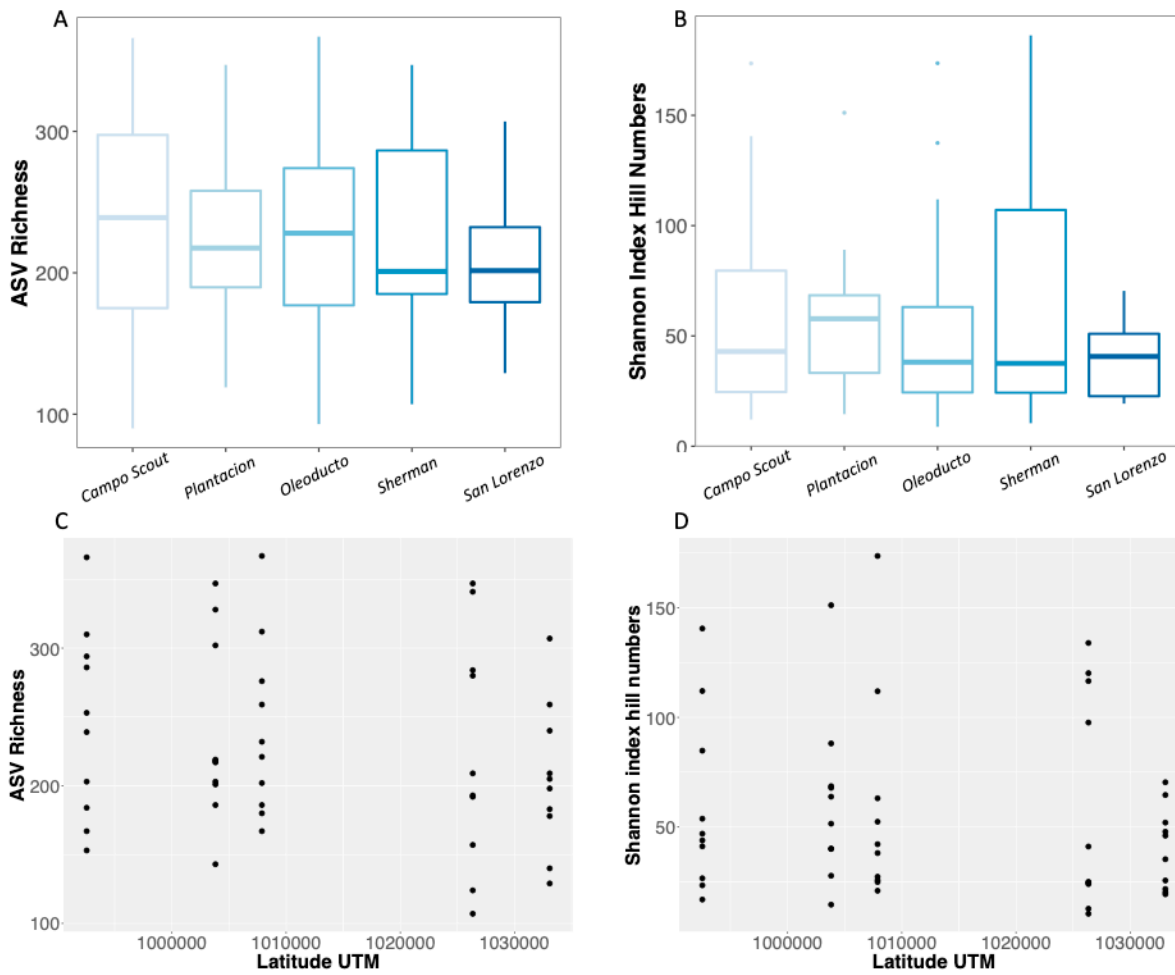
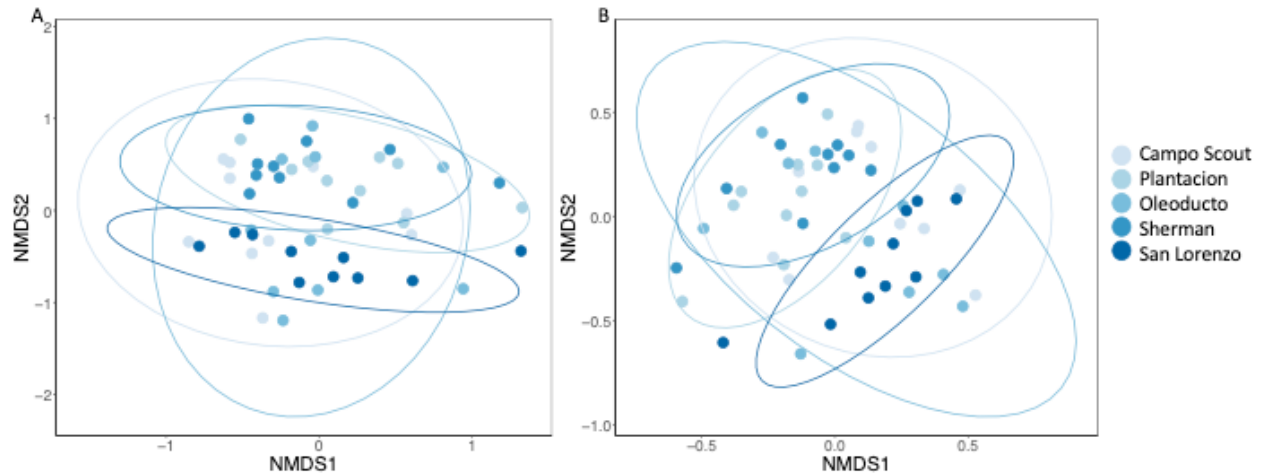


Figure 2. ASV richness and evenness across sites along the latitudinal gradient at the Panama Canal Watershed.



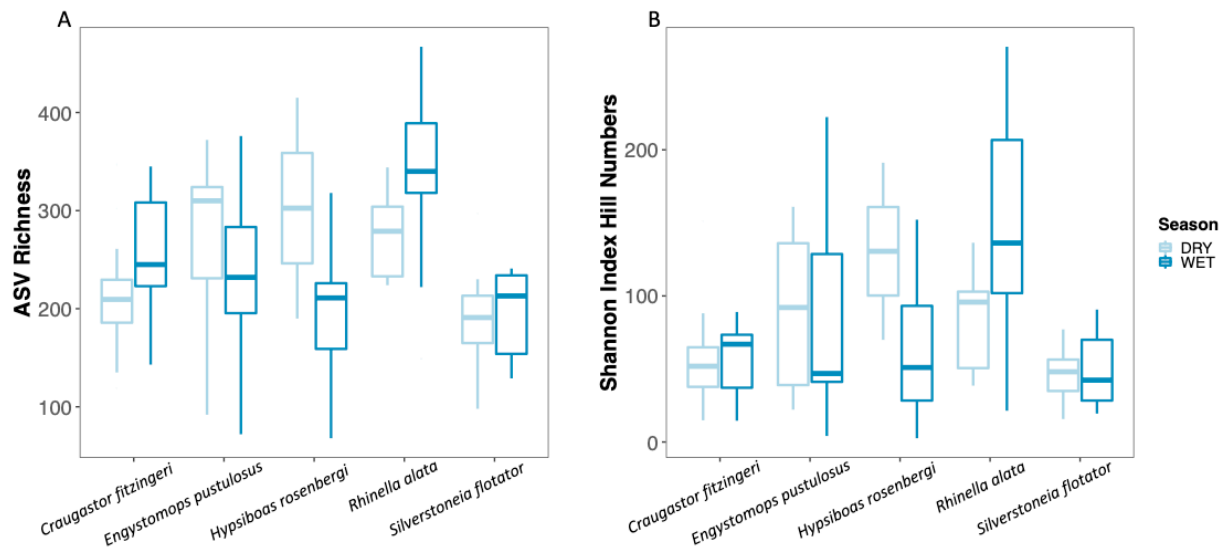
Alpha diversity metrics of ASV Richness (A) and Shannon Index Hill Numbers (B) across sites along the rainfall gradient of the Panama Canal Watershed (N = 10 *C. fitzingeri* per site). Sites are color-coded to represent the rainfall gradient from light (driest site, CS) to dark colors (wettest site, SL). There was also no correlation between ASV Richness (C) and Shannon index hill numbers (D) and latitude along the gradient.

Figure 3. Site variation of bacterial community structure across along the Panama Canal Watershed



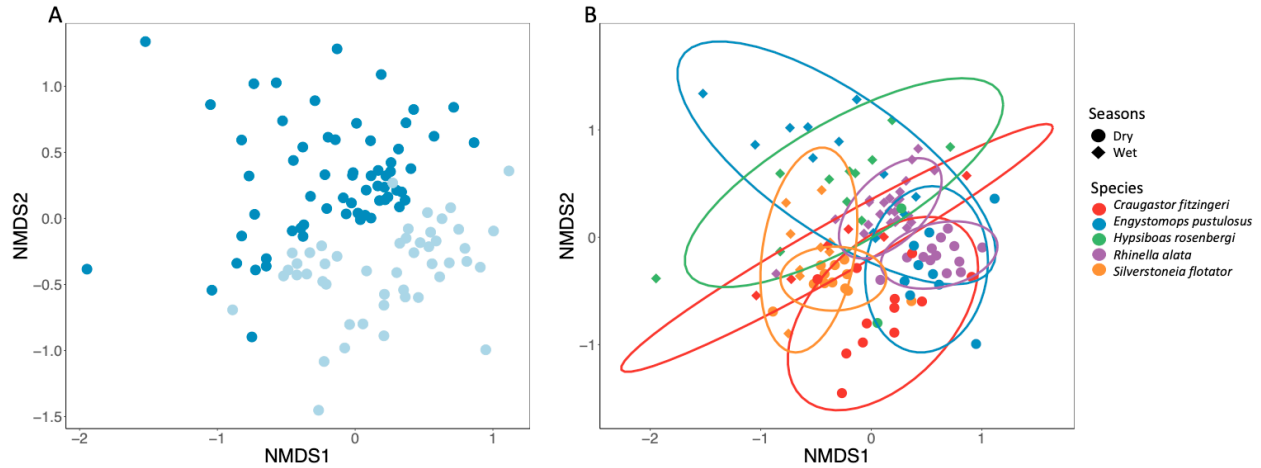
Non-metric multi-dimensional scaling (NMDS) plot of Bray-Curtis (A) and Jaccard (B) distances in skin bacterial community structure on *C. fitzingeri* across sites along the rainfall gradient. Sites are color-coded to represent the rainfall gradient from light blue (driest site, CS) to dark blue (wettest site, SL).

Figure 4. ASV richness and evenness between seasons within amphibian species



Alpha diversity metrics of ASV Richness (A) and Shannon Index Hill Numbers (B) of bacterial communities sampled in the dry (light blue) and the wet season (dark blue) within amphibian species.

Figure 5. Seasonal variation of bacterial community structure across amphibian species



Non-metric multi-dimensional scaling (NMDS) plot of Bray–Curtis distance matrix in skin bacterial community structure between dry (light blue) and wet (dark blue) at Camino de Plantacion (A). Seasonal variation on community structure within host species at a single site (B).

Figure 6. Relative abundance of shared ASVs per amphibian species

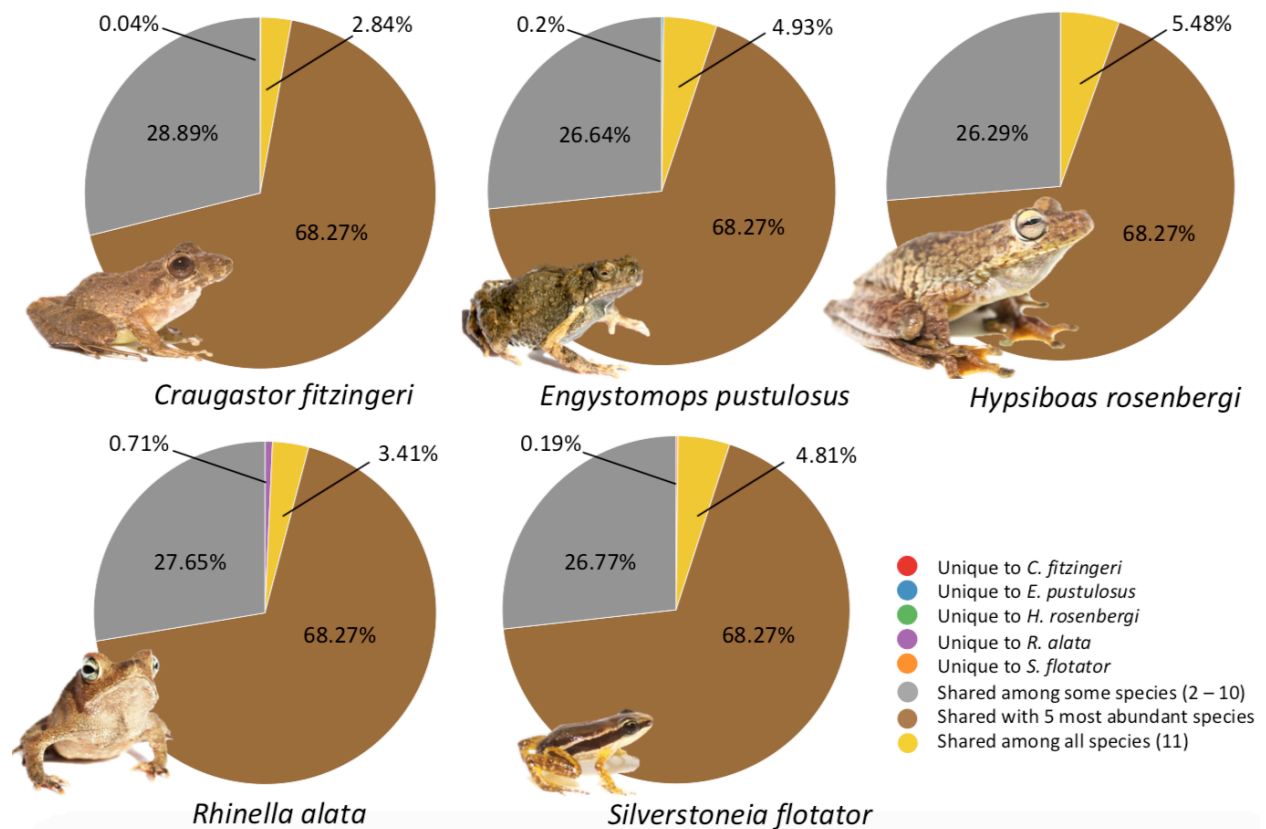


Table 2. Disease prevalence of amphibian species in the Panama Canal Watershed.

Species	Infected/Total	Prevalence
<i>Allobates talamancae</i>	7 / 93	7.53
<i>Craugastor fitzingeri</i>	12 / 275	4.36
<i>Colostethus panamansis</i>	0 / 12	0
<i>Dendrobates auratus</i>	0 / 3	0
<i>Diasporus diastema</i>	0 / 10	0
<i>Diasporus vocator</i>	0 / 3	0
<i>Engystomops pustulosus</i>	6 / 47	12.77
<i>Hyalinobatrachium fleishmani</i>	0 / 9	0
<i>Hypsiboas rosenbergi</i>	0 / 19	0
<i>Hypsiboas rufitelus</i>	0 / 3	0
<i>Leptodactylus savagei</i>	0 / 7	0
<i>Lithobates warszewitschii</i>	3 / 5	60
<i>Pristimantis gaigei</i>	2 / 4	50
<i>Rhinella alata</i>	27 / 189	14.29
<i>Rhaebo haemaetiticus</i>	0 / 2	0
<i>Rhinella marina</i>	0 / 1	0
<i>Silverstoneia flotator</i>	10 / 47	21.28
<i>Smilisca phaeota</i>	0 / 6	0
<i>Smilisca sila</i>	0 / 3	0
<i>Teratohyla spinosa</i>	2 / 4	50
Total	742	11.19

Supplementary Material

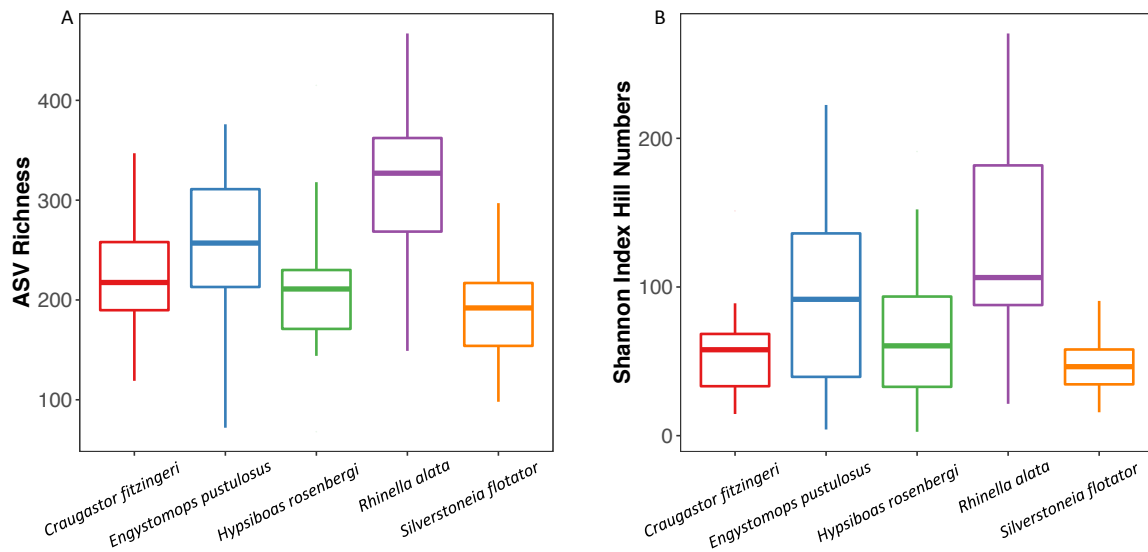
Table S1. Permutational multivariate analysis of variance (PERMANOVA) of skin bacterial communities on *C. fitzingeri* across sites along the rainfall gradient of the Panama Canal Watershed.

PERMANOVA	R ²	F stat	df	P value
Bray-Curtis				
Site	0.15354	2.1815	4	0.007
Site : Season	0.10743	2.0352	3	0.428
Site : Day	0.40512	1.487	17	1
Jaccard				
Site	0.12889	1.7312	4	0.006
Site : Season	0.08936	1.6004	3	0.539
Site : Day	0.38512	1.2918	17	1

Table S2. Indicator ASVs associated with *Craugastor fitzingeri* across sites along the rainfall gradient of the Panama Canal Watershed.

Site	ASV ID	Phyla	Class	Order	Family	Genus	stat	p.value
Campo Scout	4e8816e59e35ce95b4d2117d85e356f3	Firmicutes	Bacilli	Bacillales	Family XII	Exiguobacterium	0.853	0.001
	efc7b3a1912590d4de3de1c9a9d5773	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	Chromobacteriaceae	Vogesella	0.619	0.004
	8b5de61b51fee6d382de99cfeca85bf	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	0.669	0.006
	995ea060a4289b99164611e7d0a8c5f7	Firmicutes	Bacilli	Bacillales	Planococcaceae	Chryseomicrobium	0.702	0.007
	6c0fb36340add8c9020b7292d662b067	Bacteroidetes	Bacteroidia	Chitinophagales	Saprospiraceae	uncultured	0.632	0.008
	c44817754db79eb247f129890e4bb8146	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	0.683	0.017
	25227b9990d199ec86e61777da74525e	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	Chitinimonadaceae	Chitinimonas	0.611	0.025
	e54de8f948e4f1d2a3c9e5a394c5bc37	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	0.562	0.033
	904be71fe0bc6c92101c3c8f0b1c435	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Gemmobacter	0.543	0.039
	d1e46d42f4d09a1d844e732735b2eb21	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	Burkholderiaceae		0.546	0.039
	5e91c08749688980ba118988c5e0b879	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	Nitrosomonadaceae	Ellin6067	0.513	0.046
	6d80e3698883ee1b292e9477f396ae0	Actinobacteria	Actinobacteria	Streptomycetales	Streptomycetaceae	Streptomyces	0.696	0.002
	bd7277fc236d04b3ee5c74b3a741f1fa	Actinobacteria	Actinobacteria	Streptomycetales	Streptomycetaceae		0.747	0.002
	000529f90409472c1e671133ec0c25bf	Actinobacteria	Actinobacteria	Pseudonocardiales	Pseudonocardiaceae	Amycolatopsis	0.695	0.004
	7b90c4356e615e84daa1d061857447e8	Proteobacteria	Alphaproteobacteria	Rhizobiales	Xanthobacteraceae	Pseudolabrys	0.629	0.006
	e907c08afc269d0af44f5c96e1bac60	Actinobacteria	Actinobacteria	Streptomycetales	Streptomycetaceae	Kitasatospora	0.572	0.011
	83e8f941ebfaf966ed1b7d81e21e43a6	Bacteroidetes	Bacteroidia	Flavobacteriales	Weeksellaceae	Chryseobacterium	0.593	0.013
	e15505ebd8a99ca88fb32da56342e372	Actinobacteria	Actinobacteria	Micrococcales	Promicromonosporaceae	Promicromonospora	0.604	0.022
	268fd35b9458156c6e77e5824d32cdf	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	0.548	0.031
	781e12a6bb9dc6f6485291f0fd2c37	Actinobacteria	Actinobacteria	Micrococcales	Micrococcaceae	Nesterenkonia	0.542	0.039
	aa5c03067876b461bf9cd30928ebd53f5	Actinobacteria	Actinobacteria	Streptomycetales	Streptomycetaceae	Streptomyces	0.538	0.042
	f7de597197d0ac53a8ea6ea45c806cf	Proteobacteria	Alphaproteobacteria	Rhizobiales	A0839		0.509	0.044
Oleoducto	4f37e7672f091ad5abf26be29e5c3447	Proteobacteria	Deltaaproteobacteria	Myxococcales	Sandaracinaceae	uncultured	0.634	0.018
	d781f096db08fd6ea489f16a3e969d8	Proteobacteria	Alphaproteobacteria	Rhizobiales			0.537	0.044
Plantacion	bd7277fc236d04b3ee5c74b3a741f1fa	Actinobacteria	Actinobacteria	Streptomycetales	Streptomycetaceae		0.815	0.001
	7c2b975fd40ef244673d3ba3e2ae29117	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Luteimonas	0.763	0.007
	12534616632742aa327b73a7ba944d43	Actinobacteria	Actinobacteria	Propionibacteriales	Nocardioidaceae	Kribbella	0.754	0.001
	271b576cc1f2ce84b35d8374ed909db4	Verrucomicrobi	Verrucomicrobiae	Chthoniobacteriales	Chthoniobacteraceae	Candidatus Udaeobacter	0.740	0.011
	e15505ebd8a99ca88fb32da56342e372	Actinobacteria	Actinobacteria	Micrococcales	Promicromonosporaceae	Promicromonospora	0.733	0.001
	f7de597197d0ac53a8ea6ea45c806cf	Proteobacteria	Alphaproteobacteria	Rhizobiales	A0839		0.720	0.001
	6d80e3698883ee1b292e9477f396ae0	Actinobacteria	Actinobacteria	Streptomycetales	Streptomycetaceae	Streptomyces	0.702	0.003
	3111013c2c746f02f10f7f99e09b9b3c	Actinobacteria	Actinobacteria	Propionibacteriales	Nocardioidaceae	Aeromicrobium	0.701	0.001
	5b84f9aefbea73054bc5ebbf3904893	Actinobacteria	Actinobacteria	Micrococcales	Intrasporangiaceae		0.687	0.008
	805e633f669e5e954d57037440aa8f67	Actinobacteria	Actinobacteria	Micrococcales	Micrococcaceae		0.672	0.008
	83e8f941ebfaf966ed1b7d81e21e43a6	Bacteroidetes	Bacteroidia	Flavobacteriales	Weeksellaceae	Chryseobacterium	0.664	0.003
	a5c03067876b461bf9cd30928ebd53f5	Actinobacteria	Actinobacteria	Streptomycetales	Streptomycetaceae	Streptomyces	0.632	0.005
	b757da9a5b56247367c4be7bb9cd0a8a	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Lysobacter	0.628	0.02
	c3b9e9a07d3524d9447dfcd38e89a000	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	0.595	0.011
	e630e95789c62a8372e499eac271c90	Actinobacteria	Actinobacteria	Streptomycetales	Streptomycetaceae	Streptomyces	0.586	0.006
	8735493dec597938cbf9e275f63976e	Proteobacteria	Gammaproteobacteria	Gammaproteobacteria	Unknown Family	Acidibacter	0.577	0.045
	3a5ac7a65a6bc7d5777a5ca61b7d533	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Halomonadaceae	Halomonas	0.565	0.046
	676518ab3b38f3df5cfc01936df300b	Actinobacteria	Actinobacteria	Pseudonocardiales	Pseudonocardiaceae	Prasereella	0.548	0.028
	7ad3adeb9d4dc4c35b6383d04e59d55	Bacteroidetes	Bacteroidia	Sphingobacteriales	Sphingobacteriaceae	Olivibacter	0.548	0.029
	83b379f23da1b1dc7549ed6a2a2d88c6	Actinobacteria	Actinobacteria	Streptomycetales	Streptomycetaceae	Streptomyces	0.548	0.028
	a0548d411e04ef2888794af10c35b25	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	0.548	0.024
	a4ac89e57119b8b63c193fee24d1774	Bacteroidetes	Bacteroidia	Sphingobacteriales	Sphingobacteriaceae	uncultured	0.548	0.024
	8ccf0989b4c943e60d1020b0e82b5539	Bacteroidetes	Bacteroidia	Sphingobacteriales	Sphingobacteriaceae	Olivibacter	0.529	0.033
	d9d980e45e7118c91301ed3c168b947	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	Burkholderiaceae		0.528	0.05
	0498748ae39f33b7362be00a2010197	Proteobacteria	Deltaaproteobacteria	Myxococcales	Sandaracinaceae	uncultured	0.524	0.027
	1b60037e8dbac9689965bc3437c4d5c4	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae		0.508	0.039
Sherman	9527d20cd9d35a433e36b983b21fae92	Actinobacteria	Actinobacteria	Micrococcales	Microbacteriaceae	Curtobacterium	0.834	0.001
	c540efa822da874b7ed6b8d9ee89695	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	0.833	0.001
	49a1897b3e8a961a219716df9d1892f5	Actinobacteria	Actinobacteria	Pseudonocardiales	Pseudonocardiaceae	Actinomycetospira	0.770	0.002
	7d2f290758f1f8da52cd2cea3a924d04	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae		0.767	0.002
	609cfe075d3af9506da2e29bd06b59fe	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	0.782	0.002
	443ed63d256a53a303f58081aeb52f5	Proteobacteria	Alphaproteobacteria	Rhizobiales	Xanthobacteraceae		0.653	0.003
	b05061756663fa102d4d81f59e9c52a1	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae	Methylobacterium	0.710	0.003
	37d7579dd5e2b512286fdeb19ec47ae	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae		0.625	0.005
	523be1ac6d15745ded2d82f7b5cb9cf4	Bacteroidetes	Bacteroidia	Sphingobacteriales	Sphingobacteriaceae	Mucilaginibacter	0.735	0.005
	3eaf2c77e8c5773f6ae8ea974fc1b04e	Bacteroidetes	Bacteroidia	Sphingobacteriales	Sphingobacteriaceae	Mucilaginibacter	0.705	0.006
	3ad14316fc0452b674ab0bb2d71d1170	Actinobacteria	Actinobacteria	Micrococcales	Microbacteriaceae	Schumannella	0.671	0.009
	1e9f60664b433e12dce87674a5fec7f6	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae	Bosea	0.639	0.009
	36019bb30374dc721286587a3a7e0662	Bacteroidetes	Bacteroidia	Sphingobacteriales	Sphingobacteriaceae	Pedobacter	0.590	0.015
	5ba3e92bae7a119df5f9aa553ce1b56	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae		0.630	0.016
	90a9d4d5f86538db13546b1af5a3c12d	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	Burkholderiaceae		0.637	0.017
	ca6fb399eb48d3648a66c1b0ca8bd54b	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	0.613	0.022
	debcd050c59c4910a8f82ac5bb0117e8	Proteobacteria	Deltaaproteobacteria	Myxococcales	Birli41	metagenome	0.596	0.025
	f2074f088173743e5c8a8114114dce3	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	0.669	0.026
	d0d64bc98f5271ced87d8f0f8af01d42	Actinobacteria	Actinobacteria	Micrococcales	Micrococcaceae	Arthrobacter	0.548	0.033
	758f721b5f0c970a4f9b711ab8356889e	Bacteroidetes	Bacteroidia	Sphingobacteriales	Sphingobacteriaceae	Mucilaginibacter	0.533	0.036
	7138e91e395cf49ec69c5e65a60a4d4a	Actinobacteria	Actinobacteria	Pseudonocardiales	Pseudonocardiaceae		0.592	0.044
San Lorenzo	ac5ac33a28f0db51f9d321d2c9161a	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Ochrobactrum	0.707	0.001
	84e1b47343569760380db8f8191a13a	Bacteroidetes	Bacteroidia	Flavobacteriales	Weeksellaceae	Chryseobacterium	0.754	0.001
	9300f96e4700122e212eeebf488ca2f	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	0.900	0.001
	891019e319f5d8d088ed4f9379fd3ef	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Stenotrophomonas	0.819	0.001
	860cd2f2d2f1549faa2f5d093f9bd91	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae		0.814	0.002
	b354e84f92bd2d8864ba0519e800cfc99	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Novosphingobium	0.743	0.003
	b9d751a7ec8e838ff1c1543bac2684eb	Bacteroidetes	Bacteroidia	Sphingobacteriales	Sphingobacteriaceae	Nubella	0.680	0.003
	5dc17465e58548886dee16c2364f8be3	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae		0.742	0.004
	7fa0ea4914d95fd2426a8a2ea9e4f87	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	Neisseriaceae	Neisseria	0.701	0.006
	df23f58f1f04e8ba41fe15a88414e	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Mesorhizobium	0.557	0.022
	5b4f8b625d8fbb1268863be7dbcd4bd5d	Firmicutes	Negativicutes	Selenomonadales	Veillonellaceae	Veillonella	0.674	0.025
	88378cbe63a218c8ffcc33a01d24ca9	Bacteroidetes	Bacteroidia	Chitinophagales	Chitinophagaceae	Taibaiella	0.532	0.038

Figure S1. ASV richness and evenness across amphibian species



Alpha diversity metrics of ASV Richness (A) and Shannon Index Hill Numbers (B) of bacterial communities associated with the skin of five most abundant amphibian species in Camino de Plantacion.

Tables and Figures

Table 1. Amphibian species sampled in the wet and the dry season at Camino de Plantacion

Family	Species	Habitat	Activity	Conservation Status	Wet Season N	Dry Season N	Total N	Microbiome studies
Craugastoridae	<i>Craugastor fitzingeri</i>	Terrestrial	Nocturnal	Least Concern	6	12	18	Belden et al. 2015; Rebollar et al. 2016, 2018, 2019; Abarca et al. 2018
Bufonidae	<i>Rhinella alata</i>	Terrestrial	Diurnal	Data deficient	25	13	38	This study
Leptodactylidae	<i>Engystompos pustulosus</i>	Aquatic	Nocturnal	Least Concern	12	9	21	This study
Hylidae	<i>Hypsiboas rosenbergi</i>	Arboreal	Nocturnal	Least Concern	11	2	13	This study
Dendrobatidae	<i>Silverstoneia flotator</i>	Terrestrial	Diurnal	Least Concern	9	12	21	Medina et al. 2017

Table S3. Dominant and total number of ASVs across all amphibians sampled at Camino de Plantacion

SPECIES	Dominant ASV	TOTAL ASVs
<i>Craugastor fitzingeri</i>	Pseudomonas (0.04)	841
<i>Dendrobates auratus</i>	Pseudomonas (0.21)	150
<i>Diasporus diastema</i>	Burkholderiaceae (0.26)	216
<i>Engystomops pustulosus</i>	Enterobacteriaceae (0.05)	879
<i>Hyalinobatrachium fleishmani</i>	Burkholderiaceae (0.24)	87
<i>Hypsiboas rosenbergi</i>	Acinetobacter (0.07)	785
<i>Leptodactylus savagei</i>	Shinella (0.05)	581
<i>Pristimantis gaigei</i>	Enterobacteriaceae (0.14)	338
<i>Rhinella alata</i>	Enterobacteriaceae (0.05)	904
<i>Rhinella marina</i>	Micrococcales (0.24)	137
<i>Silverstoneia flotator</i>	Pseudomonas (0.07)	813

Dominant ASVs were defined as those with the highest mean relative abundance for each host species.

Table S4. Dominant bacterial taxa and total number of ASVs across all amphibians sampled at Camino de Plantacion

SPECIES	PHYLA	DOMINANT PHYLA	CLASS	DOMINANT CLASS	ORDER	DOMINANT ORDER	FAMILY	DOMINANT FAMILY	GENUS	DOMINANT GENUS	TOTAL ASVs
<i>Craugastor fitzingeri</i>	14	Proteobacteria (62%)	23	Alphaproteobacteria (50%)	66	Betaproteobacteriales (59%)	124	Burkholderiaceae (9%)	241	Sphingomonas (4%)	841
<i>Dendrobates auratus</i>	6	Proteobacteria (58%)	8	Gammaproteobacteria (31%)	27	Rhizobiales (21%)	47	Rhizobiaceae (11%)	55	Pseudomonas (10%)	150
<i>Diasporus diastema</i>	5	Proteobacteria (59%)	9	Alphaproteobacteria (29%)	35	Rhizobiales (16%)	63	Rhizobiaceae (8%)	102	Pseudomonas (7%)	216
<i>Engystomops pustulosus</i>	15	Proteobacteria (67%)	26	Alphaproteobacteria (32%)	71	Rhizobiales (17%)	136	Burkholderiaceae (25%)	256	Sphingomonas (4%)	879
<i>Hyalinobatrachium fleishmani</i>	7	Proteobacteria (56%)	11	Gammaproteobacteria (44%)	28	Pseudomonadales (25%)	44	Pseudomonadaceae (17%)	50	Pseudomonas (17%)	87
<i>Hypsiboas rosenbergi</i>	14	Proteobacteria (64%)	21	Alphaproteobacteria (32%)	63	Rhizobiales (18%)	116	Burkholderiaceae (10%)	223	Sphingomonas (4%)	785
<i>Leptodactylus savagei</i>	12	Proteobacteria (60%)	19	Alphaproteobacteria (33%)	59	Rhizobiales (23%)	108	Rhizobiaceae (10%)	188	Undetermined Rhizobiaceae (4%)	581
<i>Pristimantis gaigei</i>	16	Proteobacteria (62%)	26	Alphaproteobacteria (30%)	71	Rhizobiales (19%)	136	Burkholderiaceae (10%)	256	Pseudomonas (4%)	338
<i>Rhinella alata</i>	15	Proteobacteria (62%)	26	Alphaproteobacteria (31%)	70	Rhizobiales (16%)	132	Burkholderiaceae (10%)	248	Sphingomonas (4%)	904
<i>Rhinella marina</i>	7	Proteobacteria (61%)	11	Alphaproteobacteria (47%)	32	Rhizobiales (37%)	52	Rhizobiaceae (20%)	67	Undetermined Rhizobiaceae (8%)	137
<i>Silverstoneia flotator</i>	14	Proteobacteria (63%)	22	Alphaproteobacteria (32%)	64	Rhizobiales (18%)	123	Burkholderiaceae (9%)	236	Undetermined Rhizobiaceae and Sphingomonas (Both 4%)	813

Percentages represent the dominant bacterial phyla, class, order, family and genus defined as those with the highest proportion (%) of bacteria belonging to each taxon.

Table S5. Disease prevalence and infection load of amphibian species in the Panama Canal Watershed.

Species	Infected/Total	Prevalence (%)	Mean infection load (z.e.)
<i>Allobates talamancae</i>	7 / 93	7.53	22.5247
<i>Craugastor fitzingeri</i>	12 / 275	4.36	519.9769
<i>Engystomops pustulosus</i>	6 / 47	12.77	72.4146
<i>Lithobates warszewitschii</i>	3 / 5	60	172,464.7658
<i>Pristimantis gaigei</i>	2 / 4	50	11.1775
<i>Rhinella alata</i>	27 / 189	14.29	15.5113

Mean Bd infection loads are presented in zoospores equivalents (z.e.).

Chapter 4. Body condition, skin bacterial communities and disease status: Insights from the first release trial of the endangered *Limosa harlequin* frog, *Atelopus limosus*.

Abstract

Many endangered amphibian species survive in captive breeding facilities. To rebuild sustainable wild populations of these species, we will have to reintroduce offspring from these programs back into the wild, but there have been remarkably few attempts to do this. We used a release trial of captive-bred, highly endangered *Limosa harlequin* frogs, *Atelopus limosus*, which are highly susceptible to the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (Bd), to understand changes associated with the transition from captivity to the wild. Specifically, we assessed changes in the body condition, skin-associated bacterial communities and disease status following a soft-release to a site where *A. limosus* historically thrived prior to the arrival of Bd. Captive-reared frogs were housed individually in mesocosms at the field site and monitored for 28 days. Overall, the skin microbiome of captive-born frogs, based on assessment of 16S rRNA gene amplicons, changed following exposure to natural conditions; after only two weeks, frogs in mesocosms and wild conspecifics had similar skin bacterial communities. This suggests that the skin-associated microbiome of captive-born amphibians can be quickly restored. Body mass decreased in females and males after soft-release, swiftly approaching the mass of wild conspecifics. The overall Bd prevalence in wild stream-dwelling frogs was 13%-27%, and 15% of the *A. limosus* in mesocosms became infected with Bd, but we did not observe any mortality of infected animals or any visible disease signs. We conclude that mesocosms are a suitable tool for systematically and repeatedly observing amphibians during release trials, that microbiomes can be rapidly restored to wild-type after release, and that captive-reared *A. limosus* remain vulnerable to infection from the amphibian chytrid fungus in their natural habitat.

Introduction

Increasing environmental change from human activities has accelerated the loss of biodiversity (Ehrlich 1988; Vitousek *et al.*, 1997). As a result, *in situ* and *ex situ* conservation approaches, including captive breeding programs (CBP), have become a common practice to save endangered species for which threats cannot be mitigated in their natural habitat (Snyder *et al.*, 1995; Seddon *et al.*, 2007; IUCN 2013; Scheele *et al.*, 2014). However, the establishment of assurance colonies under captive conditions is challenging, largely due to the maintenance and management of genetic diversity of poorly known species (Frankman 2008). Additionally, a number of aspects associated with captive environments can facilitate adaptation to captivity with potential negative effects on individual fitness (Frankman 2008), which could worsen over successive generations in captivity (Wallace 2000; Hakansson and Jensen 2008; Williams and Hoffman 2009). For example, captive salmon can experience a reduction or a complete loss of antipredator behavior (Roberts *et al.*, 2011), and as observed in more than 40 different species, reproductive success is often diminished in captivity (Farquharson *et al.*, 2018). Overall, chances of survival after release depend on whether captive-born individuals can adapt to the natural environment, and whether they are able to withstand emergent factors, such as infectious disease (Mathews *et al.*, 2005). Despite numerous challenges, CBPs are integral for the recovery of endangered species, as 15% of threatened vertebrate species are currently kept in captive

facilities around the world (Conway 2010). Ultimately, the reintroduction or the movement of animals from captivity to their natural habitat is among the main goals of CBPs. However, attempted releases of captive-reared species have had mixed results (Griffiths and Pavajeau 2008), while others remain unpublished, and release methods for many species are determined through a process of trial and error.

The rate of release success of captive-born vertebrates can increase with an appropriate assessment focused on conservation outcomes at the population, species, and ecosystem level (Beck *et al.*, 1994, IUCN 2013). However, for species fighting infectious diseases, success rates are still poor (Griffith *et al.*, 1989; Griffiths and Pavajeau 2008). Amphibians are facing disease-related population declines and extinctions worldwide. The emergence of chytridiomycosis, an infectious disease caused by the lethal fungal pathogens, *Batrachochytrium dendrobatidis* (Bd) (Longcore, Pessier & Nichols 1999) and *B. salamandrivorans* (Bsal) (Martel *et al.*, 2013), is recognized as a major threat to amphibians (Harding *et al.*, 2015, Lips 2016). There has been substantial research focused on these chytrid fungi, leading to the development of adaptive management frameworks that include interventions to prevent further extinctions (Scheele *et al.*, 2014; Langwig *et al.*, 2015). Likewise, effort has focused on reducing Bd in the environment (Garner *et al.* 2016) and reducing infection in amphibian hosts (McMahon *et al.*, 2014; Walke and Belden 2016), while also improving the capacity of populations to persist despite increased disease-related mortality (Scheele *et al.*, 2014). In areas where Bd is enzootic, additional pressures, such as climate change and habitat degradation, can compromise populations already struggling to persist (Langwig *et al.*, 2015). Since threats faced by amphibians are complex and often synergistic, mitigating threats in the wild remains a major challenge for reintroduction programs.

Besides chytrid infections, the survival of captive-bred amphibians after release could be affected by factors associated with captivity, including changes in behavior, body condition, and skin-associated microbial communities. While body condition is used as a metric to assess overall health (Stevenson and Woods 2006), host-associated microbial communities can provide protection against pathogen infections (Redford *et al.*, 2012; Bletz *et al.*, 2013; Walke and Belden 2016). In addition, comparisons of body condition and microbial communities between captive and wild conspecifics might be used as indicators of post-release health and survival probability. For amphibian host-associated microbes, host species identity and environmental conditions and substrates are the main drivers for variation of community composition (Loudon *et al.*, 2013; Belden *et al.*, 2015; Kueneman *et al.*, 2019). Differences in the skin-associated microbial communities have been broadly detected between captive and wild individuals of multiple species (McKenzie *et al.*, 2017), including amphibians (Becker *et al.*, 2014; Xie *et al.*, 2016; Bletz *et al.*, 2017; Flechas *et al.*, 2017). However, it is unknown whether differences between captive and wild microbiomes will be maintained after captive-reared individuals are released to the natural habitat. Likewise, it is not clear if these shifts can impact the protective function of host-associated microbial community.

Harlequin frogs (genus *Atelopus*) have experienced dramatic population declines and local extinctions throughout their distributional range in Central and South America (La Marca *et al.*, 2005). Most *Atelopus* species are endemic, with relatively small distribution ranges, and are highly susceptible to Bd infection, which has prompted their prioritization in CBPs (Gratwicke *et*

al., 2015). Harlequin frogs also appear to be important in Bd dynamics in Neotropical amphibian communities. For instance, DiRenzo *et al.*, (2014) suggested that individuals of *Atelopus* spp. could act as "acute supershedders", producing exceptionally high numbers of Bd zoospores prior to death, and potentially amplifying disease risk for other amphibians in the community; however, a subsequent experimental study failed to prove this "supershedder" hypothesis (DiRenzo *et al.*, 2018). Additionally, there are marked differences in skin-associated bacterial communities between captive and wild *Atelopus* species (Becker *et al.*, 2014; Flechas *et al.*, 2017). Despite well documented changes in the captive microbiome of harlequin frogs, we are unsure of how these changes may affect the health of animals in a reintroduction scenario.

We assessed changes in body condition, skin bacterial community composition, and Bd infection status in captive-born *A. limosus* following soft-release in field enclosures (mesocosms) to a site where the species once thrived prior to Bd arrival. We compared these data with surveys of persisting wild *A. limosus* to understand the amphibian-Bd-microbiome interactions during the transition from captive to wild conditions.

Methods

Ethics statement

This study was conducted under ethics approval of the Institutional Animal Care and Use Committees of Virginia Tech (11-105-BIOL) and the Smithsonian Tropical Research Institute (2013-0401-2016-A3). As individuals were reared and housed at the Panama Amphibian Rescue and Conservation Project prior to soft-release, their implementation and research committee reviewed and established that the proposal met all animal welfare requirements. Fieldwork was completed under permit SE/A-47-12, granted by the Panama Ministry of Environment (MiAmbiente).

In-situ rearing conditions

Wild *A. limosus* founders were collected at the Upper Mamoni Valley Watershed (hereafter, Mamoni) and were brought into the CBP in 2011-2012. All rescued individuals were treated for chytridiomycosis and maintained in quarantine until they tested negative for Bd. Captive populations were separated by sex and housed in groups inside tanks at 22-24°C, with 12 hr lighting cycles including UVB, and automated misting systems. Frogs were bred in reproduction tanks (76 cm L x 33 cm W x 30 cm H) with rocks and coarse gravel submerged in 10-15 cm water that occupied 75% of the tank's substrate. An external pump (MJ-900 Cobalt® 870LPH and 120V) was used to replicate stream conditions and water flow at natural breeding sites. Once eggs were laid, adults were removed and the water was raised 10 cm. After hatching, tadpoles were fed daily with rehydrated algae until metamorphosis. Postmetamorphs were placed in small plastic tanks lined with wet paper towels and were fed springtails (*Collembola*) and fruit flies (*Drosophila melanogaster*). As individuals grew, they were kept in larger plastic containers until relocated to permanent glass tanks. Sub-adult frogs were separated by sex and housed in small groups of 8-10 individuals per tank (Cikanek *et al.*, 2014). All sub-adult frogs were fed three times a week, mainly with vitamin gut-loaded fruit flies and crickets. Frogs used in our study (N

= 30, 15 females and 15 males) were F1 individuals belonging to the same clutch and were eleven months old. Sexually-dimorphic traits allowed accurate sexing of individuals before the trials. Prior to release, individual digital photographs were taken, and their ventral markings were used for easy identification.

Field conditions and experimental design

Mamoní includes several streams and rivers flowing south into the Pacific Ocean in the Chepo district, Panama province. Two sections of a stream (~500 m total) with suitable *A. limosus* habitat were selected for our trial.

Mesocosms (76 cm X 76 cm X 46 cm) were built from a non-toxic, pliable, yet semi-rigid polyethylene mesh (0.6 mm) used in commercial aquaculture (Fig. 1). We placed rocks, palm leaves, branches and leaf litter from the site in each mesocosm to provide cover and habitat structure that would encourage natural behavior and movement. In April, 2017, we placed 30 captive-born *A. limosus* (15 females, 15 males) in individual mesocosms distributed along the stream edge. No supplemental feeding was done, as the mesh allowed small arthropod prey to enter, while reducing mortality risk from predators. Frogs were monitored for 28 days after placement in the mesocosms.

Body condition estimates

We recorded body mass (g), snout-vent length (SVL, mm) and signs of infection or injuries on a weekly basis after release (days 0, 1, 7, 14, 21, 27). We prepared an expected length-weight curve based on wild frogs surveyed in 2011-2012 during the initial rescue efforts using a regression of log body mass vs log SVL for male and females, respectively. With the wild regression equations, we used the SVL of each captive-born individual at all sampling days (0, 1, 7, 14, 21, 27) to predict the body mass of an individual of the same SVL in 2011-2012. The difference between the actual and the predicted body mass represented the residual body condition index (residual BCI, Denoël *et al.*, 2002) of each captive-born frog. We visualized changes in body condition by plotting captive-born frogs before (day 0) and after (day 27) release against the line of the fitted regression model of wild 2011-2012 *A. limosus*. Individuals above the regression line were heavier than 2011-2012 individuals of the same body size, whereas individuals below the regression line lighter.

Sampling for bacterial community and disease status

We swabbed the skin of mesocosm-housed individuals and the wild *A. limosus* we encountered (N = 8) with a sterile rayon swab to collect a sample of the bacterial community and to assess Bd infection status, as described by Belden *et al.*, (2015). Swabs of captive-born frogs were collected while still in captivity (day 0) and thereafter on a weekly basis (days 0, 1, 7, 14, 21, 27). Wild individuals were swabbed when encountered, photographed dorsally and ventrally for individual identification purposes and released immediately after sampling. At two time points (Day 2 and Day 30), we surveyed and swabbed the amphibian community at the study site by conducting nocturnal surveys of the two 500m stream transects and swabbing all amphibians encountered to assess the incidence of Bd at the community level (N= 75 at day 2 and 51 at day

30). Lastly, we sampled the environmental bacterial communities that the frogs were in contact with in the mesocosms by swabbing rocks, soil, branches and leaf litter in a subset of 12 mesocosms on day 28 following methods in Rebollar *et al.*, (2016). Amphibian and environmental swabs were placed in 1.5 ml sterile microcentrifuge tubes and were kept frozen at the field station until they could be moved to a -80°C freezer.

DNA extraction and sequence processing for skin bacterial communities

Extraction of DNA from captive-born *A. limosus* swabs for bacterial community composition and Bd detection and quantification was done with the Qiagen DNeasy blood and tissue kit (Valencia, CA) following the manufacturer's protocol with a lysozyme pre-treatment. 16S rRNA gene amplicon sequencing of the bacterial V4 region was used to assess bacterial community structure (Caporaso *et al.*, 2012). Sample preparation for sequencing followed Estrada *et al.*, (2019).

Samples for this study were part of two Illumina MiSeq runs (single end 250bp) at Harvard's Dana Farber Cancer Institute genomics facility. Raw forward read and barcode files were imported into QIIME2 (Bolyen *et al.*, 2018) and demultiplexed. Samples from each run were quality-filtered, denoised, and chimera-checked with DADA2 (Callahan *et al.*, 2016), with all reads less than 251 bp removed, and any reads with Phred quality scores < 20 removed. Following that, sequence data from the two runs were merged into a single feature table, as were the representative sequences for each run. With these merged files, an OTU table was created with all the samples based on 97% *de novo* clustering using vsearch (Rognes *et al.*, 2016). We further filtered out any OTUs that had fewer than 0.01% of the total reads (Bokulich *et al.*, 2013). Taxonomy was assigned to the remaining OTUs with a machine-learning based method (sklearn, Pedregosa *et al.*, 2011) using the pre-trained SILVA database classifier (Quast *et al.*, 2013, Bokulich *et al.*, 2018). OTUs assigned as chloroplast or mitochondria were then filtered out, leaving only bacterial OTUs. We examined a rarefaction plot to determine the appropriate rarefaction depth for the dataset, and chose to rarefy at 10,000 reads/sample. This resulted in the loss of 5 samples from the initial dataset that had lower read counts. The final OTU table contained 513 OTUs across a total of 120 amphibian and environmental samples (101 from captive-born *A. limosus*, 8 from wild frogs and 12 from environmental bacterial communities).

Batrachochytrium dendrobatidis detection and quantification

We used quantitative TaqMan PCR (qPCR; Boyle *et al.*, 2004, Hyatt *et al.*, 2007) to quantify Bd prevalence and infection load in both the *Atelopus* and the amphibian community samples. We extracted the DNA of the skin swabs from the amphibian community by adding to each sample 50 µl of PrepMan Ultra with 30-40 mg of 0.5 mm zirconium/silica beads, which were then homogenized in a Mini-Beadbeater-96 (Biospec Products) for 1 min, followed by a centrifugation at 13,000 x g for 30 s. We repeated homogenization and centrifugation, boiled the samples for 10 min, centrifuged them at 13,000 x g for 3 min, and collected all supernatant (20-40 µl). We stored these sample extracts at -20°C for later use.

For the qPCR assays, we used 20 µl reactions (Kriger *et al.*, 2006) with a Roche LightCycler 96 System. Each reaction contained 5 µl of DNA template and 15 µl of a master mix containing 10

μl of Roche FastStart Essential DNA Probes Master, 0.25 μl of Roche LightCycler® Uracil-DNA Glycosylase to avoid PCR carry over contamination, 0.8 μl of 18μM ITS1-3 Chytr and 0.8 μl of 18 μM 5.8S Chytr primer solutions, 1 μl of 5 μM ChytrMGB2 probe solution, 2 μl of 10x Exo IPC mix and 0.4 μl of 50x Exo IPC DNA TaqMan® Exogenous Internal Positive Control Reagents (Applied Biosystems No. 4308323). The internal control reagent was added to the sample mix to avoid false negatives due to inhibition of the reaction (Hyatt *et al.*, 2007). Extract dilutions without DNA were run to detect initial low infection loads and discard false positives. Samples were run for 2 min at 50°C and 10 min at 95°C, followed by 50 cycles of 15s at 95°C and 1 min at 60°C. We tested the samples in triplicate, along with five negative and two Bd positive controls on every plate (Kriger *et al.*, 2006a, b). Samples with two or three positive reactions were considered positive for Bd. Samples with inconsistent results were repeated up to three times, and considered negative when more than 50% of wells gave negative results. We quantified the infection load of Bd positive samples based on the number of zoospore equivalents (Boyle *et al.*, 2004). We used the JEL 423 Bd strain to make the standards, and the serial dilution of the standards consisted of 1×10^4 to 1×10^{-1} zoospore equivalents. We averaged the quantified values, and multiplied them by 20 or 100 to compensate for the extraction and dilution of the samples using the Qiagen DNeasy® and Prepman Ultra methods, respectively.

Statistical Analysis

Body condition estimates

All body condition analyses were separated by sex, as growth trajectories for males and females of *A. limosus* are different; adult females are larger and heavier than males (See Table 1 for biometrics of females and males under different conditions). Additionally, analysis excluded individuals that died during the field trial to standardize sample sizes for females (N = 14) and males (N = 12) throughout sampling time points. To evaluate changes in body mass and SVL in captive-born frogs, we compared biometrics before (day 0) and after (day 27) soft-release into mesocosms using a Wilcoxon rank sum test for paired data. To determine differences in residual BCI between captive-born frogs and wild counterparts, we used a Kruskal-Wallis test. For significant results, we then conducted multiple comparisons using the Dunn test, and p-values were adjusted with the Benjamini-Hochberg method.

Change in skin bacterial communities after release

We assessed skin bacterial communities on the skin of *A. limosus* before and after soft-release into the mesocosms and compared them with that on wild individuals from the extant population. We computed alpha diversity metrics with QIIME2 (Bolyen *et al.*, 2018) using OTU richness and the Shannon index (considers both richness and evenness). Generalized linear mixed models (GLMMs) were fitted to OTU richness and the Shannon index using negative binomial and Gamma error distributions, respectively (glm.nb function, package lme4, Bates *et al.*, 2015). Correlations between all predictor variables (collection day, sex and SVL) were examined to avoid collinearity. Considering that mass and SVL were strongly correlated with collection day and to each other ($P < 0.01$), collection day and sex ($R^2 = 0.22$, $P = 0.10$) were ultimately used as fixed effects in the selected model, and individual frog IDs were assigned as a random effect. We used a likelihood ratio test to estimate P-values of fixed factors. For significant GLMMs, post-

hoc Tukey tests for multiple comparisons were conducted using function `glht`, package `multcomp` (Hothorn *et al.*, 2008).

We used permutational multivariate analysis of variance (PERMANOVA) to analyze variation in bacterial community composition before and after soft-release into mesocosms, and to compare mesocosm individuals to wild frogs. To visualize the overall change in community structure throughout the soft-release, we used principal coordinate analysis (PCoA) on Bray-Curtis and Jaccard distance matrices, which included all the samples from frogs in captivity (day 0), mesocosms (day 27), and wild conspecifics. For a subset of 12 samples (i.e., those from mesocosms from which the environmental samples were taken), we tracked the changes on the skin bacterial communities through time (days 0, 1, 7, 14, 21 and 27) and examined the variation between these communities and environmental bacterial communities in the mesocosms (at day 28). We also identified key bacterial taxa responsible for observed differences between frogs in captivity (day 0), frogs in mesocosms (day 27) and wild individuals, and conducted a linear discriminant analysis of effect size (LeFSe, Segata *et al.*, 2011).

Bd disease infection status

We quantified Bd infection in a total of 262 frogs: 136 *A. limosus* (including mesocosm individuals) and 126 from the amphibian community. Only four captive-born *A. limosus* tested positive for Bd infection after placement in mesocosms; hence, trends we see in Bd results should be interpreted with caution. For Bd positive *A. limosus* samples (N = 12 total across all sampling days), we tested for differences in log transformed-Bd infection intensity between individuals collected at different days in the mesocosms and wild individuals from the extant population (N = 8). We also compared log transformed-Bd infection loads among species from the amphibian community. For all comparisons of Bd infection intensity, we used a Kruskal-Wallis test for non-normal distributions.

Results

Captive-born *A. limosus* lose body condition in mesocosms, but mortality is low

All captive-born frogs lost body mass after spending 27 days in the mesocosms. Captive-born females lost 13% of total body mass (Wilcox test, $V = 105$ $P = 0.0001$, Fig. 2A). Similarly, males lost 4% of total body mass, but differences between captive and wild individuals in mass were not statistically significant (Wilcox test, $V = 54$ $P = 0.2261$, Fig. 2A). The body size (SVL) of captive-born frogs did not change from captivity to day 27 in mesocosms (Wilcox test for females $V = 44$ $P = 0.6257$ and males $V = 30$ $P = 0.5186$, Fig. 2B). Of the 30 frogs, 4 (13%) died during the mesocosm trial; one was potentially lost due to predation by army ants; the three other deceased animals swabbed negative for Bd and the cause of death was unknown.

Residual BCI of captive-born frogs decreased after spending 27 days in the mesocosms (Kruskal-Wallis for females $\text{Chisq} = 19.124$, $\text{df} = 6$, $P = 0.0039$, Fig. 3A and males $\text{Chisq} = 95.554$, $\text{df} = 6$, $P < 0.0001$, Fig. 3B). Multiple comparisons revealed different body condition dynamics between females and males (see Dunn test results on Table S1). In general, all females in captivity were heavier than wild conspecifics. On day 27, some females had lower residual

BCI, representing lower body mass than wild females of the same SVL (Fig. 4A). All captive-born males maintained higher residual BCI throughout the field trial, suggesting that they remained heavier than wild conspecifics of the same body size ($P > 0.0001$, Fig. 4B).

The skin bacterial community on captive-born *A. limosus* reverts to wild conditions. Our dataset consisted of 513 unique bacterial OTUs in amphibian and environmental samples, which included 136 bacterial families from 13 phyla. OTUs associated with amphibian skin predominantly belonged to the phyla Proteobacteria, Bacteroidetes, Actinobacteria, Firmicutes and Verrucomicrobia. The 35 bacterial families with the highest relative abundance ($> 1\%$) represented 91% of the bacterial community associated with frogs in captivity, in the wild and in environmental samples (Fig. S1). Changes in bacterial community composition on captive-born *A. limosus* after placement into mesocosms were mainly driven by variation in the bacterial families with the highest relative abundances: Sphingobacteriaceae (day 0: 47%/day 27: 17%), Rhizobiaceae (12%/10%), Moraxellaceae (4%/0.8%), Staphylococcaceae (0.1%/8%) and Pseudomonadaceae (1%/6%). Wild frogs were enriched by Rhizobiales (13%), Sphingobacteriaceae (12%), Weeksellaceae (10%), Intrasporangiaceae (9%) and an unknown family in the order Micrococcales (7%). Environmental samples were not dominated by a single family; Sphingomonadaceae and Rhizobiales (both 12%), along with Beijerinckiaceae and Chitinophagaceae (both 4%) were the most abundant.

The bacterial diversity, of captive-born frogs while still in captivity (day 0), as indicated by OTU richness, was about 79% of that observed in wild frogs, but community diversity and evenness on the skin of all captive-born *A. limosus* increased after soft-release into mesocosms and became similar to those on wild conspecifics (OTU richness $\text{Chisq} = 17.668$; Fig. 5A and Shannon index $\text{Chisq} = 19.254$, $P < 0.001$ Fig. 5B). Sex was a significant predictor of OTU richness ($\text{Chisq} 6.576$, $P = 0.037$); males had lower diversity of skin bacteria than females (z value = -2.54 $se = 0.064$, $P = 0.011$). No differences between sexes were observed for Shannon index ($\text{Chisq} = 3.606$, $P = 0.165$). Analysis of time series data showed an increase in bacterial diversity through time immediately after placement into mesocosms, followed by no detectable differences from day 14 to day 27. Additionally, alpha diversity metrics of environmental samples were distinct from the bacterial communities on captive-born and wild *A. limosus* (OTU richness $\text{Chisq} = 38.622$, $P < 0.001$; Fig. S2.A and Shannon index = 13.746 , $P = 0.008$; Fig. S2.B; Tukey test multiple comparisons in Tables S2 and S3).

There was a significant shift in bacterial community structure of captive-born *A. limosus* after soft-release into mesocosms, ultimately overlapping the community found on wild conspecifics (Bray-Curtis Pseudo-F = 6.988 $R^2 = 0.376$, $P < 0.001$, Fig. 5C; Jaccard Pseudo-F = 8.699 $R^2 = 0.441$, $P < 0.001$ Fig. 5D). Time series analysis showed that sampling day had a significant effect on community structure (Bray-Curtis Pseudo-F = 6.988 $R^2 = 0.376$, $P < 0.001$; Jaccard Pseudo-F = 8.699 $R^2 = 0.441$, $P < 0.001$). Lastly, bacterial community structure of the environmental samples was significantly different than that of skin bacterial communities of frogs in mesocosms at all sampling days (Bray-Curtis Pseudo-F = 8.335 , $R^2 = 0.438$, $P < 0.001$; Fig. S2.C and Jaccard Pseudo-F = 5.374 $R^2 = 0.334$, $P < 0.001$; Fig. S2.D).

LefSE analysis identified 16 bacterial taxa, with the highest linear discriminant analysis (LDA) scores associated with *A. limosus* in captive conditions, 25 taxa associated with *A. limosus* in the

mesocosms, and 26 taxa associated with wild *A. limosus* from the extant population (Fig. 6). See Table S4 for a full list of OTUs identified by LefSE. Overall, some key OTUs were almost exclusively found under captive conditions at day 0 sampling (e.g., genus *Rodococcus*, family Nocardiaceae, phylum Actinobacteria) or were significantly more abundant (e.g., genus *Pedobacter*, family Sphingobacteriaceae, phylum Bacteroidia). Other OTUs were detected or increased their relative abundance only after frogs were soft-released into mesocosms (e.g., genera *Staphylococcus* and *Jeotgalicoccus*, family Staphylococcaceae, phylum Firmicutes). Fewer OTUs had higher relative abundances solely on the skin of wild frogs (e.g., order Micrococcales, phylum Actinobacteria). See figure S3 for examples of relative abundance of OTUs on frogs in captivity, in mesocosms and in the wild.

Bd prevalence and infection loads of reintroduced *A. limosus* were within the range of those of wild conspecifics and in the rest of the amphibian community.

All *A. limosus* tested negative for Bd infection while in captivity. After one month of exposure to natural conditions in mesocosms, 15% (4/26) of captive-born individuals were Bd positive, and the mean infection intensity of positive individuals by day 27 was 5,838 zoospore equivalents (range: 5 - 528,500 z.e.) (Table 2). Infection was first detected on two individuals on day 14, and both prevalence and infection intensity increased over time after placement into the mesocosms (Table 2). The overall prevalence of Bd and intensity in captive-reared frogs was not markedly different from that of the wild riparian frog community (13-27% prevalence with a range of 12-491,600 z.e., Table 3)

Discussion

Our ability to resample individuals across time, an advantage of the soft-release design, gave us a unique opportunity to determine changes in body condition, skin-associated bacterial communities and disease status on the individuals.

Body mass loss after soft-release

Captive-born *A. limosus* started losing body mass soon after placement into mesocosms. Both increases and decreases in body mass have been observed after amphibian releases (Brannelly *et al.*, 2015; Zhang *et al.*, 2016) without a significant effect on survival. In our study, individual survival was high (87%), and we did not need to employ supplemental feeding because body condition did not drop below the baseline wild body condition index. The loss of body mass was greater in females than males, but as these were not sexually mature animals it is unlikely to have been related to female reproductive condition. We did not conduct diet studies, but observed fecal pellets and potential prey items (e.g. ants, termites, mites, springtails) inside the mesocosms suggesting that natural feeding was occurring.

By slightly modifying the residual BCI to allow between population comparisons, we were able to assess body mass of captive-born frogs before, during and after soft-release relative to the condition of wild conspecifics collected at the site in 2011-2012. We found that captive-born males and females had BCIs above the predicted mass of wild frogs, suggesting that they were heavier than wild conspecifics of the same size. There is little evidence that suggests a link

between higher body mass (better BCI) in captivity and survival after release (McCarthy *et al.*, 2012). However, in amphibians, BCI can also provide evidence for intrinsic and extrinsic factors that can impact reintroduction outcomes (Bancilla *et al.*, 2010). After placement into mesocosms, body condition of females fluctuated, while that of males remained stable. Studies in rodents have found that residual BCI limits the ability to predict body mass based on sex (Labocha *et al.*, 2013). Since only captive males had reached adult size, we suggest that the differences in BCI dynamics between the sexes potentially resulted from the small sample size, differing average adult size, and reproductive status of wild females. Therefore, values for mass were predicted from a regression equation based exclusively on size and no other factors (i.e. age, gravidity) that could be equally or more important for estimating body condition of females. Ultimately, if the release habitat is suitable and the period of acclimatization is overcome, body condition should not decline in either sex (Bartolero and Oro 2009). Importantly, despite observed BCI decline, most captive-born frogs approached the condition of wild individuals. As wild individuals are adapted to local habitat conditions, we suggest that a criterion for natural or “ideal” body condition after release should approximate those of wild conspecifics. Overall, these results highlight the need to monitor body condition to inform possible animal husbandry adjustments prior to release, and to determine how body condition in captivity relates to survival post-release and thus overall success of reintroductions efforts.

Change in skin-bacterial communities after soft-release

Changes in skin-associated bacterial communities were observed soon after placing captive-born *A. limosus* into mesocosms, and by the end of the trial communities were indistinguishable from those of wild conspecifics from the extant population. Our results are consistent with those that found lower bacterial community diversity under captive conditions (Loudon *et al.*, 2014, Bataille *et al.*, 2015, Sabino-Pinto *et al.*, 2016, Kueneman *et al.*, 2016, Flechas *et al.*, 2017). Captive *A. limosus* had 78% of the OTU richness of extant wild frogs, similar to the pattern observed in a prior study of captive-bred Panamanian golden frog, *A. zeteki*, which after 8 years in ex-situ CBPs, shared 70% of their bacterial taxa with wild frogs (Becker *et al.*, 2014). Less diverse bacterial communities in captivity are attributed to artificial and sterile environments, less diverse diets, and the use of antibiotics and antifungals (reviewed by West *et al.*, 2019).

The effects of captivity on host-associated microbial communities have been described for a wide range of vertebrate species (McKenzie *et al.*, 2017), yet bacterial community shift after release has been less explored. Here, we provide evidence that there is a rapid shift of the skin microbiome from a captive-type to a wild-type after placing captive-bred frogs in the natural environment. Several conservation interventions have focused on manipulating the skin microbiome in laboratory trials to prevent Bd infection (e.g., Becker *et al.*, 2011; 2015). While these interventions have been unsuccessful to date, our observations of a rapid shift in the skin microbiome pose additional challenges that will need to be considered if probiotic manipulations are to be successful. *Atelopus* species are highly susceptible to Bd infections (Woodhams *et al.*, 2006; Richards-Zawacki 2010; Becker *et al.*, 2012; McMahon *et al.*, 2014), but recent evidence suggests that some persisting populations of *Atelopus* have evolved anti-Bd skin secretions that have allowed survivors to persist, and perhaps even recover from the disease (Voyles *et al.*, 2015). As we discover more about the importance and potential fitness consequences for animals related to the microbiome (Yong 2016; Bahrndorff *et al.*, 2016), concern has been raised that the

potential loss of wild microbiomes in captivity may make it challenging or impossible to ever fully recover from a prolonged period in captive conditions (Redford *et al.*, 2012; Kueneman *et al.*, 2016; Passos *et al.*, 2018; Trevelline *et al.*, 2019; West *et al.*, 2019). While further studies about the restoration of microbiome function would be required, the rapid recovery of a wild-type microbiome somewhat alleviates these concerns.

Our findings are consistent with previous research that has identified differences between captive and wild conditions in the main bacterial taxa associated with the amphibian skin (Becker *et al.*, 2014; Flechas *et al.*, 2017; Hernandez-Gomez *et al.*, 2018). However, until now, it was unknown if the effects of captivity on amphibian skin microbiomes were permanent (Holden *et al.*, 2014). Our study provides insight into the long-term effects of exposure to captive conditions on amphibian skin bacteria. For example, we found that captive-microbiomes were strongly dominated by Sphingobacteriaceae (0.47) and that after soft-release into mesocosms, there was a significant reduction in their relative abundance (0.17), yet it remained the most abundant taxa in the community. The steep decline in relative abundance in this group may be primarily responsible for the observed changes in bacterial community diversity and structure after release. By the end of the trial, captive-born frogs had similar proportions of their bacterial community dominated by Sphingobacteriaceae (0.17 and 0.12). In addition, *Pedobacter* sp. (Sphingobacteriaceae) was identified as a key OTU associated with captive conditions on *Atelopus* skin. Becker *et al.*, (2014) suggested that high relative abundance of *Pedobacter* could be a good predictor of low evenness in captive-microbiomes; thus, its reduction in relative abundance might explain, in part, the restoration of community evenness after soft-release.

Additional OTUs, such as *Staphylococcus* sp. and *Jeotgalicoccus*, only emerged or increased in relative abundance after frogs were exposed to their natural habitat in mesocosms. While common members of the Staphylococcaceae family have been intensely investigated due to their association with severe infections in mammals and antibiotic resistance in humans (Ludwig *et al.*, 2008), they are also widely distributed in the environment (Schwaiger *et al.*, 2010). In amphibians, members of this group have been identified, for example, as core members in the skin of *Plethodon cinereus* (Loudon *et al.*, 2014) and *Eleutherodactylus coqui* (Longo *et al.*, 2015). Although a great number of species within the Staphylococcaceae family can have direct effects on host health, there is no evidence linking them with amphibian skin pathogen infections.

Within the environmental samples, the family Sphingomonadaceae exhibited substantially higher relative abundance than in any amphibian skin sample. In addition, families that were dominant on frog skin (Sphingobacteriaceae, Rhizobiaceae, Chitinophagaceae) were found in lower abundance in the environment. These results reiterate previous findings suggesting that amphibians select for rare bacteria in the environment (Walke *et al.*, 2014; Rebollar *et al.*, 2016; Bletz *et al.*, 2017). Moreover, since differences were also observed between environmental samples and wild *A. limosus* from the extant population, the results also highlight the selective pressure posed by the host (i.e., habitat filtering) on their associated bacterial communities, regardless of host origin (captive vs. wild). Apart from the anti-fungal properties of amphibian skin microbes (Woodhams *et al.*, 2015), the influence of symbiotic communities on amphibian host health remains largely unknown, and would be needed to further interpret these data.

Bd infection after soft-release

Captive breeding facilities keep pathogen-free populations with strict biosecurity protocols to reduce disease risk (Murray *et al.*, 2011; Pessier and Mendelson 2017). While released animals were all Bd-negative at the beginning of the trial, we detected the first Bd positive individuals after just 2 weeks in the mesocosms. In total, four captive-born frogs (15% prevalence) were infected with Bd over the 27 days. This infection timeframe is consistent with laboratory Bd-infection trials of *Atelopus* that have detected Bd positive samples one week after experimental inoculation, with a rapid increase in infection intensity over time (DiRenzo *et al.*, 2014; Ellison *et al.*, 2014; DiRenzo *et al.*, 2018). Frogs were maintained somewhat isolated in the mesocosms (small frogs could pass through the mesh), and were set away from flowing water, yet still some were infected. Though our trial spanned a short period of time, our surveys of wild frogs indicate that Bd is present in the environment, and capable of infecting susceptible species and naïve individuals (captive-born) soon after exposure to their natural habitat. Concern has been raised that releases of highly susceptible species into the environment may increase Bd-prevalence in the frog community persisting in the wild (DiRenzo *et al.*, 2014). A recent follow up experimental test of this hypothesis found that increases in *Atelopus*' infection were mainly driven by pathogen replication within an individual, and not from amplification of Bd zoospores coming from an environmental source (DiRenzo *et al.*, 2018).

In the wild community, Bd prevalence estimates ranged from 9-32% over the course of our trial. Infection intensity during that time varied across amphibian species at the study site. Specifically, we found higher Bd infection intensity in *Colostethus panamansis*, *Incilius coniferus* and *Pristimantis cruentus*; some of these species have suffered population declines at higher elevations (Lips *et al.*, 2006). Within this context, the co-occurrence of an *Atelopus* species with other members of the community harboring a relatively high Bd prevalence has been found previously, including in Panama. For instance, at mid-elevations (~500 m) in Panama, Perez *et al.*, (2014) detected high Bd prevalence on *C. panamansis* (24%), *R. haemitticus* (42%), *S. flotator* (33%) and *Smilisca spp.* (100%) at a site where *Atelopus varius* still persists. Overall, despite our inability to detect changes in disease status, before and after the release trial, in the rest of the amphibian community in Mamoni, our results suggest that amphibians in this site are surviving with Bd in an enzootic infection stage, and that some species are more susceptible to infections than others. Understanding disease risk before, during and after reintroductions is critical to achieve long-term conservation goals not only for *Atelopus* species, but also for other declining Neotropical amphibians. While we have a limited ability to make strong inferences from a handful of infected soft-released individuals and their skin-associated bacterial communities or body condition, we would like to encourage research evaluating links between these factors in natural settings to better inform future reintroduction efforts.

Conclusion

Amphibian reintroductions of species that disappeared because of the amphibian chytrid fungus cannot be expected to succeed until the original threat has been mitigated through elimination of the pathogen, or more resilient animals are available for release (Muths & McCallum 2016).

Many Neotropical amphibian reintroduction efforts are at a high risk of failure, mainly because Bd is now endemic in many regions (Garner *et al.*, 2016), and virulence has not attenuated (Voyles *et al.*, 2018). Additional challenges, such as climate change, pollution, and habitat degradation place additional pressures on these populations (Langwig *et al.*, 2015). However, some species that were thought to have experienced local extinctions are rebounding (Gonzalez-Maya *et al.*, 2013; Perez *et al.*, 2014), and new adaptive management approaches suggest conservation research involving the use of surplus animals from CBPs (Lewis *et al.*, 2019). The goal of this release trial was not to restore wild populations of *Atelopus limosus*, but to better understand some of the challenges faced by frogs as they transition from captivity back into the wild for adaptive management purposes.

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Tables and figures

Figure 1. Mesocosm along the stream at Mamoni.

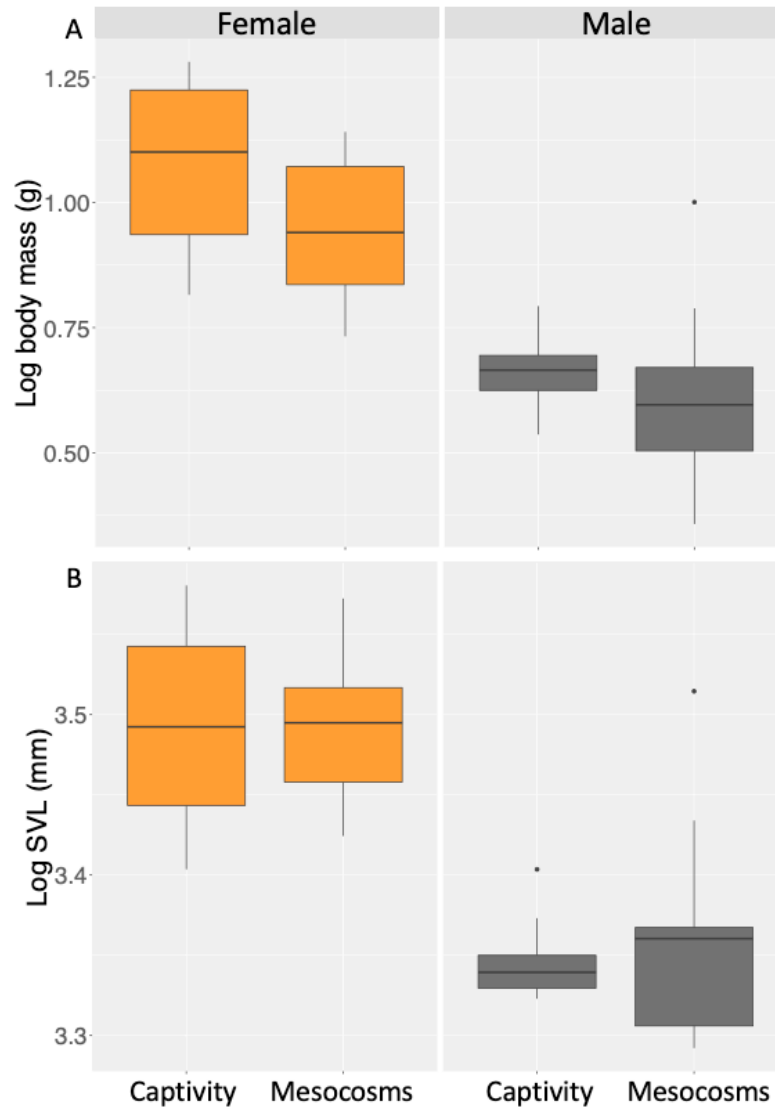


Table 1. Biometrics of captive-born and wild (2011-2012) *A. limosus* by sex and origin.

	FEMALES			MALES			Total N
	Mass mean, sd (g)	SVL mean, sd (mm)	N	Mass mean, sd (g)	SVL mean, sd (mm)	N	
Captivity	2.96, 0.47	32.73, 1.87	15	1.93, 0.15	28.31, 0.75	15	30
Mescosoms	2.58, 0.37	32.84, 1.54	14	1.86, 0.34	28.82, 1.89	12	26
Wild	2.83, 0.90	35.15, 6.07	10	1.19, 0.11	27.44, 1.04	53	63

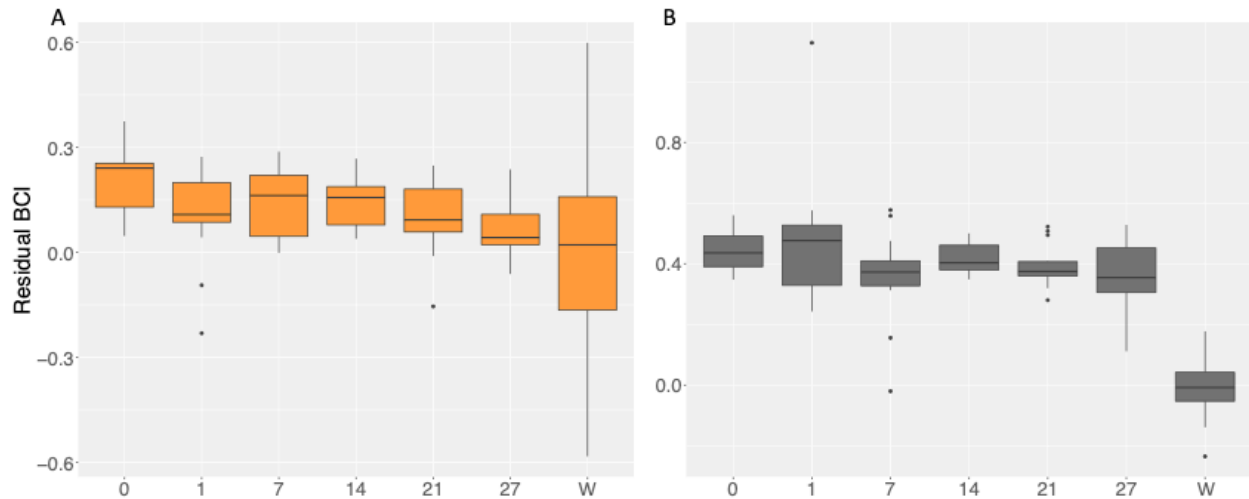
* Based on data from captive-born *A. limosus* in captivity (day 0), in mesocosms (day 27) and from wild individuals sampled in 2011-2012 during a previous study at Mamoni.

Figure 2: Body mass and SVL of captive-born *Atelopus limosus* before and after soft-release



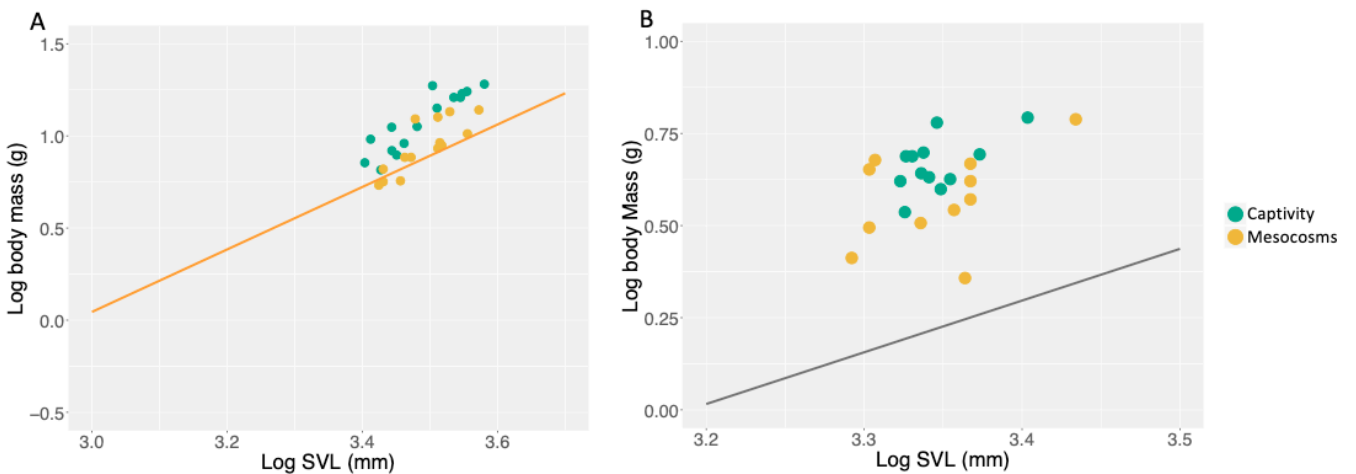
Comparisons of body mass (g) (A) and body size (SVL, mm) (B) of females and males between captivity (day 0) and after placement in to mesocosms (day 27).

Figure 3. Change in residual body condition index through time



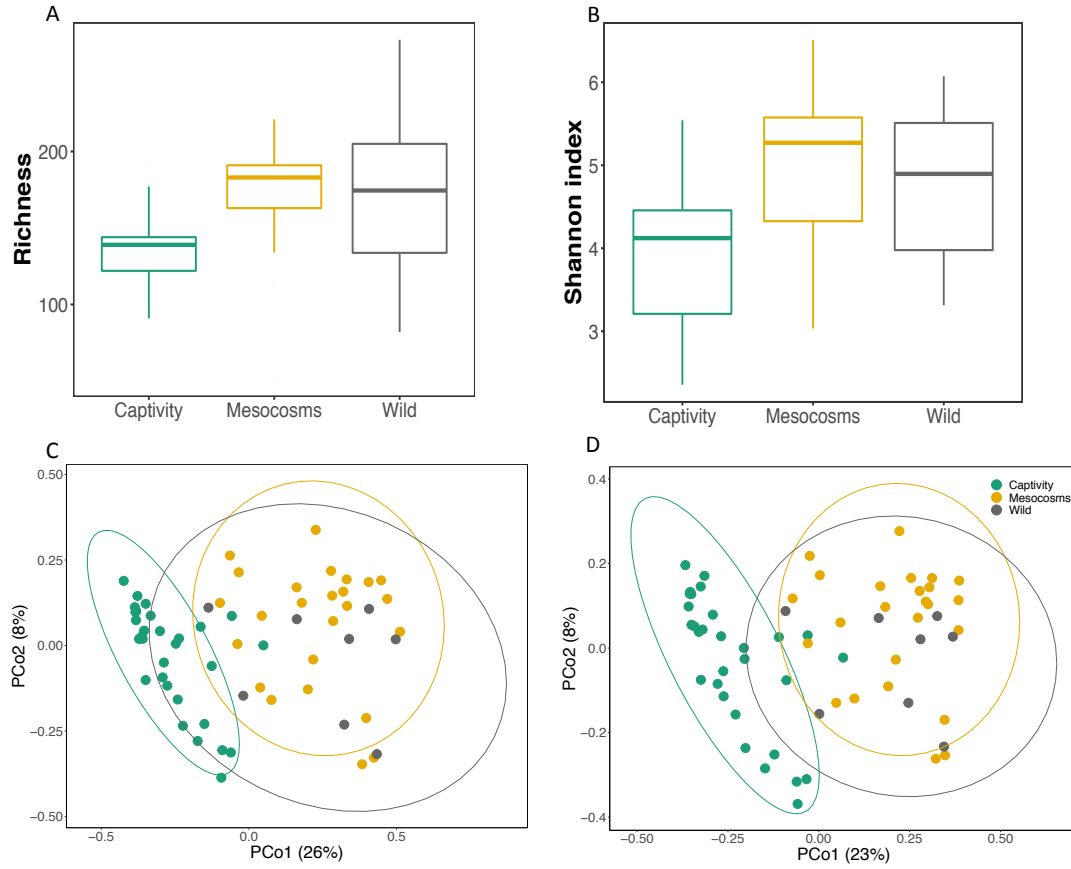
Comparisons of residual body condition index (BCI) of captive-born females (A) and males (B) at all sampling days (0, 1, 7, 14, 21 and 27 and wild conspecifics measured in 2011-2012 (W). Residual BCI of captive-born individuals was calculated from the regression equation of log body mass (g) vs log SVL (mm) from wild individuals sampled in Mamoni in 2011-2012. The BCI for wild frogs are the residuals of the linear regression.

Figure 4. Change in body condition of soft-released *Atelopus limosus* compared to wild conspecifics from the extant population.



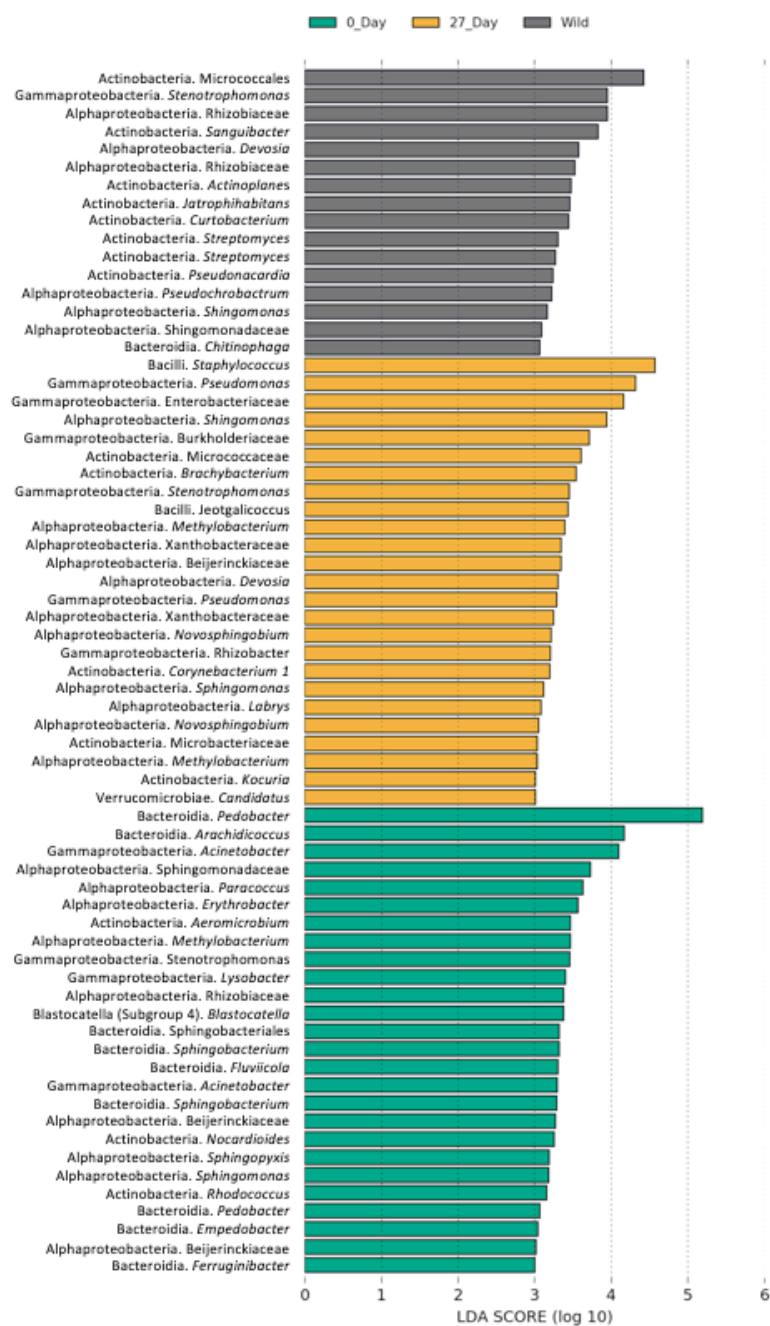
Change in body condition of soft-released frogs compared to wild conspecifics. The body mass of females (A) and males (B) in captivity on day 0 (green) and in mesocosms on day 27 (yellow). The regression lines ($\text{Mass}_F = -5.0410 + 1.6953 \cdot \text{SVL}$, $\text{Mass}_M = -4.8028 + 1.5010 \cdot \text{SVL}$) were conducted with biometric values from wild *A. limosus* surveyed in a previous study in 2011-2012. Each dot represents an individual *A. limosus* data point, and dots above or below the regression line indicate that the indicated individual had either a higher or lower body mass respectively compared to wild conspecifics of the same body length.

Figure 5. Bacterial community diversity and structure on the skin of captive-born *Atelopus limosus* before and after soft-release and on wild conspecifics



Alpha diversity as represented by OTU richness (A) and the Shannon index (B). PCoA showing beta diversity pattern based on Bray-Curtis (C) and Jaccard (D) dissimilarities. In the ordinations, each circle represents the bacterial community structure on the skin of one frog. All figures show data from frogs in captivity (N = 30) sampled on day 0 and in mesocosms (N = 26) sampled on day 27. Frogs in the wild (N = 8) were sampled, when encountered, throughout the present study.

Figure 6. Key OTUs associated with *A. limosus* before and after placement in mesocosms and of wild conspecifics



Linear discriminant analysis effect size (LeFSe). Operational taxonomic units (OTUs) that best defined the bacterial community structure of *A. limosus* before and after placement in mesocosms and of wild conspecifics. Bars represent significant LDA scores (≥ 3) associated with the key OTUs. OTU taxonomy is represented at the lowest taxonomic level available in the SILVA database.

Table 2. Infection prevalence and intensity from captive-born *A. limosus* at all sampling days.

Sampling day	Number of <i>Bd</i> + frogs	Number of total frogs	Prevalence (%)	Mean <i>Bd</i> intensity (z.e.)	<i>Bd</i> intensity range (z.e.)
0	0	30	0	0	0
1	0	30	0	0	0
7	0	28	0	0	0
14	2	28	7	15.5	2 - 29
21	2	28	7	280.5	70 - 491
27	4	26	15	5,838.50	5 - 23,100

Mean and range of *Bd* infection intensities are presented in zoospores equivalents (z.e.).

Table 3. Total number of individuals and *Bd* positive individuals across species and sampling days.

Species	10-19 April 2017		8-9 May 2017		Both	
	Prevalence (No. of <i>Bd</i> +/No. of total)	Mean <i>Bd</i> intensity (z.e.)	Prevalence (No. of <i>Bd</i> +/No. of total)	Mean <i>Bd</i> intensity (z.e.)	Prevalence (No. of <i>Bd</i> +/No. of total)	Mean <i>Bd</i> intensity (z.e.)
<i>Allobates talamancae</i>	0 / 2	0	0 / 4	0	0 / 6	0
<i>Atelopus limosus</i>	0 / 2	1,489.25	0 / 2	0	0 / 4	1,489.25
<i>Colostethus panamensis</i>	0 / 7	0	6 / 9	1,607.50	6 / 16	1,607.50
<i>Colostethus pratti</i>	3 / 12	51.91	2 / 4	317.1	5 / 16	105.84
<i>Craugastor crassidigitus</i>	0 / 0	0	0 / 1	0	0 / 1	0
<i>Diasporus quidditus</i>	0 / 6	0	0 / 3	0	0 / 9	0
<i>Hyalinobatrachium colymbiphylum</i>	0 / 0	0	0 / 2	0	0 / 2	0
<i>Hyalinobatrachium fleischmanni</i>	0 / 1	0	0 / 0	0	0 / 1	0
<i>Incilius coniferus</i>	1 / 3	1,669	2 / 4	43,865.50	3 / 7	29,800
<i>Pristimantis cerasinus</i>	0 / 1	0	0 / 0	0	0 / 1	0
<i>Pristimantis cruentus</i>	2 / 4	247,390.50	0 / 1	0	2 / 5	247,390.50
<i>Rhaebo haematiticus</i>	2 / 5	127.19	0 / 0	0	2 / 5	127.19
<i>Sachatamia albomaculata</i>	1 / 14	47.03	2 / 13	336.3	3 / 27	239.88
<i>Silverstoneia flator</i>	1 / 7	279.5	0 / 4	0	1 / 11	279.5
<i>Smilisca sila</i>	1 / 2	258.3	0 / 0	0	1 / 2	258.3
<i>Teratohyla spinosa</i>	1 / 9	59.87	0 / 4	0	1 / 13	59.87
Total	16% (12 / 75)	41,459	24% (12 / 51)	8,202	19% (24 / 126)	24,830
95% binomial confidence interval	9 - 26%	0 - 131,530	13 - 37%	0 - 22,321	13 - 27%	0 - 67,342

Mean and range of *Bd* infection intensities are presented in zoospores equivalents (z.e.).

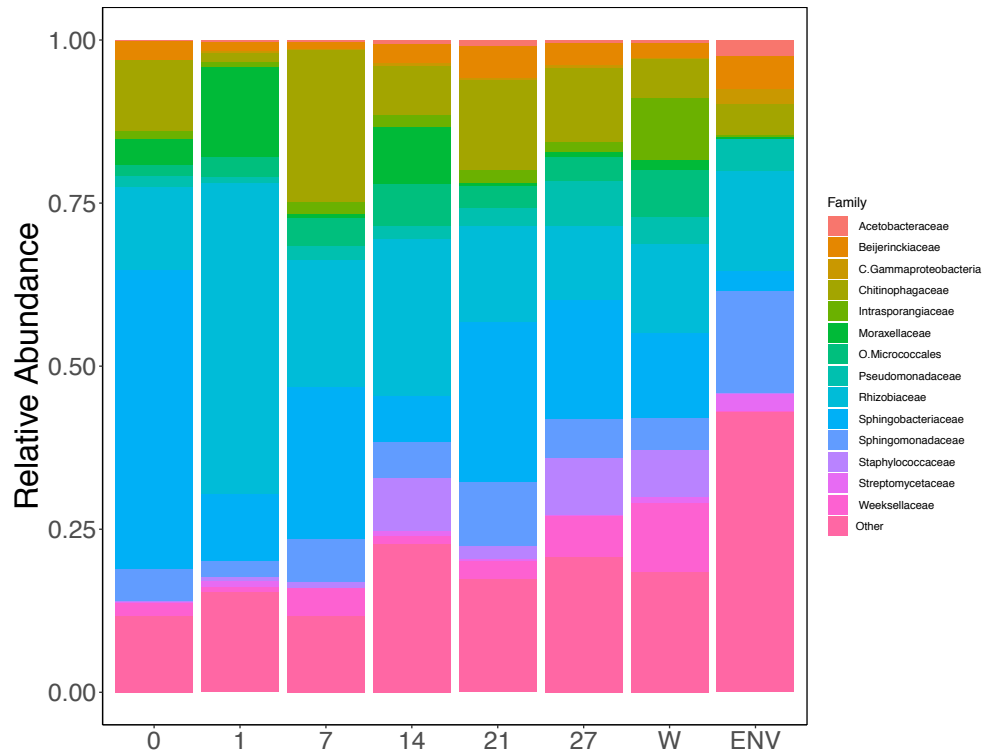
Supplementary material

Table S1. Multiple comparisons with Dunn tests for differences of residual body condition index across all sampling days and wild frogs.

Day Comparison	FEMALES			MALES		
	Z	P.unadj	P.adj	Z	P.unadj	P.adj
0 - 1	2.0923	0.0364	0.1912	0.3314	0.7403	0.9145
0 - 7	1.7766	0.0756	0.2269	1.3176	0.1876	0.5629
1 - 7	-0.2793	0.7800	0.9100	0.9920	0.3212	0.7495
14 - 7	-0.0381	0.9696	1.0000	0.9555	0.3393	0.7126
21 - 7	-0.8422	0.3997	0.5595	0.3575	0.7207	0.9459
27 - 7	-1.9640	0.0495	0.2080	0.0619	0.9506	0.9982
0 - 14	1.6558	0.0978	0.2053	0.2937	0.7690	0.8971
1 - 14	-0.2156	0.8293	0.9166	-0.0187	0.9851	0.9851
0 - 21	2.6332	0.0085	0.0592	0.9540	0.3401	0.6493
1 - 21	0.5773	0.5637	0.6964	0.6283	0.5298	0.7947
14 - 21	0.7307	0.4650	0.6102	0.6120	0.5406	0.7568
0 - 27	3.7742	0.0002	0.0034	1.2014	0.2296	0.6027
1 - 27	1.7183	0.0857	0.2001	0.8889	0.3740	0.6546
14 - 27	1.7548	0.0793	0.2082	0.8611	0.3892	0.6287
21 - 27	1.1218	0.2620	0.4232	0.2816	0.7782	0.8602
0 - W	3.4217	0.0006	0.0065	6.6582	<0.0001	<0.0001
1 - W	1.5503	0.1211	0.2119	6.2445	<0.0001	<0.0001
14 - W	1.6120	0.1070	0.2042	5.7353	<0.0001	<0.0001
21 - W	1.0105	0.3123	0.4684	5.3005	<0.0001	<0.0001
27 - W	-0.0136	0.9892	0.9892	4.6357	<0.0001	<0.0001
7 - W	1.7793	0.0752	0.2632	4.8508	<0.0001	<0.0001

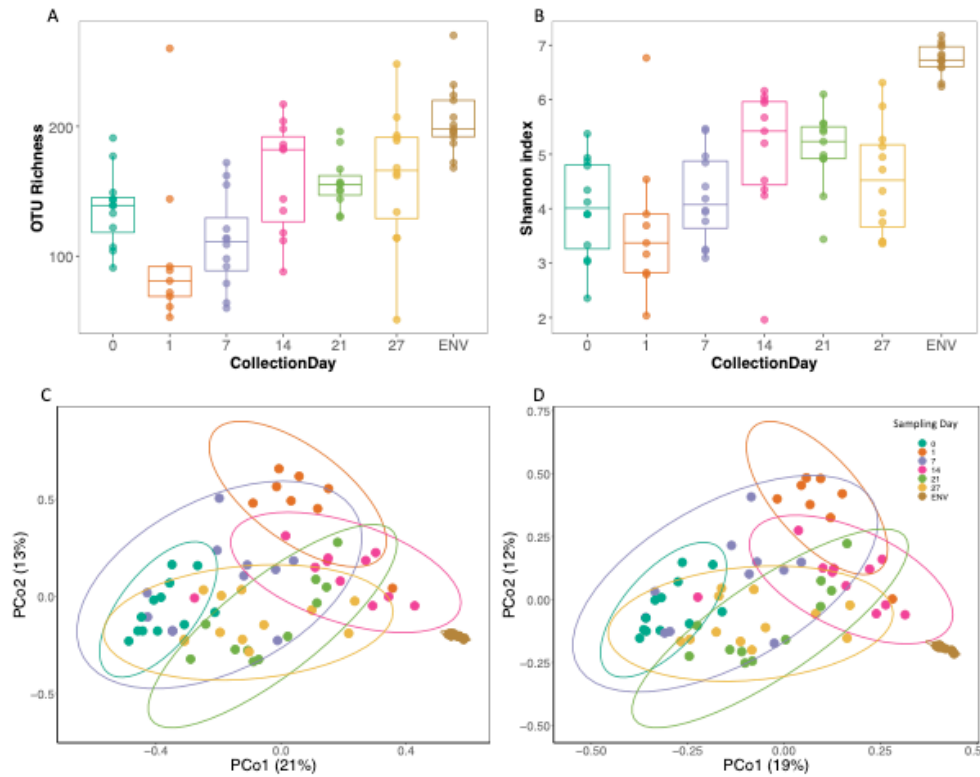
Bold numbers highlight significant P.values adjusted with the Benjamini-Hochberg method.

Figure S1. Relative abundance of bacterial families on frogs before and after soft-release, on wild frogs, and in the environment.



Bacterial families with highest relative abundance (each >1%; total 91%) in the skin of captive-born *A. limosus* in captivity (day 0) and in mesocosms (day 27). Bacteria on wild frogs and in environmental samples are also included.

Figure S2. Bacterial community diversity and structure on the skin of captive-born *Atelopus limosus* before and after soft-release, on wild conspecifics and in the environment.



Alpha and beta diversity on time series and environmental samples. Alpha diversity as represented by OTU richness (A) and Shannon index (B). PCoA showing beta diversity pattern based on Bray-Curtis (C) and Jaccard (D) dissimilarities. In the ordinations, each circle represents the bacterial community structure on the skin of one frog or from one mesocosm (for environmental samples). All figures show data from a subset of frogs (N = 12) sampled through time after placement into mesocosms. Their corresponding mesocosms were sampled for environmental (ENV) bacterial communities on day 28 (N = 12).

Table S2. Multiple comparisons with Tukey tests for differences of OTU richness across all sampling days, and with wild frogs and environmental samples.

Comparisons	Estimate	Std. Error	z value	P
1 - 0	-0.323381	0.126396	-2.558	0.1694
7 - 0	-0.203562	0.114653	-1.775	0.6347
14 - 0	0.161439	0.116188	1.389	0.8614
21 - 0	0.139587	0.116041	1.203	0.9307
27 - 0	0.162891	0.11347	1.436	0.8396
W - 0	0.228518	0.133397	1.713	0.6766
ENV - 0	0.404815	0.111531	3.63	<0.01
7 - 1	0.119819	0.127252	0.942	0.9818
14 - 1	0.48482	0.128234	3.781	<0.01
21 - 1	0.462968	0.128432	3.605	<0.01
27 - 1	0.486273	0.125987	3.86	<0.01
W - 1	0.5519	0.143905	3.835	<0.01
ENV - 1	0.728196	0.124416	5.853	<0.01
14 - 7	0.365001	0.117193	3.115	0.0385
21 - 7	0.343149	0.117213	2.928	0.0662
27 - 7	0.366453	0.114614	3.197	0.03
W - 7	0.43208	0.133921	3.226	0.0267
ENV - 7	0.608377	0.112581	5.404	<0.01
21 - 14	-0.021853	0.118303	-0.185	1
27 - 14	0.001452	0.115981	0.013	1
W - 14	0.067079	0.135423	0.495	0.9997
ENV - 14	0.243376	0.114179	2.132	0.3912
27 - 21	0.023305	0.115847	0.201	1
W - 21	0.088932	0.135407	0.657	0.998
ENV - 21	0.265228	0.113997	2.327	0.2771
W - 27	0.065627	0.133116	0.493	0.9997
ENV - 27	0.241923	0.111007	2.179	0.3621
ENV - W	0.176296	0.130524	1.351	0.878

Bold numbers highlight significant P.values.

Table S3. Multiple comparisons with Tukey tests for differences of Shannon index across all sampling days, and with wild frogs and environmental samples.

Comparisons	Estimate	Std. Error	z value	P
1 - 0	0.022622	0.023778	0.951	0.9798
7 - 0	-0.012812	0.020341	-0.63	0.9984
14 - 0	-0.050935	0.019117	-2.664	0.1289
21 - 0	-0.052229	0.019073	-2.738	0.1071
27 - 0	-0.029403	0.019604	-1.5	0.801
W - 0	-0.041105	0.022426	-1.833	0.5875
ENV - 0	-0.101528	0.017091	-5.94	<0.001
7 - 1	-0.035434	0.023396	-1.515	0.7928
14 - 1	-0.073558	0.022277	-3.302	0.0202
21 - 1	-0.074851	0.022239	-3.366	0.0167
27 - 1	-0.052025	0.022742	-2.288	0.2915
W - 1	-0.063728	0.025138	-2.535	0.1744
ENV - 1	-0.12415	0.020569	-6.036	<0.001
14 - 7	-0.038123	0.018632	-2.046	0.4411
21 - 7	-0.039417	0.018586	-2.121	0.3915
27 - 7	-0.016591	0.019122	-0.868	0.9882
W - 7	-0.028293	0.021916	-1.291	0.8984
ENV - 7	-0.088716	0.016528	-5.367	<0.001
21 - 14	-0.001293	0.017217	-0.075	1
27 - 14	0.021532	0.017811	1.209	0.9264
W - 14	0.00983	0.020914	0.47	0.9998
ENV - 14	-0.050592	0.015005	-3.372	0.0166
27 - 21	0.022826	0.017765	1.285	0.9007
W - 21	0.011123	0.020866	0.533	0.9995
ENV - 21	-0.049299	0.014953	-3.297	0.0209
W - 27	-0.011702	0.021329	-0.549	0.9993
ENV - 27	-0.072125	0.015618	-4.618	<0.001
ENV - W	-0.060423	0.018824	-3.21	0.0278

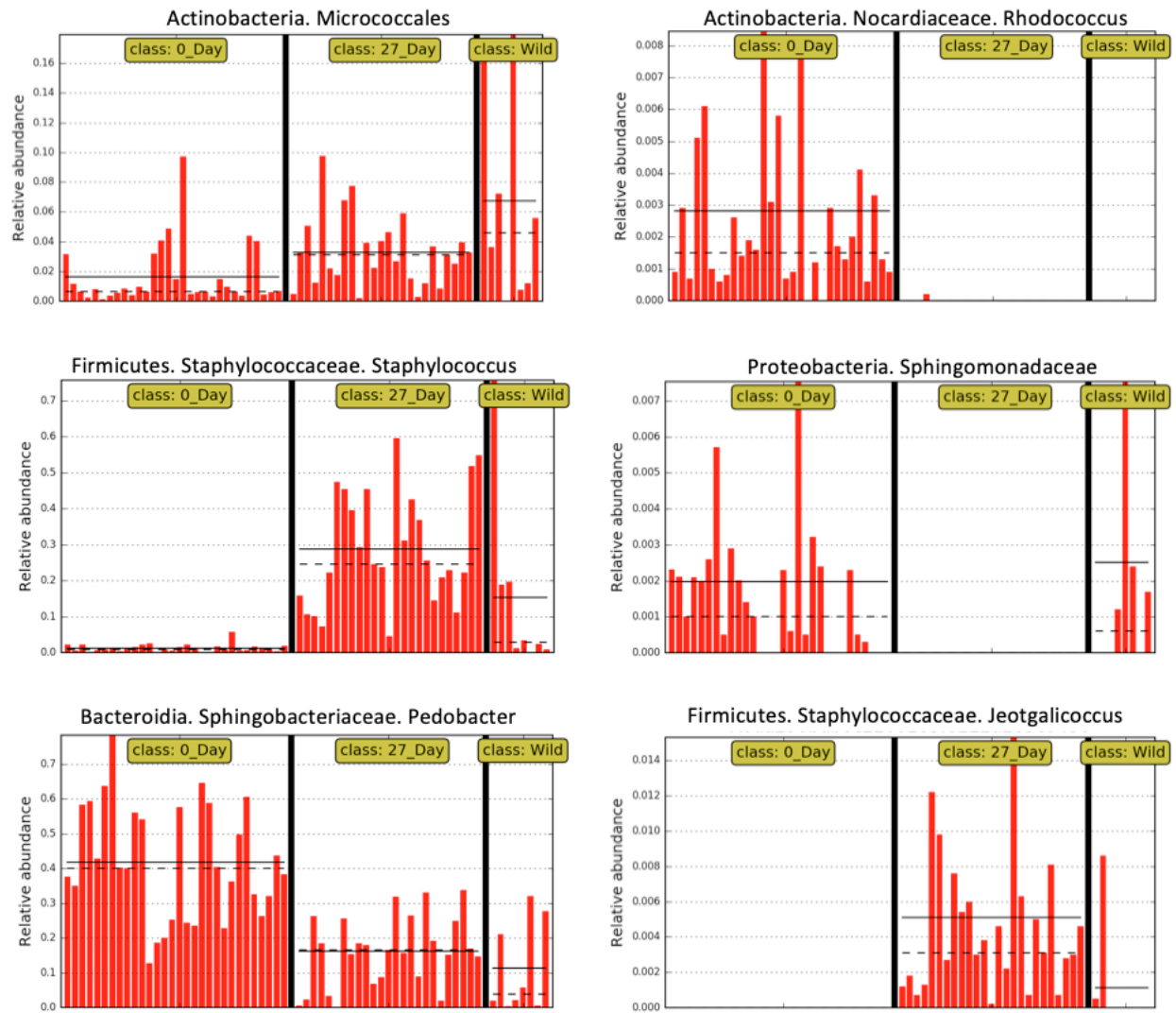
Bold numbers highlight significant P.values.

Table S4. Linear discriminant analysis effect size (LeFSe). Operational taxonomic units (OTUs) that best define bacterial community structure in *A. limosus* before and after release and on wild conspecifics.

OTU_ID	Phyla	Class	Order	Family	Genus	Species	Day	value	score	p.value
2d2996f7631b0e34f282b2ed38d1b346	Actinobacteria	Actinobacteria	Micrococcales				Wild	4.82720768	4.42702033	0.00392066
d0099ee91db9830ee9ca81d0e85747	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Stenotrophomonas	Stenotrophomonas rhizophila	Wild	4.30400555	3.9512085	4.30E-08
3e00a33b844a56c2e0a0cedff43b5e	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae			Wild	4.39619935	3.95092592	5.43E-06
3ab466c5dc2d8ee0ad2fd2c28a198e	Actinobacteria	Actinobacteria	Micrococcales	Sanguibacteraceae	Sanguibacter	uncultured bacterium	Wild	4.161368	3.82523461	1.92E-07
37263866e850776c55a8d5745c2119	Proteobacteria	Alphaproteobacteria	Rhizobiales	Devosiaceae	Devosia	uncultured bacterium	Wild	3.98340074	3.57767673	0.00142778
2639a2bf3f00f36938cdcb126252c5	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae			Wild	3.90512099	3.52648465	6.80E-08
38810e2f99779b8185b5aa25eb8cd3	Actinobacteria	Actinobacteria	Micromonosporales	Micromonosporaceae	Actinoplanes	uncultured bacterium	Wild	3.77451697	3.47366373	5.91E-08
a8b40f00ce58f3eb4d30bf7b9d5fa85	Actinobacteria	Actinobacteria	Frankiales	Frankiaceae	Jatrophihabitans	uncultured bacterium	Wild	3.75108656	3.45908154	2.40E-09
e6e024215a635bef49488eb7fa1b8617	Actinobacteria	Actinobacteria	Micrococcales	Microbacteriaceae	Curtobacterium		Wild	3.81706932	3.4474664	2.26E-05
09d2bace120d5c6c6bce2f94b8efbdf4d	Actinobacteria	Actinobacteria	Streptomycetales	Streptomycetaceae	Streptomyces		Wild	3.62710971	3.30913441	4.16E-07
5a9ca01e6ea962f83f5b63a2dd5c28f	Actinobacteria	Actinobacteria	Streptomycetales	Streptomycetaceae	Streptomyces		Wild	3.61410591	3.27546159	0.00011641
78c07a380fde312c999698b581e8c5f	Actinobacteria	Actinobacteria	Pseudomonadales	Pseudomonadaceae	Pseudonocardia	uncultured bacterium	Wild	3.63598611	3.24317254	5.55E-06
c81bf6c7381e95d025bebd3434620749	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Pseudochrobactrum	uncultured bacterium	Wild	3.50514998	3.20465637	7.05E-06
c540fefa822da874b7ed68d9ee89695	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas		Wild	3.45484486	3.16478865	2.73E-06
702f797c35040a2a3780677411a1030c	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae			Wild	3.40010607	3.09641566	3.21E-06
f589039fde730089bda3f653206fd67	Bacteroidetes	Bacteroidia	Chitinophagales	Chitinophagaceae	Chitinophaga	uncultured bacterium	Wild	3.42934847	3.07043868	0.00032873
asd1217aa1c154c499796f9040d954	Firmicutes	Bacillia	Bacillales	Staphylococcaceae	Staphylococcus		27	4.8661667	4.56902983	9.43E-10
7b5fac21508102af6c81750c6efbdf8f2	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas		27	4.73333025	4.31752365	1.20E-05
677929ea3c2e081cd8e08a09a70eb2	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae			27	4.49986893	4.16287567	4.74E-08
1f4fa82b05eccf36104734520166e	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas		27	4.44197515	3.94050157	2.59E-06
03074960b88424990b08a196244860	Proteobacteria	Alphaproteobacteria	Betaproteobacteriales	Burkholderiaceae			27	4.4056268	3.71322834	0.00579461
109f463e7355f97cf225888cd3dda	Actinobacteria	Actinobacteria	Micrococcales	Micrococcaceae			27	4.07820537	3.60793582	0.01539644
c360a7e1096c42329a5275f02974e44e	Actinobacteria	Actinobacteria	Micrococcales	Dermbacteriaceae	Brachybacterium	uncultured bacterium	27	3.82611171	3.54595596	1.25E-09
43f41dd4045f31804b51faecd53ba41	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Stenotrophomonas		27	3.83559346	3.45407121	2.43E-06
0da296b4b8c2200183f8222a28e9450	Firmicutes	Bacillia	Bacillales	Staphylococcaceae	Jeetgallibacter	uncultured bacterium	27	3.70772089	3.40408919	8.80E-12
13732ba3b63c8dbf5169bf74a69673	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae	Methylobacterium	Methylobacterium radiotolerans	27	3.9009099	3.39670846	6.29E-05
e3874604d4ed0507c274f1c24113983a	Proteobacteria	Alphaproteobacteria	Rhizobiales	Xanthobacteraceae	uncultured		27	3.70997914	3.3466065	1.48E-06
fc9d9de5a09a6a632f52620b151ff5b8	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae			27	3.77088451	3.34648372	8.79E-06
e6e57c26107207468660e16e3f0881cd	Proteobacteria	Alphaproteobacteria	Rhizobiales	Devosiaceae	Devosia		27	3.73146899	3.30858564	1.04E-06
4123e98613052b1445d0f879eb430693	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	Pseudomonas putida	27	3.61364875	3.28988787	0.03072709
f952b4a7db5ef3da66f023cc071356ef	Proteobacteria	Alphaproteobacteria	Rhizobiales	Xanthobacteraceae			27	3.57242204	3.25237277	5.44E-06
da00a09609c3a7a4c3d43a50c271b1	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Novosphingobium		27	3.54410332	3.21516352	6.62E-08
fcba3c512956a20a371b35e6b2da5c	Proteobacteria	Gammaproteobacteria	Burkholderiales	Burkholderiaceae	Rhizobacter		27	3.66769215	3.21057834	0.00681662
d6a0c6d0de17ed04d43387f72ac246a	Actinobacteria	Actinobacteria	Corynebacteriales	Corynebacteriaceae	Corynebacterium 1		27	3.7547586	3.20216825	0.02730778
9c0f13da7e122b66fbcf8ed9323c6214	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	metagenome	27	3.47482591	3.11862021	2.86E-06
ecf99b89071363030cb08a343b406d	Proteobacteria	Alphaproteobacteria	Rhizobiales	Labraceae	Labrys		27	3.39308209	3.08443334	3.88E-07
54580f940f267b8e94283e28ad1f13f	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Novosphingobium	uncultured bacterium	27	3.45212118	3.04934387	0.00247723
aa75f0c425c12ce3009d5059234eaab7	Actinobacteria	Actinobacteria	Micrococcales	Microbacteriaceae			27	3.42034599	3.03557498	0.00250898
aa3d92f9aea44c4c43e19a80aa53577	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae	Methylobacterium	Methylobacterium aquaticum	27	3.32884354	3.03365838	6.78E-09
6628b08e13117c52417f5dfef409aef7	Actinobacteria	Actinobacteria	Micrococcales	Micrococcaceae	Kocuria		27	3.25724805	3.01484951	3.71E-10
271b576cc1f2ce84b35d8374ed909d4b	Verrucomicrobia	Verrucomicrobiae	Chthoniobacteriales	Chthoniobacteraceae	Candidatus Udaebacter		27	3.3312431	3.01275443	2.17E-05
358c365247ae643e2971c42b09197be	Bacteroidetes	Bacteroidia	Sphingobacteriales	Sphingobacteriaceae	Pedobacter		0	5.62086609	5.18919554	1.21E-07
99ca48329a3136667f4d1e2da6228e0a	Bacteroidetes	Bacteroidia	Chitinophagales	Chitinophagaceae	Arachidicoccus	uncultured bacterium	0	4.54273394	4.17263792	4.43E-06
107aa7e6b56274803bffd1ccff5b2ee6	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter		0	4.4815686	4.09541428	7.02E-08
e6c333964fe450a50ba4835114fce943	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae			0	4.05285448	3.73404178	6.29E-09
e58f4afd38d5d2317e576701ea6c32a4	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Paracoccus		0	3.97931243	3.62939961	6.41E-10
aa8dbbebae412f35580375554464d02c	Actinobacteria	Actinobacteria	Sphingomonadales	Sphingomonadaceae	Erythrobacter	uncultured bacterium	0	3.87042229	3.56758666	6.90E-10
184a2fa24dc9ca4bf09abd1a18553b11	Actinobacteria	Actinobacteria	Propionibacteriales	Nocardioidaceae	Aeromicrobium	uncultured bacterium	0	3.8850928	3.46844812	3.18E-05
899a13d9709418800bd1c612fe0b23e2	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae	Methylobacterium	uncultured bacterium	0	3.9529789	3.46564647	0.00103884
631250b5c547cb1529044e9dd009761	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Stenotrophomonas		0	3.78949375	3.46377051	1.13E-09
6c3fae2cb1742e578d9cb5cd65543beb	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Lysobacter		0	3.63941396	3.40588604	8.77E-06
5935b7424d68dab9ca4706b22832f8e	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae			0	3.74977453	3.38257083	8.79E-09
6623b5a374b329c5c7eae4f15383989	Acidobacteria	Blastocatellia (Subgroup 4)	Blastocatelliales	Blastocatellaceae	Blastocatella	uncultured bacterium	0	3.68615421	3.38227017	1.80E-10
f15072c6db15cdf9aea5522979e8cd0f	Bacteroidetes	Bacteroidia	Sphingobacteriales	env.OP5 17			0	3.6353907	3.32164827	0.00223245
3bdf3962a4d6b9738dc1bfa095f6cc8e	Bacteroidetes	Bacteroidia	Sphingobacteriales	Sphingobacteriaceae	Sphingobacterium	Sphingobacterium multivorum	0	3.61774996	3.320098	4.50E-08
96f0e85d4d1083107ed4704b14ef718	Bacteroidetes	Bacteroidia	Flavobacteriales	Crocinitomicaceae	Fluvicola	uncultured bacterium	0	3.61441425	3.30371752	0.01429451
0eb8e63cb87cb7aa58b403dc29da7de	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter		0	3.51769507	3.2949521	0.01091996
332984af6a246195771ec1ab95e19d0d	Bacteroidetes	Bacteroidia	Sphingobacteriales	Sphingobacteriaceae	Sphingobacterium	uncultured bacterium	0	3.56502183	3.29025921	9.08E-08
e272199023312ee49dbac55946150a2e	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae			0	3.75109113	3.27041318	0.02158414
0079bc0fadf3a30edc95d12ed0305be	Actinobacteria	Actinobacteria	Propionibacteriales	Nocardioidaceae	Nocardioides		0	3.61592938	3.25587937	2.22E-05
e94194d60112452524885c4d46533a1b	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingopyxis	uncultured bacterium	0	3.54951912	3.19434083	5.49E-06
1282ac478e61eae5f1051bb73c10f0a	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas		0	3.5440622	3.17985072	2.77E-05
c6943bf094da0955330c258096040a	Actinobacteria	Actinobacteria	Corynebacteriales	Nocardiaceae	Rhodococcus	uncultured bacterium	0	3.44938414	3.16294109	4.25E-11
5d8f1bcb154d9f4666f0228bcf25d911	Bacteroidetes	Bacteroidia	Sphingobacteriales	Sphingobacteriaceae	Pedobacter	uncultured bacterium	0	3.45100653	3.07189469	3.67E-07
1b30163271d634a3e9fcdaf5f9455ca90	Bacteroidetes	Bacteroidia	Flavobacteriales	Weeksellaceae	Empedobacter	uncultured bacterium	0	2.78783765	3.04429821	6.63E-07
8f28b7185e38f2d0edfed8e7fddfeb93	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae			0	3.34983767	3.02005109	2.99E-08
151f7e9f5b7684814d6783039d5d66	Bacteroidetes	Bacteroidia	Chitinophagales	Chitinophagaceae	Ferruginibacter		0	3.16635731	3.00798789	0.01834318

Scores represent linear discriminant analysis (LDA).

Figure S3. Examples of changes in relative abundance of OTUs selected by linear discriminant analysis effect size (LeFSe).



Chapter 5. Summary Conclusion.

Amphibians around the world are disappearing due to habitat loss coupled with a myriad of synergistic factors, such as overexploitation, pollution, the introduction of non-native species and infectious diseases (Collins 2010). Massive population declines and local extinctions are often linked to chytridiomycosis outbreaks, a lethal infectious disease caused by a pathogenic fungus, *Batrachochytrium dendrobatidis* (Bd), that infects amphibian skin. While Bd-related declines in wild amphibian populations are difficult to halt, some of the most promising conservation interventions include the use of beneficial bacteria to boost amphibian immune responses and the establishment of captive assurance colonies for the most vulnerable of species (Mendelson *et al.*, 2006; Bletz *et al.*, 2013). Although probiotic therapies have proven to be successful in some human applications, the utilization of such technologies across multiple host species and across different pathogen strains have yielded mixed results (Walke and Belden, 2016). For example, while bacteria isolated from red-backed salamanders can provide defense against Bd infections (Harris *et al.*, 2009), the application of the same bacterium on Panamanian golden frogs failed to persist in the skin and did not provide protection against chytridiomycosis (Becker *et al.*, 2011; 2014). Other studies also support the idea that individual bacteria isolated from the skin of amphibians vary in their ability to inhibit pathogen infections (Antwis *et al.*, 2015). Some of the inconsistencies are attributed to the context-dependency of symbiotic interactions; the influence of host identity and the effect of environmental conditions on symbiotic microbes through time and space remain poorly understood. To address this knowledge gap, my dissertation research provides evidence for natural spatio-temporal variation in bacterial communities associated with the skin of more than a dozen lowland amphibian species found in the Panama Canal Watershed that may be surviving with enzootic Bd (Woodhams *et al.*, 2008; Kilburn *et al.*, 2010; Rebollar *et al.*, 2014).

Many amphibian species persist in lowland sites across the Neotropics despite Bd occurrence. Although there is little evidence for population declines and extinctions directly linked with Bd epidemics, species inhabiting leaf litter in dry forests in the lowlands have been experiencing declines in the last four decades mostly linked to climate change (Ryan *et al.*, 2015). In general, some lowland sites are thought to be Bd refugia for amphibian species that can endure the characteristically higher temperatures of the region (Puschendorf *et al.*, 2009; Zumbado-Ulate *et al.*, 2014), a physical factor that tends to limit Bd growth and spread. Thus, conservation programs are identifying potential reintroduction sites within the region to release captive-born species, making my dissertation research in the region of particular pertinence. Most of my dissertation work was conducted in the Panama Canal Watershed, a narrow corridor of land and fresh water inhabited by a great diversity of amphibian species across its elevation and rainfall gradients (Condit *et al.*, 2001; Ibáñez *et al.*, 2002). In this watershed there are strong wet and dry seasons affecting all amphibian hosts, their skin-associated microbiomes, and Bd. Much research focuses on understanding the interplay of host species identity and environmental conditions on these host-microbiome-Bd dynamics. Additionally, in collaboration with the Panama Amphibian Rescue and Conservation Project that maintains assurance colonies of amphibian species found west and east of the watershed, my study aided in the development of methodologies to release captive amphibians into their natural habitat.

In **Chapter 2**, I tracked changes in the skin-bacterial communities of two treefrog species, *Agalychnis callidryas* and *Dendropsophus ebraccatus*, across different temporal scales (i.e. across years, seasons, and days) and detected low interannual stability of the bacterial communities on these amphibians. Moreover, variation in these skin bacterial communities was strongly linked with rainfall and temperature patterns on the day of sampling. Hence, day explained most of the variation across seasons and years. These two sympatric species encounter the same environmental conditions, which enabled me to determine that these general patterns of temporal microbiome variation are analogous across both species. Lastly, seasonal effects in bacterial community composition varied between treefrogs, likely due to behavioral differences between the two species.

In **Chapter 3**, I expanded on my Chapter 2 results assessing the effects of seasonality on skin microbiomes of individual amphibian species to examine entire amphibian communities. In this instance, my results were supported by research determining species-specific microbiomes in co-occurring host species (Kueneman *et al.*, 2014; Belden *et al.*, 2015; Rebollar *et al.*, 2016; Flechas *et al.*, 2017; Varela *et al.*, 2018). Importantly, in this chapter, I provided strong evidence for host species-specific responses of skin bacterial communities to seasonal fluctuations in environmental conditions. Through this project, I was also able to characterize the skin bacterial communities of several lowland amphibian species that had not been previously described, including: *Pristimantis gaigei*, *Leptodactylus savagei*, *Rinhela alata*, *Hipsiboas rosenbergi* and *Engystomops pustulosus*. For amphibian species with known microbiomes, *Craugastor fitzingeri* and *Silverstoneia flotator*, this research extended our knowledge of the microbiome repertoire across environmental gradients (for *C. fitzingeri*) and between seasons (for *S. flotator*). Particularly, the skin of *C. fitzingeri*, a common species inhabiting the forest floor, had been recently studied to characterize skin microbial communities and metabolite production (Belden *et al.*, 2015; Rebollar *et al.*, 2016; Abarca *et al.*, 2018) as well as Bd susceptibility (Rebollar *et al.*, 2014). I found less variation in bacterial community richness and evenness, and high variability in bacterial community structure on *C. fitzingeri* skin across sites. My field surveys across a rainfall gradient and wet and dry seasons added valuable information on spatio-temporal variation on skin bacterial community of *C. fitzingeri* that could potentially explain their persistence across Bd prevalent sites.

The results of these first two chapters suggested that despite strong host filtering on skin-associated bacteria that colonize the skin, temporal and spatial variation among hosts is likely mediated by differences in environmental conditions at the time of sampling. I also found low Bd prevalence in the lowlands (11%). Although the function of bacterial community composition was not assessed, future studies should explore functional traits in these communities with the use of high-throughput -omics technologies (i.e. genomics, metabolomics) across temporal and spatial gradients *in-situ* or under laboratory conditions reproducing natural environmental conditions. Since these bacterial communities are vastly diverse and dynamic, the identification and manipulation of the core microbiome, or key bacteria, to create synthetic microbial communities to test the potential protective function will be an informative initial approach. Most attempts to test amphibian probiotics have used single bacterial strains, but suites of bacteria may be more effective. As I mentioned previously, the science around probiotic treatment in amphibians requires a deeper understanding of not only the ecology of Bd and its hosts, but of the microbial communities associated with different host species and their natural

variation across time and space. This variation becomes even more important for those amphibian species that are currently maintained in captive breeding programs. Several interventions (i.e., probiotics) are being considered for these captive species prior to reintroduction that could increase survival and permit the establishment of self-sustaining wild populations. My first two chapters evaluated temporal variation in skin microbiomes on non-Bd susceptible amphibians at sites with a low prevalence of Bd. Therefore, to truly understand the potential implication of natural temporal variability in amphibians that are highly susceptible to Bd, I attempted the first field exposure study that investigated microbiome and disease changes with an individual-based approach and replicated measurements. The extensive historic records of Bd-related declines and extinctions (Lips 1999; Lips *et al.*, 2015; Lips *et al.*, 2006; Woodhams *et al.*, 2008; Crawford *et al.*, 2010), the large amount of work characterizing, testing and isolating bacteria from amphibian skin (Walke *et al.*, 2011; Woodhams *et al.*, 2015; Belden *et al.*, 2015; Rebollar *et al.*, 2016; Medina *et al.*, 2017), and the pioneering conservation work related to *in-situ* captive breeding in Panama (Zippel 2002; Gagliardo *et al.*, 2008; Murphy and Gratwicke, 2017), allowed me to address important applied questions in the amphibian-Bd-microbiome system.

For **Chapter 4**, I used field enclosures (mesocosms) to better understand some of the challenges faced by amphibians as they transition from captivity back into the wild. The use of mesocosms allowed for repeated monitoring and data collection under natural environmental conditions to study body condition, the microbiome and Bd infection dynamics in a more natural setting. My collaborators continue to develop management tools that may mitigate the threat of chytridiomycosis, and my project was an important contribution to that effort. I found that exposure to natural conditions led captive-born *Atelopus limosus* to become more similar to wild conspecifics from the extant population based on three indicators of post-release health and survival: body condition, skin microbiome and disease status. That is, after one month following soft-release into field enclosures, captive-born frogs lost mass and overall body condition decreased to become more similar to wild conspecifics, skin-associated bacterial communities reverted to a "wild-type", and 15% of frogs became infected with Bd, similar to the level of infection observed in the existing amphibian community at the site. However, no mortalities were associated with body condition or disease status during our study. Together these results suggest that: a) captive-born amphibians will inevitably lose body mass after release, especially without supplemental feeding; b) the skin-associated microbiome of captive-born amphibians can be quickly restored to the microbiomes that occur on wild frogs; and c) without pre-release Bd-mitigation interventions, captive-born *Atelopus* remain susceptible to Bd infections. I learned that short-term use of mesocosms is a suitable tool for systematically and repeatedly monitoring amphibian skin disease dynamics following release, and I recommend the use of them in future trials looking to answer relevant ecological and applied research questions for the protection of these treasured animals.

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Appendix A

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