Fatal chytridiomycosis and infection loss observed in captive toads infected in the wild

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Abstract:

A parasitic fungus, *Batrachochytrium dendrobatidis* is now recognised as an important factor in the amphibian biodiversity crisis. Toad species of the genus *Bufo* are among those susceptible to infection by the pathogen in Europe. The aim of this study was to observe the presence and impact of infection in adults of two toad species collected for captive breeding. The total number of animals included in the study was 162, but only subsets were used for sampling at different occasions (35 specimens in the initial sampling in summer 2011, 48 post hibernation during winter 2011, and 31 in summer 2012, after all toads in captivity were treated with itraconazole). We performed TaqMan real-time quantification PCR to detect and quantify the pathogen. We found that a large infection load was linked to mortality in a single adult green toad (*Bufo viridis)*. However, low infection loads observed in five *B. viridis* and five natterjack toads (*B. calamita)* were lost over time, with no apparent adverse effect. Intraconazole treated animals were all clear of infection. As infection in these two toad species either led to mortality or recovery, it seems unlikely they could act as permanent carriers of *B. dendrobatidis* and therefore persistence of the pathogen is likely maintained by different host species. This is the first study to date that has detected infection and observed its impact and persistence in wild-infected toads in Europe.

Batrachochytrium dendrobatidis, chytridiomycosis, Bufo, amphibian conservation

Batrachochytrium dendrobatidis is an important fungal pathogen of amphibians, which causes the cutaneous disease chytridiomycosis (Longcore et al. 1999). Whether infection persists and develops into disease is modulated by both external and intrinsic factors, including climatic conditions and host immunity (Fisher at al. 2007). The disease disrupts electrolyte transport through the skin and is often fatal (Voyles et al. 2009). The clinical signs include lethargy, dehydration, uneven skin shedding, loss of appetite, occasionally ulceration or necrosis of digits and neurological defects (Duffus and Cunningham 2010). However, these symptoms are non-specific and infection needs to be proven in the laboratory (Duffus and Cunningham 2010). The question of the geographic origin of the pathogen remains unanswered, but the fungus has already reached global distribution and infects over 350 amphibian species (Fisher et al. 2009). It is believed to be one of the causes of the current amphibian diversity crisis (Skerratt et al. 2007). As the extinction risk is different between various taxa of amphibians (Bielby et al. 2008), it is important to discriminate between the immune-, carrier- and susceptible-species in each region. Laboratory experiments and observations on species susceptibility are vital for understanding the outcome of infection in different taxa (Searle et al. 2011), and also enable data to be gathered which would often be unavailable in a field environment.

Presence of *B. dendrobatidis* in the Czech Republic was detected in 2008 (Civiš et al. 2012) with the documented host species including water frogs of the genus

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Pelophylax, the European fire-bellied toad (*Bombina bombina*), the yellow-bellied toad (*Bombina variegata*) and the common toad (*Bufo bufo*). All European species of the genus *Bufo* (taxonomy following Speybroeck et al. 2010) are known to be susceptible to infection (Garner et al. 2005; Ohst et al. 2011) and *B. bufo* has proven to be a great model in experimental studies (Garner et al. 2011). However, mortalities linked to *B. dendrobatidis* infection in toads of the genus *Bufo* from Europe are uncommon and limited to specific conditions (e.g. Bosch and Martinez-Solano 2006). All three European *Bufo* species (common toad *Bufo bufo,* natterjack toad *B. calamita,* green toad *B. viridis*) are present within the Czech Republic, and it is therefore an ideal area to study the interactions between *B. dendrobatidis* and the different species of this genus. *Bufo bufo* was shown to harbour the pathogen at low prevalence in the Czech Republic, however, there is no information available on *B. dendrobatidis* infection status and its impact to *B. calamita* and *B. viridis*, even though they are recognised as endangered species by national law and are included in numerous conservation and surveillance schemes.

The aim of this study was to monitor the pathogen in an *ex-situ* breeding project *B. calamita* and *B. viridis* to search for cases of acute chytridiomycosis and observe infection persistence in time.

Materials and Methods

In total, 162 adults of two toad species, *B. calamita* (n = 113) and *B. viridis* (n = 49) were collected from two localities in an area of open-pit mining in the western part of the Czech Republic (N50.212767°, E12.605683° and N50.245044°, E12.671663°) post breeding in May 2011. Toads were captured as part of a project which focuses on amphibian captive breeding in artificial basins, by a company specialising in amphibian and reptile conservation (NaturaServis Ltd., Hradec Králové, www.naturaservis.net). The animals were housed in the basins throughout summer and autumn 2011. Up to 10 animals were accommodated in each of the 7 small basins (size 8 m^2) while 5 large basins (size 16 m^2) housed both species, up to 23 animals per basin. The basins were filled with substrate from the sites of collection. Dry land covered 2/3 of each basin and contained various hiding spots and plants. The remaining 1/3 of the basin was a pool of water. The animals were fed crickets and sweep-netted insects twice a week.

Due to limited funding, only a subset of all animals could be sampled for *B. dendrobatidis* detection. Therefore two individuals were sampled per small basin and four individuals per large basin. One lethargic *B. viridis* male was housed separately and also sampled. This first sample set, collected in May 2011 thus contained 35 animals (27 *B. calamita* and 8 *B. viridis*).

All toads in captivity were put to hibernation in November 2011. They were sorted into groups of 4–5 individuals and placed in refrigerators at 4–5 °C in boxes with moistened substrate from the original locality. A subset of individuals from both species was kept at a lowered temperature of 1–2 °C (near-freeze treatment) for 10 days during December 2011.

The second sampling phase took place in February 2012. The presence of infection by *B. dendrobatidis* was checked in all animals from the six basins where infection had been detected during initial sampling (48 specimens). Following the "near-freeze treatment", toads in this group were sampled individually (n = 24), but the samples were pooled for qPCR detection so DNA samples from all animals per hibernation box were used together as one sample. Although this could dilute the target DNA of the pathogen, it should not lower the chance of detection of positive individuals as was shown by Hyatt et al. (2007). Toads that were not near-freeze treated were also sampled individually (n = 24); if found positive, they were housed separately and resampled once a week until the end of hibernation (in total seven sampling occasions).

The toads were moved to breeding tanks in April 2012. In order to clear any potential *B. dendrobatidis* infection, all toads (n = 162) were treated with Itraconazole post hibernation by daily bathing in a 0.01% itraconazole solution, for 11 successive days (as in Forzan et al. 2008). In order to stimulate any remaining infection, the water level in the tanks was increased for a period of 2 weeks in July (as advised by Trenton Garner, Zoological Society of London). Altogether 31 toads (24 *B. calamita* and 7 *B. viridis*) from basins that contained positive individuals after initial sampling in May 2011 were randomly selected for *B. dendrobatidis* detection, two weeks after this increased water treatment.

All samples in this study were collected by swabbing the skin using sterile rayon swabs MW100 (Medical Wire). Sampling and DNA extraction using PrepMan Ultra (Applied Biosystems, UK) followed the standard protocols (Boyle et al. 2004; Hyatt et al. 2007). Sample homogenisation was performed by MagNA Lyser (Roche Diagnostics, CZ). *B. dendrobatidis* detection was performed by real-time qPCR (Boyle et al. 2004), but with the addition of bovine serum albumin (BSA) to lower PCR inhibition (Garland et al. 2010), reduction of volume of the reaction and running the reactions in triplicates (Hyatt et al. 2007)

using Light Cycler 460 II system with original Roche reagents. DNA quantification standards were provided by the Institute of Zoology, Zoological Society of London. The standards are made by isolation of the pathogen's DNA from exactly counted zoospores. Comparing the results of unknown samples to standard curve based on the standards allows quantification of the pathogen in the sample with the unit being GE = genomic equivalent of one zoospore (Boyle et al. 2004). The method represents the most commonly used option in studies that need high sensitivity and ability to distinguish the infection intensities among sampled amphibians.

Results

During the initial field sampling period in May 2011 (n = 35), an infection prevalence of 18.5% was detected in *B. calamita* (5 out of 27; 95% CI = 7.6–36.9) and 87.5% in *B. viridis* (6/8; 95% CI 50–99.4). Infection intensity ranged from 0.3–58 GE in *B. calamita* and 1.3-13,750GE in *B. viridis*. The individual *B. viridis* with the highest score was in poor physical condition at the time of swab collection. This animal was quarantined, but died the following day with no further tests/analysis carried out on its carcass.

The second sampling period, carried out in February 2012 during hibernation (n = 48) gave one positive result from *B. calamita* with a GE of 1.9 (SD 0.4). All other animals tested were negative during that time. The positive animal was not included in the initial sampling period, and was not among those that received the near-freeze treatment. Infection load in the positive animal decreased during the period of hibernation from 1.9 GE to 0.63 GE on 28th February and 0.40 GE on 6th March. The following sampling of this animal that was done once per week (from 13 March until 4 May) gave a negative result.

All animals survived hibernation in good condition. No animals tested were found positive during the third sampling occasion (n = 31) in July 2012, following the water increase treatment.

Discussion

Keeping wild collected animals in captivity allows observation of processes that would otherwise go undetected. It is reasonable to expect that mortality of infected individuals occurs in times of climatic stress or during hibernation, when the animals are hidden. Mortality in such conditions is especially difficult to study in a field environment and it is possible that important population regulating factors go undetected. Naturally infected animals carry a local strain of the pathogen and naturally occurring infection dose, therefore their observation brings better insight of the infection result in realistic conditions.

High infection load found on one specimen of *B. viridis* was linked with lethargy and later, mortality. Although no histological examination was performed, it can be presumed this mortality was a results of fatal chytridiomycosis, as such high levels of infection (> 10,000 GE) are often regarded as a sign of acute chytridiomycosis (Vredenburg et al. 2010; Kinney et al. 2011). Our findings also imply that intense infections can be linked to mortality in adult *B. viridis* and similarly in *B. calamita* (Bosch and Martinez-Solano 2006), both species are able to clear mild infection or suppress it to levels undetectable by qPCR. As our sampling effort had apparent limitations in sample size and timing of sampling, our data did not show exactly when the loss of infection occurred and we were not able to track the individual changes of infection over time. However, our data still bring interesting results. The single individual that was found positive during hibernation lost any detectable infection before the end of overwintering, surprisingly at a time when the amphibian immune system is suppressed by temperature (Ribas et al. 2009). It is possible that relatively low humidity of the skin and temperature below optimal caused stress to

the fungus and lead to a loss of infection or reduction of its quantity in the outer skin layer below a detectable level. Our results highlight a very intriguing question as to where the fungus overwinters and which species contribute to its survival during winter. Dormant stages of the fungus could allow survival of harsh conditions independently from the host but to our knowledge, this life stage has not been described in *B. dendrobatidis* so far. Based on our findings, toads seem unlikely to be permanent reservoirs of *B. dendrobatidis*. If overwintering and long term survival of this fungal pathogen is maintained by a limited number of species, it may help to direct future mitigation attempts, at least in countries with temperate climate.

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