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Efeitos de ambientes artificiais no perfil da comunidade microbiana cutânea de *Scinax alcatraz* (Anura: Hylidae)

Effects of artificial environments on the profile of cutaneous microbial community of Scinax alcatraz (Anura: Hylidae)

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Orientador: Prof. Dr. Carlos Arturo Navas Iannini

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Prof. Dr. Carlos Arturo Navas Iannini

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À minha família.

"An understanding of the natural world and what's in it is a source of not only a great curiosity but great fulfillment."

Sir David Attenborough

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Índice

Resumo Geral

Anfíbios possuem uma microbiota cutânea que os protege contra patógenos. Essa proteção se dá pela produção de moléculas antimicrobianas e pela competição por espaço e nutrientes contra patógenos. Alterações na composição da microbiota, causadas por fatores bióticos e abióticos do ambiente e por fatores ecofisiológicos do hospedeiro, podem afetar a resistência dos anfíbios à doenças. Assim, é possível que ambientes artificiais, por conter condições ambientais diferentes dos naturais e por alterarem aspectos ecofisiológicos dos indivíduos, devem modular a microbiota cutânea de animais mantidos e nascidos em cativeiro. Nós avaliamos diferenças inter e intra-populacionais no perfil da comunidade bacteriana de *Scinax alcatraz* entre três grupos: indivíduos selvagens; indivíduos nascido em cativeiro; e indivíduos mantidos em cativeiro por dois anos. Também verificamos o efeito temporal de ambientes artificiais no perfil da microbiota cutânea entre e dentre indivíduos selvagens mantidos em cativeiro ao longo de 312 dias. Os parâmetros microbiológicos utilizados foram riqueza de morfotípos bacterianos e abundância de colônias bacterianas. As diferenças encontradas entre populações apontam para o ambiente como um importante modulador da microbiota cutânea. No entanto, as diferenças encontradas entre indivíduos de uma mesma população apontam para a importância de aspectos fisiológicos do hospedeiro na modulação. Por fim, a avaliação temporal foi importante para mostrar que tanto aspectos ambientais quanto aspectos ecofisiológicos atuam juntos na modulação da comunidade bacteriana cutânea de anfíbios mantidos em cativeiro.

Palavras-chave: Conservação; declínios populacionais; doenças emergentes; microrganismos; perereca-de-alcatrazes

Abstract

Amphibians harbor a skin microbiota that provides protection against pathogens. This protection happens by production of antimicrobial substances and by competition for space and nutrients against pathogens. Changes in the microbiota composition, caused by biotic and abiotic factors of the environment and ecophysiology factors of the host, may affect disease resistance of amphibians. As artificial environments contain different environmental conditions compared to natural ones and may alter physiological aspects of individuals, it may modulate the cutaneous microbiota of captive animals. Our study evaluated inter- and intra-population differences in the profile of bacterial community of *Scinax alcatraz* from three distinct groups: wild individuals; individuals born in captivity and individuals kept in captivity for two years. We also investigated the temporal effects of artificial environments on the cutaneous microbiota profile between and within wild individuals kept in captivity over 312 days. Microbiological parameters analyzed were richness of bacterial morphotypes and abundance of bacterial colonies. The differences found between populations show that the environment may be an important modulator of the microbial community. However the differences between individuals within a population demonstrate the importance of physiological aspects of the host for the composition of the microbiota. Finally, the temporal evaluation performed was important to show that both environmental and ecophysiological aspects act together in modulating the cutaneous microbiota community of amphibians kept in captivity.

Keywords: Alcatraz snouted treefrog; conservation; emerging diseases; population declines; microorganisms

Introdução Geral

1. Declínios populacionais, extinções e doenças patogênicas

Os anfíbios estão sofrendo declínios populacionais e extinção de espécies ao redor do mundo (Carey; Cohen; Rollins-Smith, 1999; Eterovick et al., 2005; Heyer et al., 1988; Lips; Burrowes; Mendelson III, 2005; ; Mendelson III et al., 2006; Pouds et al., 2007). Das quase 7.500 espécies de anfíbios conhecidas (AmphibiaWeb, 2016), 32% estão categorizadas como Vulnerável, Em Perigo ou Criticamente Em Perigo de Extinção (IUCN Red List, 2008) e cerca de 168 espécies são consideradas extintas (Stuart et al., 2004). Apesar de existirem há mais de 300 milhões de anos, os declínios e as extinções estão sendo reportados nas últimas décadas e são ocasionados principalmente por impactos causados por atividades humanas (Blaustein; Wake; Sousa, 1994; Stuart et al., 2004). Destruição, alteração e fragmentação de habitat, poluição ambiental, introdução de espécies exóticas e sobre-exploração são ações antropogênicas responsáveis pelo cenário de declínio mundial dos anfíbios (Berger et al., 1998; Blaustein; Wake; Sousa, 1994; Pounds; Carnaval; Corn, 2007.) No entanto, mesmo em locais sem atividade humana e relativamente preservados, muitas populações de anfíbios declinaram e espécies foram extintas (Bradford; Graber; Tabatabai, 1994; Lips, 1999). Nesses casos, mudanças climáticas e doenças infecciosas são consideradas as principais ameaças (Carey; Cohen; Rollins-Smith, 1999; Carey, 2000; Daszak et al., 1999; Lips, 1999; Pounds; Crump, 1994).

Apesar de patógenos sempre existirem na natureza e terem um papel fundamental na dinâmica de populações (Anderson; May, 1986), os recentes declínios e mortalidade em massa dos anfíbios estão associados à doenças emergentes (Carey; Cohen; Rollins-Smith, 1999; Carey, 2000; Daszak et al., 1999), ou seja, patógenos causadores dessas doenças que podem ter aumentado sua

incidência, sua patogenicidade e sua área de ocorrência (Daszak; Cunningham; Hyatt, 2000). Exemplos de patógenos causadores de doenças emergentes em anfíbios incluem fungos, vírus e bactérias (Cunningham et al.,1996). A quitridiomicose é uma doença causada pelo fungo patogênico *Batrachochytrium dendrobatidis* (*Bd*) (Pessier, 1999). Esse fungo infecta partes queratinizadas de anfíbios, como por exemplo a pele de indivíduos adultos e órgão bucal de girinos, e é responsável por declínios populacionais de anfíbios na Austrália, na America Central e nos Estados Unidos (Berger et al., 1998; Longcore; Pessier; Nichols, 1999; Pessier et al., 1999). Exemplo de vírus responsável por declínios de anfíbios em diversos lugares do mundo é o *Ranavirus*, um tipo de iridovirus que, assim como o *Bd*, infecta a pele dos anfíbios (Cunningham et al., 1996). A síndrome da pata vermelha é causada pela bactéria *Aeromonas hydrophila* e também pode infectar o indivíduo pela pele e pelo trato intestinal (Hunsaker; Potter, 1960).

Apesar desses patógenos estarem amplamente distribuídos pelo mundo, nem todas as populações e espécies de anfíbios são infectadas ou adquirem as doenças. Por exemplo, Daszak et al. (2004) mostrou que a espécie *Lithobates catesbeianus* (rã-touro) pode ser infectada pelo fungo *Bd* porém não apresenta sintomas, enquanto que outras espécies que habitam o mesmo local podem desenvolver a doença. A variação na susceptibilidade ao fungo *Bd* também pode ser observada entre populações de uma mesma espécie de anfíbio. Por exemplo, duas populações das espécies *Rana muscosa* e *R. sierrae* foram extintas em parques nacionais da Califórnia por causa da doença quitridiomicose enquanto que outras populações das mesmas espécies, que habitam diferentes locais, continuaram persistindo mesmo infectadas pelo fungo *Bd* (Vredenburg et al., 2010). A variação na susceptibilidade à infecções de indivíduos, populações e espécies pode ser devido tanto à diferenças

na virulência do patógeno em diferentes áreas geográficas quanto a mecanismos de defesa dos anfíbios (Carey; Cohen; Rollins-Smith, 1999).

2. Mecanismos de defesa inato, microbiota cutânea e fatores moduladores da comunidade microbiana cutânea

Os primeiros mecanismos de defesa dos anfíbios contra microrganismos patogênicos incluem respostas imunológicas inatas da pele e do sistema digestivo (Carey; Cohen; Rollins-Smith, 1999). Como microrganismos patogênicos normalmente infectam os anfíbios pela pele, a mesma se torna a primeira barreira contra infecção (Carey; Cohen; Rollins-Smith, 1999). Além de conferir uma barreira física, as defesas da pele incluem mecanismos bioquímicos e biológicos (Assis, 2012). A defesa bioquímica faz parte do sistema imune inato e provém da secreção de peptídeos antimicrobianos pelas glândulas granulares (Carey; Cohen; Rollins-Smith, 1999; Zasloff, 2002). A defesa biológica, considerada uma extensão do sistema imune inato, é conferida pela comunidade microbiana associada a pele dos anfíbios (Woodhams et al., 2007). A pele dos anfíbios é considerada um substrato adequado para o estabelecimento de microrganismos simbiônticos, pois através da secreção de muco pelas glândulas mucosas, confere umidade e nutrientes necessários para o crescimento de microrganismos (Harris et al., 2006; Lauer et al., 2007). Enquanto a pele oferece condições favoráveis para o estabelecimento de microrganismos, os mesmos conferem proteção contra patógenos, tanto por produzir moléculas com potencial antimicrobiano, como também por competir com os patógenos por espaço e por nutrientes (Lauer et al., 2008).

A comunidade microbiana cutânea dos anfíbios é composta por uma variedade de fungos e bactérias (Austin, 2000; Culp; Falkiniiam; Belden, 2007), e a composição adequada destes microrganismos é possivelmente importante para a

proteção do hospedeiro contra patógenos (Loudon et al., 2014). A composição inicial da microbiota cutânea dos anfíbios deve ocorrer pelo contato do hospedeiro com o ambiente, pois os microrganismos presentes na pele de alguns anfíbios foram também identificados na água, no solo e em plantas presentes no habitat do indivíduo (Culp; Falkiniiam; Belden, 2007; Loudon et al., 2014). A transmissão também deve ocorrer pelo contato com outros indivíduos da mesma espécie, por exemplo no cuidado parental e na reprodução, e pelo contato com indivíduos de outras espécies (Banning et al., 2008; Lauer et al., 2007; Walke et al., 2011). No entanto a comunidade microbiana cutânea não é estática, podendo ser modulada por fatores que serão abordados a seguir:

2.1. Fatores ecofisiológicos do hospedeiro

Como explicado anteriormente, a pele dos anfíbios possui substâncias antimicrobianas e muco produzidos por glândulas granulares e mucosas respectivamente. Os peptídeos antimicrobianos são responsáveis por inibir o crescimento de certos microrganismos na pele dos animais, sendo estes patogênicos ou não (Rollins-Smith et al., 2002; Woodhams et al., 2006). Como cada espécie de anfíbios produz diferentes perfis de peptídeos antimicrobianos (Conlon et al., 2007), as comunidades microbianas cutâneas dessas espécies poderão ser diferentes entre si. Já o muco presente na pele dos indivíduos atua como fonte de nutrientes para os microrganismos crescerem (Duellman; Trueb, 1994; Brizzi; Delfino; Pellegrini, 2002). Assim, alterações nessa secreção, que podem ser ocasionadas por aspectos nutricionais do hospedeiro, também poderão modular a composição de microrganismos na pele.

2.2 Fatores ambientais

Os microrganismos são sensíveis a fatores ambientais como temperatura, pH, umidade e radiação e necessitam de condições adequadas para sobreviverem (Madigan et al., 2009). Com isso, mudanças nos fatores abióticos do ambiente no qual o microrganismo vive irão afetar sua taxa de crescimento. Dito isso, como a pele dos anfíbios atua como substrato para colonização de microrganismos, alterações abióticas, tanto na pele quanto no microambiente que o hospedeiro habita, poderão modular a comunidade microbiana presente na pele dos indivíduos. Por fim, os fatores ambientais podem atuar em sinergismo com fatores ecofisiológicos do hospedeiro. Por exemplo, mudanças nos fatores abióticos do ambiente podem afetar a disponibilidade de alimentos e, consequentemente, afetar a composição do muco secretado pelas glândulas mucosas na pele dos anfíbios. Não só a composição das secreções, mas também alterações nas taxas de secreções, tanto das substâncias mucosas quanto dos peptídeos antimicrobianos, poderão afetar a composição da comunidade microbiana cutânea dos anfíbios. As alterações nas taxas de secreção do muco podem ser causadas por mudanças na termoregulação do indivíduo (Lillywhite, 1974) e as alterações nas taxas de secreção de peptídeos antimicrobianos podem ser acarretadas por exemplo, pela contaminação do indivíduo por poluentes presentes no ambiente (Davidson, 2007). Dado todos os fatores que podem compor e modular a comunidade microbiana cutânea dos anfíbios, é provável que espécies, populações e até indivíduos possuam distintas comunidades microbianas entre si.

3. Conservação *ex situ* de anfíbios e contextualização do trabalho

Uma das estratégias para a proteção de espécies animais que sofrem risco imediato de extinção é a utilização de programas de reprodução e manutenção em cativeiro. Este tipo de conservação assegura o estabelecimento de populações para

que, se houver necessidade, as mesmas sejam reintroduzidas na natureza (Griffiths; Pavajeau, 2008; Mendelson III et al., 2007). Essa estratégia têm sido amplamente utilizada ao redor do mundo por zoológicos, aquários e jardins botânicos. (Conde et al., 2011; Gordon; Zippel, 2008; Hutchins; Conway, 1995). No entanto, condições de cativeiro podem implicar em alterações fisiológicas e comportamentais nos indivíduos, o que pode comprometer o estabelecimento dessas populações na natureza.

No Brasil, somente a espécie *Scinax alcatraz* é contemplada em programas de conservação *ex situ* (Lisboa; Vaz, 2012). Essa espécie de anuro é considerada criticamente ameaçada de extinção (Rodrigues; Cruz, 2004) e é endêmica da Ilha dos Alcatrazes, situada a 35km da costa de São Sebastião (SP, Brasil) (Brasileiro, 2008). Atualmente a espécie sofre com possíveis ameaças como perda de hábitat por ações humanas, desastres naturais, variações climáticas e introdução de doenças (Lisboa; Vaz, 2012). O "Projeto de conservação *ex situ* de *S. alcatraz*", realizado pela Fundação Parque Zoológico de São Paulo (FPZSP), visa manter e reproduzir a espécie em cativeiro para, caso necessário, realizar uma suplementação ou reintrodução de indivíduos cativos na natureza (Rodrigues; Cruz, 2004; Lisboa; Vaz, 2012). Dado que, fatores ambientais e ecofisiologicos do hospedeiro influenciam a composição da microbiota cutânea dos anfíbios, é possível que animais selvagens mantidos em cativeiro e animais nascidos em cativeiro apresentem uma composição microbiana cutânea diferente de animais selvagens. Além disso, dado todos os fatores que podem compor e modular a comunidade microbiana cutânea dos anfíbios, é provável que espécies, populações e até indivíduos possuam distintas comunidades microbianas entre si. Com isso, a presente pesquisa teve como objetivo investigar os efeitos de ambientes artificiais no

perfil de comunidades microbianas cutâneas de *S. alcatraz*, resultando em dois capítulos distintos.

O foco do primeiro capitulo foi verificar possíveis diferenças no perfil da microbiota cutânea entre três populações distintas de *S. alcatraz*: população selvagem; população nascida em cativeiro; e população de indivíduos selvagens mantidos em cativeiro por cerca de 2 anos. Também verificamos se, ao longo do tempo, ocorrem variações na microbiota cutânea de indivíduos selvagens mantidos em cativeiro (de 0 a 312 dias em cativeiro). O segundo capítulo teve como foco principal investigar variações individuais na composição da microbiota cutânea de *S. alcatraz*. Para isso, investigamos diferenças entre indivíduos de uma mesma população (variação inter-individual) e diferenças dentre cada indivíduo ao longo do tempo (variação intra-individual). Investigando variações intra-individuais, foi possível também verificar se a comunidade microbiana cutânea é estável e resiliente, ou seja, se após uma mudança na composição, a comunidade é capaz de se recompor. Para a realização da pesquisa, utilizamos técnicas de cultivo bacteriano, e as comparações foram baseadas na riqueza de morfotípos bacterianos e abundância de colônias bacterianas.

Capítulo 1

Captive environments modify the cutaneous bacterial community of anurans

Captive environments modify the cutaneous bacterial community of anurans

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Abstract

Amphibians harbor a symbiotic microbial community on their skin that protects them against pathogenic microorganism. Given that biotic and abiotic factors affect the characteristics of the cutaneous microbiota of amphibians, we investigated if the translocation of specimens from their natural environment to captive environments as well as breeding individuals under artificial conditions may alter the composition of symbiotic skin bacteria of amphibians. Using culturing methods, we compared bacterial morphotype richness and abundance of skin bacteria colonies of *Scinax alcatraz,* taking into consideration captive and wild animals, and we followed changes in the composition of this community through time in wild individuals placed in captivity for 39, 82, 116, 312 and 717 days. From 34 frogs, totalizing 78 samples, we isolated 575 morphotypes and estimated a range of 400 to 2000 colonies per frog. The richness of bacterial morphotypes in individuals kept in captivity decreased over time and captive born individuals presented fewer bacterial morphotypes on their skin when compared with wild individuals. The abundance of colonies did not vary among all grups. Although we do not understand the specific effects of these changes in the cutaneous microbiota diversity of amphibians yet, we suggest that this important amphibian defense mechanism should be considered in *ex situ* conservation

programs because it may have an important role in the sustainability of reintroduced populations.

Keywords: captive breeding, disease susceptibility, amphibian population declines, skin-associated bacteria.

1. Introduction

Amphibians are experiencing population declines and extinction worldwide and one of the causes that has been associated with these events is the emergence of infectious diseases caused by pathogenic microorganism such as fungus (Berger et al., 1998; Carey; Cohen; Rollins-Smith, 1999; Lips, 1998), virus (Daszak et al., 2007) and bacteria (Carey, 1993). Because this is not a universal trend, and some amphibian populations are able to coexist with such pathogens without acquiring diseases (Carey; Cohen; Rollins-Smith, 1999), variation in sensitivity to infections must exist among species. This variation possibly occurs due to differences in the host's adaptive and innate immune system responses (Belden; Harris, 2007).

Besides the immune system of amphibians, aspects of skin morphology combined with ecophysiological traits enhance protection against pathogens (Carey; Cohen; Rollins-Smith, 1999). The skin of amphibians, a physical barrier, acts as a first line of defense against pathogenic microorganisms. Additionally, it hosts a symbiotic microbiota (Harris et al., 2006; Harris et al., 2009; Lauer et al., 2007; Lauer et al., 2008) and may display antimicrobial peptides that prevent infections (Rollins-Smith et al., 2011). Such peptides can be secreted by the granular glands of the skin (Zasloff, 2002) and by symbiotic microorganisms that colonize the skin of the host (Woodhams et al., 2007). These microorganisms comprise a cutaneous microbial community consisting of bacteria and fungi that, besides secreting antimicrobial

substances, compete for space and resources, preventing alternative microorganisms, including pathogens, from invading the host's skin (Lauer et al., 2007; Lauer et al., 2008; Harris et al., 2009; Woodhams et al., 2007).

The profile of the cutaneous bacterial community of anurans may vary among and within species due to physiological, ecological and behavioral aspects of the host, including not only skin physiology (e.g. temperature, pH, and resources available to microorganisms in the skin) (Meyer et al., 2012) but possibly also microhabitat selection and patterns of activity. For example, a likely physiological aspect modulating cutaneous microbial communities is the secretion profile of granular and mucous glands. This is so for secretions may provide nutrients for symbiotic bacteria to stabilize on the skin (Belden; Harris, 2007), and simultaneously may inhibit the growth of certain bacterial types (Loudon et al., 2014). In turn, at least in some species, the pattern of secretion of the glands seems to vary in the context of behavioral thermoregulation, with enhanced secretions as temperatures rise (Lillywhite, 1974). In addition, species may differ in individual life areas and habitats, which matters because soil and water are reservoirs of microorganisms. Finally, the contact of the host with other individuals (e.g. parental care and mating) and other species may also modulate the composition of the cutaneous bacterial community of the host (Belden; Harris, 2007; Lauer et al., 2008; McKenzie et al., 2012; Woodhams et al., 2007).

Because the above mentioned characteristics of the host and of the environment may be direct and indirect modulators of the cutaneous bacterial community of amphibians, changes are expected when individuals are moved to new habitats or bred under artificial conditions. If this premise holds, practices used in conservation such as translocation of specimens from their natural environment to

facilities, and breeding individuals in captivity, may alter the bacterial communities found in the skin of these individuals, relative to wild counterparts (Antwis et al., 2014; Loudon et al., 2014). These alterations may be due to husbandry conditions, which likely change the physicochemical traits of the skin not only for the reasons discussed above but also for captive animals usually access a lower range of food items. Given that theory predicts changes in the microbiota of amphibians kept in captivity and that one of the approaches to minimize amphibian's population declines and extinctions is *ex situ* conservation programs, we aim to understand how the transfer of individuals from natural habitat to captive environments affects the cutaneous bacteria community of amphibians.

Our focal species is *Scinax alcatraz*, a highly endemic and insular hylid species that has been bred in an artificial environment as part as an *ex-situ* conservation program hold at the São Paulo Zoo. Using conventional culturing methods, we characterize and compare the cutaneous bacterial community of wild and captive-born individuals of *Scinax alcatraz,* focusing on bacterial morphotype richness and abundance of colonies. We also analyzed changes in the bacterial community of wild animals transferred and maintained in captivity over time. In addition, we investigated if gender and life stage could influence the profile of the bacterial community.

Given differences between environmental bacterial communities and abiotic aspects under each condition, shifts in skin bacterial communities were expected, although current theory does not allow anticipating which changes may occur. We analyze the dynamics of these changes to enhance understanding on the effects of captivity and maintenance in the target species, and to propose generalizations to be tested in other anuran species.

2. Methods

2.1. Species information

Scinax alcatraz (Lutz, 1973) is a bromelicolous tree frog, endemic of Ilha dos Alcatrazes (São Sebastião, São Paulo, Brazil) and is listed as “Critically Endangered” on the International Union for the Conservation of Nature (IUCN) Red List (Rodrigues; Cruz, 2004). Due to the limited natural occurrence, this species is susceptible to threats that can decimate the population, such as anthropogenic habitat destruction, natural disasters or the introduction of new predators or diseases (Brasileiro, 2008; Lisboa; Vaz, 2012; Rodrigues; Cruz, 2004). Because of these treats, in 2008, the São Paulo Zoo initiated an *ex situ* conservation program for this species, with *in situ* monitoring and has succeed in reproduce and maintain the species in captivity.

2.2. *Ex situ* maintenance

Sao Paulo Zoo, under permission of SISBIO (19200-2), collected eleven wild animals in 2011 to be the founders of the captive population we used to carry out this project. In addition, we collected eleven animals directly from the island in October 2013. The captured animals were treated using the protocols approved by the São Paulo Zoo. In short, animals are maintained in an isolated room to avoid contamination by contact with other amphibian species of the zoo collection or the native species of the zoo's forest area. To guarantee this isolation, all incoming material is disinfected and standard clothing practices applyied (Pessier; Mendelson, 2010). The animals are held in glass aquariums with some plants for refuge and a water pot that is replaced every two days. They are feed with newborn crickets (*Gryllus* sp.) dusted with Repashy Superfoods Calcium Plus ICB® vitamins twice a week. During this study, room temperatures ranged from 11.5°C to 27.1°C (M =

21°C) and the ambient humidity ranged from 54% to 99% (M = 86.37%). For the reproduction of the species the same protocol developed with *S. perpusillus* was used (Lisboa; Vaz, 2012).

2.3. General approach

To investigate if the profile of the cutaneous bacteria is different between individuals of *S. alcatraz* in natural and artificial habitat conditions, we compared morphotype richness and abundance of skin bacteria colonies between wild animals and two groups of captive animals. To obtain the microbiota profile for wild animals we collected bacterial samples from the skin of eleven individuals straight in the island. One captive group was composed by animals born in captivity in 2012 (N=17) and the other composed by animals (N=6) collected from the island in 2011 and sampled after 717 days of life in captivity (from now on will be called *Transported Group*) under the same conditions than Zoo-born individuals. All the animals sampled in the wild and the captive born animals were juveniles, with no gender distinction. In the transported group there were two females, three males and one juvenile with no gender distinction. All bacterial samples were taken in October 2013.

To investigate if the profile of the cutaneous bacteria could be affected by captive environment in a time course, we sampled the wild animals placed in captivity in October, November and December 2013 and in February and August 2014 (0, 39, 82, 116 and 312 days in captivity respectively).

2.4. Bacterial sampling

Fresh sterile gloves were used to handle each animal in order to avoid any sort of contamination. Prior to bacterial sampling, the animals were rinsed with sterile ultrapure water to remove any transient bacteria (Lauer et al., 2007). Sampling

consisted of swabbing the animals, with a sterile cotton swab (Assis, 2011) five times throughout the dorsal surface, including the head and five times throughout the ventral surface including the throat region. This was repeated two times for each animal. For culturing and isolating bacteria the swabs were streaked onto different plates containing low nutrient Difco R2A media (Lauer et al., 2007) with antifungal solution. The plates were stored for 24 and 48 hours in room temperature (M = 23C, +- 2C) for bacterial growth. We characterized all different morphotypes to estimate the bacterial richness and counted colonies by plate to analyze bacterial density (Assis, 2011). The characterization of morphotypes was based on color, border format, brightness and surface appearance.

2.5. Statistical analysis

All statistical analyses were carried out using IBM SPSS statistic (version 23 for MAC). To address whether morphotype richness and abundance of colonies varied between wild, captivity born and transported individuals we applied normality tests and compared the results using one-way ANOVA analysis. For the comparison between each group the student t-test was applied. To determine if time in captivity affected the bacterial community of individuals we conducted one-way ANOVA variance analysis between all the groups and used a paired t-test to compare each situation between each group.

3. Results

From 34 frogs, totalizing 78 samples, we found 575 bacterial morphotypes. We isolated 2 – 20 morphotypes per individual (M = 6,9; SD = 3,56) and estimated a range of 400 – 2000 colonies per frog (M = 1143,75; SD = 356,29). The amount of

bacterial morphotypes varied between wild and captive animals (ANOVA, $F_{2,31}$ = 13,203, *P* = 0,001), so that wild animals displayed the most diverse microbiota from 2 to 20 morphotypes per individual (M = 11,18, SD = 5,41). Both groups kept in captivity displayed much less morphotype richness, and the transported group exhibited with 3 – 5 morphotypes per frog (M= 4,16; SD = 0,75) (Figure 1).

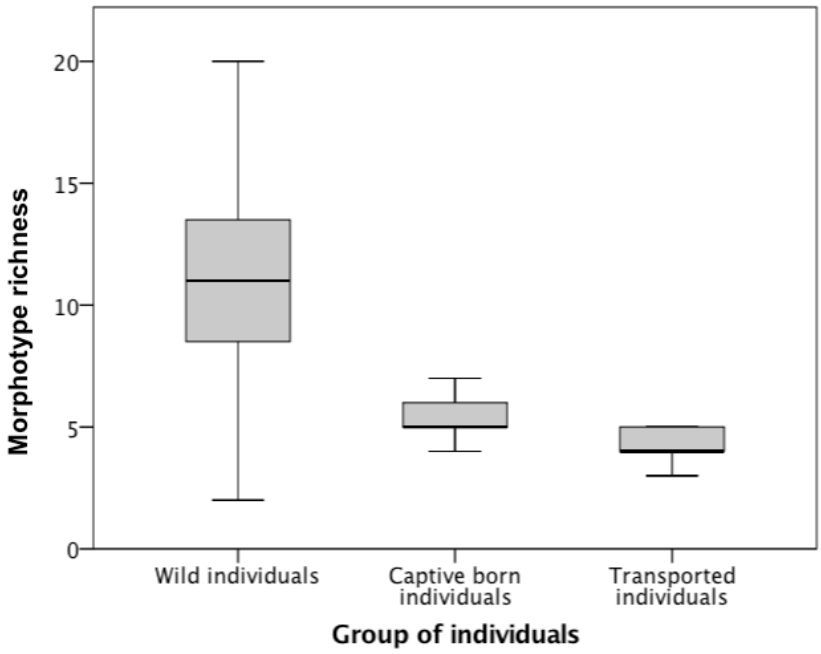

Fig 1. Morphotype richness of bacterial isolated from the skin of wild, captive born and transported individuals of *Scinax alcatraz*. The number of morphotypes of wild animals differed significantly compared with captive born animals (two-sample t(11) = 3,4, *P* = 0.005) and with transported animals (two-sample t(10) = 4,1, *P* = 0,002). The number of morphotypes of animals born in captivity did not differed when compared with transported (two-sample t(21) = 1,9, *P* = 0.068). Dispersions bars represent the greatest and least values of all data.

The bacterial community varied significantly over time in captivity (ANOVA, $F_{4,36}$ = 5,261, *P* = 0,02). The number of morphotypes in wild animals was highest after capture and declined thereafter and remained mostly constant throughout time (with one unexplained episode of fluctuation on day 82, Figure 2). It is possible to

observe that there was a difference in response when comparing the groups pairwise (Figure 2).

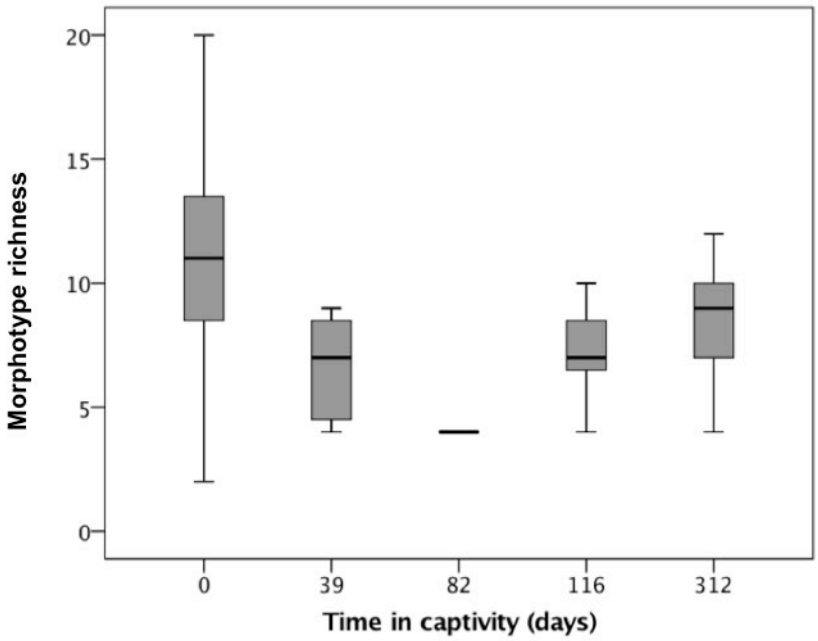

Fig. 2. Morphotype richness of bacterial isolated from the skin of individuals of *Scinax alcatraz* kept in captivity over time. There was a significant variation on the morphotype richness between all groups (ANOVA, $F_{4,36}$ = 5,261, *P* = 0,02), however, when comparing pairwise, the numbers of morphotypes differed significantly between individuals from 0 days in captivity with individuals kept in captivity for 82 days (two-sample t(6) = 2,9, *P* = 0.028) and for 312 days (two-sample t(8) = 3,2 *P* = 0.012). There was no significantly difference in the number of morphotypes of individuals kept in captivity for 39 days (two-sample t(6) = 0,96 *P* = 0.375) and for 116 days (two-sample t(6) = 0,95 *P* = 0.38). Dispersions bars represent the greatest and least values of all data.

Life stage or gender influenced neither the morphotype richness (ANOVA $F_{1,20}$ = 1,75, *P* = 0,21) nor the abundance of colonies (ANOVA, $F_{1,20}$ = 4,26, *P* = 0,069) and the number of colonies did not differ among all groups sampled (ANOVA $F_{1,20}$ = 0,01, *P* = 0,91).

4. Discussion

We demonstrate that captivity influences the cutaneous bacteria community of one amphibian species. The cutaneous bacterial community of wild individuals of *S. alcatraz* changes after captive conditions are imposed. Similarly, Kung et al. (2014) described a gradual change in the cutaneous bacteria of the frog *Colostethus panamansis* after 48 days of maintenance in captivity, and the skin microbial communities of the salamander *Plethodon cinereus* also changed from after being kept in captivity (Loudon et al., 2014). However, despite initial changes in captivity, Loudon et al., (2014) verified that the cutaneous bacterial community of the salamander *Plethodon cinereus* remains stable in individuals maintained in captivity for 28 days. Our study does not support such pattern of stability and the cutaneous microbiota of *S. alcatraz* may shift over time even under rather constant conditions in captivity. However, the salamanders in the above cited study were maintained in contact with their natural substrate, a possible pool of bacteria closely related to natural conditions, whereas individual *S. alcatraz* were maintained in sterile substrates. Generalizations are not yet possible, but physiological changes to captivity (Berner; Heil; Romero, 2013), changes in environmental bacterial communities (Loudon et al., 2014) and changes in environmental conditions possibly matter. The overall data available stresses the importance of environmental bacterial communities in the microhabitat as a possible reservoir influencing cutaneous bacteria communities in amphibians (Loudon et al., 2014).

It is possible that the relation between cutaneous and environmental bacterial communities are a main component explaining why captive born individuals of *S. alcatraz* present lower skin bacterial morphotype when compared with wild animals. The cutaneous bacteria of captive born individuals must come from the contact of

individuals with substrate, food and housing material, resulting in comparably lower diversity (Loudon et al., 2014). In contrast, wild animals are likely exposed to a higher diversity of bacterial sources in the environment (McKenzie et al., 2012). However, indirect effects cannot be discarded as partial explanations to the observed pattern. Wild amphibians have access to a broad range of dietary items and nutrients that likely influence production of antimicrobial peptides and mucous (Antwis et al., 2014), which provide nutrition and alter the chemical skin environment for cutaneous bacteria (Brizzi; Delfino; Pellegrini, 2002; Lauer et al., 2007). Captive individuals of *S. alcatraz* receive monotypic diets which may modify the skin secretions and consequently change the composition profile of the bacterial community. Finally, given that at least in some amphibians the secretion of mucous glands is also affected by thermoregulation (Lillywhite; Licht, 1975), changes in the thermal environment may affect the amount of nutrients secreted and thus the structure of cutaneous bacterial communities. Thermal environment changes between natural habitat and captivity may explain the fluctuations of the bacterial community of individuals of *S. alcatraz* after being maintained in captivity overtime.

Overall, our study demonstrates an unambiguous pattern, that the community of *S. alcatraz* changes over time in captivity decreasing morphotype richness, and that captive-born individuals have comparatively simpler cutaneous bacterial communities. As the cutaneous bacterial community of amphibians is an important defense mechanism in disease resistance (Belden; Harris, 2007; Lauer et al., 2008; Harris et al., 2009), and that *ex situ* conservation programs are an important strategy to minimize population declines and protect amphibian species from extinction (Conde et al., 2011; Gordon; Zippel, 2008; Hutchins; Conway, 1995; Mendelson III et al., 2007), further researches to understand which mechanisms (e.g. changes in

environmental bacterial communities, changes in diet, shifts in thermal environment, or combinations of all these factors) influence the bacterial community of *S. alcatraz* in captivity need to be addressed.

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Capítulo 2

Inter and intra-individual differences and temporal variation in the composition of the cutaneous microbial community of amphibians

Inter and intra-individual differences and temporal variation in the composition of the cutaneous microbial community of amphibians

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Abstract

Skin microbial community of amphibians is an important defense mechanism against pathogenic microorganism and the correct assemblage of skin microbes is important in disease resistance. Environmental factors and host aspects may be relevant on the modulation of the community between and within individuals and differences on the cutaneous microbiota of individuals may affect protection. Herein we analyzed inter and intra-individual differences on the cutaneous bacterial community of *Scinax alcatraz.* To investigate differences between individuals, we compared the bacterial morphotype richness of each animal in three different groups (wild individuals; captive born individuals; and wild individuals living in captivity. To investigate differences within individuals and consistence, we compared morphotype richness from wild animals placed in captivity from overtime (0, 39, 82, 116 and 312 days in captivity). The community composition of the animals was different between each other in the three groups analyzed. However the group of wild animals presented greater number of bacterial morphotypes when comparing to the other two. The cutaneous bacterial community also differs within individuals over a period of time and it is not consistent. In most of the cases, bacterial morphotype richness decreased but some individuals increased in number of bacterial morphotype over time. Overall this study demonstrated that differences in the microbiota community

occur between and within individuals and these differences may be due to both environmental and host factors.

Keywords: *ex situ* conservation; host-bacteria associations; immune defenses; infection

1. Introduction

Symbiotic relationships with microrganims are important in disease resistance of several animals and plants (Dethlefsen; Mcfall-Ngail; Relman, 2007; Gil-Turnes; Hay; Fenical, 1989; Kaltenpoth et al., 2005). Overall, amphibians harbor a symbiotic cutaneous microbiota that, along with other defense mechanisms (e.g. immune system) protects them against pathogenic microrganisms. This protection happens through the production of antimicrobial substances and through competition for space and resources against pathogens (Becker; Harris, 2010; Brucker et al., 2008; Harris et al., 2006; Lauer et al., 2007; Woodhams et al., 2007). Despite this protection, populations and species of amphibians can differ in their susceptibility to pathogens (Belden; Harris, 2007; McKenzie et al., 2012; Woodhams; Bigler; Marschang, 2012). While some amphibians are susceptible to infections, some are resistant and others are known to be infected but do not develop the disease (Daszak et al., 2004; Woodhams; Bigler; Marschang, 2012). This variation in disease resistance may be associated with differences in the immune system responses and in the cutaneous microbiota community of the individuals (Brucker et al., 2008; Flechas et al., 2012; Harris et al., 2009; Robinson; Bohannan; Young, 2010; Woodhams et al., 2007).

The skin microbiota of amphibians is composed by a diverse group of fungi and bacteria (Lauer et al., 2007), and the adequated assemblage of microganisms

(e.g. density and diversity of microbial species) is probable to be important for protecting them from infectious disease (Loudon et al., 2014). This assemblage must be acquired through transmission between the host and the environment and through the interaction between the host and other individuals (Antwis et al., 2014). However, the bacterial community is not static and may be modulated by environmental aspects and ecophysiological factors of the host. Examples of environmental factors affecting cutaneous microbiota of amphibians are the microbes found in the host's microhabitat and abiotic factors of the environment. As the environmental microbial community acts as a reservoir for the microbiota of the host, different environmental conditions might modulate host microbiota assemblages. Also, abiotic factors of the environment, such as temperature, humidity, pH and radiation levels affect growth of microganisms, modifying the skin microbiota community of the host. The ecophysiological aspect, that may modulate the cutaneous microbiota community, is related to the skin secretion of amphibians. This secretion contains nutrients, that are known to be important for bacteria to grow, and antimicrobial substances that may select the microorganisms that will get to be established on the host skin. Therefore, changes on the secreted substances may select different microorganisms. Characteristics that can affect the secreted substances are, the nutrition aspect of the host, which may influence the profile of the secretions, and the environmental temperature level, which may affect the rate of secretion by mucous glands and the frequency of skin sloughing (Meyer et al., 2012), causing an effect on the abundance of skin microorganisms (Brucker et al., 2008; Harris et al., 2006; Harris et al., 2009; Rollins-Smith, 2009).

Since environmental and host factors may be modulators of the cutaneous community, individuals, population and species may exhibit distinct skin bacterial

assemblages and these differences may be important for disease resistance. Studies of cutaneous microbiota of amphibians are being performed to investigate differences in the microbiota community profile between population and species (Antwis et al., 2014; Assis, 2011; Flechas et al., 2012; McKenzie et al., 2012). Nevertheless, little is known about variation between and within individuals. Therefore, we aim to investigate the patterns of skin bacterial community variations, between individuals of the same microhabitat (inter-individual differences) and within individuals (intra-individual differences) over a period of time. Using bacterial culture methods, we characterize and compare the cutaneous bacterial community between individuals from natural environments and individuals from artificial environments. We also analysed intra-individual temporal differences on the bacterial community of captive individuals of *S. alcatraz.* Comparisons were based on the richness of bacterial morphotypes. Given that ecophysiological characteristics of the host as well as environmental aspects are important in the composition of the cutaneous microbiota, we expect differences on the skin bacterial profile between individuals. In addition, since artificial environments do not harbor a variety of microrganisms as natural environments and abiotic factors in captivity may be different from natural habitats, we expect the display of temporal disruption on the cutaneous microbiota of captive individuals. We also investigated if life stage, gender and weight have an effect in the composition of bacterial morphotype between individuals and if length and weight could influence the profile of the bacterial community within individuals over time.

When analyzing inter and intra-individual variation, we will be able to understand aspects that may modulate the skin bacterial communities of captive amphibians, if the skin bacterial community is resistant to changes or if it is resilient (Robinson; Bohannan; Young, 2010). Our species of interest is the Alcatraz Snouted

Tree Frog (*Scinax alcatraz* Lutz, 1973). This species was chosen because there are already two populations of individuals living in artificial environment: a population with captive born individuals; and a population of wild individuals transported to captivity. In addition, their cutaneous bacterial community has already been investigated in other studies. Futhermore this species is endemic of Ilha dos Alcatrazes (Sao Sebastiao, Sao Paulo, Brazil) and is considered “critically endangered” by IUCN red list (Rodrigues; Cruz, 2004). Due to the degree of threat, it is very important to study ecological, behavioral and physiological aspects of captive individuals to ensure a healthy population if reintroduction of the species in the natural environment is necessary.

2. Methods

2.1. Animal collection and husbandry

To achieve our goals, we used 34 individuals of *S. alcatraz* from three different populations: six animals collected by Sao Paulo Zoo in 2011, seventeen animals born in captivity in 2012 and we collected eleven animals from the island in October 2013. To avoid contamination from other species of the zoo collection and from native species of the zoo's forest area, the animals are being held in an isolated room and biosecurity measures are implemented. The animals are maintained in glass aquariums with plants for refuge and a water pot, which is replaced every two days. They are fed with newborn crickets (*Gryllus* sp.) dusted with Repashy Superfoods Calcium Plus ICB® vitamins twice a week. During the study, temperature levels varied from 11.5°C to 27.1°C (M = 21°C) and the ambient humidity ranged from 54% to 99% (M= 86.37%). For the reproduction of the species the same protocol developed with *S. perpusillus was used* (Lisboa; Vaz, 2012).

2.3. General approach

2.3.1. Inter-individual differences of bacteria morphotypes

To investigate whether there are differences in the profile of cutaneous bacterial community between individuals, we compared richness of bacterial morphotypes of each animal in three different groups: 1) eleven wild individuals; 2) seventeen captive born individuals; 3) six wild individuals sampled after 717 days in captivity (hereafter called *transported individuals*). All the animals sampled in the wild and the captive born animals were juveniles, with no gender distinction. The transported group had two females, 3 males and 1 juvenile, also with no gender distinction. All bacterial samples were taken in October 2013.

2.3.2.Temporal intra-individual differences and consistence of bacteria morphotypes

To investigate whether the profile of the cutaneous bacteria is consistent over time, we compared the bacterial morphotypes richness from wild animals placed in captivity from time to time. For this, after sampling the wild individuals directly in the island (0 days in captivity), the animals were transported in sterile plastic containers to the isolated facility at Sao Paulo zoo and maintained separated from other individuals. These animals were sampled in November and December 2013, February and August 2014 (39, 82, 116 and 312 days in captivity respectively).

2.4. Skin bacteria collection and isolation

Fresh sterile gloves were used to handle each animal in order to avoid contamination. Prior to bacterial sampling, the animals were rinsed with sterile ultrapure water to remove transient bacteria not associated with the skin (Lauer et al., 2007). Upon capture, each animal was swabbed with a sterile cotton swab, five times

on the dorsal surface, including the head and five times on the ventral surface, including the throat region (Assis, 2011). For culturing and isolation of bacteria, the swabs were streaked onto different plates containing low nutrient Difco R2A media (Lauer et al., 2007) with antifungal solution. The plates were stored for 24 and 48 hours in room temperature (mean = 23C, +- 2C) for bacterial growth. We characterized all the different morphotypes found in each plate based on color, border format, elevation, brightness and smooth or rough surface.

2.5. Statistical analysis

All statistical analyses were carried out using IBM SPSS statistic (version 23 for MAC). Normality tests were applied and all the data were normally distributed. To determine if life stage, gender, length and weight could influence the bacterial morphotype richness between and within individuals, we applied linear regression tests. Furthermore, in order to investigate inter-individual differences, we compared the number of bacterial morphotypes of each individual with others from the same group (wild individuals, captive born individuals, transported individuals). Additionally, to analyze temporal intra-individual differences of bacteria morphotypes we conducted one-way ANOVA variance analysis. Finally, to verify whether there is consistence in the bacterial profile over time, we performed intra-class correlation analyses and *Pearson* product-moment *correlation* coefficient.

3. Results

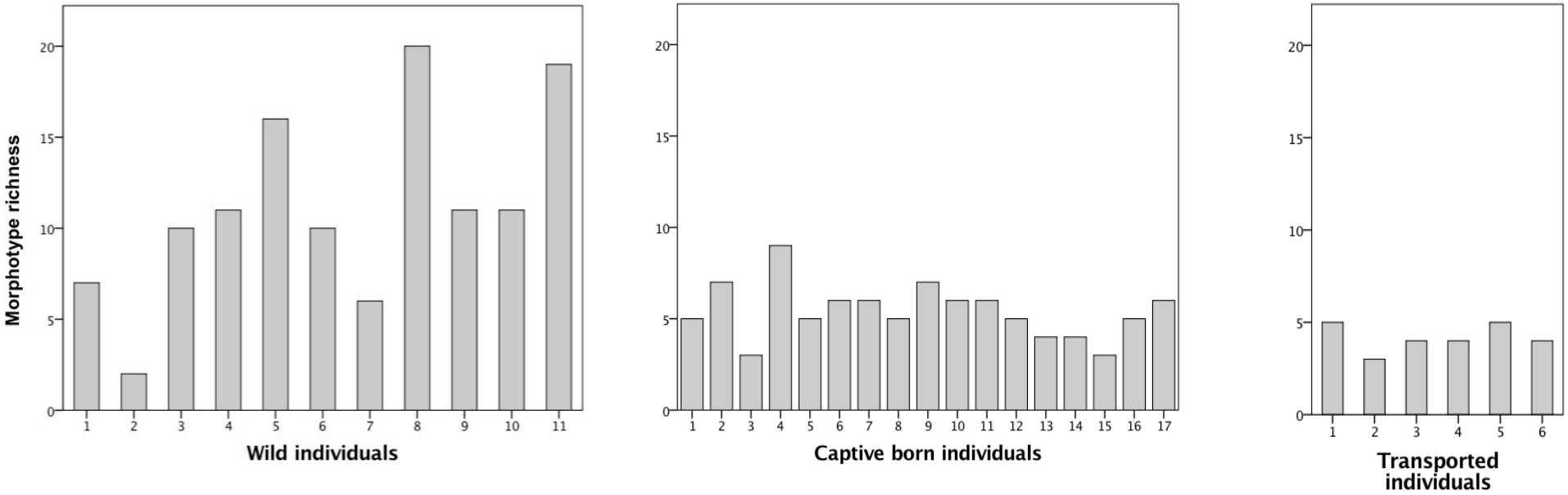
3.1. Inter-individual differences of bacteria morphotypes

From 34 frogs, considering all the individuals from 3 groups (wild animals, captive born individuals and transported individuals) we found 240 bacterial

morphotypes and we isolated 2 – 20 morphotypes per frog (mean = 7,06; SD = 4,32). Frogs that had the highest (20) and the lowest number (2) of morphotypes were from the wild group, which showed the highest mean and standard deviation (M = 11,18; SD = 5,42), in relation to number of bacterial morphotypes when compared with the captive born group (M = 5,41; SD = 1,50) and with the transported group (M = 4,17; SD = 0,75). When comparing inter-individuals differences, there was a significant difference in the bacterial morphotype richness between each individual in all 3 groups (Figure 1). Life stage ($F_{1,25}$ = 0,320; p = 0,577), sex ($F_{1,5}$ = 2,755; p = 0,158) and weight ($F_{1,19}$ = 0,005; p = 0,944) did not influence the number of bacterial morphotypes between individuals.

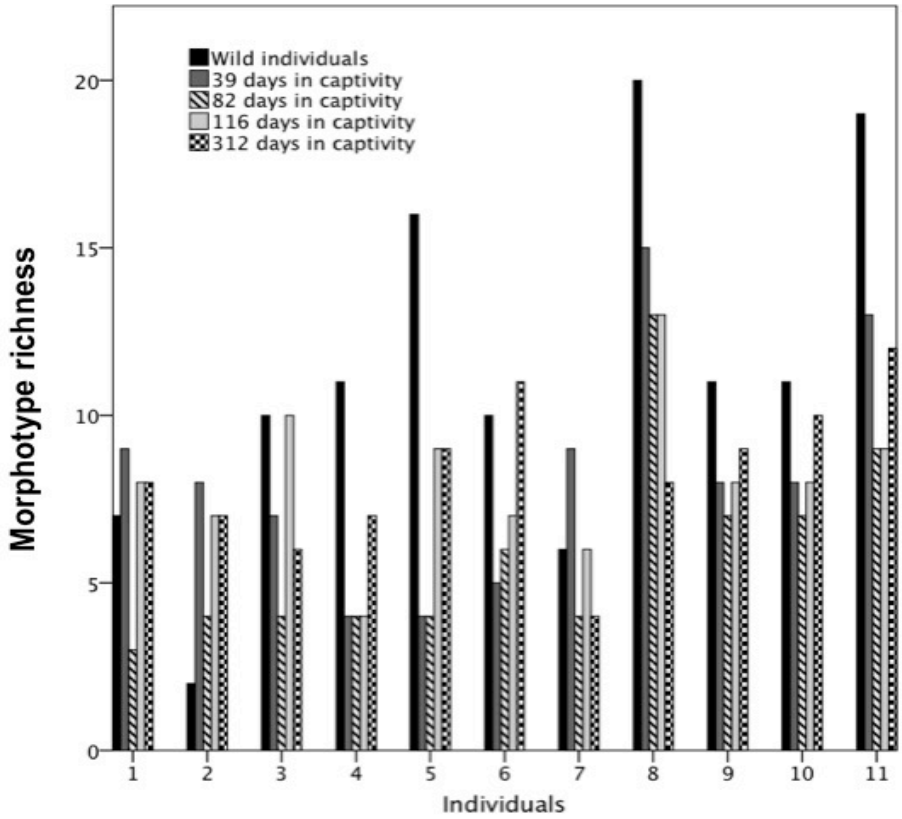
3.2. Temporal intra-individual differences and consistence of bacteria morphotypes

From 11 frogs, we found 458 morphotypes and isolated 2 – 20 morphotypes per frog (M = 7,93; SD = 3,99). Frogs that were sampled directly in the wild had the highest (20) and lowest number (2) of morphotype (Figure 2). The number of bacterial morphotypes from wild individuals placed in captivity over time was not consistent (Icc = 0,024, p = 0,42). In fact, there was a significant temporal intra-individual variation on the number of morphotypes (ANOVA, $F_{10,29}$ = 2,411, p = 0,032) (Figure 2). Length ($F_{1,28}$ = 0,138; p = 0,713) or weight ($F_{1,28}$ = 0,161; p = 0,692) did not influence the number of bacterial morphotypes.

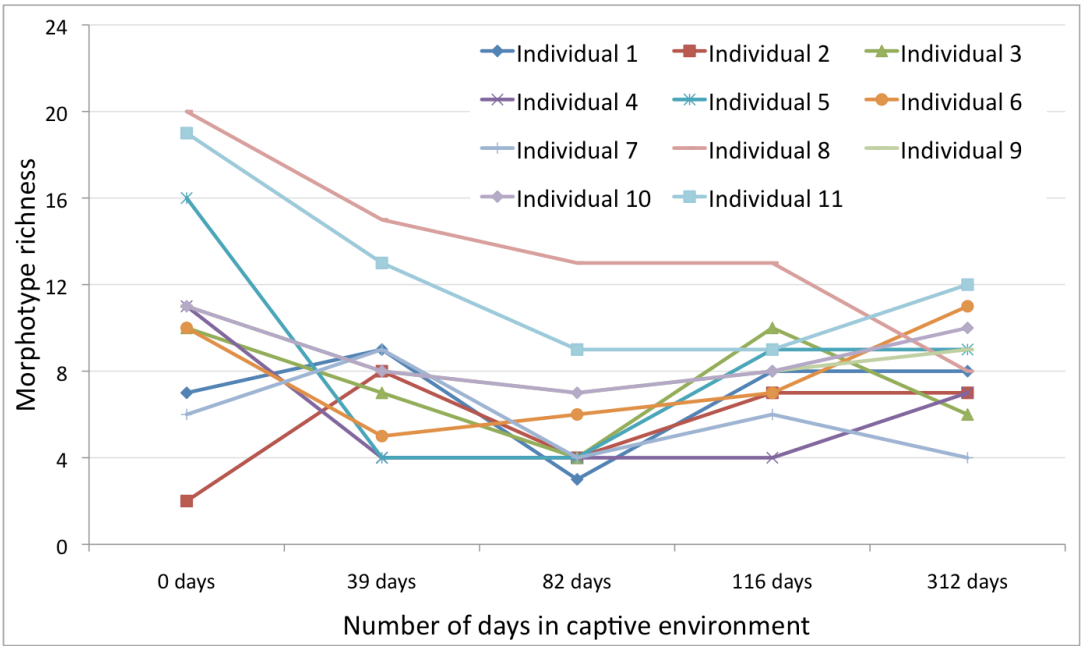


a b c

Fig. 1. Inter-individual variability of cutaneous bacterial morphotypes richness of *Sinax alcatraz* under three different conditions. In all cases, there were significant differences between individuals. a) Bacterial morphotype richness from the skin of 11 wild animals sampled directly in the wild (M = 11,18; SD = 5,42), t(10) = 6,84, *p* = 0,000; b) Bacterial morphotype richness from the skin of 17 captive born animals (M = 5,41; SD = 1,50), t(16) = 14,85, p = 0,000; c) Number of bacterial morphotype from the skin of 6 transported (M = 4,17; SD = 0,75), t(5) = 13,56, *p* = 0,000.



a



b

Fig. 2. a) Variation on bacterial morphotype richness of each individual considering each treatment. Significant intra-individual variation on the number of morphotypes over time (ANOVA, $F_{10,29}$ = 2,411, *P* = 0,032); b) Temporal intra-individual variation in bacterial morphotype richness of each individual.

4. Discussion

The cutaneous microbiota of amphibians plays a great role on disease susceptibility (Rollins-Smith et al., 2011) and the correct assemblage of microbes' matters. In this study, it was possible to demonstrate that the cutaneous bacterial community of individuals of *S. alcatraz* differ between and within individuals. The bacterial community composition, assessed against the richness of morphotypes, differed between animals of the same group in the three microhabitats analyzed (wild, captive born and transported groups). Except for the wild group, the transported animals and animals born in captivity were kept under the same conditions in each group. Thus, the microbiota of the environment was the same for all individuals of the same group, suggesting that the differences found may be due to physiological aspects, such as different secreted substances between individuals or behavior of the individual, for example, if the individual stays longer in contact with the water, with the soil or with other individuals. Therefore, host characteristics are likely to be important for the composition of the skin microbiota.

Although hosts factors seem to be important for the composition of the skin bacterial community, Loudon et al. (2014) suggest that the stability of the community depends on the environment. Our results demonstrated that the cutaneous bacterial community differs within individuals over a period of time and is not consistent. Therefore, our results do not show a pattern, since some individuals increased in number of bacterial morphotype over time and some decreased their number. In most of the cases, the number of bacterial morphotypes decreased in captivity, which may be caused by the lack of bacterial reservoir from the environment, since the water provided for the animals is sterilized and the aquariums are cleaned from time

to time. Individuals that enhanced their quantities of bacteria probably acquired new morphotypes by contact with others individuals. Thus, the temporal variation we found in this study is more likely to be due to environmental conditions than to host factors. Indeed, Loudon et al., (2014) found out that, even though bacterial community of salamanders shift after being place in captivity, the bacterial community of salamander maintained in contact with natural soil was stable over time while the bacterial community of salamanders placed in sterile environment was not.

Overall, this study demonstrated that both environmental and host factors matter as modulators of the microbial community of *S. alcatraz*. Environmental may be more important for the stability of the community and, host factors plus the environment might be important for the composition of the initial assemblage. This study is at forefront of attempts to seek temporal differences within individuals in artificial environments. However, it is important to investigate temporal changes in the composition of the cutaneous microbiota of amphibians in their natural habitat, in order to understand if the temporal changes we found here follow the same pattern as in natural habitats. Therewith, it may be possible to comprehend the natural dynamics of the association between amphibians and symbiotic bacteria.

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Discussão geral e conclusões

Através de nossa pesquisa foi possível demonstrar que tanto fatores ambientais quanto fatores ecofisiológicos do hospedeiro podem modular a comunidade microbiana cutânea de *S. alcatraz*. Um primeiro indicativo de que o ambiente modula comunidades microbianas foi o achado de que populações distintas de *S. alcatraz* possuem perfis de comunidade bacteriana cutânea diferentes. Como animais nascidos e animais mantidos em cativeiro apresentam menor riqueza bacteriana quando comparados com animais selvagens, é possível dizer que o ambiente no qual a população está inserida seja o principal fator modulador das comunidades nessas populações estudadas. Além de diferenças entre populações, a microbiota cutânea também difere entre indivíduos de uma mesma população. Nesse caso, o ambiente não pode explicar essas diferenças, pois os indivíduos habitavam o mesmo microhabitat, porém podemos dizer que essa diferença se dá por aspectos ecofisiológicos de cada indivíduo. Por fim, podemos observar claramente o efeito temporal de ambientes artificiais em indivíduos selvagens mantidos em cativeiro. Apesar da comunidade microbiana da população de indivíduos ter decrescido ao longo do tempo, as variações intra-individuais nos mostram que não há um padrão nesses decréscimos. Assim sendo, a diminuição na riqueza de morfotipos pode ser explicada tanto pelo ambiente pobre em microrganismo quanto por variações ecofisiologicas de cada indivíduo.

Apesar deste trabalho não explicar quais fatores ambientais e ecofisiologicos modulam a comunidade microbiana cutânea de *S. alcatraz*, podemos concluir que ambientes artificiais modulam a comunidade microbiana cutânea de anfíbios. Além disso, através das investigações temporais, pudemos verificar que a comunidade microbiana cutânea de *S. alcatraz* não é estável em ambientes artificiais. Pesquisas adicionais para investigar se as diferenças encontradas neste estudo também são

observadas na natureza são importantes para entender a dinâmica da relação entre microganismos simbiônticos e anfíbios. Além disso é importante investigar como se dá a recolonização de bactérias na pele dos indivíduos cativos para que medidas preventivas para a reintrodução de espécies na natureza sejam tomadas.

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