



Pathogen load and body condition correlate with conservation status of syntopic and closely related frogs

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Abstract. Mitigating global amphibian declines requires a thorough understanding of stressors that differentially impact amphibian populations. We compared body condition and occurrence of the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) in syntopic frogs, belonging either to the small range, vulnerable species *Pelophylax shqipericus* or the widespread *P. ridibundus*. *Bd* was detected more frequently in *P. shqipericus* than in *P. ridibundus* (15.6% and 2.9%, $p < 0.05$, respectively). Infections were characterized by low infection intensity, absence of clinical signs and lack of any obvious impact on body condition. However, body condition in *P. shqipericus* was significantly lower than in *P. ridibundus*. Relatively high pathogen prevalence and poor body condition in the threatened *P. shqipericus* is worrying and urges the need for close monitoring, risk analysis and population assessment.

Key words. Amphibia, Anura, Ranidae, *Pelophylax shqipericus*, *P. ridibundus*, *Batrachochytrium dendrobatidis*, Montenegro.

Introduction

Chytridiomycosis is the most widespread fungal disease in amphibians caused by the potentially lethal skin pathogen *Batrachochytrium dendrobatidis* (*Bd*) and considered to be an important driver of worldwide population declines and extinction of species of this most threatened vertebrate class (e.g. WAKE & VREDENBURG 2008, SCHEELE et al. 2019). This infectious disease may be particularly detrimental to spatially constrained (endemic) species, contributing to their rapid decline, especially when acting synergistically with other stressors (BURROWES et al. 2004, MALHOTRA et al. 2007).

Although worldwide effort to collect data about *Bd* incidence over the last few decades has been intense (OLSON et al. 2013, SCHEELE et al. 2019, FISHER & GARNER 2020), amphibian populations from large parts of Europe (central, southern and eastern Europe) were less intensively sampled. The genus *Pelophylax* has often been included in studies of *Bd* infection among European amphibians (e.g. OUELLET et al. 2012, BALÁŽ et al. 2014a,b, VOJAR et al. 2017, AZMANIS et al. 2018, KOSTANJŠEK et al. 2021). They exhibited spatial heterogeneity in the presence of *Bd* and at positive sites they usually had higher infection prevalences compared to other amphibians, but without obvious adverse or lethal effects (BALÁŽ et al. 2014a, MALI et al.

2017, VOJAR et al. 2017, AZMANIS et al. 2018). Consequently, *Pelophylax* species were recognised as potential *Bd* reservoirs and transmitters and promising candidates for further *Bd* research (OUELLET et al. 2012, BALÁŽ et al. 2014b).

Almost half of the currently recognised species and hybrids of the genus *Pelophylax* in Europe are endemics of the southern peninsulas, with threatened conservation statuses and decreasing population trends (SPEYBROECK et al. 2016, 2020). Such populations may be more vulnerable to negative effects of infections. Up to now, *Bd* has been identified in two endemic species – *P. perezi* (Iberian Peninsula) (WALKER et al. 2010) and *P. epeiroticus* (Balkan Peninsula) (AZMANIS et al. 2018), while *P. cretensis*, endemic to the Greek island of Crete, tested negative for *Bd* (in AZMANIS et al. 2018). So far, the Albanian water frog (*P. shqipericus*), an endemic Balkan species restricted to wetlands of the coastal part of Albania and southeastern Montenegro, has not been tested for the presence of *Bd*. Although VOJAR et al. (2017) conducted a survey of *Bd* occurrence in samples of water frogs from the *P. shqipericus* range, *Bd* detection was not attributed to the individual species level and *Pelophylax* sp. was considered as a single taxon. The high infection rate in *Pelophylax* sp. from the Balkan Peninsula (VOJAR et al. 2017), stressed the need for further research to gain additional information about possible role of fungal infections in the decline of *P. shqipericus*. The species has

been categorized as ‘Vulnerable’ according to IUCN criteria (Iucn Ssc Amphibian Specialist Group 2020), due to the small area of occupancy and a number of threats such as increasing destruction, alteration and pollution of its habitats and overharvesting. In southeastern Montenegro the Albanian water frog shares the habitat with the Marsh frog (*P. ridibundus*). Although the Marsh frog is under the same threats as its cohabitant is, for now it is assessed as ‘Least Concern’ species mainly due to its wide distribution in Europe and western Asia (KUZMIN et al. 2009).

The aims of our study were to (i) investigate the presence of *Bd* in two syntopic water frogs *P. shqipericus* and *P. ridibundus* in a sample location from southern Montenegro, (ii) check whether there are differences in *Bd* prevalence between species, and (iii) test whether *Bd* infection status is associated with frog body condition.

Materials and methods

Study site and species

The study was conducted in the hinterland of Velika Plaža beach in southeastern Montenegro.

Being a part of the Eastern Mediterranean Key Biodiversity Area “Bojana/Buna River Delta” (DARWALL et al. 2014), the site is characterized by a diverse complex of unique and endangered habitats and species, and as such is recognised as an important area for nature protection both at national and international level (ČAKOVIĆ & MILOŠEVIĆ 2013). Velika Plaža beach hinterland is a marsh area with white willow and white poplar riparian forests, temporary ponds and humid dunes. Yet, these wetlands are exposed to a variety of threats, mainly due to inadequate waste management and expansion of touristic infrastructure (LJUBISAVLJEVIĆ & IKOVIĆ 2020).

At the study site, two species of water frogs *P. shqipericus* and *P. ridibundus* inhabit temporary ponds and marshy edges of floodplain forests. Although recent molecular analyses suggest a certain differentiation of the Marsh frog populations inhabiting the southeastern part of Montenegro, assigning them to the Balkan water frog (*P. kurtmuelleri*) (VUCIĆ et al. 2018), the validity of this species has not been widely accepted (e.g. SILLERO et al. 2014, SPEYBROECK et al. 2016, 2020, CRNOBRNJA-ISAILOVIĆ et al. 2018), due to a small genetic distance and the lack of clear characteristics on the basis of which the Balkan water frog could be distinguished from the Marsh frog. In accordance with these arguments, the study population of the Marsh frog will be considered as *P. ridibundus*.

Field methods

Sampling was conducted in several interconnected ponds at Gornji Štoj locality in the hinterland of Velika Plaža beach within a period of five consecutive nights in the middle of May 2021. The specimens were collected using flash lights, caught by hands and processed using a

new pair of disposable gloves for each individual to prevent infection transmission between animals (FORZÁN et al. 2008). Individuals were assigned to the species on the basis of external morphological diagnostic characters and specific male advertisement calls (SPEYBROECK et al. 2016, LJUBISAVLJEVIĆ & IKOVIĆ 2020). Further analyses included only adult specimens with snout–vent length (SVL) larger than 55 mm (*P. ridibundus*) and 41 mm (*P. shqipericus*), on the basis of the size at maturity reported for these species in previous studies (DŽUKIĆ et al. 2003, MIKULIČEK et al. 2014), to avoid possible ontogenetic variation in susceptibility for *Bd* infection (VAN ROOIJ et al. 2015) and body condition (BRODEUR et al. 2020).

At least thirty adult individuals per species were collected to ensure confidence in *Bd* detection (DIGIACOMO & KOEPEL 1986). Samples were taken by rubbing the abdomen, thighs and feet at least ten times (e.g. KRIGER et al. 2006) using a rayon tipped swab (551 C, Copan Italia S.p.A., Brescia, Italy). The swabs were stored in a small amount (1 ml) of 96% ethanol (LASTRA GONZÁLEZ et al. 2021) and transported to the Faculty of Veterinary Medicine, Ghent University, Belgium where laboratory analyses were performed.

Each specimen was sexed based on the presence or absence of vocal sacs and nuptial pads on thumbs, weighed to the nearest 0.01 g with a portable electronic balance and measured for the body length (from snout to vent, SVL) to the nearest 0.01 mm with a digital calliper to assess body condition index (BCI). Body measurement was taken by the same person (KL). After measuring and checking for the clinical signs of disease such as lethargy, skin discoloration, skin roughening, hyperkeratosis, accumulation of sloughed skin over the body or limbs, and excessive sloughing of the skin (MUTSCHMANN 2015), all frogs were released at their point of capture.

Laboratory and data analyses

DNA was extracted from swabs using 100 µl of Prepman Ultra DNA extraction buffer (BOYLE et al. 2004, HYATT et al. 2007). The extracted DNA samples were diluted 1/10 in HPLC water for reducing PCR inhibition and processed using the *Bd*-specific real-time PCR described by BOYLE et al. (2004) on a CFX96 real-time system (Bio-Rad Laboratories, Hercules, CA). Real-time PCR results (expressed as genomic equivalents (GE) of *Bd* zoospores of *Bd* strain JEL423) were corrected for the applied dilution factor and converted to loads per swab, with a detection limit of 20 GE/swab.

Body condition is considered both a non-invasive measure of individual fitness and an indicator of environmental stress (e.g. BÂNCILĂ et al. 2010). To test the potential effect of *Bd* infection on the frog’s body condition, scaled mass index (SMI) was used. PEIG & GREEN (2009) proposed the use of a scaled mass index (SMI) to determine body condition from mass and length estimates because its use of model II linear regression (standardized major axis regression – SMA). This method was shown to be a better pre-

Table 1. Incidence of *Bd* infection and body size in two water frog species at Gornji Štoj locality in southeastern Montenegro. SVL – snout–vent length (in mm), N – sample size, *Bd*+ – number of *Bd* positive specimens, Prevalence – proportions of *Bd* positive specimens in percent with 95% confidence intervals (CI), GE – genomic equivalents of zoospores, SE – standard error of the mean.

Species	Sex	SVL (mean±SE)	N/ <i>Bd</i> +	Prevalence [%] (95% CI)	GE (mean±SE)
<i>P. shqipericus</i>	males	53.94±1.24	27/5	15.6 (6.5–29.5)	106.6±25.6
	females	59.99±2.38	18/2		
<i>P. ridibundus</i>	males	83.29±3.22	14/1	2.9 (0.1–15.3)	57
	females	83.35±4.95	20/0		

dictor of fat and energy reserves in a variety of organisms compared to other, more traditional, morphometric condition indices (MACCRACKEN & STEBBINGS 2012, CAMIZULI et al. 2014, MASSE et al. 2016).

Scaled mass index (Mi), for each individual frog, was calculated using the following equation: $Mi = M \times [SVL_i / SVL_o]^{bSMA}$ (Equation 1), where M and SVL are the body mass and the snout–vent length of the individual, respectively, bSMA is the scaling exponent estimated by the standardized major axis (SMA) regression of log M on log SVL, SVL_o is the mean SVL of the population, and Mi is the predicted body mass for individual i when the linear body measure is standardized to SVL_o . Comparison of the scaled mass indices (Mi) between species with the significant difference in body mass is inappropriate as mass has a strong impact on calculated Mi (see Eq. 1). In this study, preliminary analysis showed that *P. ridibundus* is significantly heavier than *P. shqipericus* (mass mean ± SE in g – *P. shqipericus* 15.86 ± 1.36, *P. ridibundus* 64.03 ± 3.19). Therefore, the relative scaled mass index (rMi) for each individual has been calculated by dividing individual Mi with max Mi for given species, which gives values $0 < rMi \leq 1$ (near zero – individuals with the lowest Mi, 1 – individual with the highest Mi for given species). As potential outliers in Mi distribution can impact rMi calculation profoundly, we used Grubbs test with overall alpha level of 10% to detect more potential outliers than those with smaller alpha levels. Only one *P. shqipericus* individual with the highest Mi (25.21) was excluded from the dataset as outlier.

The *Bd* prevalence between species and sexes were compared using a G test. Difference in rMi, with the *Bd* prevalence and species as factors, was tested with the ANOVA tests. Correlation of *Bd* load with rMi was tested with the Spearman's correlation test. Statistical analyses were performed in R version 4.0.2 (R Core Team 2021) using the package smatr (WARTON et al. 2012), and in Statistica 10 (StatSoft Inc., www.statsoft.com). All statistical tests were performed considering $p < 0.05$ as the level for significance.

Results

Bd prevalence and load

In total, 45 and 34 adult specimens of *P. shqipericus* and *P. ridibundus*, respectively, were tested for *Bd* infection. Of these, seven individuals (15.6%) of *P. shqipericus* and one

individual (2.9%) of *P. ridibundus* were infected (Table 1). The proportions of *Bd* positive and *Bd* negative samples between the two species were marginally significantly different (G test, $df = 1$, $p = 0.0489$). Although there were more infected males than females, the difference in prevalence was not statistically significant both within species ($df = 1$, $p = 0.4942$ in *P. shqipericus*, $df = 1$, $p = 0.1775$ in *P. ridibundus*) or at genus level ($df = 1$, $p = 0.1580$). The *Bd* infection intensity was low, with the GE loads ranging from 45 to 242 (mean 106.6 ± 25.6) in *P. shqipericus*, and 57 in infected specimen of *P. ridibundus* (Table 1).

Scaled mass index

The ANOVA test for body size (SVL) showed the sexes within each species do not differ ($SVL_f/SVLM$, $p > 0.05$ for both species, Table 1), which justifies to calculate species SVL_o with pooled sexes (*P. shqipericus* $SVL_o = 56.36$, *P. ridibundus* $SVL_o = 83.32$) for use in Eq. (1). The sexes did not differ in their SMA slopes in either species (*P. shqipericus* females – model fit $R^2 = 0.90$, $p < 0.0001$, $b = 3.26$; males – model fit $R^2 = 0.73$, $p < 0.0001$, $b = 3.94$; bf/bm $p = 0.1690$; *P. ridibundus*: females – model fit $R^2 = 0.98$, $p < 0.0001$, $b = 3.03$; males – model fit $R^2 = 0.96$, $p < 0.0001$, $b = 3.09$; bf/bm $p = 0.7810$), therefore justifying the use of a common slope with sex pulled data in order to examine the scaling relationship between body mass and body size. Species (sexes pooled within) statistically significantly differ in their SMA slope (*P. shqipericus*: model fit $R^2 = 0.84$, $p < 0.0001$, $b = 3.67$; *P. ridibundus*: model fit $R^2 = 0.97$, $p < 0.0001$, $b = 3.04$). These two species slopes (*P. shqipericus*: $b = 3.67$; *P. ridibundus*: $b = 3.04$) were substituted for bSMA in Eq. 1 in order to calculate scaled mass index.

The ANOVA test showed that non-infected *P. shqipericus* did not differ in the relative scaled mass index (rMi) from infected *P. shqipericus* (rMi mean ± SE, non-infected 0.60 ± 0.02 , infected 0.64 ± 0.05) (Fig. 1). Such a test was not performed for *P. ridibundus*, as only one individual has been infected. In addition, same test showed that non-infected *P. shqipericus* had significantly smaller rMi than non-infected *P. ridibundus* (rMi mean±SE, non-infected *P. shqipericus* 0.60 ± 0.02 , non-infected *P. ridibundus* 0.81 ± 0.02) (Fig. 1). Relative scaled mass index rMi was not correlated with the chytrid infection loads in *P. shqipericus* (Spearman's correlation $r = 0.14$, $p > 0.05$).

Discussion

The results confirm the presence of *Bd* in *P. shqipericus* for the first time and indicate differences in the prevalence of infection between *P. shqipericus* and *P. ridibundus*. The prevalence of *Bd* infection is higher in small range *P. shqipericus* than in the widely distributed *P. ridibundus*. This pattern could be the result of a range of biotic and abiotic factors, such as host, pathogen or microhabitat differences (e.g. DASKIN et al. 2014, HEARD et al. 2014). Although a higher prevalence of *Bd* infection in *P. shqipericus* may present an additional threat to this already vulnerable endemic species, further research is necessary to determine the possible role of dynamics of closely related syntopic species in *Bd* infection. However, *P. shqipericus* also exhibited significantly lower body condition index than *P. ridibundus*, which may be an additional evidence that the species is currently stressed and the *Bd* infection is just another, additional stressor, or evidence of decreased host defences under persistent infections. Low values of body condition index in frogs were proven to be linked to immunological and physiological stresses experienced by animals in anthropogenically polluted freshwater habitats (e.g. ZHELEV et al. 2017, 2020).

Infected individuals had low infection loads and did not show clinical signs of chytridiomycosis that may lead to mortality (e.g. WALDMAN et al. 2001). However, it should be noted that our sample was restricted to adult specimens which may be less susceptible for infection than post-metamorphic or sub-adult ones (e.g. BALÁŽ et al. 2014b). Low level *Bd* infections without obvious decline in body condition have been previously reported in water frogs (e.g. MALI et al. 2017, VOJAR et al. 2017, KÄRVEMO et al. 2020). A resistance to disease, with exceptional mortality even in cases of high *Bd* infection loads in experimental conditions (WOODHAMS et al. 2012) indicated water frogs as *Bd* reservoirs and vectors (BALÁŽ et al. 2014b). Although studies to date suggest that *Pelophylax* species are unaffected by *Bd* infection, some populations may coexist with *Bd*, tolerate a low-level, chronic infection and have slow population decline, but still with an overall negative effect (PILLIOD et al. 2010).

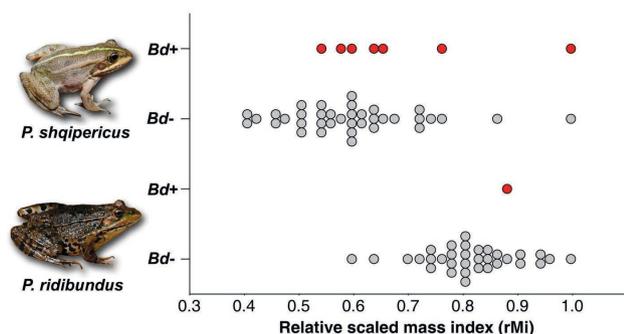


Figure 1. A dot plot showing the distribution of the relative scaled mass index values for non-infected (*Bd*-) and infected frogs (*Bd*+) in two water frog species, *P. shqipericus* and *P. ridibundus*. A dot represents a single individual.

The results of our study indicate that further research should include sampling throughout the range of *P. shqipericus* to elucidate spatial and temporal patterns in *Bd* infection prevalence in this species and its syntopic congener and the effect of *Bd* infection in interaction with other stressors on endemic *Pelophylax* species as carriers of the chytrid pathogen. The presence of the pathogenic *Bd* fungus in the endemic and vulnerable water frog species is worrying and indicates the need for close monitoring, urgent risk analysis, and population health assessment.

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