

Skin-mates or neighbors? A seasonal study of amphibian chytrid and dermocystid infection in *Boana pulchella* (Anura: Hylidae)

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ABSTRACT

We studied in this work the prevalence of two eukaryotic skin pathogens in a population of the frog *Boana pulchella* (Anura, Hylidae) from southeastern Uruguay, the fungus *Batrachochytrium dendrobatidis* (Rhizophydiales) and the protoctistan *Valentines rwandae* (Ichthyosporea, Dermocystida), between 2012 and 2014. In both cases higher prevalences were recorded in cooler seasons, winter and early spring for *B. dendrobatidis* (0.78 and 0.95 respectively) and autumn for *V. rwandae* (0.33 and 0.49), with mean temperatures between 11.5 and 17.8°C. No cases of infection were detected in summer. In both cases the probability of infection was strongly influenced by season (and also the year for *B. dendrobatidis*), but in neither case the occurrence of the other pathogen was significant. The parasitic cycles seem to occur independently and are likely strongly associated to environmental temperatures. Future field studies on amphibian eukaryotic pathogens from temperate areas of South America should consider seasonal variations.

Key words: *Amphibiocystidium*; *Batrachochytrium*; *Valentines*; Amphibians; Skin disease

RESUMEN

En este trabajo se estudió la prevalencia de dos patógenos cutáneos eucariotas en una población de la rana *Boana pulchella* (Anura, Hylidae) del sureste de Uruguay, el hongo *Batrachochytrium dendrobatidis* (Rhizophydiales), y el protista *Valentines rwandae* (Ichthyosporea, Dermocystida), entre 2012 y 2014. En ambos casos las prevalencias más altas se registraron en épocas frías, invierno y primavera temprana para *B. dendrobatidis* (0,78 y 0,95 respectivamente) y otoño para *V. rwandae* (0,33 y 0,49), con temperaturas medias entre 11,5 y 17,8°C. No se detectaron casos de infección en verano. La probabilidad de infección en los dos casos está fuertemente influenciada por la estación (y además por el año para *B. dendrobatidis*), pero no por la ocurrencia del otro patógeno. Ambos ciclos parasitarios parecen ocurrir independientemente, y son probablemente condicionados en gran medida de la temperatura ambiental. Futuros estudios de campo sobre patógenos eucariotas en anfibios de zonas templadas de Sudamérica deberán tener en cuenta variaciones debidas a la época del año.

Palabras clave: *Amphibiocystidium*; *Batrachochytrium*; *Valentines*; Anfibios; Enfermedad cutánea.

Introduction

The diseases of wild amphibian populations have been scarcely studied as compared with those of other vertebrates (Reichenbach-Klinke and Elkan,

1965; Mader and Divers, 2013). Currently, there is an increasing interest in the study of infectious and non-infectious diseases of amphibians due to its

implications in conservation (Gilbert *et al.*, 2013). There is a consensus among researchers about the important role of diseases in the current global decline of amphibians (Lips, 2014). However, little is known about how pathogens may interact and produce synergistic effects (Blaustein and Kieseker, 2002). Understanding coinfection patterns of different amphibian pathogens is a major goal for disease ecologists (Stutz *et al.*, 2018).

Among the infectious diseases capable to produce significant morbidity and mortality in wild populations of amphibians are those produced by *Ranavirus* (Gray *et al.*, 2009), bacteria, and eukaryotic microbes, which to a large extent affect the skin. The study of eukaryotic pathogens of amphibians has significantly grown since the discovery of chytridiomycosis, a skin disease caused by fungi of the genus *Batrachochytrium* (Rhizophydiales) associated to population declines worldwide (Berger *et al.*, 1998; Vredenburg *et al.*, 2010). Other eukaryotic pathogens potentially lethal for amphibians are protozoans of the Class Ichthyosporozoa, belonging to the Orders Dermocystida (i.e. *Amphibiocystidium*, Pascolini *et al.*, 2003; Raffel *et al.*, 2008) and Eccrinida (*Ichthyophonus*, Raffel *et al.*, 2006).

Batrachochytrium dendrobatidis grows on keratin, and once in the skin of post metamorphic amphibians or tadpole keratinized oral structures develop sporangia that release infective motile zoospores when mature (Berger *et al.*, 2005). Macroscopic lesions are evident only in clinical cases, and include skin discoloration, erythema and hyperkeratosis (Rachowicz and Vredenburg, 2004). Detection of its parasitic phase is usually made by histological methods (Berger *et al.*, 1999) or PCR (Annis *et al.*, 2004). Amphibian dermocystids present also a simple life cycle and infection targets primarily amphibian skin (Borteiro *et al.*, 2018). In this case macroscopic lesions are evident upon careful examination, noticed as small nodules, sometimes ulcerated (Raffel *et al.*, 2008; Borteiro *et al.*, 2018). Unequivocal diagnosis of the infection they cause can be achieved by histological identification of capsulated sporangia of approximately 300–500 µm, located in the dermis and filled with numerous spores whose cytoplasm is crescent shaped (Carini, 1940; Borteiro *et al.*, 2014).

The biology of amphibian chytrids and dermocystids scarcely known in temperate areas of South America, from where epidemiological studies focused on individual populations of native amphibians

are lacking. Previous studies in wild amphibians from Uruguay have identified chytrid fungi (*B. dendrobatidis*; Borteiro *et al.*, 2009), dermocystids (*Valentines rwandae* as *Amphibiocystidium* sp., Borteiro *et al.*, 2014; Borteiro *et al.*, 2018), and eccrinides (*Ichthyophonus* sp., Borteiro *et al.*, 2015).

In the present work, we hypothesize that the frequency of infection of coexisting skin pathogens in amphibians may be constrained by climatic factors, as well as by their interactions within a host. In this sense, infection by a pathogen would predispose the host to be infected by a second one, in a process of facilitation by diminishing defenses and/or infection mechanism. We predict that: 1) the number of infected individuals in a population would change among different seasons, and 2) the probability of infection by one pathogen would increase when the other is present. The aim of the present research was to explore the occurrence of seasonal variation of chytrid fungi (*B. dendrobatidis*) and dermocystid infection (*V. rwandae*) and their possible association, in a population of the ubiquitous frog species *Boana pulchella* (Hylidae) from southern Uruguay.

Materials and methods

Study area and specimens. The studied species *Boana pulchella* (Hylidae) is a medium-sized anuran widely distributed in central-eastern Argentina, Uruguay, southern Brazil and Paraguay (Ceï, 1980; Brusquetti and Lavilla, 2006). Adult specimens measure about 37–50 mm snout-vent length, are terrestrial, nocturnal, and breed on several occasions throughout the year (Gallardo, 1961; Ceï, 1980). This frog is fairly abundant and widespread in Uruguay, and is considered among species of least conservation concern (Canavero *et al.*, 2010). Study specimens were collected along route 10, in a 2.5 km transect at Laguna de Rocha, Departamento de Rocha, in southern Uruguay (34°39' 54°13'S; 34°38' 54°12'W; 5–42 m altitude). Frogs were captured at breeding temporary ponds and transported to the laboratory individually, in plastic bags; they did not present abnormal behaviors or postures indicative of disease other than macroscopic skin lesions due to dermocystid parasites (see below). Climate in the study area is temperate with well-marked four seasons, whose mean temperatures are 18, 12, 16 and 21°C for autumn, winter, spring and summer respectively; accumulated precipitation for each season usually ranges between 280 and 330 mm

(www.inumet.gub.uy). During the study period (2012-2014) there were no exceptional episodes in regard to temperature or pluviosity.

Field sampling. Non-systematic field visits to the study site were done, as part of disease surveys in Uruguayan wild amphibian populations (Borteiro, 2016). A total of 139 frogs were captured in different seasons, early spring (September 2012, n = 19), late spring (November 2013, n = 32), summer (February 2014, n = 31), autumn (April 2013, n = 29; April 2014, n = 9), and winter (August 2013, n = 19). Amphibian collection permits were issued by División Fauna – MGAP (Res. No. 01/2010 and 199/2013).

Pathogen detection. Endpoint polymerase chain reaction (PCR) on skin swab material was used for the detection of *B. dendrobatidis* following Annis *et al.* (2004). Skin swabs were made by gently rolling polyvinyl tipped swabs (Puritan[®]) five times over the ventral surface of abdominal skin, hands and feet, and stored in dry tubes at -20 °C until their DNA load was extracted with DNeasy Blood & Tissue Kit (Qiagen[®]). Positive control was DNA of strain JEL423. PCR sensitivity was about 10 pg DNA, as previously indicated (Annis *et al.*, 2004). After swabbing, frogs were euthanized, by cutaneous application of benzocaine gel (Chen and Combs, 1999); handling protocol was authorized by Comisión de

Ética en el Uso de Animales de Experimentación (CEUA - MNHN).

Carcasses were immediately examined under a stereoscopic microscope at 10X for the identification of dermocystid nodules in the skin due to *V. rwandae*, which was made after Borteiro (2016) and Borteiro *et al.* (2014), Fig. 1. The identity of dermocystids in the studied population of *B. pulchella* was confirmed in previous work using histology and DNA sequencing (Borteiro *et al.*, 2014; Borteiro *et al.*, 2018). Dermocystid nomenclature follows Borteiro *et al.* (2018).

Statistical analyses. Infection prevalence and confidence intervals were calculated following Wilson (1927) formulae. The association between both infectious agents (coinfection) in the same individual was evaluated with Spearman correlation coefficient (ρ) (Siegel, 1956).

In addition, we explored the data with a generalized linear model of binomial distribution (infected/non-infected) in which the presence of each pathogen was considered as the dependent variable. Season and year of sampling were considered as independent variables, as also the presence of the other pathogen. Variable contributions to the models were compared by means of analysis of variance, ANOVA-LRT (Zuur, 2009), and removed when not statistically significant ($\alpha \geq 0.05$). Summer



Figure 1. Frogs of the species *Boana pulchella* (Hylidae) at Laguna de Rocha, Uruguay, infected by *Valentines rwandae* (Dermocystida). Left: male calling at a breeding site. Right: ventral view of another male in life. Notice the skin nodules that bear dermocystid sporangia (arrows).

was not included in the models because all sampled hosts were negative for both pathogens during this season. Differences between pathogen frequencies in relation to season were explored with the Hochberg factor test using the software R (R Core Team, 2016). No sex differences were evaluated as the sample contained only 3 females.

Results

Overall prevalence (P) of *B. dendrobatidis* infection was 0.41 at PCR, as 56 frogs were positive during the whole study period. Prevalence was higher in winter and early spring, with no cases detected in summer. Dermocystids were identified in only 26 frogs (P = 0.19), but cases occurred also with marked seasonal variation. Prevalence peaked up in autumn when almost half of specimens were infected (i.e. Apr. 2013, P = 0.49) and lowered in spring and winter; like chytrids, they were not detected in summer. The association of both pathogens in a given individual (coinfection) regardless year or season, was not significant, $\rho = 0.117$ ($p_{0.05} = 0.173$). Seasonal variation of prevalence is summarized in Table 1 for both pathogens. The GLM showed a significant contribution of season for chytrids (explained deviance = 23.6; AUC = 65 %) and dermocystids (explained deviance = 8.6; AUC = 39 %), and also the year in the case of *B. dendrobatidis*. Both pathogens exhibited their respective higher frequencies in different seasons, chytrids in winter and dermocystids in autumn (in this last case with no statistically significant

differences with respect to winter). In neither case, the prevalence of infection by the other pathogen significantly contributed to the model (Table 2). The probability model is shown in Fig. 2.

Discussion

Seasonal variation of *B. dendrobatidis* infection with high incidence during cooler periods agrees with several studies, in which chytrid prevalence showed an increase in winter and early spring (i.e. Berger *et al.*, 2004; Woodhams and Alford, 2005; Kriger and Hero, 2006). Our high and variable prevalence data recorded in autumn, winter and early spring in absence of disease and mortality (0.22–0.95), resemble enzootic infection by *B. dendrobatidis* in absence of disease-driven population declines (Retallick *et al.*, 2004, 0.18–0.28; Borteiro, 2016, 53–0.88). The only report of wild Neotropical anurans that apparently died from chytridiomycosis in temperate lowland habitats in South America correspond to some specimens of *Leptodactylus latrans* (Leptodactylidae) found dead during winter in Buenos Aires Province, Argentina (Herrera *et al.*, 2005). Temperatures around 17–23 °C seems to favor amphibian chytrid virulence, while immune response of some hosts could be diminished (Berger *et al.*, 2004; Ribas *et al.*, 2009). In less favorable periods for growth and development like summer, chytrids may persist at rather low intensities of infection in amphibian skin (i.e. beyond our present PCR sensitivity), and without causing disease (Retallick *et al.*, 2004). Temperatures above the optimum for chytrid growth would aid host defensive mechanisms and clearance of the pathogen (Woodhams *et al.*, 2003; Chatfield and Richards-Zawacki, 2011; Forrest and Schlaepfer, 2011). Alternatively, *B. dendrobatidis* may survive in ponds infecting non-amphibian hosts like fish and crustaceans (McMahon *et al.*, 2013; Liew *et al.*, 2017).

Concerning dermocystid prevalence in amphibians, available literature shows broad variations from 0.04 to 0.52, usually in winter and spring, but data mostly correspond to single or scattered observations throughout the year (Guyénot and Naville, 1922; Remy, 1931; Carini, 1940; Brož, 1944; Brož and Přivora, 1952; Jay and Pohley, 1981; Pascolini *et al.*, 2003; Curtois *et al.*, 2013). As in our study case, most work also indicate strong seasonal fluctuations. For instance Pérez (1907; 1913) observed dermocystid infection in European newts mainly in winter (Fe-

Table 1. Prevalence of infection (P) by *Batrachochytrium dendrobatidis* (Bd) and *Valentines rwanadae* (Vr) in *Boana pulchella*, expressed as positive (+) individuals in of total in each sampling event (n). CI, confidence interval ($p < 0.05$).

	Bd+ / n (P; CI)	Vr+ / n (P; CI)
Spring 2012	18 / 19 (0.95; 0.75–0.99)	3 / 18 (0.17; 0.06–0.39)
Autumn 2013	12 / 28 (0.43; 0.27–0.61)	14 / 29 (0.49; 0.31–0.66)
Winter 2013	14 / 18 (0.78; 0.55–0.91)	3 / 19 (0.16; 0.06–0.38)
Spring 2013	5 / 32 (0.16; 0.07–0.32)	3 / 32 (0.09; 0.03–0.24)
Summer 2014	0 / 31 (0; 0–0.11)	0 / 31 (0; 0–0.11)
Autumn 2014	2 / 9 (0.22; 0.06–0.55)	3 / 9 (0.33; 0.12–0.65)

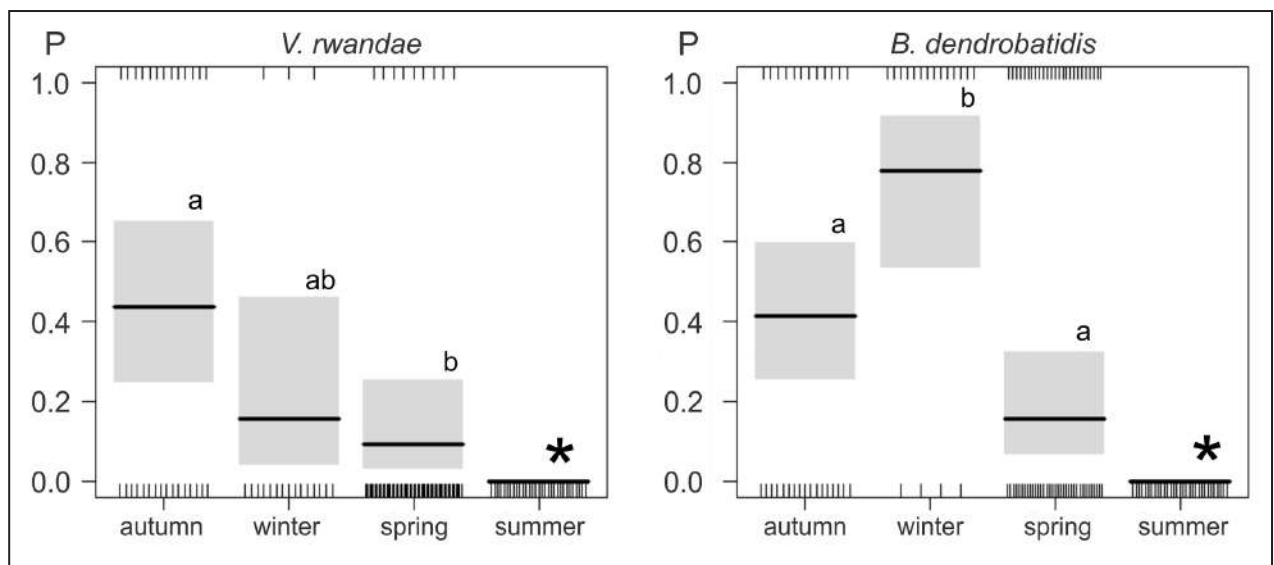
Table 2. Generalized linear model ANOVA for the prevalence of infection by *Batrachochytrium dendrobatidis* (*Bd*) and *Valentines rwandae* (*Vr*) in *Boana pulchella*.

	<i>B. dendrobatidis</i>		<i>V. rwandae</i>		
	Deviance	P	Deviance	p	
Season	140	0.02	107	0,009	
Year	113	2.7 x 10⁻⁷	106	0.41	
<i>Vr</i>	113	0.62	<i>Bd</i>	106	0.53

bruary and March) during a three-year period, and Poisson (1937) reported a peak in winter and spring (March and April) over two years. Likewise, infection in other European amphibians was reported in winter and spring (Guyénot and Naville, 1922; Remy, 1931; Brož and Přivora, 1952; Curtois *et al.*, 2013). In North America, *Dermocystidium penneri* was found infecting the toad *Anaxyrus americanus* (Bufonidae) in autumn and winter (Jay and Pohley, 1981), and high prevalences of *Dermotheca viridescens* in the newt *Notophthalmus viridescens* (Salamandridae) were detected in winter and early spring (Raffel *et al.*, 2008). Similarly, prevalence of infection by *V. rwandae* in *B. pulchella* was high in cooler seasons. Its absence in summer could be explained also by low prevalence during periods of high environmental temperatures that would favor host defenses. The occurrence of sporulated infective forms in the environment has not yet been demonstrated. In both fish and amphibians, the major incidence of fungal and dermocystid infections during cooler periods (i.e. winter) could be the result of depressed immune response of the hosts and/or optimal environmental

temperatures for the growth of the pathogen (Bly *et al.*, 1993; Raffel *et al.*, 2008).

The finding of concomitant infection by *B. dendrobatidis* and other eukaryotic pathogens is not common in the literature. Chytrid-dermocystid coinfection in *B. pulchella* was reported for some localities in Uruguay, including the present study site (Borteiro *et al.*, 2014; Borteiro *et al.*, 2018). This seems to have been first observed about a century ago in the European newt *Lissotriton helveticus* (Salamandridae) by De Beauchamp (1914), who describes infection by a pathogen similar to *Dermocystidium pusula*, plus the presence of small sporangia that produced zoospores. The author identified the later as chytridiomycetes (*Batrachochytrium*?) but did not discard the production of motile spores by dermocystids. This possibility was also suggested later by Poisson (1937), who made similar observations in *L. helveticus*, as also did Grassé (1926). It is noteworthy that the putative presence of amphibian chytrids in Europe predates by several decades the oldest known record of chytrid infection in this continent (1997, Bosch *et al.*, 2001).

**Figure 2.** GLM binomial models for infection frequencies of the pathogens *Valentines rwandae* and *Batrachochytrium dendrobatidis* in *Boana pulchella* (see text for explanation). Different letters indicate statistically significant differences; * indicates that season does not apply.

The first of our predictions in the present work was confirmed, as both pathogens presented well-marked seasonal peaks, but the second did not. In both cases, the parasitic cycles seem to occur independently from each other. We are unaware of previous work testing for such an association between skin pathogens in wild frog populations. In a previous work, we suggested the possibility of a relationship between chytrid and dermocyetid infection in *B. pulchella*, given mainly the keratin disruption putatively produced by the first that would facilitate entry of dermocyetids (Borteiro *et al.*, 2014). Unexpectedly, the results presented herein argue on the contrary. Furthermore, high dermocyetid prevalence in autumn occurs after a period when chytrids were not detected. In our study case, the influence of environmental temperatures on the parasitic life cycles of these eukaryotic pathogens needs further exploration.

This report shows that both chytrids and dermocyetids could be present and easily identified in wild *B. pulchella* without severe disease and mortality, despite of high prevalence. The absence of disease outbreaks in this species during further surveys at the study site (C. Borteiro and F. Kolenc, pers. obs.), suggests the occurrence of normal seasonal variation of endemic pathogens. Furthermore, the role of disease in local amphibian declines, particularly chytridiomycosis, is not clear (Kolenc *et al.*, 2009). The remarkable abundance and ubiquity of *B. pulchella*, which is active in all four seasons, makes it a good candidate host for studying biological cycles of amphibian parasites. Finally, it must be pointed that studies on wild amphibian pathogens from temperate lowland and eventually high-altitude mountain habitats in South America should consider season as a significant source of variation.

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