Farides del Carmen Lamadrid Feris

Relações entre corticosterona, cativeiro como estressor e a resposta imune inata em anuros

Relationship between corticosterone, captivity as stressor and the immune innate response in anurans

São Paulo, 2021

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Orientador: Prof. Dr. Fernando Ribeiro Gomes

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Prof. Dr. Fernando Ribeiro Gomes
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“A Deus e a minha família, os quais fizeram possível com seu apoio e amor aquilo que em solidão parecia impossível”

“Courage is like — it’s a habitus, a habit, a virtue: you get it by courageous acts. It’s like you learn to swim by swimming. You learn courage by couraging.”

(Dra. Marie Maynard Daly)

“*Uma nuvem não sabe por que se move em tal direção e em tal velocidade. Sente apenas um impulso que a conduz para esta ou aquela direção. Mas o céu sabe os motivos e os desenhos por trás de todas as nuvens, e você também saberá, quando se erguer o suficiente para ver além dos horizontes*”

(Richard Bach)

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RESUMO GERAL

Anfíbios anuros enfrentam um drástico declínio populacional em nível mundial, devido às mudanças ambientais, perda de habitat e doenças infeciosas emergentes. Essas ameaças representam estressores capazes de gerar respostas crônicas com consequências letais aos anuros. As respostas fisiológicas a estressores podem gerar efeitos imunomodulatórios complexos, que dependem da duração e da intensidade do aumento da concentração plasmática de glicocorticoides (GCs) em sua decorrência. Os efeitos tendem a ser imunoestimulatórios em resposta a estressores agudos e imunossupressores em resposta a estressores crônicos. O objetivo dessa tese foi compreender o impacto do aumento experimental da concentração plasmática de GCs e do cativeiro como estressor, sobre a resposta imune inata e microbiota da pele dos anfíbios anuros. No primeiro capítulo da tese, foi investigado se é possível emular experimentalmente níveis crônicos de corticosterona (CORT) para avaliar seus efeitos sobre a imunidade inata em indivíduos de *Rhinella ornata*, espécie de sapo associada a regiões florestadas do Brasil. Para tanto, sapos foram implantados subcutaneamente com tubos silásticos contendo CORT (grupo experimental) e tubos silásticos vazios (grupo controle). Amostras de plasma foram obtidas a cada 5 dias após o implante, durante 15 dias, para estimar a concentração de CORT e a capacidade bactericida plasmática (CBP) contra *Aeromonas hydrophila*. O tratamento aplicado aumentou a concentração plasmática de CORT ao longo do período experimental e resultou em aumento do CBP do dia 1 ao dia 10 pós-implante, e uma redução da CBP no dia 15 pós-implante. Esses resultados evidenciaram que os implantes de CORT elevaram a concentração de CORT no plasma de maneira crônica, levando à imunomudulação complexa ao longo do tempo experimental. No segundo capítulo da tese, foi investigado se a manutenção em cativeiro de indivíduos da perereca das folhas (*Phyllomedusa distincta)* resultou em estresse, gerando impactos sobre a imunidade inata e a composição de bactérias da pele. Brevemente, indivíduos de *P. distincta* foram mantidos em cativeiro por 28 dias e amostrados a cada 7 dias. Foram coletadas amostras de sangue, secreções cutâneas, e microbiota da pele. A manutenção em cativeiro não alterou a concentração plasmática de CORT, mas reduziu a CBP, incrementou a riqueza de sinais iônicas nas secreções

cutâneas e alterou a estrutura do bacterioma da pele, diminuindo a diversidade e abundância gerais. As bactérias potencialmente benéficas contra *A. hydrophila* e *B. dendrobatidis* aumentaram em cativeiro. O bacterioma das amostras do campo foi mais parecido com o do último dia em cativeiro, evidenciando uma tendência à resiliência das comunidades. Após a submissão experimental aos estressores (tubos silásticos e cativeiro), as duas espécies mostraram diminuição da CBP, mas só os sapos implantados mostraram aumento da CORT. Esses resultados indicam que o uso de implantes de CORT é um estressor para os sapos, sendo capaz de ativar o eixo HHI, aumentando a secreção de CORT e estimulando a função imunitária no inicio e diminuindo ela com o tempo. Adicionalmente, as pererecas de folha apresentam uma função imune inata plasmática menor se comparada à dos sapos, e elevada ao nível cutâneo o que poderia aumentar seu valor adaptativo no seu ambiente nativo e favorecer o sucesso frente a patógenos emergentes.

Palavras chave: Anuros, Corticosterona, Cativeiro, Microbiota cutânea, Secreções Cutâneas.

GENERAL ABSTRACT

Amphibians anuran face a dramatic population decline worldwide, because of environmental changes, loss of habitat, and emerging infectious diseases. These threats represent stressors capable of producing chronic responses with lethal consequences to frogs. The physiological responses to stressors can generate complex immunomodulatory effects, which depend on the duration and intensity of the increase in the plasma concentration of glucocorticoids (GCs). The effects lean to be immunostimulatory in response to acute stressors and immunosuppressive in response to chronic stressors. The aim of this thesis was to understand the impact of the experimental increase in the plasma concentration of GCs and captivity as stressors, on the innate immune response and skin microbiota of anuran amphibians. In the first chapter of this thesis, we investigated whether it is possible to emulate experimentally chronic levels of corticosterone (CORT) to evaluate its effects on innate immunity in individuals of *Rhinella ornata*, a species of toad associated with forested regions of Brazil. For this purpose, we implanted the toads subcutaneously with silastic tubes containing CORT (experimental group) and empty silastic tubes (control group). We got plasma samples every 5 days after implantation, for 15 days, to estimate CORT concentration and plasma bactericidal capacity (BKA) against *Aeromonas hydrophila*. The applied treatment increased the plasma CORT concentration throughout the experimental period and resulted in an increase in BKA from day 1 to day 10 post-implantation, and a reduction in BKA on day 15 post-implantation. These results showed that the CORT implants chronically increased the concentration of CORT in the plasma, leading to complex immunomodulation over the experimental time. The second chapter of the thesis investigated whether the maintenance in captivity of individuals of the leaf frog (*Phyllomedusa distincta*) resulted in stress, generating impacts on the innate immunity and the composition of skin bacteria. Briefly, individuals of *P. distincta* were

kept in captivity for 28 days and sampled every 7 days. Blood samples, skin secretions, and skin microbiota were collected. Maintenance in captivity did not alter the plasma concentration of CORT, but reduced BKA, increased the richness of signals in skin secretions, and altered the structure of the skin bacteriome, decreasing overall diversity and abundance. The potentially beneficial bacteria against *A. hydrophila* and *Batrachochytrium dendrobatidis* increased in captivity. The bacteriome of the samples from the field was more similar to that of the last day in captivity, showing a tendency to resilience in the communities. After experimental submission to stressors (silastic tubes and captivity), both species showed a decrease in BKA, but only implanted toads showed an increase in CORT. These results indicate that the use of CORT implants is a stressor for anurans, being able to activate the HPA/I axis, increasing the secretion of CORT and stimulating immune function in the beginning, and decreasing it through time. Additionally, leaf frogs have a lower plasma innate immune function compared to that of toads but elevated at the cutaneous level, which could increase their fitness in their native environment and facilitate success against emerging pathogens.

Keywords: Anurans, Corticosterone, Captivity, Skin microbiota, Cutaneous Secretions

GENERAL INTRODUCTION

Under stress conditions, the hypothalamus-pituitary-interrenal axis (HPA/I) is activated in vertebrates, leading to an increase in circulating levels of glucocorticoids (GCs) (Sapolsky, Romero, and Munck 2000), with corticosterone (CORT) as the main glucocorticoid present in amphibians (Rollins-Smith 2017). The production of GCs in response to stressors and their immunological effects vary depending on the duration and intensity of the stressor event. Short-term stressors activate the HPA/I axis modulating several physiological functions, and contributing to integrative and adaptive responses. Acute stress response enhances leukocytes mobilization from lymphoid organs to circulation; mainly Natural Killers cells and granulocytes (Dhabhar 2009; Viswanathan and Dhabhar 2005; Dhabhar 2002; Dhabhar and McEwen 1997; Tatiersky et al. 2015), increases the metabolic rate (DuRant et al. 2008), anti-predator behaviors (Thaker, Lima, and Hews 2009; Ohmer, Robertson, and Zamudio 2009) and suppress the reproductive hormones (Davies et al. 2016; Ricciardella et al. 2010; Zamudio et al. 2009) among other effects. Nevertheless, long-term activation of the HPA/I axis under chronic stress conditions have harmful effects, including immunosuppression (Dhabhar 2009; McEwen and Stellar 1993). Also, GCs immunosuppressive effects include inhibition of synthesis, reallocation and efficiency of various cytokines and other mediators that promote immune response and inflammatory reactions, and atrophy lymphoid tissues, particularly of the thymus (Sapolsky, Romero, and Munck 2000; Wiegers and Reul 1998; Simmaco et al. 1998; Garvy et al. 1993). In this way, GCs show complex immunomodulatory effects, including bimodal effects associated with acute and chronic responses (Dhabhar 2009; Sapolsky, Romero, and Munck 2000; Munck, Gurye, and Holbrook 1984).

Studies with experimental manipulations of GCs levels in amphibians have contributed to understanding acute and chronic stress response and its immunological

consequences (Crossin et al. 2016). Many advances in the understanding the relation between GCs and different with phenotypic traits in wild animal are correlational, but experimental manipulation of GCs is necessary to stablish causality (van Kesteren et al. 2019). In anurans, transdermal applications have been used to manipulate levels of CORT and to study immune consequences of acute increased corticosterone plasma levels (Kaiser et al. 2015; Gardner et al. 2018; Madelaire, Cassettari, and Gomes 2019; Titon et al. 2019; Assis et al. 2017, 2015). However, daily CORT transdermal application in the toad *Rhinella icterica* did not induce chronic CORT plasma levels (Assis et al. 2017). Experimental treatments capable of maintaining constant dose and release rate of GCs are necessary to induce chronically high plasma levels of these hormones (Quispe et al. 2015; Crossin et al. 2016). Silastic subcutaneous implants are good alternatives to induce continuous stimulation of endocrine axis, *e. g.* hypothalamic-pituitary-gonad (HPG) axis (Quispe et al. 2015; Koresh, Matas, and Koren 2016; Tamaru et al. 1990), and the HPA/I axis (Juneau, Gilmour, and Blouin-Demers 2015; Falso et al. 2015; Bonier et al. 2009). Using endocrine treatments for observing immunomodulatory consequences is an interesting approach. Previous studies suggested that researches with experimental manipulation of GCs must have an appropriate theoretical framework through which can correctly interpret the increased produced by this method (L. Romero 2004; Crossin et al. 2016). *Rhinella ornata*, is a non-threatened Brazilian toad from the Atlantic forest (Baldissera 2010), for which it was previously observed a negative association between plasma bacterial killing ability (BKA) and CORT plasma levels (F. R. Gomes et al. 2012). It is also known that dehydration triggers a pronounced stress response (Barsotti et al. 2019), exposure to short and mid-term stressors results in immuno-enhancing effects, and long-term captivity produce immunosuppressive effect (Titon et al. 2019) in *R. ornata*.

Based in all that information, we chosen this species as the model in our research about experimental manipulation of CORT levels.

The chronic activation of HPA/I axis and its immune consequences is a topic particularly relevant for amphibians because of the worldwide declining of their populations caused by climate change, habitat loss and emerging infectious diseases (Carey, Cohen, and Rollins-Smith 1999; Daszak et al. 1999; Daszak, Cunningham, and Hyatt 2003). Diseases like Chytridiomycosis (caused by the fungus *Batrachochytrium dendrobatidis*) and red-leg syndrome (bacterial septicemia that include *Aeromonas hydrophila* (Pessier 2002)) have decimated and disappeared entire populations and species (Daszak, Cunningham, and Hyatt 2003; James et al. 2015; Scheele et al. 2019) forcing to transfer the survivors populations to captivity conditions. Besides being a source of amphibian declines, these diseases share the skin as the target organ. The skin is the first physical barrier and protect against predators, injuries and pathogens (Varga, Bui-Marinos, and Katzenback 2019; Haslam et al. 2014). In amphibian anurans, the skin produce several substances as antimicrobial peptides (AMPs) (Conlon, Iwamuro, and King 2009), together with alkaloids (Toledo and Jared 1995; A. A. Gomes et al. 2007), lipids and polysaccharides (Elkan 1968). AMPs are considered effector molecules of innate immunity (Varga, Bui-Marinos, and Katzenback 2019). Stress, injury, or infection can induce the secretion of constitutive AMPs from granular glands in anurans (Heimlich, Harrison, and Mason 2014; Pask, Woodhams, and Rollins-Smith 2012). Cortisone treatment blocked de novo synthesis of all AMPs, blocking nuclear factor kappa-beta (NF-kB) by induction of inhibitor of NF-jB (IkBa) synthesis according to (Boman 2003). Then, the AMPs can be altered by the activation of the HPA/I axis (Radek et al. 2010) responding to increased circulating GCs levels (Tatiersky et al. 2015; Simmaco et al. 1997).

Together with the immune system, the skin microbiome can regulate pathogens, through competition by resources and the producing antimicrobial metabolites (Barnes, Carter, and Lewis 2020; Byrd, Belkaid, and Segre 2018; Kearns et al. 2017; Colombo et al. 2015; Myers et al. 2012) conferring it to skin microbial communities a protective role (Bletz et al. 2013; Jiménez and Sommer 2017; Burkart et al. 2017; Colombo et al. 2015). Nowadays, the perception of skin as an ecosystem with living biological and physical components occupying diverse habitats granted the advanced understanding of the delicate balance between host and microorganism (Grice and Segre 2011). Many studies with anuran skin microbiome revealed several commensal bacteria produced metabolites capable to inhibit the growth of known pathogens (Woodhams et al. 2016; Burkart et al. 2017). Researches with skin commensal bacteria in anurans sought antifungal bacteria and documented them in the Antifungal Isolates Database (Woodhams et al. 2015), considering that the fungus *B. dendrobatidis* is the major cause of worldwide anuran declines (Scheele et al. 2019). The bacterial metabolites can act synergistically with cutaneous AMPs to inhibit the skin pathogens (Myers et al. 2012). Several morphotypes of four families of commensal skin bacteria were identified in *Phyllomedusa distincta* with activity against bacterial pathogens (Brito de Assis et al. 2016), although the possible inhibitory effects on the pathogen fungus *B. dendrobatidis* remain unexplored. Also, this species of frog have different AMPs against bacteria, such as Phylloseptins and Dermaseptins (Brito de Assis et al. 2016; C. V. . Batista et al. 2001; Silva et al. 2008; Roberto et al. 2008). Thus, *P. distincta* represents an ideal model for investigation of multifaceted innate immunity approaches, which is critical to explaining the complex host-pathogen-environment interactions at the skin interface and their part in amphibian susceptibility to emerging infectious diseases (Varga et al., 2019). The physiological state of the amphibian host must be considered to understand their ability to deal with emerging

infectious diseases, given that allows to discern the direct consequences of physiological mediator levels on immunity and therefore on the microbiome of the skin. Also, since captivity may be a long-term stressor for anurans (Titon et al. 2017), it is important to understand its immune consequences to guarantee the viability of *ex-situ* programs and the eventual success of reintroduction initiatives (Zippel et al. 2011; McMahon et al. 2014; Alford and Richards 1999). Captivity may be a chronic stressor for toads, decreasing BKA and testosterone levels (Assis et al. 2017). Although, many studies with amphibians were focused on acute stress responses and its immune consequences (Narayan, Cockrem, and Hero 2013; Madelaire, Cassettari, and Gomes 2019; Assis et al. 2015; Ricciardella et al. 2010; Homan, Reed, and Romero 2003).

In the first chapter (*Emulating chronic stress: A practical approach and its immunological consequences*) we investigated if the use of subcutaneous silastic implants of CORT allows achieving chronically elevated CORT levels in *R. ornata*, and if this endocrine treatment can promote changes on a parameter of the innate immune response, the BKA. In the second chapter (*Consequences of captivity on innate immunity and skin bacteriome of São Paulo leaf frog*) we evaluated how captivity maintenance affects physiological state, immune function, and the structure of skin bacteriome, focusing especially on potentially beneficial bacteria against emerging pathogens.

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CHAPTER 1. — EMULATING CHRONIC STRESS: A PRACTICAL APPROACH AND ITS IMMUNOLOGICAL CONSEQUENCES

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1.1. Abstract

Under stress conditions, increasing levels of glucocorticoids (GCs), and other physiological mediators in vertebrates, influence a suite of physiological functions that include immunity. Depending on the lasting and intensity of the stress response, it could lead to immune enhancement when acute, or immunosuppressive when chronic, in most cases. However, study chronic stress responses are experimentally difficult for most vertebrate groups because the temporal pattern and its effects are not completely understood. Here, we evaluated (1) the use of subcutaneous silastic implants of corticosterone (CORT) as a method to emulate a chronic stressor in amphibians anurans under laboratory conditions and (2) to test if chronically increased levels of CORT achieved through this method are related to changes on a parameter of the innate immune response (plasma bacterial killing ability—BKA). For this, we tested two sizes of CORT silastic implants, 7mm and 15 mm, in adult males of *Rhinella ornata* (Bufonidae) for 15

days. We found that CORT implants are capable of chronic increase the CORT plasma levels. The sustained increase of CORT achieves by the implants allowed us to see the split on time between acute and chronic effects. The CORT increase reached by the treatment enhances the BKA from day 1st to 10th, and on 15th produces a BKA impairment.

Keywords: Anurans; BKA; Corticosterone, Innate immunity; *Rhinella ornata*.

1.2. Introduction

Different stressors such as environmental changes and emerging diseases could trigger the stress response (Homan et al. 2003; Rollins-Smith et al. 2011; Reeve et al. 2013). The Hypothalamic-Pituitary-Adrenal/Interrenal (HPA/I) axis is activated in response to stressors, leading to increased circulating levels of GCs in vertebrates (Selye 1950; Chrousos 2002; Miller 2018). The increased secretion of GCs during a stress response influence several physiological functions, including immunity (Sapolsky, Romero, and Munck 2000; Webster, Tonelli, and Sternberg 2002). Acute stress response, usually lasting from minutes to hours, can be adaptive and lead to immune enhancement, increasing, for instance, cell effector function, antibody production, and pro-inflammatory cytokines (Dhabhar and McEwen 1997). In contrast, chronic stress response, persisting for several hours per day, for weeks, or months, can be immunosuppressive (Sapolsky, Romero, and Munck 2000; McCormick, Shea, and Langkilde 2015) decreasing cellular (Garvy et al. 1993; Kaiser et al. 2015) and humoral response (Brooks and Mateo 2013; Assis et al. 2015), and delaying cutaneous wound healing (Thomas and Woodley 2015).

Stress response is highly complex, encompassing changes in multiple mediators and effectors, which also depend on time of exposition to stressors (McEwen and Stellar 1993; Sapolsky, Romero, and Munck 2000; Dhabhar 2009). Even emulating changes in

one mediator of the stress response, such as GCsids, is challenging under chronic conditions (Boonstra 2013; Falso et al. 2015). For this, one of the experimental approaches conceived is multiple transdermal applications of GCsids, such as corticosterone (CORT; the main GCs in amphibians) (Wack, Lovern, and Woodley 2010; Assis et al. 2015). However, this method frequently results in CORT elevations that are not continuous through time, limiting the interpretation of emulating patterns of HPI axis activation during chronic stress (McCormick, Shea, and Langkilde 2015; Assis et al. 2015; Kaiser et al. 2015; Assis et al. 2017).

In that way, the experimental solution to induce chronically increased CORT levels must include the use of techniques allowing control of time, concentration, and rate of hormone release. Subcutaneous implants using silicone tubing (Tamaru et al. 1990; Salvante and Williams 2003; Quispe et al. 2015; Koresh, Matas, and Koren 2016), cocoa butter (Juneau, Gilmour, and Blouin-Demers 2015), or beeswax implants (Beck et al. 2016) represent valuable methods to get continuous release rate and elevated levels of steroid hormones for prolonged periods in vertebrates (*e.g.,* birds, Salvante and Williams, 2003; mammals, Koresh et al., 2016; and snakes, Claunch et al., 2017). For instance, silastic implants maintained high doses of CORT for at least one month in reptiles (Morici, Elsey, and Lance 1997), and raised CORT plasma levels for up to 90 days in birds (Pravosudov 2003). In anurans, silastic implants increased estradiol plasma level for 30 days (Vandorpe and Kühn 1989); testosterone plasma levels for 26 weeks (Regnier and Herrera 1993), and CORT plasma levels up to 28 days (Falso et al. 2015). However, it is necessary to calibrate volumes and releasing rates for each studied species and life-history context (Sopinka et al. 2015; Crossin et al. 2016).

The impact of chronic stress on the immune response is particularly relevant for amphibians, given these vertebrates have suffered elevated rates of population declines

associated with environmental changes interacting with emerging infectious diseases caused by bacteria as *Aeromonas hydrophila* (Tiberti 2011; Rivas 2016), iridoviruses (Chen and Robert 2011), fungus as *Batrachochytrium dendrobatidis* (Rollins-Smith et al. 2011), and parasites as trematodes (Pessier 2002). Using subcutaneous silastic implants as a source of CORT to increase plasma levels in anurans would enable us to test the efficacy of this method to reach CORT chronic levels and to assess its immune effects. *Rhinella ornata* (Bufonidae), is a nonthreatened Brazilian toad from the Atlantic forest (Baldissera 2010), for which it has been previously shown that intense acute stressors, such as restraint of movements and dehydration can induce immunosuppression (F. R. Gomes et al. 2012; Barsotti et al. 2019). Recently, immuno-enhancing effects following short and mid-term stressors and immunosuppressive effects following captivity maintenance as a long-term stressor and long-term CORT transdermal application were demonstrated for these toads (Titon et al. 2019). Here, we used subcutaneous silastic implants of CORT in the Brazilian cururuzinho toad (*R. ornata*) to evaluate: (1) if this method allows achieving chronically elevated CORT levels, and (2) if this endocrine treatment can promote changes on a parameter of the innate immune response (plasma bacterial killing ability - BKA). We expected chronically increased CORT plasma levels and decreased capacity of plasma to limit bacterial proliferation in response to subcutaneous silastic implants of CORT.

1.3. Materials and Methods

1.3.1. Animals and Study Site

Rhinella ornata is a neotropical toad, family Bufonidae of the lowland Atlantic forest of Brazil. This toad uses temporary pools of the forest for reproduction and seems tolerant to habitat modification because there are populations in human intervened areas (Baldissera, Caramaschi, and Haddad 2004; Baldissera 2010). We

used 12 adult males collected in fragments of Atlantic forest, at the Botanical Garden of São Paulo (23.6416° S, 46.6234° W) and in the campus of the University of São Paulo (23°33'45''S, 46°43'40''W). Specimens collection was under the authorization of IBAMA (SISBIO process 178951) and experimental procedures were under the approval of the Ethics Committee on the Use of Vertebrate Animals in Experimentation (protocol 259/2016 (Proc. 16.1.319.41.1)).

1.3.2. Captive Maintenance Conditions.

The specimens were individually maintained into plastic containers [20.0 L – 43.0 L x 28.5 W x 26.5 H cms]. with a substrate of dry leaves for hiding, and free access to a recipient of water. The lids of the containers had holes to allow air circulation. Lighting conditions and temperature kept constant (LD 13h [light turned on at 06:30 am] and 11h of dark [light turned off 07:30 pm] and 22 ± 2°C). The containers were cleaned, and toads were fed with cockroaches once per week throughout the experiment. The toads were kept in these conditions for one month (habituation period) and subsequently for the 15 days of experiment.

1.3.3. Collecting and Processing Blood Samples.

We collected blood samples (about 100 µl) via cardiac puncture by using 1ml syringes and previously heparinized 26Gx1/2" needles. Only blood samples collected within 3 min from any disturbance were considered for analyses, to avoid any influence of the stress of capture and manipulation on hormone levels (L Michael Romero and Reed 2005). Blood samples were labeled and kept in ice up to 10 minutes until centrifugation. Plasma samples (a range of 20–80 µl) were obtained by centrifugation (4 min at 604g) and stored at -80°C for posterior hormone assays and BKA analyses. These procedures were repeated on days 1, 5, 10, and 15 after implantation of the silastics tubes (see 2.5).

1.3.4. Experimental Design

We used 12 adult males for this experiment. Blood samples were collected for each individual one day before silastic tubes were implanted (day 0), and on days 1, 5, 10, and 15 after implantation. After blood sampling on day 0, we weighed (16.3 g) and measured the snout-vent length (5.62 cm) of toads. Then, we assigned randomly four toads to each treatment group (I7 and I15) or the placebo group (Pl), with no statistical differences in weight or size between treatments (df = 2; $F_{2, 9} = 0.5338$; $P = 0.6038$). We subsequently manipulated the circulating levels of CORT during 15 days using silastic tubes (see 2.5).

1.3.5. Silastic Implants

We used implants of 7 mm (I7) and 15 mm (I15) of size, filled with CORT to test for possible differences in resulting CORT plasma levels. We used empty 7 mm implants in the placebo group (PL). For each type of implant (empty and filled) we cut pieces of silicone tubing (Perfimed ®, Brazil; 1.50mm inner diameter, and 2mm outer diameter), then we sealed one end with nontoxic silicone glue (Pulvitec Polystic Ref. 106612), and we filled them with crystalline corticosterone (Sigma - 27840). All implants were washed with ethanol (70%) and allowed to dry overnight for sterilization. After collecting a blood sample, as mentioned before, we performed a minor surgery in each individual to insert one implant subcutaneously on the right side of the abdomen. We made a superficial cut of 2,5 mm by using an ophthalmic scissor, sliding the implant below the skin, and closing the incision externally with tissue adhesive (Ethyl-2-Cyanoacrylate, Epiglu®). To avoid infections, we made all the procedures with sterilized gloves and dissection equipment, and we left the animals fasting 24 hours after the surgeries.

1.3.6. Hormonal Assay

We extracted plasma samples with ether according to (Mendonça et al. 1996; Titon et al. 2019; Assis et al. 2015). Thereby, 3 ml of ether added to 10 µl of each sample, and vortexed for 30 seconds and centrifuged (4°C, 9 min, at 583 G). Next, the samples decanted in -80°C freezer for 7min and the liquid phase transferred to another tube. These tubes were kept in laminar flow hood at room temperature (20 ± 2°C) until all the ether had evaporated (approximately 24h). Then, we resuspended the samples in EIA buffer and assayed CORT using EIA kits (number 500655, Cayman Chemical - AnnArbor, MI, USA), according to the manufacturer's instructions and previous studies of (Assis et al. 2017; Titon et al. 2019). Intraassay variation (estimated from sample duplicates) and inter-assay variation (estimated from the average of four intermediate values from the standard curve, as recommended by the kit instructions), were 6,3%. and 15.77%, respectively. The sensitivity of the assays, calculated as 80% B/B0 curve value, was 35.72 pg/mL.

1.3.7. Bacterial Killing Ability (BKA)

This assay characterizes humoral innate immune response that involves the action of opsonizing proteins (Demas et al. 2011), to recognize and promote cell lysis of a foreign microorganism (Millet et al. 2007; Matson, Tieleman, and Klasing 2006). BKA was performed using an anuran opportunistic pathogen, the gram-negative bacteria *Aeromonas hydrophila* (IOC/FDA 110–36), following the protocol proposed to *Escherichia coli* by Assis and collaborators (2013) with the modifications for *A. hydrophila* proposed by Moretti and collaborators (2019). A sample of stock cultured (bacteria + tryptic soy broth [TSB] + glycerol at −80 °C) was resuspended in 5 mL sterile TSB and incubated overnight at 37 °C. Then, 24 hours later, bacteria concentration was measured in a spectrophotometer (Spectra Max 250, at 595 nm).

Serial dilutions in sterile amphibian Ringer solution were performed to obtain a working concentration of 2.5×10^7 microorganisms x mL^{-1}. As follow, plasma samples diluted (1:20) in amphibian Ringer's solution (10 µL plasma:190 µL Ringer) were mixed with 10 µL of bacteria working solution (2.5×10^5 microorganisms). Then, samples and controls were incubated for one hour at 37ºC. Positive controls consisted of 10 µL of bacteria working solution in 200 µL of Ringer's solution, and negative controls contained 210 µL of Ringer's solution. After the incubation period, 500 µL of TSB was added to each sample. The bacterial suspensions were thoroughly mixed and 300 µL of each was transferred (in duplicate) to a 96 wells microplate. The microplate was incubated at 37 °C for 1 h, and thereafter the optical density of the samples was measured hourly in a plate spectrophotometer (wavelength: 595 nm), for a total of four readings. The plasma BKA was evaluated at the log-phase growth (bacterial exponential growth phase) and calculated according to the formula: 1 - (optical density of sample / optical density of positive control), which represents the proportion of killed microorganisms in the samples compared to the positive control.

1.3.8. Statistical analysis

We first submitted all data to descriptive statistics and tests of normality (Shapiro-Wilk test). To test if silastic implants of each group (I7, I15, and PL) affected CORT plasma levels and plasma BKA throughout time, we analyzed the data by fitting a mixed-model analysis of repeated measures data. This mixed model uses a compound symmetry covariance matrix, fitted by Restricted Maximum Likelihood (REML). Considering that we did not assume sphericity because of our design is of repeated measures in time, we used the correction of Geisser-Greenhouse as proposed by Maxwell & Delaney, (2013). We also performed multiple comparisons tests using Bonferroni correction to compare values between days within each treatment, and

between treatments each day. We used Prism version 8.3.1 for Windows, GraphPad Software, San Diego, California USA (www.graphpad.com) to conduct all the analyzes. All values showed in this research correspond to the mean ± standard deviation (SD) and values in the graphs to mean ± standard error (SEM).

1.4. Results

The CORT silastic implants increased CORT plasma levels in interaction with time post-surgery, independently of implant size (Table 1; Fig. 1). CORT plasma levels in I7 and I15 group were higher than those from PL group on days 1, and 5, and on day 15 for I7 implants (Table 1, Fig. 1). Throughout the experiment, CORT plasma levels of both CORT-implanted groups were on average 14.4 times higher than those from the placebo group, with higher values up to day 5 and subsequently lower values by the end of the experiment (Fig. 1). CORT plasma levels remained constantly low throughout the experiment on PL toads (Fig.1). CORT implants also affected plasma BKA in interaction with time post-surgery (Table 2). Plasma BKA of CORT implanted toads (I7 and I15) was higher than those from placebo toads on day 10 post-surgery (Fig. 2, Table 2). On the other hand, plasma BKA from I7 CORT-implanted toads was lower than those from toads that received empty implants on day 15 post-surgery (Table 2, Fig. 2).

Table 1.4.1. Descriptive fixed effect test from mixed-effects model, with days as a random effect for corticosterone plasma levels of *Rhinella ornata* with subcutaneous silastic implants empty (PL) and filled with corticosterone (I7 and I15). CORT: Corticosterone; PL: Placebo group; I7: CORT silastic implant of 7 mm; I15: CORT silastic implant of 15 mm; REML: Restricted maximum likelihood. *p < 0.05, **p<0.001.

CORT plasma levels		
Mixed-effects model (REML)		
	P value	F-statistic
Day	<0.001**	$F_{2,15} = 13.76$
Treatment	<0.001**	$F_{2,9} = 19.31$
Day x Treatment	0.003*	$F_{8,31} = 3.845$
Goodness of fit		
Degrees of freedom	38	
REML criterion	132.1	
Bonferroni's multiple comparisons test		
Day 1st		$t_{(DF)}$
PL vs. I7	0.002*	$t_{(4)} = 7.342$
PL vs. I15	0.029*	$t_{(3)} = 4.244$
Day 5th		
PL vs. I7	0.009*	$t_{(3)} = 6.793$
PL vs. I15	<0.001**	$t_{(4)} = 11.53$
Day 15th		
Table 1, continued		
PL vs. I7	0.042*	$t_{(3)} = 4.201$

Table 1.4.2. Descriptive fixed effect test from mixed-effects model, with days as a random effect for the plasma BKA of *Rhinella ornata* with subcutaneous silastic implants empty (PL) and filled with corticosterone (I7 and I15). BKA: Bacterial Killing Ability; PL: Placebo group; I7: CORT silastic implant of 7 mm; I15: CORT silastic implant of 15 mm; REML: Restricted maximum likelihood. *P < 0.05.

BKA values		
Mixed-effects model (REML)		
	P value	F-statistic
Day	0.0190*	$F_{(2, 20)} = 4.586$
Treatment	0.3667	$F_{(2, 9)} = 1.124$
Day x Treatment	0.0180*	$F_{(8, 35)} = 2.754$
Goodness of fit		
Degrees of freedom	42	
REML criterion	130.6	
Bonferroni's multiple comparisons test		
Day 10		$t_{(DF)}$
PL vs. I7	0.0017*	$t_{(6)} = 6.196$
PL vs. I15	0.0290*	$t_{(4)} = 3.775$
I7		
Day 0 vs Day 15	0.0249*	$t_{(3)} = 9.481$
Day 10 vs Day 15	0.0220*	$t_{(4)} = 9.890$

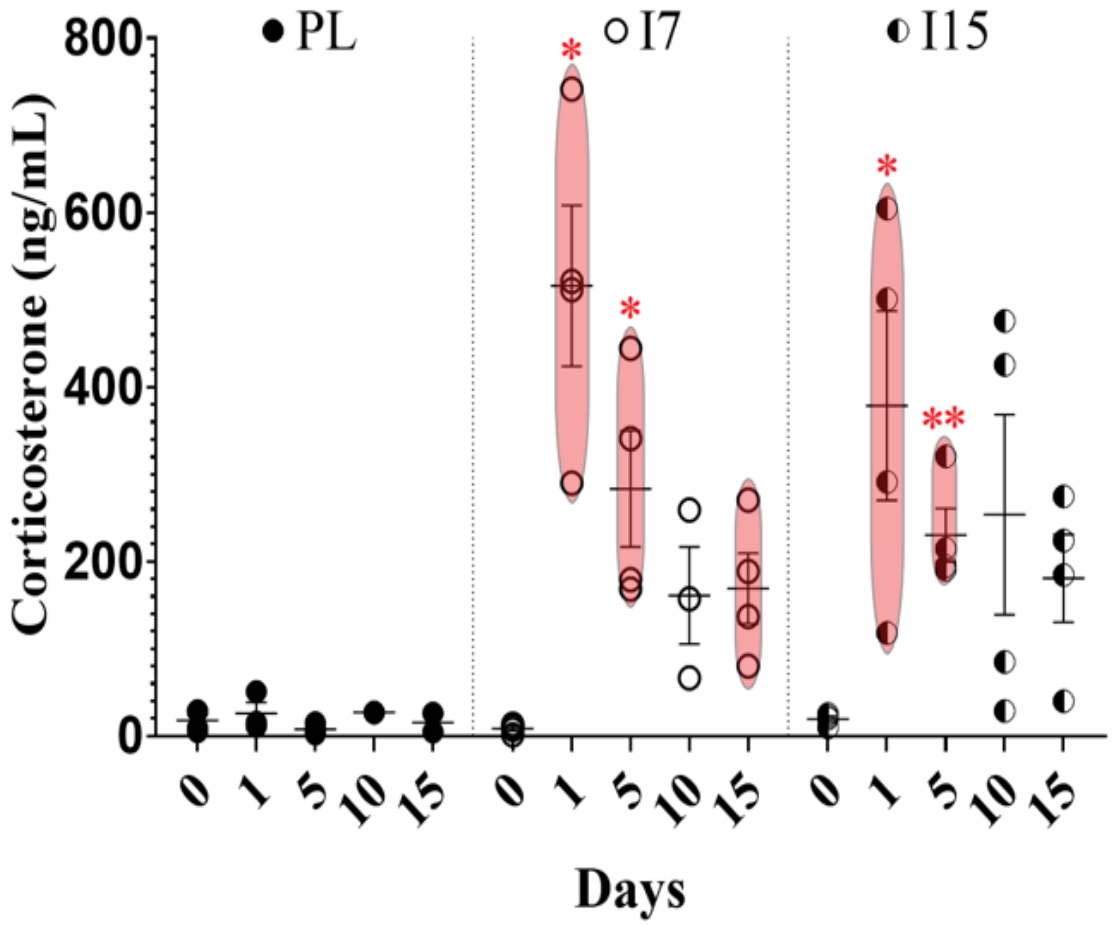

Figure 1.3. Corticosterone plasma levels of *Rhinella ornata* toads before (0) and after (1,5,10 and 15 days) corticosterone silastic implants. (**PL**) placebo with 7mm empty implant, (**I7**) 7mm implant filled with corticosterone and (**I15**) 15mm implant filled with corticosterone. Bars represent mean ± standard error. Statistical differences between groups and days were calculated by post hoc comparisons using Bonferroni adjustments in Mixed-effects models. Differences between placebo (**PL**) and experimental groups (**I7** and **I15**) within each day are shown in red and with asterisks (*) for $p< 0.05$ and (**) for $p<0,001$.

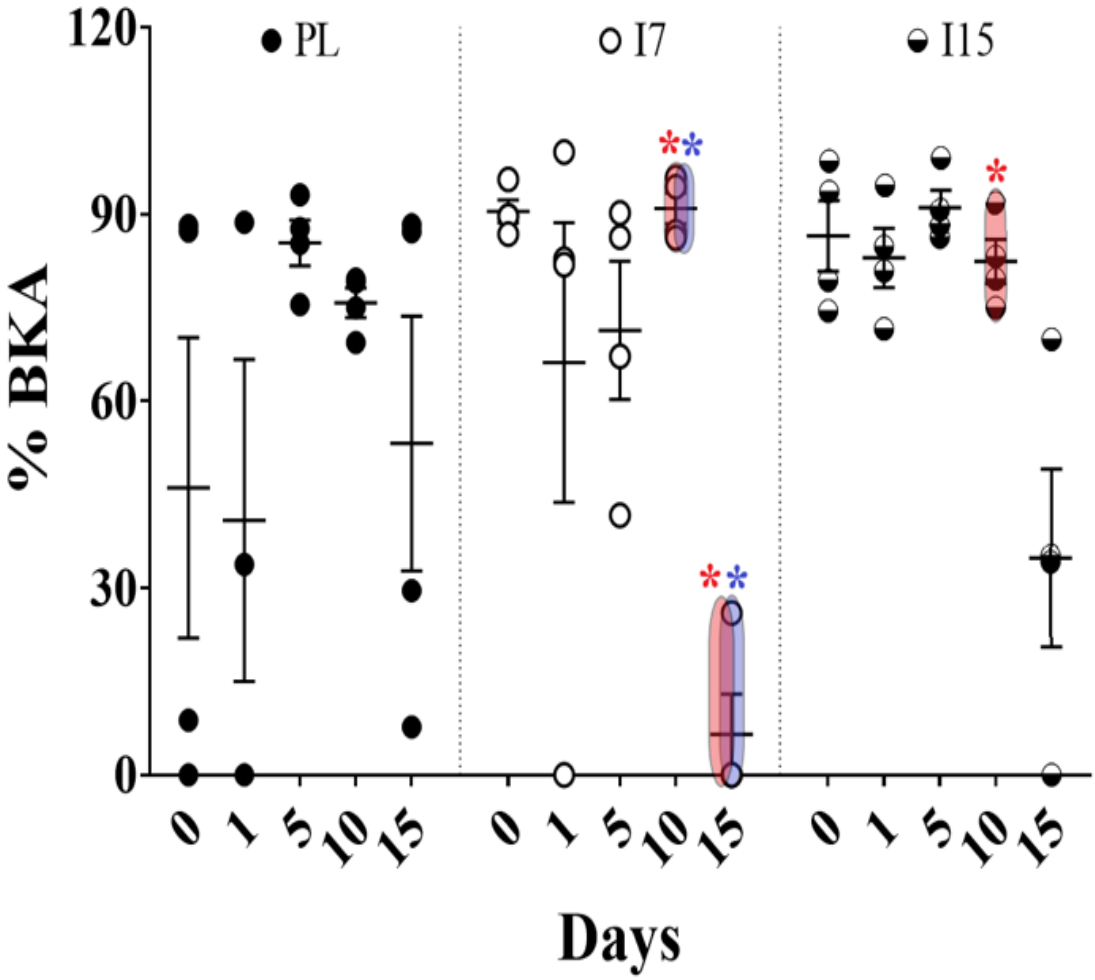

Figure 1.4. Plasma Bacterial killing assay (BKA) of *Rhinella ornata* toads before (day 0) and after (1,5,10 and 15 days) corticosterone silastic implants. (**PL**) placebo with 7mm empty implant, (**I**7) 7mm implant filled with corticosterone and (**I1**5) 15mm implant filled with corticosterone. Bars represent mean ± standard error. Statistical differences between groups and days were calculated by post hoc comparisons using Bonferroni adjustments in Mixed-effects models. Differences between placebo (PL) and experimental groups (I7 and I15) within each day are shown in red. The statistical difference between days 0 and 10 within I7 group, and between day 10 and day 15 post-surgery are shown in blue. The asterisks (*****) represents $p < 0.05$.

1.5. Discussion

The treatment with CORT silastic implants increased the plasma CORT levels of *R. ornata* by 14.4 times (average of all days), which are similar to those values previously measured in *R. ornata* submitted to CORT transdermal application (14 times higher than the control group (Titon et al. 2019)), dehydration (14.9 times higher in mean than the control group (Barsotti et al. 2019)) and restriction of movements (10.53 times higher than the baseline values (F. R. Gomes et al. 2012)). However, higher CORT plasma levels achieved by CORT implants were not constant throughout the experiment. On day 1 after implants, plasma CORT values in CORT implanted toads were 38.5 times higher than those measured in PL toads. These supraphysiological CORT plasma levels acutely achieved after surgery were not fatal and decreased subsequently through time, achieving constant values within the post-stressor physiological range (F. R. Gomes et al. 2012; Barsotti et al. 2019; Titon et al. 2019). In this way, our results suggest a relevant role of CORT silastic implant for future studies testing the physiological effects of chronically elevated CORT plasma levels.

This pattern of peaking CORT plasma levels just after CORT implants, followed of decreased values through time could be a result of using silastic implants with at least one open end, which allow rapid release of CORT at the beginning of the experiment (e. g., Birds, two sides open: (Goutte et al. 2010); Snakes, one open side and a punched hole: (Claunch et al. 2017)). Otherwise, using implants with both sides sealed usually shows lower releasing rates that depend only on material permeability (Richards 1985). Additionally, previous studies suggested than silastic implants allow continuous hormone release, but not necessarily a consistent releasing rate over time (Fusani 2008). Based on our results, to get a finely describe CORT plasma levels pattern it would interest test

CORT implants for a more prolonged time to specify the immunological effects of CORT chronic increase.

GCs are immunomodulatory, they enhance or impair immunity (Dhabhar and Mcewen 1999). On day 10 the effect of the CORT implants seems immune enhancer because the BKA values ballooned when compared to the PL group levels (Fig. 1). However, on day 15 the effects becoming chronic impairing the BKA. We expected that the chronic increase of CORT plasma levels achieved by the implants would decrease the capacity of plasma to limit bacterial proliferation. As expected, lower BKA values were observed in CORT-implanted toads after 15 days of surgery, statistically significant for the I7 group. The plasma BKA depends on lysozymes known as the complement system to kill pathogens (Merchant et al. 2003; Matson, Tieleman, and Klasing 2006; Millet et al. 2007) such as the bacteria *Aeromonas hydrophila* used here. The complement system is a thermolabile component, and the responsible of plasma BKA, increasing the opsonization and death of pathogens, playing an important role in coating them and facilitating their destruction by antibodies and phagocytic cells (Mayilyan et al. 2008; Speth et al. 2008; Murphy et al. 2010; Elvington, Liszewski, and Atkinson 2016). Elevated GC levels have an inhibitory effect on complement activity (Packard and Weiler 1983) through transcriptional mechanisms meditated intracellularly (Boumpas et al. 1991) and the inhibition of convertase (Gewurz et al. 1965; Jennings and Taylor 1964; Weiler and Packard 1982). Previous studies showed that confinement stress increased CORT levels in toads and decreased BKA values (Graham et al. 2012; Assis et al. 2015), suggesting that stress of movement or confinement may affect complement-mediated BKA. Lower BKA in toads implanted with CORT for a prolonged period (15 days) is consistent with immunosuppressive effects of previously observed after chronic exposition to stressors, such long-term captivity treatment (90 days), or transdermal

application of CORT for 20 consecutive days in *R. ornata* (Titon et al. 2019). Suppressing BKA by prolonged exposition to increased CORT may be because of changes in transcriptional processes that take longer to manifest (Boumpas et al. 1991; Lappin and Whaley 1991; Padgett and Glaser 2003).

1.6. Conclusions

The CORT silastic implants are a useful tool for elevated CORT plasma levels in amphibians. The sustained increase of CORT achieved by the implants induced mid-term immunoenhancing and long-term immunosuppressive effects on the ability of plasma of toads (*Rhinella ornata*) to kill *Aeromonas hydrophila*, an anuran opportunistic pathogen.

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CHAPTER 2. — CONSEQUENCES OF CAPTIVITY ON INNATE IMMUNITY AND SKIN BACTERIOME OF SÃO PAULO LEAF FROG

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2. 1 Abstract

The skin is the first barrier against invasion and colonization of pathogens. The microbiome (microorganisms occurring inside or on the host) is part of this barrier and a collaborator of the immune system. The fast decline of amphibian populations around the

world is in part driven by the emerging infectious diseases that alter the physiology of the amphibian host and its microbiome. Captivity is an especially relevant stressor in conservation studies, since threatened species are often taken and held captive to avoid outbreaks of pathogens, habitat loss, or to be reproduced in ex-situ for liberation and reintroduction goals. Physiological mediators associated with the stress and metabolic response as GCs display complex immunomodulatory functions. Corticosterone levels can regulate the immune mechanisms like the complement system, the ionic skin profile, and in consequence, reshape and regulate the skin microbiome. To test the previous hypotheses, we used the leaf frog *Phyllomedusa distincta* to evaluate how captivity maintenance affects physiological state, immune function, and the structure of skin bacteriome, focusing especially on potentially beneficial bacteria against *Aeromonas hydrophila* and *Batrachochytrium dendrobatidis*. We found that captivity conditions did not increase CORT plasma levels of *Phyllomedusa distincta*, albeit, innate immunity parameters measured shifted with captivity time, and clear differences in α-diversity and community composition were present between wild and captive individuals. Further, the potentially beneficial bacteria anti-Ah and anti-Bd increased with time in captivity. We conclude that experimental researches and conservation programs with anurans must consider captivity time and conditions. The consequences observed in the innate immune function and skin bacteriome can determine the success of future reintroduction programs.

Keywords: Corticosterone, BKA*, Phyllomedusa distincta, Aeromonas hydrophila, Batrachochytrium dendrobatidis*, skin ionic profile, core community.

2. 2 Introduction

The immune function is conceptually divided into innate (immediate and stereotyped response to all new antigens) and adaptive or acquired (slow, specific, and depending on a previous exposition to the antigen) (Abbas, Lichtman, and Pillai 2007). The elements of innate immunity are conserved among vertebrates, including external physical barriers as skin, humoral mechanisms (e.g. as antimicrobial peptides (AMPs), complement system, and lysozymes), and cellular effector mechanisms as T lymphocytes, phagocytic B cells, etc. (*Reviewed in* Riera Romo et al., 2016). The AMPs, secreted by the granular (or serous) skin glands in amphibians belong to the innate immune humoral response (Demas et al. 2011), disturbing the pathogens membranes structure to kill them (Simmaco et al. 1998; Conlon 2011), regulating the skin microbiome who can vary among species and populations of host (Jiménez and Sommer 2017; Loudon et al. 2014). The skin is the first barrier to pathogens and can also be its target. Epithelial barrier against invasion and colonization is constituted by physical barriers and by chemical shield encompassed by antimicrobial molecules like peptides and enzyme inhibitors (Radek et al. 2010; Aberg et al. 2008; Brunetti et al. 2018).

In the last years, the studies showed the microbiome as part of the barriers against pathogens and a collaborator of the immune system (Thaiss et al. 2016; Forsythe and Bienenstock 2010; Jiménez and Sommer 2017). Currently, we know the crosstalk between innate immunity and the microbiome (all microorganisms that colonize the body and their genomes) extends far beyond the achievement of a careful balance between tolerance and commensal microorganisms and immunity to pathogens (Thaiss et al. 2016). The innate immune system and the microbiome have a network of interactions affecting one another, orchestrating immunological processes and stabling microbial composition (Thaiss et al. 2014; Brunetti et al. 2018). Also, the complex ecosystem of

the microbiome is shaped by interactions with the environment and host factors that influence colonization and resilience dynamics (Costello et al. 2009; Rosenthal et al. 2011). Plenty of skin microbiota research has been culture-based and has shown that amphibian skin microbial communities mediate disease susceptibility (Smith et al. 2018; Burkart et al. 2017). Otherwise, culture-independent approaches allow the identification of uncultivable members of the community (Kueneman et al. 2014; Belden and Harris 2007; Jiménez and Sommer 2017) enabling more accurate ecological studies. Together these methods enable us to characterize the structure of the amphibian skin microbiome and its dynamics (J. Xu 2006; Scanlan and Marchesi 2008; Gilbert and Dupont 2011; Su et al. 2012; Pandya et al. 2017).

One of the principal drives to the fast decline of amphibians populations around the world are the emerging infectious diseases and the habitat disturbances (Rachowicz et al. 2005; Daszak, Cunningham, and Hyatt 2000; Blaustein et al. 2018). The chytridiomycosis produced by the fungus *Batrachochytrium dendrobatidis* (Bd) and *B. salamandrivorans* (Kilpatrick, Briggs, and Daszak 2010; Van Rooij et al. 2015), the red leg syndrome a bacterial septicemia caused by *Aeromonas hydrophila* (Ah), *Pseudomonas spp*, *Acinetobacter lwoffi*, *Citrobacter freundii*, *Chryseobacterium spp*, and others, or Iridovirus as the genus *Ranavirus* (Pessier 2002) are examples of this emerging infectious diseases affecting the amphibian populations. For that reason, emerging infectious diseases have been the principal focus of several studies for the last years. Several studies have suggested that the complement system is a key part of an early and robust immune response that confers host resistance to chytridiomycosis, but currently lacks an understanding of pathway activation (*Reviewed in* Rodriguez and Voyles, 2020). The complement response is a major contributor to the *in vitro* immune challenge classically employed in ecoimmulogical studies, known as bacterial killing ability (BKA)

(Assis et al. 2013; Liebl and Martin II 2009; Matson, Tieleman, and Klasing 2006; Millet et al. 2007).

Physiological mediators associated with the stress and metabolic response as GCs have an immunomodulatory role (Bellavance and Rivest 2014; Dhabhar 2014). Different stressors such as unpredictable weather events (L Michael Romero and Wikelski 2010; L M Romero and Wikelski 2001), predation, or captivity can evoke a stress response and increase circulating GCs levels (Narayan et al. 2011; Assis et al. 2015). Increasing GCs circulating levels by a short time (acute stress response) usually has immunoenhancing effects, such as improve dendritic cell, neutrophil, macrophage, and lymphocyte trafficking, maturation, and function (Dhabhar and Mcewen 1999; Dhabhar 2009). Otherwise, the prolonged and sustained increase in circulating GCs levels associated with chronic stress response usually led to immunosuppressive effects, including altering the cytokine balance, accelerating immunosenescence, and suppressing immunity by decreasing numbers, trafficking, and function of protective immune cells while increasing regulatory/suppressor T cells (McEwen 2017; Padgett and Glaser 2003). Increased corticosterone (CORT) plasma levels, the main GCs in anurans, and altered plasma bacterial killing ability (BKA) occurs in response to stressors (e.g. restrictions of movements (Assis et al. 2015), dehydration levels (Barsotti et al. 2019), temperature (Moretti et al. 2019; Lima et al. 2020), and captivity (Narayan et al. 2011; Titon et al. 2017, 2019; Assis et al. 2015), as well as in response to CORT treatments, such as transdermal application (Assis et al. 2015; Wack, Lovern, and Woodley 2010) and silastic implants (Lamadrid in prep.). However, studies on the effects of GCs on AMPs production are scarce and showing opposed effects. While some studies suggest that pharmacological concentrations of corticosteroids inhibit the renewal and secretion of AMPs (Simmaco et al. 1997), others suggest that these conditions facilitate the renewal

of AMPs (Tatiersky et al. 2015). In consequence, even considering there are many studies on amphibian AMPs, their focus has been its chemical nature and immunological role (Huttner and Bevins 1999; Conlon 2011; König, Bininda-Emonds, and Shaw 2014; Colombo et al. 2015), their effect on pathogens as Bd (Holden et al. 2015; Pask, Woodhams, and Rollins-Smith 2012; Woodhams et al. 2010), and its bio-prospection as antibiotics (Muñoz-Camargo et al. 2016; Nacif-Marçal et al. 2015; Lamadrid-Feris et al. 2014), leaving a gap on the modulation exerted by the physiological state on AMPs.

Captivity is an especially relevant stressor in conservation studies since threatened species are often taken and held captive to avoid outbreaks of pathogens, habitat loss or to be reproduced *ex situ* for liberation and reintroduction goals (Becker et al. 2014; Hernández-Gómez, Briggler, and Williams 2019; Antwis et al. 2014; Zippel et al. 2011). However, moving animals keeping them outside their natural habitat can impair their immunity (Titon et al. 2019; Graham et al. 2012) and modify their microbiome (K. A. Bates et al. 2019; Antwis et al. 2014; Sabino-Pinto et al. 2016). At the microbiome level, ecological concepts such as resistance (insensitivity to disturbance), resilience (the rate of recovery after disturbance) (Shade et al. 2012) and redundancy (performs like original community) are used to explain the changes in the microbial communities composition caused by disturbances like captivity (Allison and Martiny 2008; Finlay, Maberly, and Cooper 1997). Then, a better approach to understand the ability of the amphibians to deal with emerging infectious diseases must include the study of the physiological state of the amphibian host, allowing to discern the direct consequences of physiological mediators levels on immunity and therefore on the microbiome of the skin.

Frogs of the suborder Neobatrachia are by far the most important source of AMPs and over ~1000 of these have been characterized (Z. Wang and Wang 2004; G. Wang 2020). Neotropical hylid frogs that belong to the subfamily Phyllomedusinae are an excellent

source of AMPs. Studies about *Phyllomedusa* skin secretions during the last five decades unveiled a combination of biologically active peptides (Erspamer et al. 1985; Azevedo Calderon et al. 2011; C. V. F. F. Batista et al. 2001) that show striking interspecific variation, possibly associated with differences in the species niche, interactions with environment, predators, and pathogens (Azevedo Calderon et al. 2011). All these characteristics make *Phyllomedusa* an interesting model to study the relationships between anuran physiological host state, innate immune function, and skin bacteriome. Frogs from this genus secrete various antimicrobial, hormonal, and neuroactive peptides (X. Xu and Lai 2015). In particular, the leaf tree-frog (*Phyllomedusa distincta*), endemic to the Brazilian Atlantic Forest, has at least two types of AMPs, dermaseptins, and distinctins. Both are effective antimicrobial agents against several Gram-negative and Gram-positive bacteria (Nicolas, Vanhoye, and Amiche 2003; Roberto et al. 2008; C. V. . Batista et al. 2001; Brito de Assis et al. 2016). Here, we evaluated how captivity maintenance affects physiological state, immune function, and the structure of skin bacteriome, focusing especially on potentially beneficial bacteria. Specifically, our aims are to determine whether captivity (i) increases CORT plasma levels; (ii) impairs humoral innate immune parameters (plasma BKA and skin AMPs); (iii) reduces the presence and frequency of the potential beneficial bacteria anti-Bd and anti-Ah on skin bacteriome; and (iv) changes skin bacteriome structure of *P. distincta.*

2.3 Materials and methods

2.3.1 Animal Habitat Environment

We collected eleven adult males of *Phyllomedusa distincta* in their natural zone of occurrence of the Brazilian Atlantic forest ecosystem (Castanho 1994; Bertoluci and Rodrigues 2002), at night, in the emergent vegetation of permanent ponds in the

forest, where the individuals usually remain in leaves and branches above the water. We carried out the fieldwork at Intervales Natural State Park, an Atlantic Forest reserve situated in the coastal mountains of SE Brazil (SP) (24 ° 12'-24 ° 25'S, 48 ° 03'-48 ° 30'W), and located between the municipalities of Iporanga, Ribeirão Grande, and Sete Barras, between 800 and 900m of altitude. We conducted fieldwork during the mating season (October - November) in summer (Bertoluci and Rodrigues 2002; Bertoluci and Trefaut 1991) the warmer and rainy season of the year. Specimens collection was under the authorization of IBAMA (SISBIO process 54354) and experimental procedures were under the approval of the Ethics Committee on the Use of Vertebrate Animals in Experimentation (protocol 259/2016 (Proc. 16.1.319.41.1)).

We brought the individuals to the vivarium of ectotherms in the Biosciences Institute, using clean individual containers previously sterilized [20L—43.0 (L) x 28.5 (W) x 26.5 (H) cm]. Each terrarium had clean artificial green leaves and branches, plastic containers of sterile water (200 ml), and Petri plates (90 x15 mm) with wet cotton to increase the humidity and water access. The lids of the containers had a plastic mesh to allow air circulation. Lighting conditions and the temperature kept constant (LD 13h [light turned on at 06:30 am] and 11h of dark [light turned off 07:30 pm] and 22 ± 2°C). We fed the frogs two times a week at 15:00 to 17:00 with 6 - 8 nymph cockroaches. We kept the frogs in these conditions for seven days (habituation period) and subsequently for the 21 days of the experiment.

2.3.2 Sample Collection

To test the captivity effect on skin bacteriome communities and the selected innate immune parameters, we collected samples once in the field and four times in captivity conditions (Figure 1). The sampling occurred every seven days until complete 28 days in captivity conditions. We considered the first week in captivity the acclimation

period. Thus, we call day zero (D0) the beginning of sampling in captivity, and day 21 the end of them. During fieldwork, we sampled 11 individuals, capturing five and carry them to the vivarium of ectotherms. The sampling protocol comprised four steps, (i) taking skin bacteriome samples using sterile swabs for each individual, swabbing five times the dorsum and ventral skin, (ii) getting blood samples by cardiac puncture, (iii) obtaining skin secretions samples by electrostimulation, and finally (iv) measuring the body mass (Mi) and linear body measure (Li) for each individual to calculate the scaled mass index ($\widehat{Mi}$) according to the equation:

$$\widehat{M_i} = \left[\frac{L_0}{L_i}\right]^{b_{SMA}} \quad \text{(Eqn. 1.)}$$

Where Mi and Li are the body mass and linear body measurement (snout-vent length) of individual respectively; $bSMA$ is the scaling exponent estimated by the standardized major axis (SMA) regression of lnM on lnL; L_0 is an arbitrary value of L (e.g. the arithmetic mean value for the study population); and $\widehat{Mi}$ is the predicted body mass for individual i when the linear body measure is standardized to L_0 (Peig and Green 2010).

2.3.2.1 Skin bacteriome sample collection

We captured individuals by hand and rinsed with 300 ml of sterile water during 10s. Rinsing the animal before swabbing is important to ensure that the sample primarily included skin-associated microorganisms and to remove the transient bacteria of the skin (Lauer et al. 2007, 2008). Following rinsing, we swabbed the frogs with sterile cotton-tipped swabs (Model 23007, ABSORVE, São Paulo, Brazil) for 30s in a rotating motion while applying minimum pressure. We collected two swabs, sampling by both dorsal and ventral regions 6 times. Immediately after collection, we diluted one sample swab in 1 ml sterile saline

solution (NaCl, 0.9 %) and used to identification of potential beneficial morphotypes against *Aeromonas hydrophila*. We stored the other sample swab in a PowerSoil® Bead Tube, placed on liquid nitrogen, and moved to a −80 °C freezer. We use this last sample for the identification and analysis of the microbial community.

2.3.2.2 Potentially beneficial bacteria

We removed the bacteriome from swabs by vigorous vortex mixing. Then, we prepared serial dilutions in saline for inoculation on R2A medium (DIFCO), using standard aseptic techniques. We incubated the inoculated plates at room temperature for 48 hr. We obtained the isolated colonies using the streak plate technique. We classified the colonies according to morphological characteristics such as size, color, elevation, texture, opacity, and type of margin (results not shown). Finally, we selected one morphotype, which is a representative of a group of colonies that presented the same visual characteristics, and tested as potentially beneficial bacteria by antagonistic activity.

For screening of inhibitory effects against the opportunistic pathogen *Aeromonas hydrophila* – IOC/FDA 110–36, we transferred each morphotype isolated transferred into tryptic soy broth, cultured at room temperature for 24 h, and then stored at -80°C after the addition of 20% glycerol. By a modified double-layer method previously described by Westerdahl et al. 1991; Maia et al. 2001, we created macrocolonies of the isolates on tryptic soy agar plates by inoculating 5 µl droplets of 24 h culture. After incubation for 24 h at 30°C, we killed the colonies with ultraviolet light (60 min) and then we suspended 100 µl of an overnight culture of *Aeromonas hydrophila* in tryptic soy broth soft agar which and poured over the plates (15 ml per plate). Again, we incubated the plates for

24 h at 30°C before the examination. We saw the inhibition of growth of the pathogen around and/or over the macrocolony as a clear zone.

We transferred a colony of each isolate identified as positive inhibitors by the test explained above to a 1.5-ml microcentrifuge tube with 20 µl of Milli-Q water. We amplified Bacterial 16S rDNA and sequenced using the bacterial 16S rDNA primers 27F and 1492R (Lane 1991). We purified the amplified fragments and sent to Macrogen Inc. (South Korea) for Sanger DNA sequencing. We aligned forward and reverse nucleotide sequences in Geneious Pro (Biomatters, Ltd.; Drummond et al., 2010) to create a consensus sequence of approximately 500 bp. We made the alignment by comparison with the sequences at the GenBank by a basic local alignment search tool (BLAST) analysis (Altschul et al. 1990) in the National Center for Biotechnology Information (NCBI) databases. Following the alignment of sequences, we compiled a FASTA file containing a single representative of each unique consensus sequence and defined operational taxonomic units (OTUs) using qiime2 (v2019.10; (Bolyen et al. 2019)). We clustered the sequences at 99% similarity to create genus-level OTUs and assigned taxonomy using the Greengenes 13_8 reference database. Finally, we downloaded the sequences from the closely related species and aligned them through ClustalW using MEGA software (version 7.0) (Kumar et al. 2008)

2.3.2.3 Skin bacteriome

We performed the DNA extraction with the MoBio Power Soil Extraction kit (MoBio Laboratories, Carlsbad, CA, USA) using the standard MoBio protocol. We measured the concentration and purity of extracted gDNA using a NanoDrop™2000 Spectrophotometer and we stored at -20 °C until collected all the samples and then we sequenced together at the end of the sampling period.

We compared the microbial species composition between individuals through the experimental time. Sequencing by Macrogen (Seoul, (Republic of Korea)) using the Illumina MiSeq system (Illumina), according to the manufacturer's instructions.

2.3.2.4 Collecting and processing blood samples

We collected blood samples in captivity (about 50 µl) via cardiac puncture by using 1ml syringes and previously heparinized 26Gx1/2" needles. We only considered blood samples collected within 3 min from any disturbance for analyses, to avoid any influence of the stress of capture and manipulation on hormone levels (L Michael Romero and Reed 2005). We labeled the blood samples and kept them in ice for up to 10 minutes until centrifugation. We obtained the plasma samples (a range of 10–20 µl) by centrifugation (4 min at 604g) and stored at -80°C for posterior hormone assays and BKA analyses.

2.3.2.5 Hormonal assay

We extracted plasma samples with ether according to Mendonça et al. 1996; Titon et al. 2019; Assis et al. 2015. Thereby, 3 ml of ether added to 10 µl of each sample, and vortexed for 30 seconds, and centrifuged (4°C, 9 min, at 583 G). Next, the samples decanted in a -80°C freezer for 7min and we transferred the liquid phase to another tube. These tubes were kept in a laminar flow hood at room temperature (20 ± 2°C) until all the ether had evaporated (approximately 24h). Then, we resuspended the samples in EIA buffer and assayed CORT using EIA kits (number 500655, Cayman Chemical - AnnArbor, MI, USA), according to the manufacturer’s instructions and previous studies of (Assis et al. 2017; Titon et al. 2019). Intraassay variation (estimated from sample duplicates) and inter-assay

variation (estimated from the average of four intermediate values from the standard curve, as recommended by the kit instructions), were 8,55% and 11.43%, respectively. The sensitivity of the assays, calculated as 80% B/B0 curve value, was 28,27 pg/mL.

2.3.2.6 Plasma Bacterial Killing Ability (BKA)

We performed BKA assays using an anuran opportunistic pathogen, the gram-negative bacteria *Aeromonas hydrophila* (IOC/FDA 110–36), following the protocol proposed to *Escherichia coli* by Assis et al. 2013, with the modifications for *A. hydrophila* proposed by Moretti et al. 2019. We resuspended a sample of stock cultured (bacteria + tryptic soy broth [TSB] + glycerol at −80 °C) in 5 mL sterile TSB and incubated overnight at 37 °C. Then, 24 h later, we measured bacterial concentration in a spectrophotometer (Spectra Max 250, at 595 nm). We performed serial dilutions in sterile amphibian Ringer solution to obtain a working concentration of 2.5 × 107 microorganisms x mL−1. As follows, we diluted plasma samples (1:20) in amphibian Ringer's solution (10 µL plasma:190 µL Ringer) and mixed them with 10-µL bacteria working solution (2.5 × 105 microorganisms). Then, we incubated the samples and controls for one hour at 37°C. Positive controls comprised 10-µL bacteria working solution in 200 µL of Ringer's solution, and negative controls contained 210 µL of Ringer's solution. After the incubation period, we added 500-µL TSB to each sample. We thoroughly mixed bacterial suspensions and transferred 300 µL of each (in duplicate) to a 96-well microplate. Then, we incubated the microplate at 37 °C for 1 h, and after that we measured the optical density of the samples hourly in a plate spectrophotometer (wavelength: 595 nm), for a total of four readings. We evaluated plasma BKA at the log-phase growth (bacterial exponential growth

phase) and calculated according to the formula: 1 - (optical density of sample/optical density of positive control), which represents the proportion of killed microorganisms in the samples compared to the positive control.

2.3.2.7 Cutaneous secretions

We collected skin secretions from each frog using a transcutaneous amphibian stimulator (TAS; Grant and Land, 2002), which applies a weak electric current to the skin, causing the secretion of the contents of their granular glands. We standardized the TAS treatment among frogs (Frequency: 50 Hz; Pulse width: 2 ms; Amplitude: 6 V; Time: 1−2 min). Next, we wash the frogs with 50 ml of Milli-Q water to collect the secretions, deposited them in a propylene tube, placed them on liquid nitrogen, lyophilized, and moved them to a −80 °C freezer. Untarget metabolomics strategies were applies to produce a skin secretions fingerprint (Bauermeister et al. 2017). The lyophilized material was diluted in a solution of 0.1% TFA in 50% acetonitrila/water to obtain a sample concentration of 5 mg.mL−1. These samples was mixed with α-cyano-4-hydroxycinnamic acid matrix diluted in the same solution in a 1:1 ratio. Then the samples were applied on a ground steel plate for MALDI (Bruker Daltonic, Bremen, Germany). Analyses were performed using a MALDI- TOF/TOF UltrafleXtreme (Bruker Daltonics, Bremen, Germany) system equipped with a smartbeam-II laser system controlled by a FleXcontrol v.3.3 (Bruker Daltonics, Bremen, Germany), employing AutoXecute mode. The samples were acquired by octuplicate in the automatic mode of analysis and all the spectra were recorded across a mass range of m/z 700−4000 and accumulate 500 shots per spectrum. A peptide calibration standard II (Bruker Daltonics, Bremen, Germany) was used as the external calibration of the mass spectrometer. The average mass deviation was below 10

ppm. One of strategies for peptides identification is mass spectrometry by MALDI (Matrix-Assisted Laser Dissociation Ionization). In this work, the untargeted study was used to allow the evaluation of the metabolomic profile in the peptides specific mass/charge region (Gorrochategui et al. 2016; Vincenti et al. 2020). In addition, we can detect other signs such as glycoprotein and carbohydrates. Therefore, it proves to be important for comparative and qualitative studies between different samples.

2.3.2.8 Presence/Absence of *Batrachochytrium dendrobatidis*

Additionally, to control and avoid the cross effects by skin pathogens we tested for the presence/absence of *Batrachochytrium dendrobatidis* in all samples. We extracted DNA from skin swabs using the protocol developed by (Boyle et al. 2004), including the changes made by (Lambertini et al. 2013). Specifically, for each Eppendorf tube containing the swab, we added 50 µL of PrepMan™ ULTRA Sample Preparation Reagent (Applied Biosystems® by Life Technologies, Warrington, UK). Then, we vortexed tubes for 45 s and centrifuged for 30 s at 12,000 rpm. We heated tubes in a boiling water bath for 10 min, cooled at room temperature for 2 min, and centrifuged again for 1 min at 12,000 rpm. We then inverted the swabs in the Eppendorf tube using sterile flanged tweezers (i.e., by applying a flame sterilization technique between samples) and centrifuged the tubes for 5 min at 12,000 rpm. Lastly, we discarded swabs, briefly centrifuged (for a few seconds) the tubes, then transferred approximately 45 µL of solution to new tubes and stored them in a freezer at -2 Before performing real-time PCR reactions (qPCRs) to detect and quantify Bd infections, we diluted the extracted DNA in a 1:10 dilution (Lambertini et al. 2013). To prepare the qPCR reactions, we made a master mix, which is also based on the protocol developed by Boyle et

al. (2004), and contains 1250 µL of Taqman Master Mix (Applied Biosystems®), 125 µL of the primer ITS1-3 Chytr (5'-CCTTGATATAATACAGTGTGCCATATGTC-3') at 18 µM, 125 µL of the primer 5.8S Chytr (5'-AGCCAAGAGATCCGTTGTCAAA-3') at 18 µM, 125f ChytrMGB2 probe (5'-6FAM CGAGTCGAACAAAAT MGBNFQ-3') at 5 µM, 275 µL of distilled water, and 100 µL of bovine serum albumin (BSA). To prepare the qPCR 96-well plate, we added 20 µL of the mix to each well and 5 µL of the extracted DNA dilution. We ran samples in singlicate. To make the standard curve, we used the Bd isolate CLFT 159, an isolate associated with a genotype from the Bd lineage GPL, from a frog of the genus *Hylodes* from the Atlantic Forest (e. g., Greenspan, et al. 2018). To make the standard curve, we diluted the Bd isolate to the concentrations 103, 102, 101, 100 and 10-1 zoospores and we ran the standards 103, 102, 101 in duplicates and the standards 100 and 10-1 in quadruplicates. We considered an individual infected (Bd+) when we detected at least one Bd genomic equivalent (≥ 1 g. e.; Kriger et al. 2007). We rounded g. e. values to integers. We calculated the intensity of infection by multiplying the values resulting from the qPCR by the dilution factor (1:10) used in the DNA extraction.

2.3.2.9 Bd-inhibitory bacterial database

Further, to determine if the bacteriome of *P. distincta* putatively inhibit, facilitate, or have no effect on Bd, we compared our OTUs to Antifungal Isolates Database (Woodhams et al. 2015) using the vsearch cluster-features-closed-reference script (Rognes et al. 2016) to identify sequences with 99% match to bacterial isolates previously shown to inhibit Bd growth in culture by at least 80% compared to controls. They constructed the anti-Bd database from 1994 isolates

of bacteria cultured from the skin of amphibians from around the globe, including boreal toads, and tested against Bd in co-culture (Woodhams et al. 2015). The sequenced anti-Bd bacteria underwent closed reference OTU picking with the Greengenes 13_8 99% OTUs reference classifier.

2.3.3 Sequence and statistical analysis

We performed microbiome bioinformatics with Quantitative Insights Into Microbial Ecology (QIIME 2) version 2019.10 (Bolyen et al. 2019). We demultiplexed the raw sequence data and filtered by quality using the q2-demux plugin followed by denoising with DADA2 (Callahan et al., 2016) (via q2-dada2). We aligned all amplicon sequence variants (ASVs) with mafft (Katoh et al. 2002) (via q2-alignment) and used to construct a phylogeny with fasttree2 (Price, Dehal, and Arkin 2010) (via q2-phylogeny). We assigned the taxonomy to ASVs using the q2-feature-classifier (Bokulich et al. 2018) classify-sklearn naïve Bayes taxonomy classifier against the Greengenes 13_8 at the standard 99% OTUs reference sequences (McDonald et al. 2012). We performed additional quality filtration on the OTU table by removing OTUs represented by fewer than 0.005% of the total read count (Bokulich et al. 2018), and to avoid including any OTUs generated by sequencer error, such as base miscalls or chimeras. We rarefied the OTU table to 53000 sequences per sample resulting in 2,577,573 reads clustered into 12,476 OUT, to standardize sequencing depth across all samples (N=25).

We transferred the rarefied OTU table and Newick phylogenetic tree to R (version 3.6.3). To evaluate alpha diversity, we calculated community richness (observed OTUs), evenness (Shannon diversity), and phylogenetic diversity (Faith's phylogenetic diversity) using the packages vegan (Oksanen et al. 2019) and picante (Kembel et al. 2010). We created a heat tree summarizing relative abundance main

results using Metacoder package in R software (Foster, Sharpton, and Grünwald 2017). Besides, we implemented the packages phyloseq (McMurdie and Holmes 2013), Vegan and Microbiome (Lahti and Shetty, n.d.) to calculate pairwise unweighted/weighted UniFrac distances and Bray-Curtis dissimilarities between samples. We chose to calculate these three measures of beta diversity as they account for phylogenetic presence/absence-based differences (unweighted UniFrac), phylogenetic abundance-based differences (weighted UniFrac), and non-phylogenetic abundance-based differences (Bray-Curtis) among samples.

We evaluated the effect of captivity on richness, phylogenetic-based, and evenness measures of alpha diversity (OTU richness, Faith's phylogenetic diversity, and Shannon index) using generalized linear models with the lme4 package (D. Bates et al. 2015). We assessed differences in community composition among the time of sampling using analysis of composition of microbiomes (ANCOM) through the package Compositions (van den Boogaart, Tolosana-Delgado, and Bren 2020). To characterize the strength and significance of differentiation among field and captivity samples, we implemented a two-way PERMANOVA test with interaction term using all three beta diversity metrics. We generated NMDS plots using weighted UniFrac, unweighted UniFrac, and Bray-Curtis dissimilarities to visualize the clustering of samples by the time of sampling. Finally, we estimated the bacterial core community through the microbiome package. We defined the core community in our study as selection of ASVs with a relative abundance of at least 0.1% and present in more than 95% of tested samples. We used the method of double principal coordinate analysis or (DPCoA), since it jointly analyzes the differences between species and the distribution of species between communities, generating an assembly of

multidimensional space that correlates species with communities (Pavoine, Dufour, and Chessel 2004; Purdom 2011; Fukuyama et al. 2012).

We submitted data on physiological parameters to descriptive statistics and tests of normality (Shapiro-Wilk test). To test if captivity time affected CORT plasma levels, $\widehat{Mi}$, plasma BKA and ionic profiles of skin secretions throughout time, we analyzed the data by fitting a mixed-model analysis of repeated measures data. This mixed model uses a compound symmetry covariance matrix, fitted by Restricted Maximum Likelihood (REML). Considering that we did not assume sphericity because our design is of repeated measures in time, we used the correction of Geisser-Greenhouse as proposed by Maxwell & Delaney, (2013). We also performed multiple comparisons tests using Bonferroni correction to compare values within each treatment by time of sampling. We performed all statistical analyses in R version 4.0.2 (2020-06-22) -- unless otherwise noted. All values showed in this research correspond to the mean ± standard deviation (SD) and values in the graphs to mean ± standard error (SEM).

2.4 Results

Captivity did not affect CORT plasma levels or $\widehat{Mi}$ (Table 2.1 and 2.2) but influenced immune variables (Table 2.2). The BKA showed no differences between day 0 to 14 in captivity, and decreased significantly on day 21 (Table 2.1, 2.2, Figure 2.2B). The total number of ions (m/z) from each individual's skin secretions increased from the field sample until day 7 in captivity and then decreased significantly until day 21 (Table 1, Figure 2D). The signals profiles from skin secretions from field to captivity samples were clearly different (Figure 3B). In Figure 3C is shown the 489 identified signals from skin secretion, among them, 106 were uniquely exhibited in the field samples. The total

number of ions (m/z) from each individual skin secretions increased from the field sample until day 7 in captivity and then decreased significantly until day 21 (Table 2.1, Figure 2.2D). The signals profiles from skin secretions from field to captivity samples were clearly different (Figure 2.3B). In Figure 2.3C is shown the 489 identified signals from skin secretion, among them, 106 were uniquely exhibited in the field samples.

We found 48 phyla and 237 families compounding the skin bacteriome of *P. disctincta* (Figure 2.4A). Regarding the phyla identified, the most dominant were Proteobacteria, Bacteroidetes, Acidobacteria and Actinobacteria. Of these four phyla, Regarding the 48 phyla identified, Proteobacteria and Bacteroidetes were more dominant in captivity, while Acidobacteria and Actinobacteria were more dominant in the field (Figure2.4B, and 2.5). Our results showed that all the classes were more abundant in the field than in captive conditions (Figure 2.4A). The alpha diversity indexes showed field samples as significantly more diverse than captivity samples (Figure 2.5 and 2.6A, Table 2.2). Only with the Simpson index the alpha diversity was significantly different between days (Table 2.2).

Frogs harbored significantly different bacterial communities in the field and in captivity (PERMANOVA for all time of samplings: Table 2.3, Figure 2.6C). Richness of the skin bacteriome declined over time in captivity (Figure 2.6C). The beta diversity analysis did not show clear clustering among the different bacterial communities; however, some trends are worth noticing. Figure 2.6 shows field communities more similar to captivity samples of day 21 and distant from captivity samples of 0, 7, and 14 days. The distribution of captivity samples on day 0 was the more dispersed in the plot, indicating higher variation within this day. Beta-diversity analysis showed that time of sampling (Table 2.3) was a significant predictor of the variation in bacterial community composition. Analysis of Composition of Microbes (ANCOM) (Mandal et al. 2015)

revealed three genera with significant differential abundance across sampling time. All the three genera belonged to the phylum Proteobacteria, *Sphingomonas* (W = 22.24, ANCOM BC, $p < 0.001$) and *Beijerinckia* (W = 14.65, ANCOM BC, $p < 0.001$) were more abundant in field samples, and *Delftia* (W= -10.54, ANCOM BC, $p < 0.001$) was more abundant in captivity samples (Figure2. 4A and Figure 2.5). The bacterial core community, defined as ASVs with relative abundance of at least 0.1% and present in 95% of samples or more, comprised 30 ASVs, classified into 10 different bacterial genera (Figure 2.7). The two most prevalent core ASVs corresponded to the genus *Propionibacterium* and *Rhodococcus*. Ten genera constituted the core skin bacteriome of *P. distincta* individuals, with Pseudomonaceae, Alcaligenaceae, and Nocardiaceae as the more prevalent families (Figure 2.7).

We found 45 morphotypes of potentially beneficial bacteria isolated by culture against *Aeromonas hydrophila* (Anti-Ah) from 257 morphotypes obtained from all sampling times (Table 4). The potential beneficial bacteria anti-Ah were identified as belonging to two phyla (Proteobacteria and Firmicutes), nine genera, and 37 species (Figure 2.8, Table 2.4). 40,5% of the isolates belong to the genus *Pseudomonas*, followed by the genus *Delftia*, with 16,21% (Figure 2.9). The Firmicutes phylum was represented by only two genera: *Paenibacillus* and *Staphylococcus*. All the potential beneficial bacteria Anti-Ah identified showed lower abundance in samples from the field (Figure2.8). Field samples showed only four genera of the potential beneficial bacteria Anti-Ah, three of them from phylum Proteobacteria, and only one from phylum Firmicutes. The genus *Paeinibacillus* was exclusively present in the field samples, albeit the 66.6% of the genus identified were exclusively present in captivity samples (Figure 2.9). The genus *Acinetobacter*, *Enterobacter,* and *Staphylococcus* appeared by the end of

the time in captivity, with *Staphylococcus* increasing its abundance almost twofold from day 14 to 21 (Figure 9B).

We evaluated the abundance of putative *B. dendrobatidis*-inhibitory bacteria (Anti-Bd) in all the samples from each sampling time and found significant differences between samples from field and captivity (Figure 2.10). We putatively identified 24 genera and 29 species in the Bd database (Woodhams et al. 2015). From the total OTUs putatively identified, we identified four phyla (Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes) considered as Anti-Bd OTUs in the database (Woodhams et al. 2015). We identified ten genera, and we identified only six OTUs until species level. The species detected were *Brevibacterium aureum, Elizabethkingia meningoseptica, Microbacterium chocolatum, Pseudomonas alcaligenes, Erwinia dispersa* and *Serratia marcescens* (Figure 10A, Figure 12). All the putatively anti-Bd bacteria identified showed lower abundance in samples of field conditions (Figure 10B). The species *Brevibacterium aureum* and *Pseudomonas alcaligenes* were present throughout all the time of sampling. Although, *Elizabethkingia meningoseptica* appeared only in field samples, *Erwinia dispersa* in field and day 0 samples, and *Serratia marcescens* only in captivity samples. *Pseudomonas, Acinetobacter*, and *Delftia* were genera present in the core bacteriome and belong to the potential beneficial bacteria group anti-Ah and Anti-Bd (Figure 9B, 10B).

Finally, we assessed all individuals for Bd presence, and six individuals were positive. We sampled and included an individual with the higher zoospore load (464.2 zoospores) in the field, but it was not brought to captivity. Other five individuals showed really low scores (2 – 3.9 zoospores) of Bd zoospores loads at least one day of sampling in captivity but were negative for the rest of the time because of that we decided included in our results (Table S1).

Table 2.1. Descriptive statistics of plasma corticosterone levels, plasma BKA and skin ionic signals richness analyzed in field and captivity conditions with *Phyllomedusa distincta* individuals.

Variables	Time of Sampling *(Day)*	N	Mean	±SD
CORT (ng/µl)	Field	—	—	—
	0	2	1,12	1,30
	7	3	2,49	1,03
	14	4	6,60	10,12
	21	3	0,78	0,76
BKA (%)	Field	—	—	—
	0	2	-1,01	0,45
	7	2	-0,72	0,13
	14	4	-1,25	0,62
	21	2	-6,66	4,57
Skin Ionic Signals Richness	Field	5	74,00	15,52
	0	2	79,50	3,54
	7	2	103,50	4,95
	14	3	91,67	16,20
	21	2	91,00	8,49
$\widehat{M\iota}$	Field	6	11,12	5,23
	0	6	10,47	3,57
	7	6	8,42	5,34
	14	6	8,17	4,94
	21	6	11,48	4,99

Table 2.2. Linear mixed model results for alpha diversity indexes values (Chao1, Simpson, and Shannon) of skin microbiota of *Phyllomedusa distincta* control group. The times of sampling were: Field = when individuals collected, and Day 0, 7, 14, and 21= days in captivity condition.

	Shannon			**Simpson**			**Chao1**		
Predictors	*Estimates*	*Conf. Int (95%)*	*P-Value*	*Estimates*	*Conf. Int (95%)*	*P-Value*	*Estimates*	*Conf. Int (95%)*	*P-Value*
(Intercept)	2.71	1.53 – 3.89	**<0.001*** **	0.82	0.76 – 0.88	**<0.001*** **	449.01	-144.23 – 1042.25	0.138
Day 14	0.96	-0.60 – 2.52	0.226	0.11	0.03 – 0.19	**0.006** **	124.32	-209.49 – 458.13	0.465
Day 21	0.81	-0.75 – 2.37	0.310	0.09	0.02 – 0.17	**0.019** **	101.49	-232.31 – 435.30	0.551
Day 7	1.10	-0.57 – 2.77	0.196	0.12	0.04 – 0.21	**0.004** **	106.55	-259.14 – 472.24	0.568
Field	2.95	1.62 – 4.28	**<0.001*** **	0.15	0.08 – 0.22	**<0.001*** **	691.09	330.51 – 1051.68	**<0.001*** **
Random Effects									
σ^2			1.09			0.00			47933.08
τ_{00}			0.00 $_{\text{Individuo}}$			0.00 $_{\text{Individuo}}$			775680.59 $_{\text{Individuo}}$
ICC						0.11			0.94
N			12 $_{\text{Individuo}}$			12 $_{\text{Individuo}}$			12 $_{\text{Individuo}}$
Observations			25			25			25
Marginal R^2 / Conditional R^2			0.552 / NA			0.426 / 0.490			0.103 / 0.948

** p<0.05 ** p<0.01 *** p<0.001*

Table 2.3. PERMANOVA results for the beta diversity analysis of skin bacteriome of *Phyllomedusa distincta* using unweighted and weighted UniFrac distances. Asterisks represent significant differences (* *p<0.05* ** *p<0.01* *** *p<0.001)*.

		Unweighted UniFrac					***Weighted UniFrac***				
	Df	Sums Of Sqs	Mean Sqs	F.Model	R2	Pr(>F)	Sums Of Sqs	Mean Sqs	F.Model	R2	Pr(>F)
Time of Sampling	4	0,012	0,003		0,33	0.03*	0,012	0,003	2,463	0,33	**0,03***
Residuals	20	0,024	0,001	NA	0,67	NA	0,024	0,001	NA	0,67	NA
Total	24	0,036	NA	NA	1	NA	0,036	NA	NA	1	NA

Pairwise Comparisons

Group 1	Group 2	Sample size	pseudo-F	p-value	q-value
0	14	7	1.218	0.165	0.275
0	21	7	1.551	0.104	0.208
0	7	7	1.232	0.218	0.311
0	Field	13	2.977	**0.003****	0.01
14	21	8	0.953	0.526	0.584
14	Field	14	2.309	**0.002****	0.01
21	Field	14	2.253	**0.001*****	0.01
7	14	8	0.781	1.0	1.0
7	21	8	0.929	0.502	0.584
7	Field	14	1.866	**0.005***	0.012

Table 2.4. Species of potentially beneficial bacteria identified in skin microbiota of *Phyllomedusa distincta* using a molecular marker for the 16S ribosomal gene.

Morphotype ID	GreenGenes ID	Top-hit strain	Genbank ID	Accession Number	Genus	Species
CO-10	*Pseudomonas monteilii*	NBRC 103158	*Pseudomonas sp. strain LIN1*	KY922972	+	-
CO-11	*Acinetobacter lwoffii*	NCTC 5866	*Acinetobacter sp. strain OE*	MH915643.1	+	-
CO-14	*Delftia acidovorans*	2167	*Delftia sp. strain JY-49*	MK332301.1	+	-
CO-15	*Pseudomonas hunanensis*	LV	*Pseudomonas plecoglossicida strain XSDHY-P*	CP031146.1	+	-
CO-18	*Delftia acidovorans*	2167	*Delftia sp. S17*	HE662648.1	+	-
CO-26	*Paenibacillus taichungensis*	BCRC 17757	*Paenibacillus sp. E12*	KT965177.1	+	-
CO-39	*Enterobacter cancerogenus*	ATCC 33241	*Yokenella regensburgei strain A4-18*	MH074808	-	-
D14-02	*Yokenella regensburgei*	ATCC 49455	*Yokenella regensburgei strain A4-18*	MH074808.1	+	+
D14-25	*Serratia quinivorans*	CP6a	*Serratia marcescens strain ZCL-01*	MH251251.1	+	-
D14-29	*Pseudomonas protegens*	CHA0	*Pseudomonas protegens strain Exi5-13*	MK235212.1	+	+
D14-30	*Delftia tsuruhatensis*	NBRC 16741	*Delftia tsuruhatensis strain AD4*	MK336721.1	+	+
D14-36	*Paenibacillus LAZU_s*	DMB20	*Paenibacillus sp. strain CCOS10*	MK005262.1	+	-
D14-52	*Pseudomonas protegens*	CHA0	*Pseudomonas sp. strain RL17-340-BIF-B*	MK373708.1	+	-
D21-04	*Pseudomonas protegens*	CHA0	*Pseudomonas protegens strain KPS-50*	MK402059.1	+	+
D21-05	*Yokenella regensburgei*	ATCC 49455	*Yokenella regensburgei strain A4-18*	MH074808.1	+	+
D21-06	*Serratia marcescens subsp. marcescens*	ATCC 13880	*Serratia marcescens strain Tjj550*	MF280125.1	+	+
D21-16	*Pseudomonas protegens*	CHA0	*Pseudomonas protegens strain Exi5-13*	MK235212.1	+	+
D21-18	*Enterobacter siamensis*	C2361	*Serratia sp. strain BR13856*	MK156446.1	-	-
D21-30	*Serratia marcescens subsp. marcescens*	ATCC 13880	*Serratia sp. CR1-2*	LC439479.1	+	-
D7-02	*Delftia acidovorans*	2167	*Delftia sp. strain JY-49*	MK332301.1	+	-
D7-13	*Paenibacillus glucanolyticus*	DSM 5162	*Paenibacillus glucanolyticus strain B05*	MH734924.1	+	+
D7-26	*Staphylococcus argensis*	M4S-6	*Staphylococcus sp. strain SW11*	MK358977.1	+	-
D7-28	*Pseudomonas protegens*	CHA0	*Pseudomonas protegens strain KPS-50*	MK402059.1	+	+
D7-49	*Enterobacteriaceae CP009451_s*	SSMD04	*Enterobacteriaceae bacterium strain T4-4-1*	MH074838.1	+	-
D7-52	*Pseudomonas protegens*	CHA0	*Pseudomonas sp. strain RL17-340-BIF-B*	MK373708.1	+	-

D7-54	*Staphylococcus sciuri*	DSM 20345	*Staphylococcus sp. strain SW11*	MK358977.1	+	-
D7-55	*Delftia acidovorans*	2167	*Delftia sp. strain TC10*	MK459473.1	-	+
DO-01	*Pseudomonas PHTD_s*	JJ3	*Pseudomonas japonica strain CH-26*	MH712955.1	-	-
DO-02	*Pseudomonas protegens*	CHA0	*Pseudomonas sp. strain MR29*	MG674320.1	+	+
DO-11	*Serratia quinivorans*	CP6a	*Uncultured bacterium OreMos_Food_304*	LC409838.1	-	-
DO-16	*Klebsiella michiganensis*	W14	*Klebsiella sp. RCB571*	KT260783.1	+	-
DO-19	*Pseudomonas protegens*	CHA0	*Pseudomonas sp. strain MR39*	MG674323.1	+	-
DO-22	*Pseudomonas protegens*	CHA0	*Pseudomonas sp. SCAU611*	KC012911.1	+	-
DO-23	*Serratia myotis*	12	*Uncultured Serratia sp. clone K-98-13-8*	KJ804058.1	+	-
DO-24	*Serratia CP015613_s*	PRI-2c	*Uncultured bacterium OreMos_Food_308*	LC409842.1	-	-
DO-25	*Delftia acidovorans*	2167	*Delftia acidovorans strain KBL21*	MG576174.1	+	+
DO-26	*Pseudomonas protegens*	CHA0	*Pseudomonas protegens strain KPS-50*	MK402059.1	+	+
DO-28	*Pseudomonas asplenii*	ATCC 23835	*Uncultured Pseudomonas sp. clone V107*	KX456361.1	+	-
DO-29	*Pseudomonas protegens*	CHA0	*Pseudomonas sp. strain MR29*	MG674320.1	+	-
DO-30	*Delftia acidovorans*	2167	*Delftia sp. strain JY-49*	MK332301.1	+	-
DO-31	*Pseudomonas protegens*	CHA0	*Pseudomonas sp. S18(2014)*	KM117221.1	+	-
DO-43	*Pseudomonas protegens*	CHA0	*Pseudomonas sp. strain MR29*	MG674320.1	+	-
DO-55	*Pseudomonas protegens*	CHA0	*Pseudomonas sp. strain RL17-340-BIF-B*	MK373708.1	+	-
DO-56	*Pseudomonas sesami*	SI-P133	*Pseudomonas protegens strain KPS-50*	MK402059.1	+	+
DO-59	*Pseudomonas protegens*	CHA0	*Pseudomonas sp. strain MR29*	MG674320.1	+	-

(-) Taxa ID different in both two databases, (+) same taxa ID assigned in both databases.

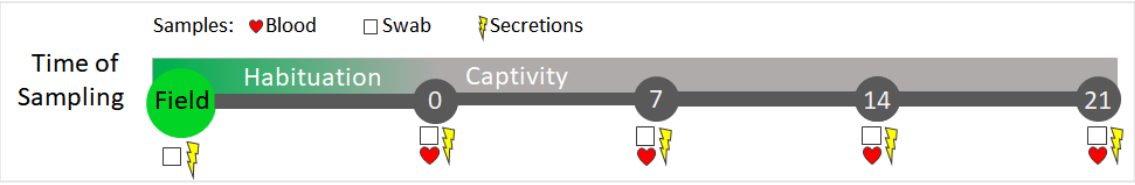

Figure 2.1: Scheme showing the time of sampling, type of samples obtained and duration of study. Blood samples were obtained by cardiac puncture (❤️), skin bacteriome by swabs (□) and cutaneous secretions by electrostimulation (⚡).

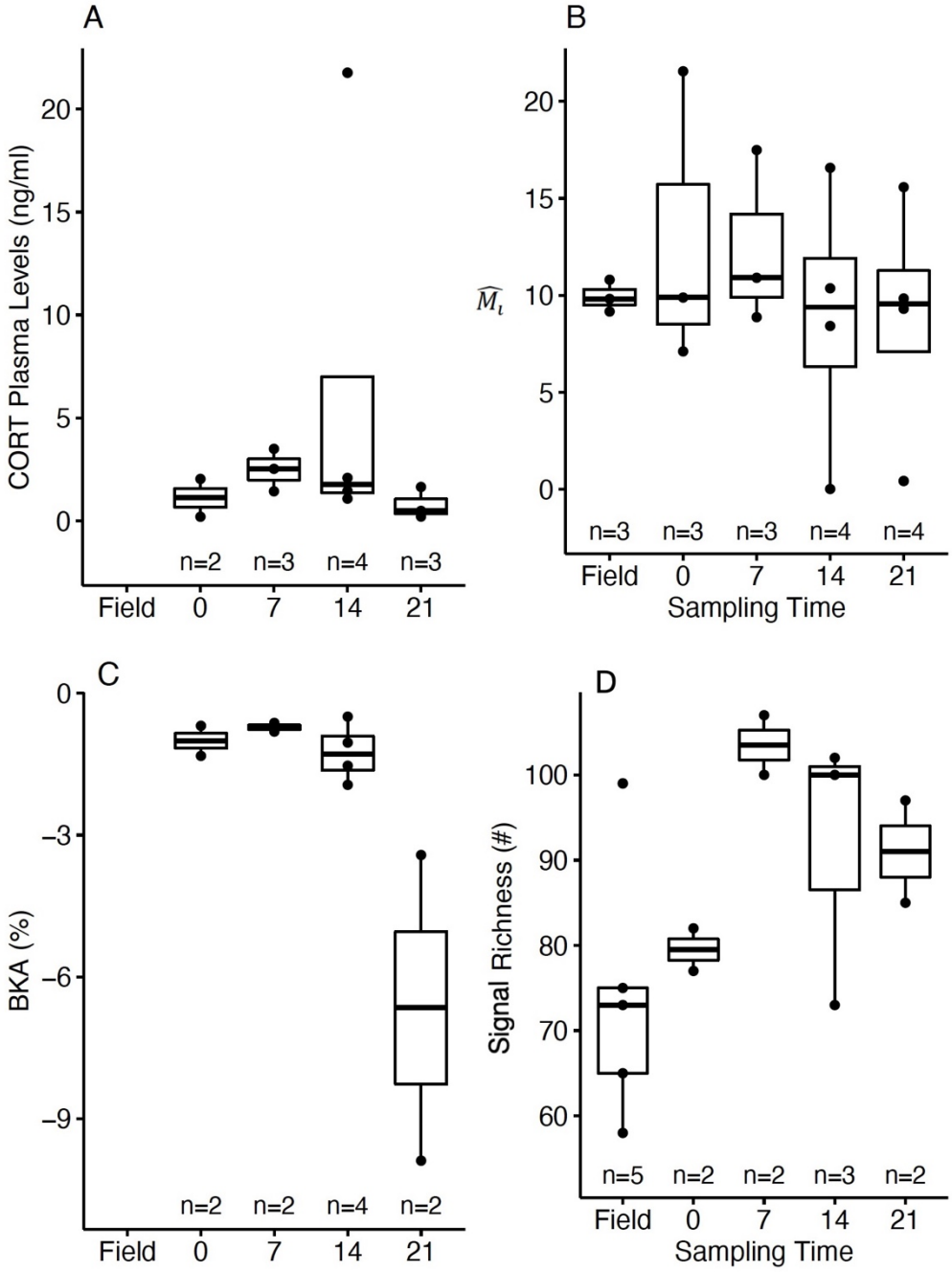

Figure 2.2: Physiological and immunological parameters measured in field and captivity conditions of *Phyllomedusa distincta* individuals. (A) Corticosterone plasma levels, (B) (C) BKA values were measured only in captivity. (D) MALDI-TOF MS corresponding to the mean of number of identified signals in each sampling time. The X-axis represent the sampling time from day 0 to 21 in captivity conditions.

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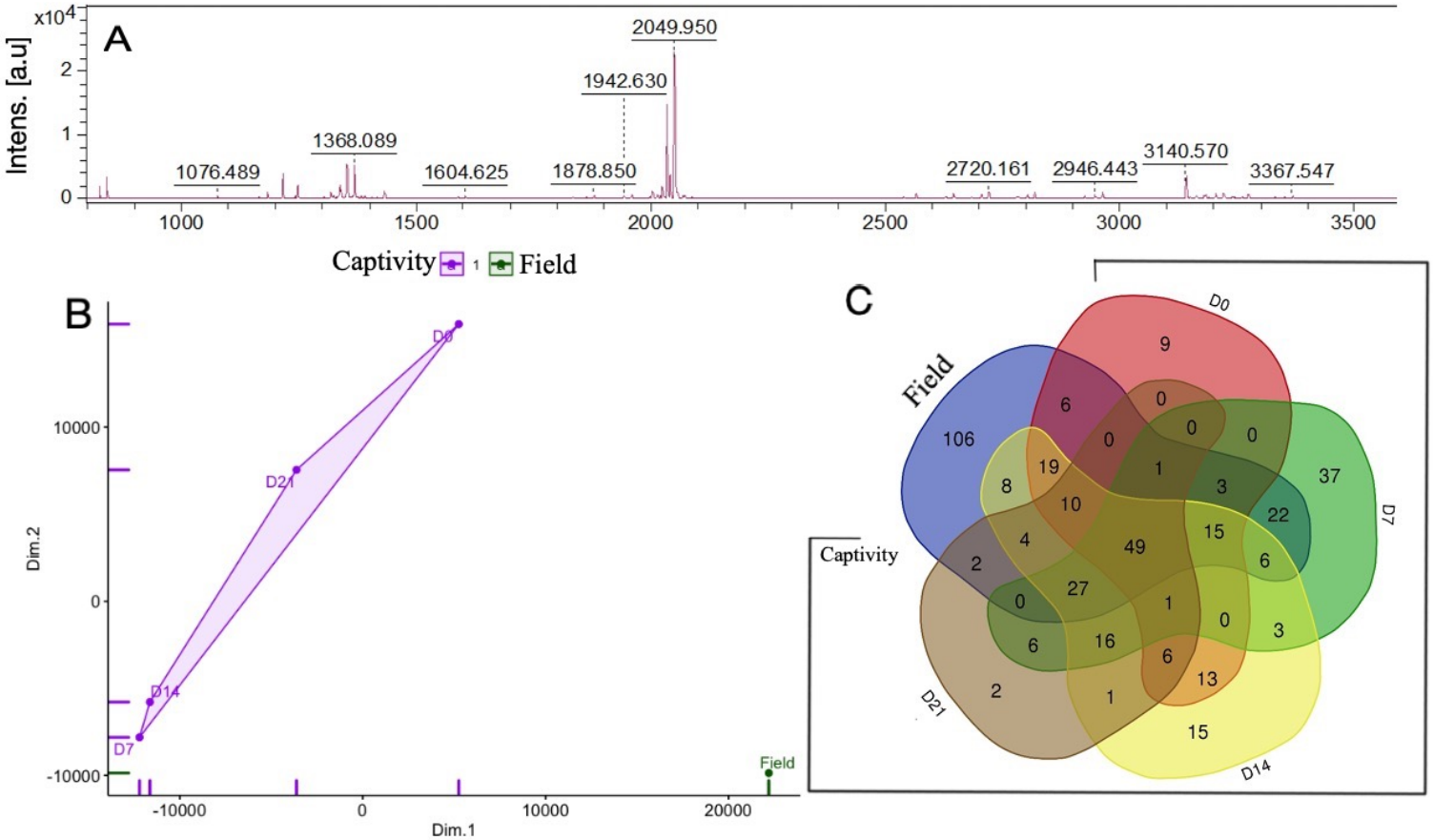

Figure 2.3: Mass spectrometry signals from skin secretions of *Phyllomedusa distincta* in field and captivity conditions. (A) A representative MALDI-TOF MS spectrum from skin secretion in field conditions. (B) Principal coordinates analysis of k-means distances between samples of skin ions (*m/z*). (C) Grouping polygon based on the similarity between samples, called Venn diagram, showing the number of shared signals between all analyzed samples

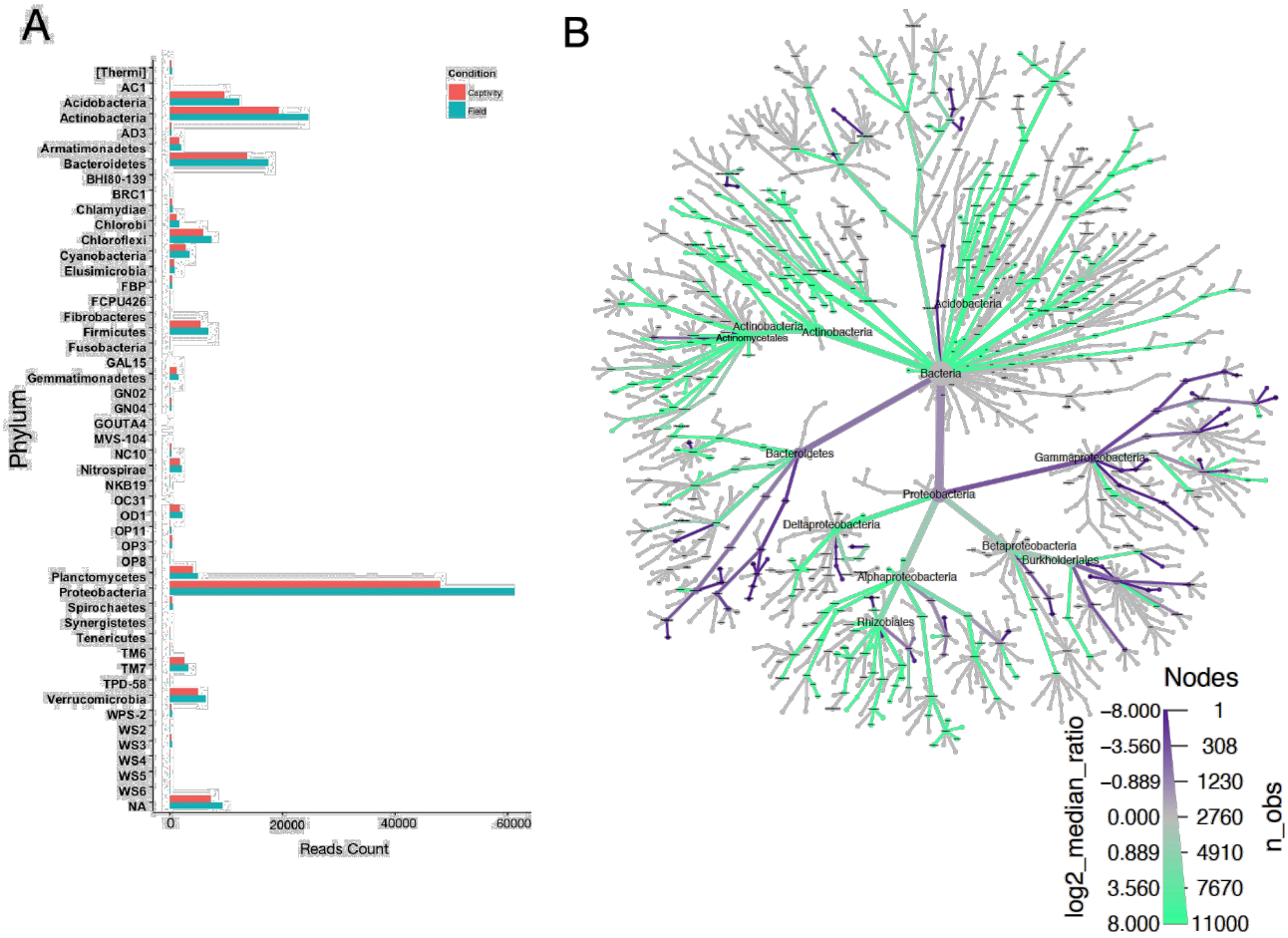

Figure 2.4: Abundance of taxa identified in the skin bacteriome of *Phyllomedusa distincta* in field and captivity conditions. (A)Bars shows the reads count of each Phylum identified in field (green) and captivity conditions (orange). (B) Heat-tree of the abundances of Phylum identified. The color of each taxon represents the log-2 ratio of difference between field (green) and captivity (purple) conditions, and the size of nodes represents the number of observations (n_obs). The lineages with more than 500 observations are marked with a bigger font size.

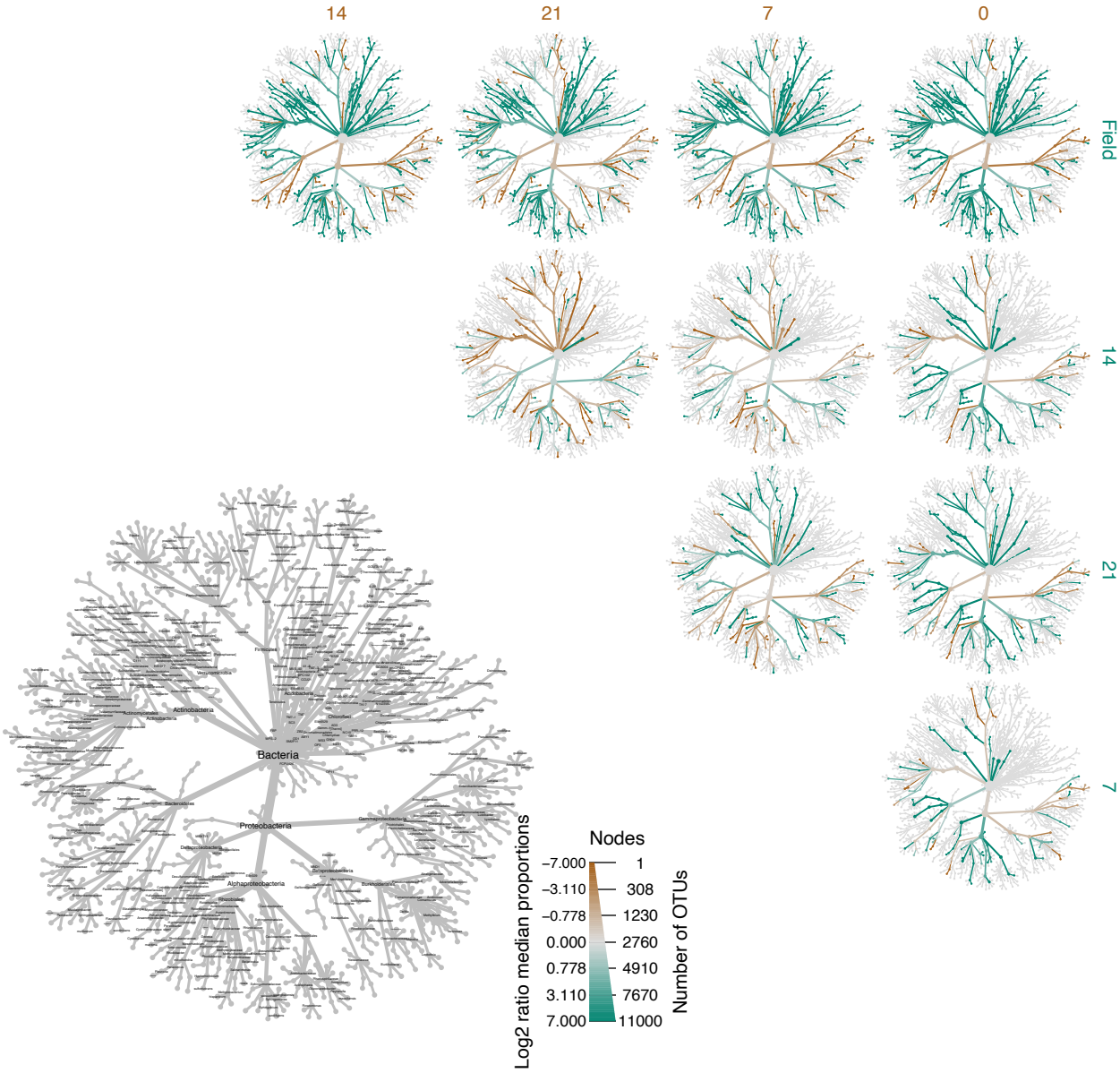

Figure2.5: Differential heat tree matrix depicting the change in taxa abundance through the different times of sampling, for skin bacteriome of *Phyllomedusa distincta*. The gray tree on the lower left shows the names of the taxa. The color of each taxon represents the log-2 ratio of median proportions of reads observed at each time of sampling. Only significant differences are colored, determined using a Wilcox rank-sum test followed by a Benjamini-Hochberg (FDR) correction for multiple comparisons. Green taxa are more abundant in the day shown in the row and those colored brown for the day shown in the column.

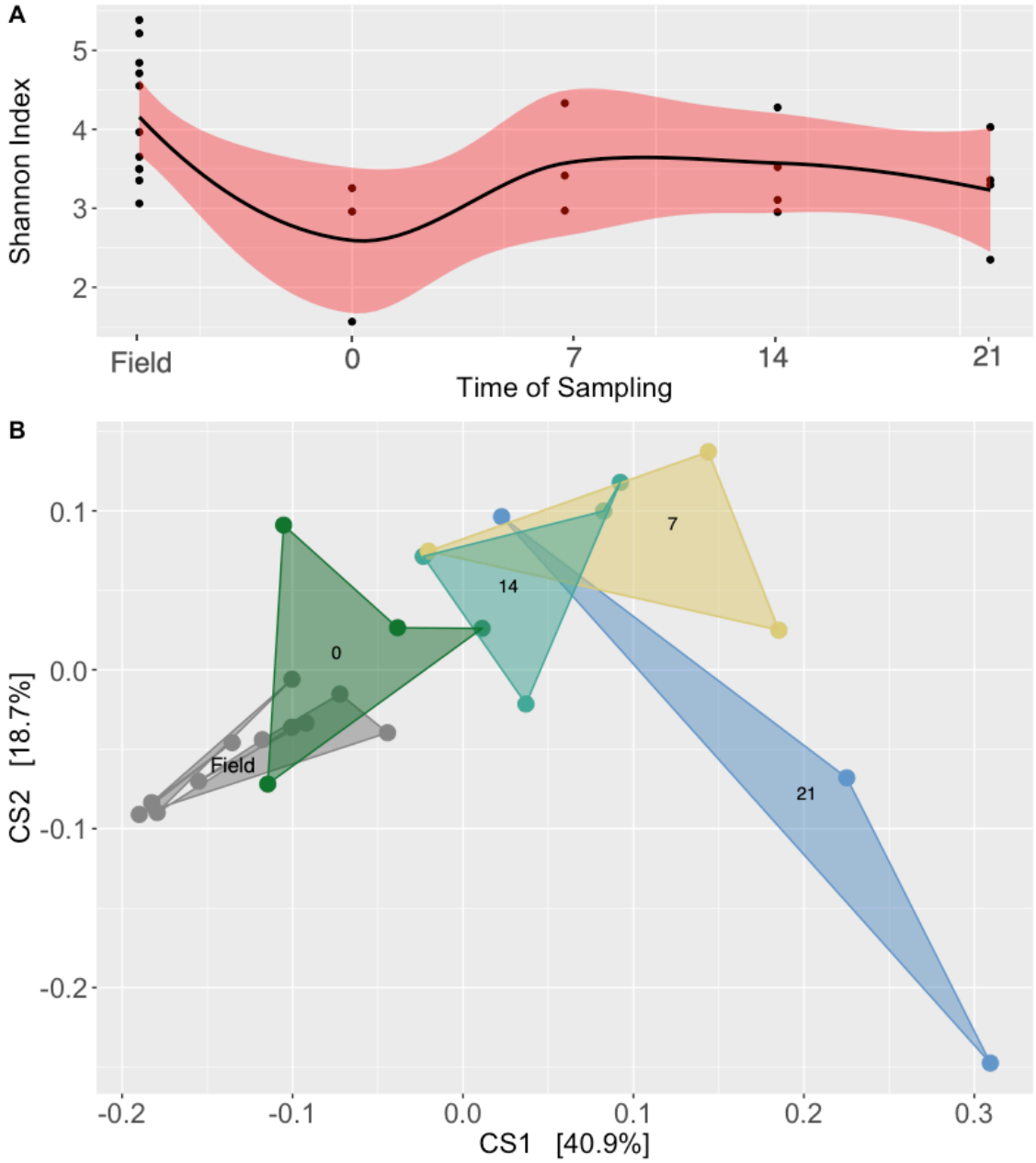

Figure 2.6: Alpha and beta diversity of skin bacteriome of *Phyllomedusa distincta* individuals through time of sampling. (A) Shannon-Weiner index values by time of sampling. (B)Double principal coordinates analysis (DPCoA) using Bray Curtis distances between time of sampling.

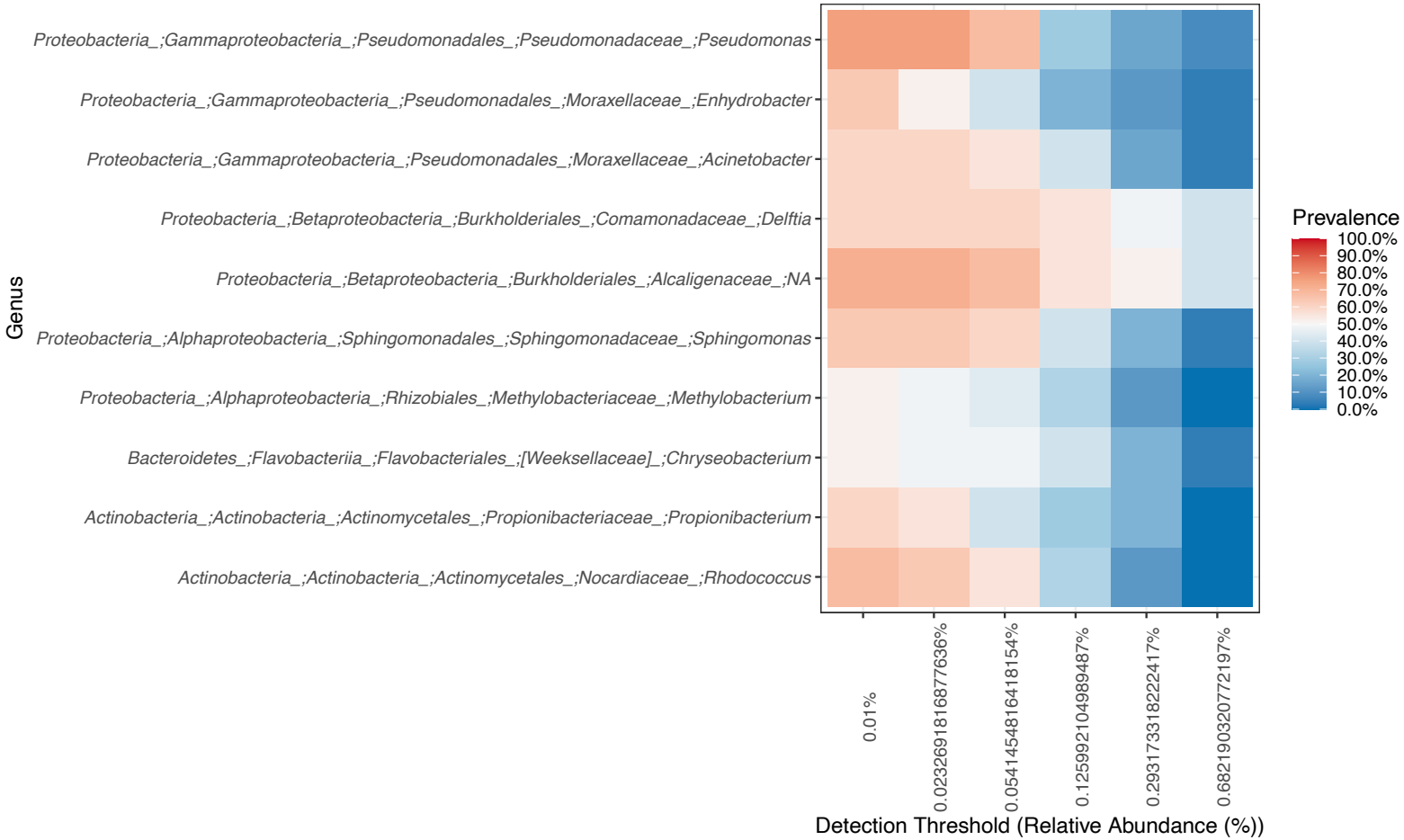

Figure 2.7: Heat-map diagram of the core skin bacteriome composition at genus level for *Phyllomedusa distincta* throughout the time of sampling.

Figure 2.8: The most abundant potential beneficial bacteria anti-*Aeromonas hydrophyla*. (A) Phyla and (B) the families in skin bacteriome of *Phyllomedusa distincta* throughout the time of samplings. The x-axis shows the times of sampling: Field =capture moment; and 0, 7, 14, and 21= days in captivity

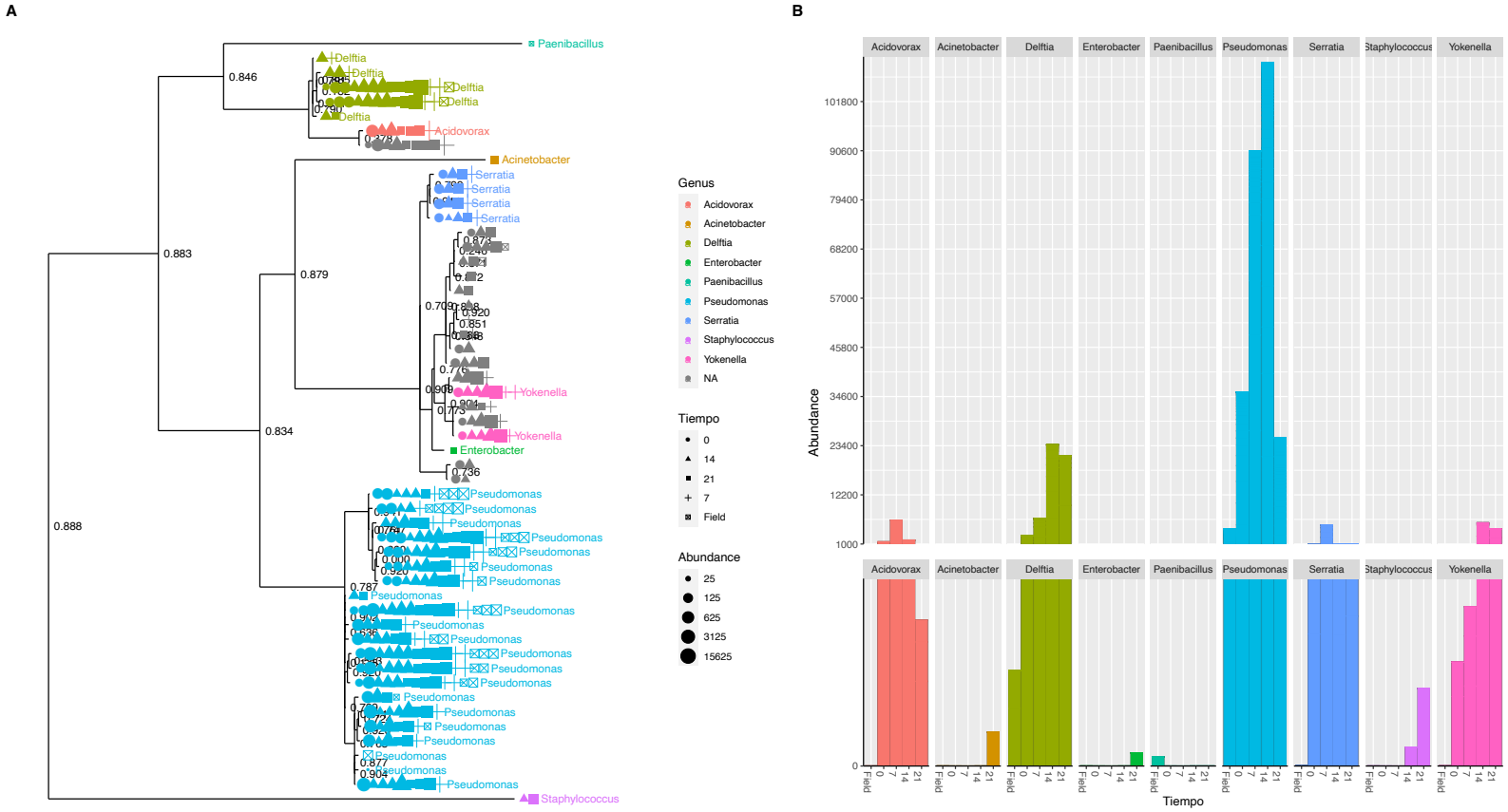

Figure 2.9: Genera of potentially beneficial bacteria anti-*Aeromonas hydrophyla* isolated and identified on skin bacteriome of *Phyllomedusa distincta*. (A) Phylogenetic tree of genera identified. The color key shows each genus identified, and the shapes represents the time of sampling. The point size represents abundance in each sample on a log scale. (B) Abundance of genera throughout the time of experiment. The x-axis shows the times of sampling: Field =capture moment; and 0, 7, 14, and 21= days in captivity**.**

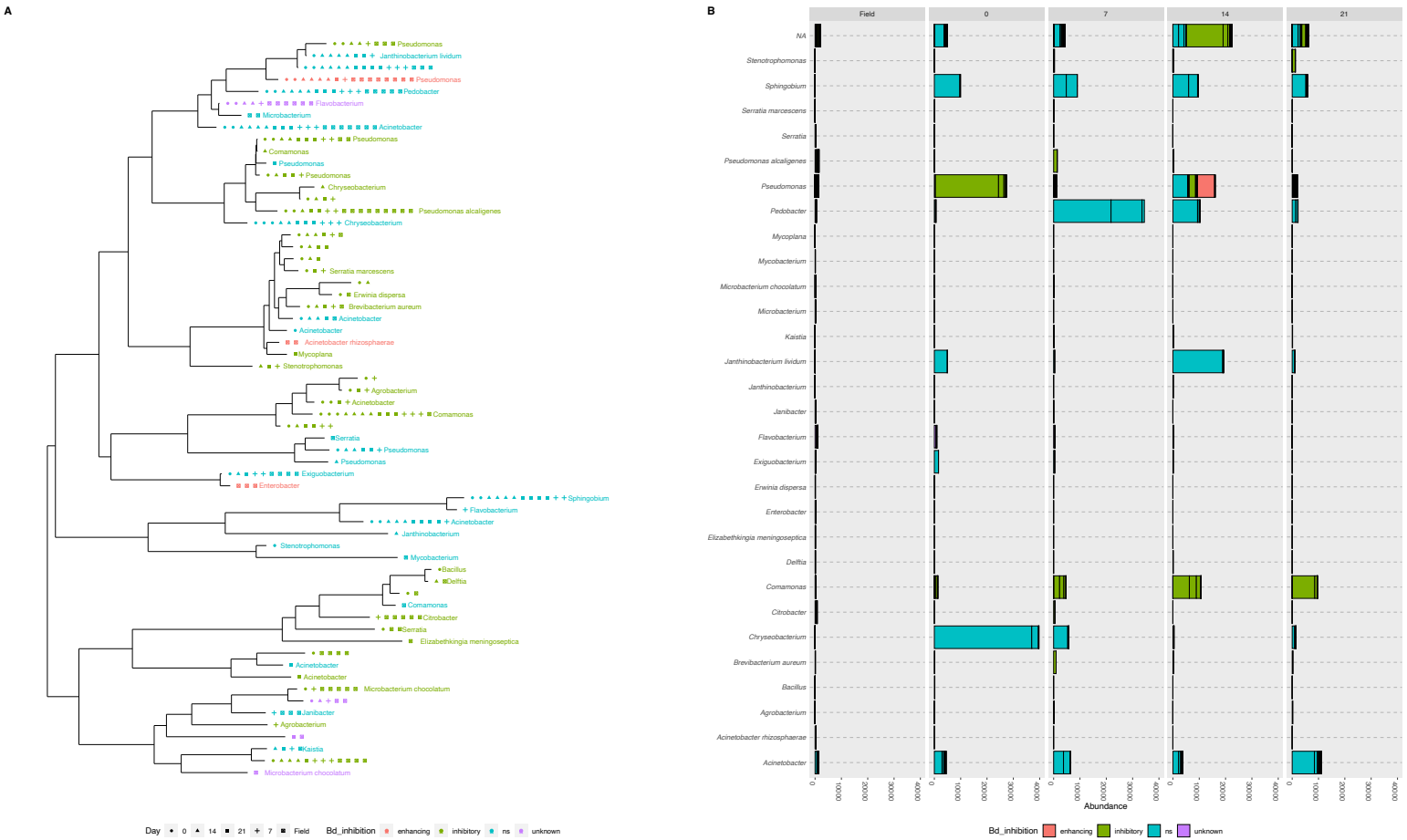

Figure 2.10. OTUs anti-*Batrachochytrium dendrobatidis* putatively identified on skin bacteriome of *Phyllomedusa distincta*. (A)Phylogenetic tree of genera identified. The color key shows the Bd-inhibition category according to the database of (Woodhams et al. 2015), and the shapes represents the time of sampling. (B) Abundance of each OTU throughout the time of experiment. The x-axis shows the times of sampling: Field =capture moment; and 0, 7, 14, and 21= days in captivity.

2.5 Discussion

We evaluated the effect of captivity on plasma BKA and skin ionic profile as innate immune parameters, the bacteriome structure of the skin, especially the presence of potentially beneficial members as pathogen inhibitors, and its relationship with plasma levels of CORT, and $\widehat{M\iota}$ as an indicator of the physiological status of *Phyllomedusa distincta*. Innate immunity parameters measured shifted with captivity time, and clear differences in α-diversity and community composition were present between wild and captive individuals. Further, the potentially beneficial bacteria anti-Ah and anti-Bd increased with time in captivity.

Captivity effects on physiological state and innate immune parameters

Against our predictions, CORT plasma levels and $\widehat{M\iota}$ did not differ in the individuals of *P. distincta* between wild and captive conditions. Thus, time in captivity did not affect the selected physiological state indicators. Classically, the stress response in vertebrates is related to increased activity of the hypothalamus-hypophysis-adrenal/interrenal axis and augmented CORT plasma levels (Dhabhar and Mcewen 1999). Studies associated captivity with transiently increased CORT levels (after 5 - 15 days in captivity), backing to baseline levels after 25 days (Narayan et al. 2011). Against our predictions, captivity did not increase CORT plasma levels neither decrease $\widehat{M\iota}$ in *P. distincta*. However, captivity shifted parameters of innate immunity. Other studies with anurans found that T was positively associated with immune parameters (Assis et al. 2017; Titon et al. 2017) and the drop in plasma T concentration was accompanied by a drop in immunocompetence with the time of captivity (Narayan, Cockrem, and Hero 2013; Marler and Ryan 1996). For that reason, we suggest measuring in future additional physiological mediators as testosterone levels.

In several vertebrates, including amphibians, stressors can change the immune parameters, taking hours to days for a full response (Webster et al., 2002; Falso et al., 2015; Fischer and Romero, 2019). Previous studies reported that BKA may respond preponderantly to captivity as a stressor in *Rhinella icterica* (Assis et al. 2015; Titon et al. 2019). After 21 days of captivity, BKA of *P. distincta* decreased significantly without apparent changes on CORT plasma levels. Considering that we used plasma rather than whole blood samples for BKA, most of the observed inhibition of growth of Ah might be associated with complement system action (Mayilyan et al. 2008; Janeway et al. 2001). Many distinct plasma proteins constitute the complement system, comprising three different pathways of activation and mechanisms of action (Janeway et al. 2001). First, generating many large activated complement proteins that bind covalently to pathogens, opsonizing them for engulfment by phagocytes. Second, through chemoattraction recruiting more phagocytes to the site of complement activation, and activating these phagocytes. Third, by creating pores in the bacterial membrane using terminal complement components (Janeway 2001). The complement system also provides, through opsonization, an important link between the two branches of the immune system, innate and the adaptive response (Murphy et al. 2010; Rodriguez and Voyles 2020). Considering that a limitation in the complement function may severely constraint the development of a robust and effective immune protection against pathogens and disease (Rodriguez and Voyles 2020), we suggest than captivity has relevant immune consequences for *P. distincta*. Nonspecific defenses as complement activation, may be key to resisting infection and protecting against lethal diseases, such as chytridiomycosis (Rodriguez and Voyles 2020; Grogan et al. 2018).

Similarly, captivity affected the innate immune response of the skin shifting the secretions ionic profiles and the richness of signals. Against our predictions, we identified

transiently increased signals by individuals in captivity than in field conditions. However, the skin ionic profiles of field samples were less diverse than in captivity. Corticosteroids under pharmacological concentrations facilitate the renewal of skin peptides in frogs according to previous studies (Tatiersky et al. 2015). Although we applied the same protocol of repeated maximal induction of peptide secretions used by Tatiersky et al. (2015), we did not include experimentally increased CORT plasma levels in *P. distincta*. However, we need to consider that the intense and repeated manipulation might have increased catecholamines and, possibly CORT, sporadically (L Michael Romero and Reed 2005). So, it is possible that transient endocrine changes occurred in response to manipulation, and we did not detect them since we always acquired blood samples before inducing the skin secretions. In this way, it is possible that cells producing peptides in the granular glands would not develop mRNAs for defensive peptides, precluding recovery of peptide profile found in the field conditions. Previous studies have found decreased BKA associated with increased CORT when anurans loose body mass throughout time in captivity (Titon et al. 2017). Contrary to those studies, *P. distincta* did not increase CORT, neither decrease body condition under captivity. In this way, the observed shifted of innate immunity (skin ionic profiles and plasma BKA) seems not to be derived from energetic limitations. Previous studies reported that exposure to pathogens resulted in increased synthesis of AMPs in *Rana esculenta* and *Bombina orientalis* (Simmaco et al. 1998; Mangoni et al. 2001). After gland depletion induced by electrostimulation, frogs maintained in a sterile environment failed to restore AMPs whereas those exposed to microorganisms did (Simmaco et al. 1998; Mangoni et al. 2001). Additionally, the host skin defense peptides possess a differential effect on their own microbiota promoting their growth or impeding colonization from non-native bacteria (Flechas et al. 2019). Thus, we suggest that the transiently increased signals detected in the skin ionic profiles was related

with the reshaped skin bacteriome, considering that more than the half of genera of potentially beneficial bacteria anti-Ah and Anti-Bd were detected only in captivity samples. The new bacterial colonization and the repeatedly skin gland depletion must be induced that changes.

Captivity effects on skin bacteriome

Studies have shown that the recovery to a pre-disturbance state, resilience, may be a key component in disease resistance (Weeks, Parris, and Brown 2020; Rebollar et al. 2016). Testing for resilience of the host microbial community may allow researchers to better predict which populations are susceptible to emerging diseases like chytridiomycosis (Weeks, Parris, and Brown 2020; Shade et al. 2012). For instance, the cutaneous microbiome of *Lithobates sphenocephalus* recovered after two biotic disturbances without inputs from environmental reservoirs, suggesting that the core community is able to remain with the animal (Weeks, Parris, and Brown 2020). Short-term exposure to an abiotic disruptor had also little impact on the skin microbiome of adult spring peepers (*Pseudacris crucifer*) (Hughey et al. 2016). In our study, transferring individuals into captivity conditions changed the structure of skin bacteriome communities according to our predictions. Interestingly, *P. distincta* skin bacteriome communities in the field were more similar in structure to those in the last time point sampling in captivity (day 21), than to the first time point sampling in captivity (day 0). This suggests that captivity is a disruptor of skin bacteriome communities and these communities seem to be resilient because they recover and tend to resemble a pre-disturbance (captivity) state (Figure 6B, 6C). In this sense, the greatest remodeling in the cutaneous bacteriome of *P. distincta* occurred at the beginning of captivity maintenance. After 7 days of being brought from the field, the number of genera composing the skin bacteriome decreased. *Azospirillum, Bacteroides, Beijerinckia, Brevundimonas,*

Comamonas, Delftia, Elizabethkingia, Flavobacterium, Massilia, Methylobacterium, Mycobacterium, and *Nocardioides* disappeared in samples from day 0. The genus *Beijerinckia* was abundant in the field and was absent on captivity samples, which suggest it as a lineage that might be sensitive to disruptions caused by both the physiological state of the host and environmental changes. *Beijerinckia* is commonly found as free-living bacteria in acidic soils, in plant rhizosphere and phyllosphere environments (Marín and Arahal 2014; Becking 2006). Many insights into the ecology of *Beijerinckia* come from research on biological nitrogen fixation in graminaceous plants in Brazil (Marín and Arahal, 2014). Although *Beijerinckia* has also been described as part of the skin microbiota of the Puerto Rican frog (*Eleutherodactylus coqui*) (Hughey et al. 2017), its physiological role in the community is not clear. This is probably associated to the fact that antibiotic potential has been the major driver of the studies with skin frog microbiota, and that characteristic has been not described for *Beijerinckia*. In contrast, *Sphingomonas* was more abundant in samples collected under captive conditions than in the field. Several indicators OTUs in the genus *Sphingomonas* were associated with Bd associated dying toads (*Atelopus zeteki*), suggesting that *Sphingomonas* suppresses the production of antimicrobial compounds produced by symbiotic bacteria (Becker et al. 2015), as observed in tomato plants (Someya and Akutsu 2009). This genus of aerobic and Gram-negative bacteria was also identified as Bd enhancer in studies conducted with four amphibian species (Walke et al. 2017). The genus *Delftia* also increased its abundance in captivity samples, but members of this genus have been shown to inhibit the growth of Bd fungus (Cikanek et al. 2014) and *Enterobacter aerogene* bacteria (B. Assis et al. 2020). *Sphingomonas* and *Delftia* were part of the core community and the most abundant genera in field samples from the individuals of *P. distincta* studied. Loudon et al. (2014) hypothesized that the core bacteria is tightly linked with immune

regulation, immune evasion, and the activation or deactivation of the host-defense pathways. It has been also suggested captivity remodeling of skin microbiome could decrease the immune ability of the host (Hernández-Gómez, Briggler, and Williams 2019). We hypothesize that as captive animals lose some genus of beneficial bacteria (e.g. *Paenibacillus*), there may be an increased recruitment and proliferation of other microbial members (e.g., *Delftia* and *Pseudomonas*), perhaps as an alternative mechanism to reduce skin dysbiosis and promote host health. Overall, our study provides many leads for important future work.

Remarkably, the identification of most potential beneficial bacteria anti-Ah and anti-Bd occurred in captivity conditions. Specifically, *Pseudomonas* genus was dominant, belonging to the core community, and prevalent throughout time evaluated. This genus is an ecologically diverse group, composed by common environmental bacteria, known pathogens, and commensals, found on the skin of amphibians (Woodhams et al. 2007), fishes (Boutin et al. 2013), mammals (Cogen, Nizet, and Gallo 2008) and plantae (Berendsen, Pieterse, and Bakker 2012) with high relative abundances (Roth et al. 2013), as seen in our study. Agriculture commonly uses *Pseudomonas* species in biological control of plagues and diseases (Philippot et al. 2013), and as antifungal for amphibians in the laboratory (Flechas et al. 2012; Madison et al. 2017). Isolates of *Pseudomonas* from *Rana cascadae* showed no anti-Bd activity (Roth et al. 2013), and *Pseudomonas* declined abundance with Bd infection in the *R. sierrae* (Jani and Briggs 2014). However, *Pseudomonas* isolates from *P. distincta* showed in vitro anti-Ah activity and putative anti-Bd functions, suggesting that even with the reshaping of skin bacteriome caused by captivity, this genus is suitable as probiotic useful in bioaugmentation. Locally occurring protective bacteria on amphibians has effectively mitigated emerging infectious diseases

like Chytridiomycosis in laboratory trials and field trials through bioaugmentation (Bletz et al. 2013; Woodhams et al. 2016).

Firmicutes phylum was represented by two genera, *Paenibacillus* and *Staphyloccocus*. Isolated from a wide range of sources, including organisms and environments, the genus *Paenibacillus* is useful in different areas because of its production of antimicrobial compounds, but with some pathogenic species to honeybees, other invertebrates and occasionally opportunistic infections to humans (Grady et al. 2016). Isolates of *Paenibacillus* from eggs and skin of salamanders showed antifungal activity (Lauer et al. 2008; Harris et al. 2006). One of the main ecological niches of *Paenibacillus* is soils (Madigan, Martinko, and Parker 2004) and it is frequent found in environments such as Brazilian natural forests (Bruce et al. 2010). In our study, we identified *Paenibacillus* as anti-Ah bacteria, and it was present only in field samples. According to that, we considered Atlantic Forest as the source of this genus of bacteria for the *P. distincta* individuals. Since this genus disappeared in captivity conditions, it was not part of the core community, highlighting the relevance of environmental sources of potentially beneficial bacteria in the protective role of the skin bacteriome. Similar protocol of maintenance and housing conditions used in our study is used for anurans in experimental research and ex situ conservation programs (Michaels, Gini, and Preziosi 2014). Considering the resilience tendency of skin bacteriome evidenced here, we recommend than *ex situ* conservation programs and experimental researches, to consider the physiological state of the anuran species of interest in field and captivity conditions because it was clear the dynamism of immune parameters and the reshaping and succession process on skin bacteriome.

2.6 Conclusions

Captivity conditions did not increase CORT plasma levels, but decreased plasma BKA, shifted skin ionic profiles and the communities of skin bacteriome in *P. distincta*. Anti-Ah bacteria were more abundant and prevalent in captivity conditions, and the putatively identified anti-Bd bacteria followed the same pattern. However, it is important to conduct in vitro assays against *B. dendrobatidis* for all the anti-Ah bacteria identified in *P. distincta*. We recommend future studies evaluate the ecological and functional relationships between potentially beneficial bacteria and amphibian host, because a comprehensive omics perspective could clear these complex relationships. Finally, we suggest future studies and conservation programs considering the time and conditions of captivity in order to minimize the negative impact on the physiology of the amphibian host and its skin bacteriome, minimizing possible health consequences.

2.7 Acknowledgments

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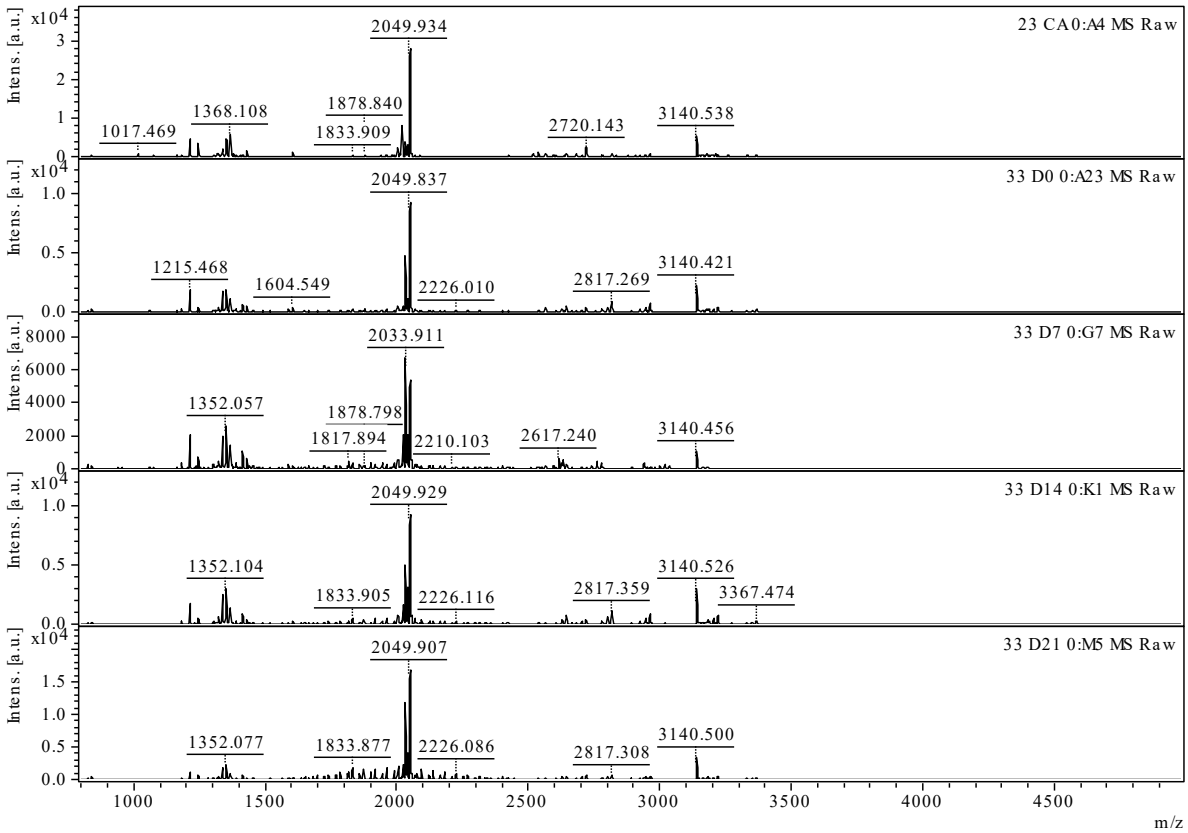
2.9 Supplementary Material

Table S1. Identification of *Batrachochytrium dendrobatidis* presence and quantification of zoospores load in *Phyllomedusa distincta* individuals.

Individual	Time of sampling	Result	Zoospore load	Bd Strain
29	Field	Negative	0	CLFT 159
28	Field	Positive	2,0	CLFT 159
27	Field	Positive	30,6	CLFT 159
26	Field	Negative	0	CLFT 159
35	Field	Negative	0	CLFT 159
25	Field	Negative	0	CLFT 159
34	Field	Negative	0	CLFT 159
24	Field	Negative	0	CLFT 159
32	Field	Negative	0	CLFT 159
23	Field	Negative	0	CLFT 159
31	Field	Negative	0	CLFT 159
22	Field	Positive	464,2	CLFT 159
33	Field	Negative	0	CLFT 159
33	0	Positive	3,9	CLFT 159
32	0	Negative	0	CLFT 159
37	0	Negative	0	CLFT 159
25	0	Negative	0	CLFT 159
34	0	Negative	0	CLFT 159
37	7	Negative	0	CLFT 159
33	7	Negative	0	CLFT 159
34	7	Negative	0	CLFT 159
25	7	Negative	0	CLFT 159
32	7	Positive	2,9	CLFT 159
34	14	Positive	2,2	CLFT 159
33	14	Negative	0	CLFT 159
32	14	Negative	0	CLFT 159
25	14	Negative	0	CLFT 159
37	14	Negative	0	CLFT 159
32	21	Negative	0	CLFT 159
25	21	Negative	0	CLFT 159
37	21	Negative	0	CLFT 159
33	21	Negative	0	CLFT 159
34	21	Negative	0	CLFT 159

Figure S2.1: Mass spectrometry signals from skin secretions of *Phyllomedusa distincta* in field and captivity conditions. Each rectangle corresponds to a representative MALDI-TOF MS spectrum from skin secretion of samples in field (23 CA), day 0 (33 D0), day 7(33D7), day 14 (33D14) and day 21in captivity conditions (33D21) of skin ions (*m/z*). the Y axis show the intensity of each signal (peak) and X-axis correspond to the time of retention (*m/z*).



GENERAL CONCLUSIONS

Corticosterone silastic implants produced sustained increased corticosterone plasma levels of *Rhinella ornata*, inducing mid-term immunoenhancing and long-term immunosuppressive effects on the ability of plasma to kill *Aeromonas hydrophila*, an anuran opportunistic pathogen. In this way, corticosterone silastic implants provide a useful experimental tool to study and unravel the underlying immunomodulation mechanisms of this hormone in anurans.

Even without changing corticosterone plasma levels, the maintenance of *Phyllomedusa distincta* in captivity shifted innate immune function. Captivity maintenance decreased the plasma ability to kill the opportunistic bacteria *A. hydrophila*, increased ionic signals on skin secretions, and increased the abundance and prevalence of Anti-Ah and Anti-Bd bacteria. Then, experimental researches and conservation programs with anurans must consider time and condition of captivity maintenance because the consequences observed in the innate immune function and skin bacteriome can determine the success of future reintroduction programs.

Considering the sharp decline that amphibian populations have been suffering, mainly due to interacting effects of climate change, emerging infectious diseases and habitat loss, it is important to assess the effects of these stressors on amphibian innate immune response and microbiota for the planning and design of conservation strategies. Future studies, involving exposure to stressors for a longer period, and their immunological consequences, would be interesting to continue understanding these relationships.

ANEXO 1 – CERTIFICADO DE APROVAÇÃO DO COMITÊ DE ÉTICA NO USO DE ANIMAIS (CEUA IB/USP)

USP Universidade de São Paulo
Instituto de Biociências

www.ib.usp.br

CERTIFICADO

Certificamos que a proposta intitulada (*)**"Efeito da corticosterona sobre a atividade imune inata anti-*Batrachochytrium dendrobatidis* em *Phyllomedusa distincta*"**, registrada com o nº 259/2016 (Proc. 16.1.319.41.1), sob a responsabilidade do Prof. Dr. Fernando Ribeiro Gomes e com a participação da colaboradora Farides del Carmen Lamadrid Feris (IB/USP), que envolve a utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica encontra-se de acordo com os preceitos da Lei nº 11.794, de 08 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009 e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovada pela Comissão de Ética no Uso de Animais – CEUA do Instituto de Biociências da Universidade de São Paulo, em reunião de 06 de julho de 2016.

Proposta inicial:

Vigência da autorização: 06/07/2016 a 05/07/2019

Finalidade: Pesquisa Científica

Nº da solicitação ou autorização SISBIO: 29896-1

Atividades: Captura e Coleta de espécimes

Espécie/Grupo Taxonômico: Anfíbio/*Phyllomedusa distincta*

Local de realização das atividades: Parque Estadual Intervales, Ribeirão Grande – SP

1º Adendo:

A Comissão de Ética no Uso de Animais – CEUA do Instituto de Biociências da Universidade de São Paulo, em reunião de 26/02/2018 aprovou alteração do título do projeto para (*)**"Atividade imune inata anti-*Batrachochytrium dendrobatidis* em anuros da Mata Atlântica"**, inclusão de local de coleta e inclusão de 40 animais da espécie relacionada abaixo:

Espécies/Grupos Taxonômicos: Anfíbio/*Rhinella ornata*

Nº de animais: 40 (M) **Peso:** não informado **Idade:** adulto **Total:** 40 animais

Local de realização das atividades: Jardim Botânico de São Paulo, São Paulo - SP

OBS.: *Qualquer intercorrência ou alteração do projeto em andamento deverá ser previamente autorizada pela Comissão de Ética no Uso de Animais – CEUA-IB.*

Prof. Dr. Pedro Augusto Carlos Magno Fernandes
Coordenador da Comissão de Ética no Uso de Animais