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Differences in chytridiomycosis infection costs between two amphibian species from Central Europe

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Abstract. *Batrachochytrium dendrobatidis* (*Bd*) causes the disease chytridiomycosis associated with amphibian declines. Response and costs of infection varies greatly between species. *Bd* can induce a stress response in amphibians resulting in elevated corticosterone (CORT). We exposed *Bombina variegata* and *Hyla arborea* tadpoles to *Bd*+ or *Bd*- *Salamandra salamandra* larvae and measured CORT release rates, *Bd* infection loads, and survival through metamorphosis. Tadpoles of both species exposed to *Bd*+ larvae had elevated CORT release rates compared to tadpoles exposed to *Bd*- larvae. *Bombina variegata* appear less resistant to infection than *H. arborea*, showing higher *Bd* loads and more infected individuals. Within species, we did not find differences in cost of infection on survival, however more *B. variegata* tadpoles reached metamorphosis than *H. arborea*. The differences in resistance may be species specific, owing to higher immunity defenses with *H. arborea* having higher overall CORT release rates, and differences in antimicrobial peptides, or to differences in *Bd* strain or other unexplored mechanisms.

Keywords: amphibian, chytridiomycosis, corticosterone, stress.

Batrachochytrium dendrobatidis (*Bd*) is a fungus that can cause the disease chytridiomycosis in amphibians (Berger et al., 1999). Infection with *Bd* has been associated with widespread amphibian declines (Stuart et al., 2004; Kilpatrick et al., 2010) and represents a global threat to amphibian biodiversity. The fungus attacks the skin of amphibians but innate antimicrobial peptides produced in their skin (Woodhams et al., 2011) and skin bacteria (Harris et al., 2006) provide protection against infection. These factors, as well ecological ones, are the principle determinant of survival against challenge by *Bd* (reviewed by Rollins-Smith et al., 2011).

Amphibian populations are declining worldwide and amphibians in Europe are particularly susceptible to chytridiomycosis (Rödder et al., 2009). Some amphibian species carry *Bd* in the absence of morbidity and may serve as reservoir hosts (e.g., American bullfrog, *Lithobates catesbeianus*; African clawed frog, *Xenopus laevis*), whereas others are highly susceptible to chytridiomycosis (Daszak et al., 2004; Rollins-Smith et al., 2009). In Europe, frogs of the genus *Rana* are unlikely to be infected whereas frogs in the family *Alytidae* and the closely related *Bombinatoridae* are significantly more likely to be infected (Baláž et al., 2014). Currently *Bd* is not causing noticeable mortalities in Central Europe and at least one ancient *Bd* lineage is present (Spitzen-Van Der Sluijs et al., 2014; Lips, 2016).

In Hungary, the yellow-bellied toad, *Bombina variegata*, was found to be the species most commonly infected with *Bd* (Baláž et al., 2014). Yet, in the field, mass mortality episodes have not been found even though there was a ∼50% prevalence of *Bd* in metamorphic toads (Baláž et al., 2014). Unlike *B. variegata*, *Hyla arborea*, the European tree frog (a sympatric species) is more resistant to *Bd* infection (Sztatecsny and Glaser, 2011). In Hungary at some locations, at higher elevation, *B. variegata* and *H.*

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arborea co-occur in ponds with fire salamanders, *Salamandra salamandra* (Solti and Varga, 1981; Dankovics, 1995; Hegyessy, 2006). Larvae of *S. salamandra* are able to overwinter in ponds (Bosch and Martínez-Solano, 2006) in high elevation areas, and overwintering larval salamanders maintain the *Bd* infection in ponds and re-infect young of the year larvae (Medina et al., 2015).

Infection with *Bd* can impose costs on amphibians. Amphibians exposed to *Bd* are predicted to have energy lost from defense against *Bd* and the costs of repair and damage caused by *Bd* (Voyles et al., 2009). Further, these costs may result in increased stress and ultimately chronic stress. The main glucocorticoid stress hormone in amphibians is corticosterone (CORT). Corticosterone and other stress hormone can be an indicator of the physiological health of wild animal populations (reviewed by Busch and Hayward, 2009; Sheriff et al., 2011). Studies have found that infected species of tadpoles have higher CORT than non-infected individuals. Warne et al. (2011) showed that tadpoles of *Rana sylvatica* infected with ranavirus had significantly higher CORT levels than non-infected tadpoles. Additionally, using water-borne CORT release rates Gabor et al. (2013; 2015) found that CORT release rates were elevated in populations of *Alytes obstetricans* that were infected with *Bd* as compared to non-infected populations. Increased CORT from infection may also affect survival to metamorphosis as CORT is already elevated during the process of metamorphosis in tadpoles (Glennemeier and Denver, 2002a) and small changes in CORT can affect amphibian fitness (Glennemeier and Denver, 2002b).

We explored differences in the response to infection with *Bd* between *B. variegata* and *H. arborea* tadpoles using *Bd* infected *S. salamandra* larvae as natural vectors. We predicted that tadpoles of *B. variegata* would have higher infection levels than *H. arborea* when reared with *Bd*+ *S. salamandra* larvae. We also predicted that tadpoles of both species infected with *Bd* would show higher CORT release rates compared to tadpoles not infected with *Bd*. We then compared the costs of infection (via *Bd* exposure and/or CORT levels) in terms of number reaching metamorphosis and survival after metamorphosis. Within species we predicted that fewer *Bd*+ individuals would reach metamorphosis and survive for 10d than *Bd*- individuals due to the costs of infection. Between species we predicted that more *H. arborea* would reach metamorphosis and survive because they may be more resistant.

We collected overwintering and young of the year larvae of *S. salamandra* in Central Spain (41.48°N, −5.45°W) in May 2015. Using quantitative real-time polymerase chain reaction (qPCR) protocol (Boyle et al., 2004; see methods below) we confirmed that the overwintering larvae ($n = 10$) were *Bd*+ (mean zoospore load per swab ± SE = 514.03 ± 69.78 GE) but the young of the year were not (mean zoospore load per swab = 0 GE). Salamander larvae were not re-tested for *Bd* infection at the end of the experiment as the temperature was maintained cold enough for *Bd* to survive for the duration of the experiment (Piotrowski et al., 2004) and, as expected, exposure was enough for infection to occur.

We collected eggs from the Mátra Mountains, Northeast Hungary, from Pásztó (47.89°N, 20.11°E) for *B. variegata* and Bárkás-tó, Recsk (47.90°N, 20.14°E) for *H. arborea* in May 2015 and brought them to the laboratory in Spain. After hatching and throughout the experiment, the tadpoles were reared on a 12:12h light cycle at 18°C and fed *ad libitum*.

Before setting up the treatments (16 days ahead) we obtained "baseline" water-borne CORT for 16 non-experimental tadpoles of each species by placing individual tadpoles in 40 ml of water in a 150 ml beaker for one hour. Following this, we used a randomized block design with five replicates of each treatment as follows: (1) *B. variegata* tadpoles ($n = 15$) and a *Bd*+ salamander larva, (2) *H. arborea* tadpoles ($n = 15$) and a *Bd*+ salamander larva, (3) *B. variegata* tadpoles ($n = 15$) with a *Bd*- salamander larva, (4) *H. arborea* tadpoles ($n = 15$) with a *Bd*- salamander larva. Fire salamander larvae could not access the tadpoles. Tadpoles were at Gosner stage 35 (Gosner, 1960) when the experiment began. Fifteen days after tanks were set up, we collected water borne CORT (15d CORT) samples from experimental tadpoles of each species ($n = 4$/tank (20/treatment)) to examine CORT release rates after exposure to *Bd*+ and *Bd*- salamander larvae. After collecting CORT samples, we measured the snout-vent length, mass, and swabbed for *Bd* on the tadpoles, and then the tadpoles were sacrificed. The remaining tadpoles were reared until metamorphosis in the presence of fire salamander larvae.

All water samples were stored at −20°C until they were thawed for extraction (Ellis et al., 2004). Hormones were extracted following Gabor et al. (2016). For both *B. variegata*

and *H. arborea*, we re-suspended the dried hormone residue in a total of 260 μl of enzyme-immuno assay buffer (EIA) and ethanol. We validated the use of water-borne CORT collection method from both *B. variegata and H. arborea* on EIA plates using a pooled sample of hormones from 10 non-experimental tadpoles (following Gabor et al., 2016). Using the pooled sample, we assessed parallelism of the serial dilution curve for six dilution samples (1:1-1:32). The CORT dilution curve was not significantly different from the standard curve for both species (comparison of slopes, *B. variegata*: $t_4 = 3.98$, $P = 0.99$; *H. arborea*: $t_2 = 0.012$, $P = 0.99$). We also spiked the pool sample with each of eight standards to determine the quantitative recovery. The minimum observed recovery for *B. variegata* was 66% and for *H. arborea* was 67%. We found a linear relationship between observed and expected slopes for *B. variegata* (slope = 0.87; $F_{1,7} = 1674.88$, $r^2 = 0.99$, $P < 0.0001$) and for *H. arborea* (slope = 0.88; $F_{1,7} = 365.92$, $r^2 = 0.98$, $P < 0.0001$).

We screened for *Bd* infection on day 15 using qPCR. We extracted nucleic acids from the whole oral disc following Boyle et al. (2004), and diluted the extractions 1:10 before qPCR amplification. Samples were examined in duplicate with *Bd* genomic equivalent (GE) standards of 100, 10, 1, and 0.1 GE. Samples were considered positive when both duplicates of an individual were positive, amplification curves presented the expected sigmoidal shapes, and the mean was above 0.1 GE.

We compared baseline CORT with 15d CORT using an unpaired *t*-test for *Bd*- exposed tadpoles of each species. We compared *Bd* infection loads (GE) between each species using a general linear mixed model (GLMM) with species as a fixed effect and tank as a random effect. All CORT data were Ln transformed. To test for effects of *Bd* treatment (*Bd*+, *Bd*-) and species (fixed effects) on CORT release rates we used a GLMM with tank as a random effect. We compared differences in the number of tadpoles reaching metamorphosis, as well the number of froglets surviving for 10d following metamorphosis, between treatments using a Log-rank Mantel-Cox test. All data met the assumptions of parametric analyses. All tests were significant at alpha = 0.05 and analyzed using JMPpro 12 and R package for the Log-rank Mantel-Cox test. To aid in visualizing the data we present non-transformed CORT values.

Baseline CORT did not significantly differ from 15d CORT release rates of *Bd*- tadpoles for both species (Unpaired *t*-test: *H. arborea*: $t_{34} = -1.87$, $P = 0.07$; *B. variegata*: $t_{25} = -1.15$, $P = 0.26$). Mean *Bd* loads (GE) on day 15 were higher in *B. variegata* than in *H. arborea* ($F_{1,6} = 6.8$, $P < 0.04$; fig. 1a). There was a significant effect of species on 15d CORT release rates ($F_{1,14} = 62.07$, $P < 0.0001$), with *H. arborea* having higher CORT release rates than *B. variegata* (fig. 1b). There was a significant effect of *Bd* infection on 15d CORT release

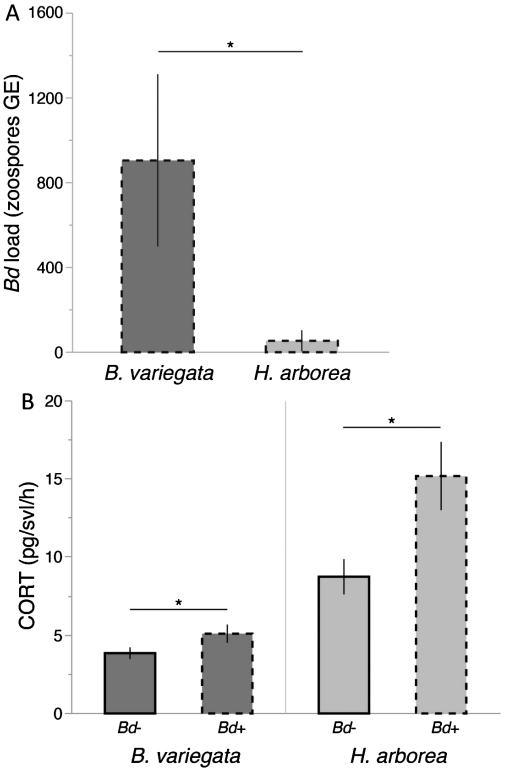

Figure 1. (A) Mean *Batrachochytrium dendrobatidis* (*Bd*) load. The mean *Bd* load (zoospores GE) ± S.E. of *Bombina variegata* and *Hyla arborea* tadpoles after exposure to *Bd*+ *Salamandra salamandra* larvae. (B) Mean corticosterone release rates. The mean corticosterone (CORT) release rates (pg/svl/h) ± S.E. of *Bombina variegata* and *Hyla arborea* tadpoles exposed to *Batrachochytrium dendrobatidis* (*Bd*)+ and *Bd*- *S. salamandra* larvae. Asterisks indicate significant differences.

rates ($F_{1,16} = 9.72$, $P = 0.0066$), with individuals of both species infected with *Bd* having higher CORT release rates than un-infected individuals (fig. 1b). There was no interaction between species and *Bd* infection ($F_{1,16} = 0.63$, $P = 0.44$). Significantly more *B. variegata* tadpoles metamorphosed than *H. arborea* tadpoles (30 vs 3%, Logrank Mantel-Cox: $\chi^2 = 39.8$, $P < 0.001$). About 50% of *Bd*- tadpoles of *B. variegata* reached metamorphosis whereas just 10% of *Bd*- tadpoles of *H. arborea* reached metamorphosis. There was no difference in the number of froglets surviving 10d after metamorphosis between *Bd*+ and *Bd*- within species (*H. arborea*, $n = 5$, 100 vs 100%, Logrank

Mantel-Cox: $\chi^2 = 2.4$, $P = 0.12$; *B. variegata*, $n = 45$, 63 vs 96%, Logrank Mantel-Cox: $\chi^2 = 0.3$, $P = 0.57$).

Batrachochytrium dendrobatidis is one of the many contributing factors to global amphibian declines (Stuart et al., 2004; Kilpatrick et al., 2010), yet knowledge of species specific effects of *Bd* is lacking despite evidence showing that some species are less resistant to *Bd* infection than others (Baláž et al., 2014, Bielby et al. 2015; Searle et al., 2013; Woodhams et al., 2007). We also found species specific differences in resistance with *H. arborea* showing higher resistance to *Bd*, which confirms their status as a reservoir. Interestingly, *H. arborea* tadpoles had higher overall CORT release rates. These differences in CORT may contribute to differential resistance because immune defenses have been linked to stress in amphibians (Pask et al., 2012). Tadpoles of both species exposed to *Bd*, via infected fire salamander larvae, had higher CORT release rates than uninfected individuals (within a species), similar to prior studies (Kindermann et al., 2012; Peterson et al., 2013; Gabor et al., 2013, 2015; Searle et al., 2015). Yet, within species, infected and uninfected animals did not differ in number reaching metamorphosis and surviving for 10 days post-metamorphosis. Differences in resistance may be owing to differences in antimicrobial peptides and/or CORT release rates (Rollins-Smith et al., 2011) but our data does not allow us to differentiate between these hypotheses.

Overall, infected tadpoles of both species had significantly higher CORT release rates than non-infected larvae, with infected larvae of *H. arborea* showing higher CORT release rates compared to infected *B. variegata* larvae. Similarly, other studies have found that CORT is higher in infected over non-infected tadpoles (Gabor et al., 2013, 2015; Warne et al., 2011). We do not think the higher CORT release rates of *H. arborea* are suggestive of higher physiological costs than for *B. variegata* because *H. arborea* CORT release rates were also higher overall for non-infected tadpoles. The higher CORT in *H. arborea* may be associated with the deeper tails that this species has (mean depth ± SE: *H. arborea*: 8.34 mm ± 0.17; *B. variegata*: 6.98 mm ± 0.14) as higher CORT is associated with deeper tails in tadpoles (Maher et al., 2013). The higher CORT released by *H. arborea* earlier in development may have contributed to the differences in their resistance because CORT can be preparative and enhance long-term immune responses (Dhabhar, 2009).

There was no difference in number of tadpoles surviving to metamorphosis between infected and non-infected individuals within species, but there was a significant difference in the number reaching metamorphosis between species. Fewer *H. arborea* metamorphosed than *B. variegata*. The dynamics between stress, immune function, and disease in amphibians is complicated and critical to understanding potential threats amphibians face from emerging pathogens and disease, yet it is relatively under studied (Savage et al., 2016). CORT is elevated in response to stress and an initial release may boost immune function (Dhabhar and McEwen, 1997), but even small changes in CORT can affect amphibian fitness (Glennemeier and Denver, 2002b). Cheatsazan et al. (2013) showed a cost of resistance to *Bd* infection most likely due to initiation of a costly immune response to infection in *Lissotriton helveticus*. *Bd* infection and subsequent immune response disrupts electrolyte transport and imposes an energy cost in *Litoria caerulea* (Voyles et al., 2009). Exposure to *Bd* imposes a cost on growth in tadpoles, which can lead to mortality before metamorphosis, even if infection is no longer detectable at time of death (Garner et al., 2009). It is possible *H. arborea* suffered greater costs due to higher CORT release rates, which may have aided in resistance but also negatively affected development, though we cannot discern this from the data and further study is needed. It is more likely that low temperatures (to maintain *Bd* infection) may have played a role in the lower survival of *H. arborea*.

We found that CORT release rates did not differ between tadpoles at Gosner stage ∼32 and ∼40 of each species. Our findings match those of Glennemeier and Denver (2002) who found that whole body CORT in *Rana pipiens* tadpoles did not increase CORT content between Gosner stages 25-40 (and those papers reviewed within). The lack of difference in baseline and 15d CORT indicate that CORT release rates are stable at least through prometamorphosis in numerous species of tadpoles even when they differ in their overall baseline CORT release rates.

There is growing evidence that there are species specific differences in susceptibility to emerging infectious diseases in amphibians (Baláž et al., 2014; Blaustein et al., 2004; Bielby et al., 2015; Searle et al., 2013; Woodhams et al., 2007). Our data support this finding and provide further evidence that amphibians infected with *Bd* show increased CORT release rates. We also found that infected fire salamander larvae were a viable transmission vector for *Bd* similar to Medina et al. (2015). Further investigation of differences in resistance in each species is warranted and an understanding of the role CORT may play will have broad implications for amphibian conservation.

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