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PRIMARY RESEARCH PAPER

High temporal and individual variation in the prevalence and intensity of chytrid infection in the southernmost Leaf Frog of the genus *Pithecopus* (Anura, Phyllomedusidae)

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Abstract *Pithecopus rusticus* is an endemic amphibian restricted to the type locality, in southern Brazil, and possibly endangered to extinction, due to habitat degradation. However, an additional threat to amphibians is the chytrid fungus, which has been associated with amphibian population declines and extinctions. Hence, we tested the hypothesis that *Batrachochytrium dendrobatidis* (Bd) prevalence and infection load varies temporally and individually in *P. rusticus*, due to the influence of climatic and intrinsic individual factors. We swabbed adult individuals during two breeding seasons. Bd prevalence and infection load differed between breeding seasons and sampled months. In the middle of the first season, we found a peak of Bd load followed by a significant decrease. Only one infected individual was found in the middle of the second breeding season. Bd load was related to air temperature and rainfall, and individuals with lower scaled mass index had higher infection load. We showed that Bd incidence is highly variable in the same wild frog population. The temporal and individual decrease in zoospore load may suggest that *P. rusticus* can reverse high infection levels, and this may be evidence of efficient immunological responses present in this leaf frog.

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Introduction

Habitat degradation and the spread of lethal diseases are the main threats to amphibians (Stuart et al., 2004; Young et al., 2004; Becker et al., 2007; May, 2010; Almeida-Gomes et al., 2016; Scheele et al., 2019). Chytridiomycosis, an emerging infectious disease caused by fungi of the genus *Batrachochytrium*, especially *B. dendrobatidis* Longcore, Pessier & Nichols (Bd) (Berger et al., 1998; Boyle et al., 2004), has been associated with amphibian population declines around the world (Carvalho et al., 2017; Scheele et al. 2019). Bd is considered a generalist pathogen (Fisher et al., 2009; Valencia-Aguilar et al., 2015; Ruggeri et al., 2018) and is currently distributed in different ecosystems (Preuss et al., 2015; Becker et al., 2016; Flechas et al., 2017).

In addition to the correlation of prevalence and infection intensity of Bd with abiotic factors (e.g., Kriger et al., 2007b; Liu et al., 2013; Longo & Zamudio, 2017), responses to infection may vary with host attributes and individual susceptibility (Woodhams et al., 2007; Gervasi et al., 2014). Thus, amphibian intrinsic characteristics (e.g., behavior and immunity) and environmental characteristics (e.g., vegetation cover, temperature, precipitation and seasonality), along with the different Bd strains that vary in virulence (Lambertini et al., 2016; Becker et al., 2017a; McDonald et al., 2020), can predict the dynamics of the disease in natural populations (Gervasi et al., 2014; Ruggeri et al., 2015; Valencia-Aguilar et al., 2015; O'Hanlon et al., 2018).

To understand Bd dynamics in the wild, it is necessary to determine the pathogen persistence in host and how individual specific responses vary temporally. Some studies indicate that seasonal variation results in different effects on the pathogen infection (Whitfield et al., 2012; Ruggeri et al., 2015; Longo & Zamudio, 2017; López et al., 2017). In these cases, temperature variation revealed to be the most predictive variable related to pathogen prevalence (Kriger et al., 2007b; Whitfield et al., 2012, 2017; Ruggeri et al., 2015; Campbell et al., 2019). Moreover, long-term studies on Bd occurrence in natural populations can provide useful information to support conservation strategies, especially for endangered species.

Pithecopus rusticus (Bruschi, Lucas, Garcia, and Recco-Pimentel, 2014) (Anura, Phyllomedusidae) is an endemic amphibian to the grasslands of the Araucarias Plateau, in the Atlantic Forest, southern Brazil (Bruschi et al., 2014). This species is known only from a small population (< 40 individuals tagged in two consecutive breeding seasons; JPB personal communication) found at the type locality, in the municipality of Água Doce, state of Santa Catarina (Lucas et al., 2010; Bruschi et al., 2014). The southern Brazilian natural grasslands suffer strong anthropogenic interference and are being replaced by livestock grazing and crops (Andrade et al., 2015; de Oliveira et al. 2017), that are potential threats to this isolated population. Herein, we investigated the temporal and individual variation of Bd infection intensity and prevalence in *P. rusticus*, during two breeding seasons, from September 2015 to February 2016, and from September 2017 to February 2018. Based on what has been observed in other studies, we tested the hypothesis that Bd prevalence and infection load vary temporally and individually, due to the effect of climatic factors, such as temperature, relative humidity and precipitation. We also discussed the importance of intrinsic factors, as differences in individual immunological systems. These data will help us understand the dynamics of Bd in the wild and improve the information for a proper evaluation of the conservation status of this microendemic leaf frog.

Methods

Study site

We sampled individuals of *P. rusticus* to their type locality, in the municipality of Água Doce, state of Santa Catarina, southern Brazil (26°35′59.90″S, 51°34′39.40″W; 1330 m above sea level; Fig. S1). The area is located in the grasslands of the Araucarias Plateau, in the Brazilian Atlantic Forest (Pillar, 2009). The vegetation is characterized by the predominance of natural grassland and includes patches of mixed ombrophilous forest. The climate is classified as subtropical humid, with higher temperatures in

summer (Alvares et al., 2013). The mean annual temperature varies between 12 and 14°C, and the mean annual precipitation varies between 1600 and 1900 mm (Alvares et al., 2013).

The species inhabits two ponds, about 100 m apart, in an area originally composed of wetlands. The area where the individuals were recorded has a perimeter of approximately 1000 m. The vegetation at the ponds is composed primarily of herbaceous plants of the families Cyperaceae, Asteraceae and Juncaceae, which are regularly managed (Lucas et al., 2010). The area around the ponds is used for cattle raising, crops, roads, housings and wind farms, all less than 100 m from the ponds (Fig. S2).

Data sampling

Fieldwork was conducted fortnightly from September 2015 to February 2016, and from September 2017 to February 2018, corresponding to the species breeding season (Boschetti et al., 2019). We used the active search method (Heyer et al., 1994), and captured individuals were handled with disposable plastic gloves. We only sampled adult individuals, since the susceptibility to Bd infection varies in different life stages (Knapp & Morgan, 2006). We swabbed every captured individual five times along the ventral, dorsal, pelvic, buccal regions and interdigital membranes of the lower and upper limbs, using smooth sweeping movements (Hyatt et al., 2007). Swabs were placed individually in a 1.5 ml microtube and stored at – 4 °C (Lambertini et al., 2013). We evaluated the presence of clinical signs of chytridiomycosis in all individuals, including lethargic behavior, abnormal posture and epidermal desquamation, as described by Voyles et al. (2011).

We measured the snout-vent length (SVL, mm) and body mass (g), and marked each individual with Visible Implant Alpha Tags (Northwest Marine Technology Incorporation, Heard et al., 2008; Courtois et al., 2013). The tag was implanted subcutaneously in the left thigh and then the individuals were released at the capture site. All procedures were approved by the University ethics committee (CEUA #008/17, Unochapecó), and sampling was permitted by the Chico Mendes Institute for Biodiversity Conservation (SISBio #14468-10).

Climatic variables, including the mean air temperature, relative humidity (RH), and accumulated precipitation of the past 10 days of both sampling periods were provided by Centro de Informações de Recursos Ambientais e de Hidrometeorologia do Estado de Santa Catarina (Ciram/Epagri).

Molecular analyses

DNA was extracted according to Boyle et al. (2004), with modifications described by Lambertini et al. (2013). We extracted Bd DNA from the swabs using PrepMan™ Ultra Sample Preparation Reagent (Applied Biosystems® by Life Technologies) and then we quantified the infection load using Taqman® qPCR Assay (Life Technologies). The stock solutions of extracted DNA were diluted in 1:10. For the qPCR, we prepared a mix containing Taqman Master Mix (Applied Biosystems®), 18 µM of the primer ITS1-3 Chytr (5′-CCTTGATATAATACAGTGTGCCA-TATGTC-3′), 18 µM of the primer 5.8S Chytr (5′-AGCCAAGAGATCCGTTGTCAAA-3′), 5 µM of the ChytrMGB2 probe (5′-6FAM CGAGTCGAA-CAAAAT MGBNFQ-3′), distilled water, and Bovine Serum Albumin (BSA) (Boyle et al., 2004). We added 20 µl of the mix and 5 µl of the diluted DNA sample to a qPCR 96-well plate. We used strain CFLT 159 (Bd-GPL) at previously determined concentrations, namely 10^3, 10^2, 10^1, 10^0 and 10^{-1} zoospore genomic equivalents (g.e.), along with negative control (distilled water). Samples were run in singlicate, the standards 10^3, 10^2, 10^1 and negative controls were run in duplicate and the standards 10^0 and 10^{-1} in quadruplicate. We considered Bd^+ all individuals with at least one zoospore g.e. detected (Kriger et al., 2007a). Infection intensity values were rounded to integer numbers.

Data analyses

We measured the infection prevalence (as the proportion of Bd^+ in relation to the total individuals examined) for each breeding season and months sampled. The infection load was defined by the number of zoospore g.e. determined by qPCR reactions, resulting in the number of zoospores for each individual (Boyle et al., 2004). The infection load values were log transformed for subsequent analyses.

We verified the difference in the infection load between both breeding seasons with independent *t* test. One-way ANOVA was run to evaluate differences in

infection load among months. When significant differences were found, a Tukey post hoc comparison was applied to determine differences among groups. We also ran *t*-test for calculating differences in the infection load between males and females. We used a multiple linear regression analysis to determine the influence of climatic variables (mean temperature, accumulated precipitation, and RH) on infection load. $P < 0.05$ was considered statistically significant (Zar, 1999). All statistical analyses were performed using Statistica 8.0 (Statsoft, 2007). Mean values are shown followed by a standard error and sample size.

To verify the relationship between SVL and body mass with infection load, we calculated the scaled mass index ($\hat{M}_i$), as a proxy to body condition, as proposed by Peig & Green (2009). We calculated by the following equation $\hat{M}_i = M_i \left[\frac{L_0}{L_1}\right]^{bSMA}$, where M_i and L_i are the body mass and SVL measurements of the individual *i*, respectively; L_0 is the arithmetic mean of SVL observed in the studied population; and M_i is the predicted body mass for the individual i when the linear body measure is standardized to L_0; *b*SMA is the scaling exponent estimated by the SMA regression of body mass (M_i) by body length (L_i). The SMA regression is an error in the model of variables that best considers the interdependence of body mass and length (Peig & Green, 2009). We performed a simple linear regression to determine the influence of the scaled mass index on infection load. For the body condition analysis, we included only Bd^+ individuals captured in both sampling periods, and no recaptures were included.

Results

We recorded a total of 50 *P. rusticus* adult individuals, 31 in the first breeding season (2015–2016) and 19 in the second (2017–2018). We analyzed 65 samples, including recaptures from the first season. The period of species activity was from October to January, in both breeding seasons. We found 61% ($n = 19$) infected individuals in the first and 5% ($n = 1$) in the second season. In the first breeding season, the prevalence ranged from zero (October 2015) to 100% (December 2015) (Table 1; Fig. 1). Females presented a prevalence of 53% (9/17) and males of 33% (11/33), considering both breeding seasons.

The infection load differed among seasons ($t = 3.26$; $P < 0.01$; Fig. 1a). We recorded a high variation among months of the first season ($F_{3,42} = 23.21$; $P < 0.01$), and December differed from the other months ($P < 0.01$). Individual Bd^+ loads ranged from 2 to over one million (62,963 ± 44,467; $n = 29$) zoospore g.e. We observed a peak in the proportion of infected individuals and in the infection load in the middle of the breeding season, December 2015 (121,373 ± 84,503 zoospore g.e.; $n = 15$; Table 1; Fig. 1b). Only one individual was recorded in the middle of the second breeding season, with a load of 4212 zoospore g.e.. We found 40% ($n = 12$) of infected individuals with less than 100 zoospores and 20% ($n = 6$) with loads over 10,000 zoospores. There was no difference in the infection load among sexes ($t = -0.02$; $P = 0.98$). Individuals with lower scaled mass index had higher infection load ($r^2 = 0.22$; $P < 0.05$; Fig. 1d).

The recapture rate was 35% ($n = 11$) in the first season (2015–2016) and 57% ($n = 12$) in the second season (2017–2018). In the first breeding season, zoospore loads of recaptured individuals varied temporally ($F_{3,22} = 24.34$; $P < 0.01$). At the beginning of the breeding season (October and November 2015), individuals that were subsequently recaptured were not infected ($n = 9$) or had a low infection load (362 ± 357 zoospores g.e.; $n = 2$). In the middle of the breeding season (December 2015), individuals had higher loads (64,200 ± 33,119 zoospore g.e.; $n = 8$), which decreased at the end of the season (January 2016) (3 ± 0.67 zoospores g. e; $n = 2$; Fig. 1d).

The infection load was related ($r^2 = 0.33$; adjusted $r^2 = 0.30$; $F = 10.43$; $P < 0.01$) negatively to air temperature ($\beta = -0.53$; $P < 0.01$) and positively with rainfall ($\beta = 0.55$; $P < 0.01$). RH did not influence Bd infection load ($\beta = 0.065$; $P = 0.60$).

Discussion

We found a high temporal variation in the prevalence and intensity of Bd infection in *P. rusticus,* within and between both breeding seasons analyzed, with a peak of prevalence and load in the middle of both reproductive periods. The temporal variation observed may be explained by a complex set of factors. Our data showed that climatic factors, as air temperature and accumulated precipitation, are correlated with this

Table 1 Infection prevalence (% infected individuals), infection load (presented as zoospores g.e. mean ± SE (min–max) of *Batrachochytrium dendrobatidis* (Bd) in *Pithecopus rusticus* and climatic variables recorded over the months sampled during the years 2015–2016 and 2017–2018, in the municipality of Água Doce, sate of Santa Catarina, southern Brazil

Month	*n*	Prevalence (%)	Load (zoospore g.e.)	Air temperature (°C)	10-day precipitation (mm)	RH (%)
Oct/2015	6	0	–	23	193.3	78.1
Nov/2015	22	54.5	381 ± 266 (5–3141)	17	36.4	94.4
Dec/2015	15	100	121,373 ± 84,503 (2–1,275,027)	17	112.8	97.1
Jan/2016	3	66.6	3 ± 1 (2–4)	24	60.2	86.2
Oct/2017	8	0	0	15	0.0	94.2
Nov/2017	9	11.1	4212	18	1.3	70.7
Dec/2017	2	0	0	21	122.8	93.2
Jan/2018	0	0	–	–	–	–

n number of individuals sampled, *RH* relative humidity

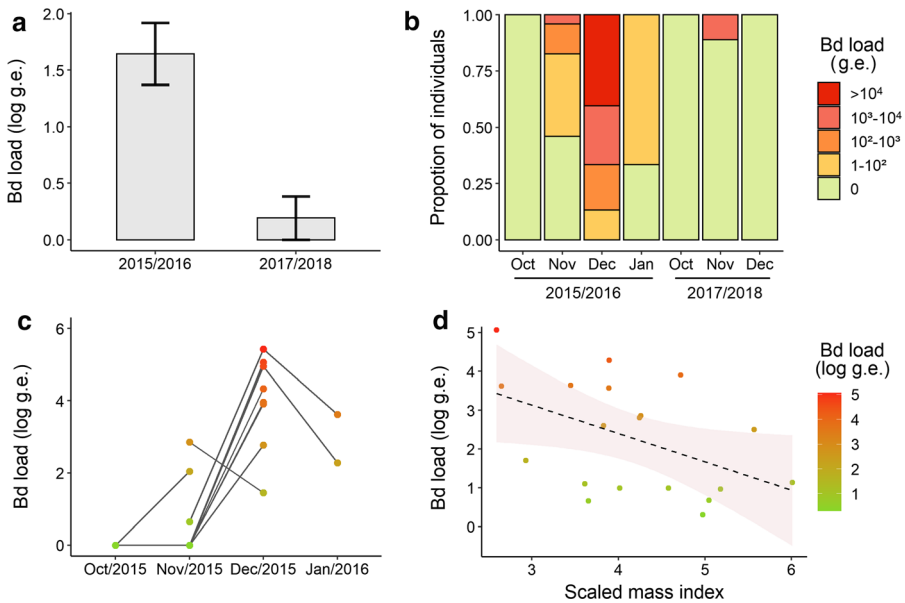

Fig. 1 Intensity of *Batrachochytrium dendrobatidis* infection in *Pithecopus rusticus* in two breeding seasons, 2015–2016 and 2017–2018, in the municipality of Água Doce, state of Santa Catarina, southern Brazil. **a** Breeding season mean infection load (log zoospores g.e. mean ± SE); **b** monthly proportion of infected individuals and their zoospores load; **c** individual variation in infection load (lines connect the same individual); **d** relationship between scaled mass index and infection load (log) ($r^2 = 0.22$; $P < 0.05$). Dashed lines represent the linear regression and the red area represents the standard error

variation. Higher infection loads were negatively correlated with air temperature, corroborating with previously observed trends (Piotrowski et al., 2004; Kriger et al., 2007b; Whitfield et al., 2012, 2017; Ruggeri et al., 2015; Campbell et al., 2019). The study area is characterized by a humid temperate climate, hot summers, cold winters and well-distributed rains throughout the year (Alvares et al., 2013). In addition, the open grassland vegetation and high altitude (1300 m) result in a considerable daily variation in temperature. Seasonal and daily variations, especially in the temperature, may influence the growth and survival of Bd (Woodhams et al., 2008; Muletz-Wolz et al. 2019), providing optimal thermal conditions of the pathogen and allowing proliferation in the host.

Our study also pointed to precipitation as an important factor that explains the infection load variations (Longo et al., 2009; Whitfield et al., 2012;

Ruggeri et al., 2015). Higher levels of precipitation allow for humid microhabitats, essential for fungus development (Longo et al., 2009; Whitfield et al., 2012). The influence of precipitation was expected since Bd is dependent on aquatic habitats (Berger et al., 2005) and intolerant to desiccation (Johnson & Speare, 2003). It has already been shown that rainfall and water availability could be associated with an increased abundance of frogs in the pond during the breeding season, and therefore more Bd zoospores in the environment (Ruggeri et al., 2015). Also, during the reproductive period, the infection load may increase progressively due to the increased interaction among individuals in the pond, facilitating the dissemination of zoospores among hosts (Piotrowski et al., 2004; Rachowicz & Vredenburg, 2004), and can be coinciding with the infection peaks observed here.

Variation in infection prevalence and infection load among breeding seasons may also be related to the persistence of the fungus in the environment or in other species that co-occur with *P. rusticus*. Considering that there is an interspecific variation in susceptibility and resistance among different species (Woodhams et al., 2007; Lenker et al., 2014; Fernández-Beaskoetxea et al., 2016), climatic variables may have different effects on each taxa. Therefore, community-level assessments will help to understand the association between prevalence and intensity of Bd infection, as well as their relationship with abiotic patterns (Whitfield et al., 2017). Moreover, differences in habitat use also determine the risk of infection (Becker et al., 2014). *Pithecopus rusticus* depends on the lentic habitat to reproduction (Bastiani et al., 2019), and the sites are deprived of forest cover, which results in direct sunlight during the day and an increase in body temperature of individuals and pond water (Raffel et al., 2010; Becker et al., 2012). This would help to control zoospore proliferation and, consequently, the infection intensity (Rowley & Alford, 2013).

We observed that individuals with lower body condition tend to show higher infection loads. Past laboratory studies with *Bufo bufo* (Linnaeus, 1758), *Rana temporaria* Linnaeus, 1758 and *Litoria caerulea* (White, 1790) reported interactions between Bd load and host body sizes, suggesting that smaller individuals demanded higher energy for their immunologic system to respond against infection (Bielby et al., 2015; Wu et al., 2018). What we observe may be indicative of the negative effects exerted by Bd infection, or even physiological resistance response against infection (Burrow et al., 2017; Wu et al., 2018), however, this needs to be further investigated. If Bd infection is not killing individuals in this region, it certainly is causing indirect sub-lethal effects that may jeopardize the maintenance of this population in the long–term. Therefore, we indicate the need for continuous monitoring of this restricted population.

We found 20% of individuals with loads over 10,000 zoospores and one individual with a load of over a million zoospores. Considering that chytridiomycosis has been identified as an important factor of unpredictable declines and extinctions (Scheele et al., 2019), although shown to be tolerant, *P. rusticus* may be susceptible to this disease. This is alarming because it is the only known population so far. However, we did not observe clinical signs of the chytridiomycosis or dead individuals in situ. Apparently, death from infection was rare to *P. rusticus*, since we obtained high rates of recapture throughout both breeding seasons. The temporal decrease in zoospore load may suggest that *P. rusticus* can reverse high infection levels. Thus, the peak followed by a decrease in Bd infection could be indicative of efficient immunological responses in this leaf frog. Despite high infection loads of individual infection, studies carried out in the Atlantic Forest with other amphibians species, including subtropical areas, did not detect population declines (Gründler et al., 2012; Ruggeri et al., 2015; Preuss et al., 2016). This may be related to variation in susceptibility and tolerance to chytrid across species or even related to Bd strains less virulent than those found in other regions (James et al., 2015; Becker et al., 2017a; Muletz-Wolz et al., 2019).

The family Phyllomedusinae, which includes *P. rusticus*, has a considerable composition of biologically active peptides secreted by the skin, such as dermaseptins and phylloseptins, which have known antifungal and antibacterial activity (Batista et al., 1999; Zhou et al., 2015; Mechkarska et al., 2018). Peptides such as dermaseptin-L1 and phylloseptin-L1 can inhibit the growth of Bd (Conlon et al., 2007) and if found in *P. rusticus*, they could have influenced the variation in infection rates observed in this study. These peptides are essential for immune defense in amphibians (Rollins-Smith & Conlon, 2005; Mechkarska et al., 2018). Furthermore, the skin microbiota can also act against Bd infection (Holden et al., 2015; Woodhams et al., 2015; Becker et al., 2017b). Both

active skin peptides and the skin microbiota can vary temporally, according to climatic and seasonal fluctuations (Bletz et al., 2017; Longo & Zamudio, 2017; López et al., 2017). The variation observed in Bd infection may be related to the composition of the skin microbiota and peptides of *P. rusticus*, and their potential against the pathogen should be investigated. However, there is evidence of population extinctions of species of the genus *Phrynomedusa* (also phyllomedusids from the Atlantic forest) probably caused by Bd (Carvalho et al., 2017). In contrast to *P. rusticus*, this suggests that members of the Phyllomedusidae family are interesting models to investigate tolerance and susceptibility to Bd.

Since the habitat of *P. rusticus* suffers intense anthropic intervention, the presence of Bd may represent an additional threat to this population. The synergy of different factors can make individuals more vulnerable to stochastic and deleterious events, which may lead to their extinction. Chytridiomycosis represents one of the greatest challenges for wildlife conservation, since there is no applicable field strategy that can control the disease in the wild (Berger et al., 2016). Our results demonstrated that the infection may be temporally highly variable, so ex situ conservation should involve long-term studies aimed at determining the real impacts of Bd on amphibian populations. Monitoring can serve as a basis for understanding the conditions that result in increased prevalence and lethality of the fungus in wild populations, thereby leading to the development of conservation strategies to avoid future declines and extinctions.

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