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Abstract

The amphibian chytrid fungus Batrachochytrium dendrobatidis (Bd) is sensitive to high temperature. Hence, exposing amphibians to high temperature may be a method to clear Bd infection. However, the effect of exposure to elevated temperature has never been tested in larval stages or temperate species. We experimentally exposed tadpoles of the toad Alytes obstetricans to low, medium and high temperatures and found that most, but not all, tadpoles lost the infection when exposed to temperatures higher than 26°C for 5 days. Thus, exposure to elevated temperatures can be used to treat tadpoles against Bd infection.
Elevated temperature clears chytrid fungus infections from tadpoles of the midwife toad, *Alytes obstetricans*

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**Abstract.** The amphibian chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) is sensitive to high temperature. Hence, exposing amphibians to high temperature may be a method to clear *Bd* infection. However, the effect of exposure to elevated temperature has never been tested in larval stages or temperate species. We experimentally exposed tadpoles of the toad *Alytes obstetricans* to low, medium and high temperatures and found that most, but not all, tadpoles lost the infection when exposed to temperatures higher than 26°C for 5 days. Thus, exposure to elevated temperatures can be used to treat tadpoles against *Bd* infection.

**Keywords:** chytridiomycosis, disease, infection, temperature, treatment.

Pathogenic chytrid fungi can drive host population dynamics (Ibelings et al., 2004). The chytrid fungus *Batrachochytrium dendrobatidis* (hereafter *Bd*) causes the amphibian disease chytridiomycosis that was characterized in the IUCN Amphibian Conservation Action Plan as “the worst infectious disease ever recorded among vertebrates in terms of the number of species impacted, and its propensity to drive them to extinction” (Gascon et al., 2007). Chytridiomycosis is thought to be one of the main reasons for the global decline of amphibians (Houlanah et al., 2000, 2001; Stuart et al., 2004; Skerratt et al., 2007; Collins and Crump, 2008). Antifungal drugs have been used successfully to clear infection with *Bd* (Nichols and Lamiranda, 2001; Parker et al., 2002; Garner et al., 2009; Tobler and Schmidt, 2010), but there are concerns that these drugs may have unwanted side effects (Nichols and Lamiranda, 2001; Woodhams, Alford and Marantelli, 2003; Garner et al., 2009). For example, when treating tadpoles of the toad *Alytes muletensis* against *Bd* infection using Itraconazole (De Beule and Van Gestel, 2001), Garner et al. (2009) reported depigmentation of the tadpoles. Therefore, Garner et al. (2009) did not generally recommend Itraconazole as an anti-*Bd* treatment because of suspected hepatotoxicity. However, Tobler and Schmidt (2010) used Itraconazole to treat tadpoles of *Alytes obstetricans* and observed no depigmentation. Furthermore, Young et al. (2007) report that no treatment using antifungal drugs is consistently effective across species. Hence, a treatment against *Bd* that has no such side effects would be of great benefit.

Because growth and survival of *Bd* are highly sensitive to high temperature (Piotrowski, Annis and Longcore, 2004), Woodhams et al. (2003) and Retallick and Miera (2007) exposed adult frogs to elevated temperature (37°C and 32°C, respectively). After being exposed to elevated temperature, frogs were no longer infected with *Bd*. Keeping frogs at high temperature may thus...
be a treatment against *Bd* infection that may be free of undesirable side effects. The use of elevated temperature to clear *Bd* infection requires further testing (Berger et al., 2010). Here, we test whether tadpoles can also be treated against *Bd* infection by keeping them at an elevated temperature. In many species, tadpoles carry infections and may be an intraspecific reservoir (Brunner et al., 2004). Additionally, tadpoles of many species are more easily accessible than adults. Hence, mitigation strategies may focus on tadpoles rather than adults (Lubick, 2010). We used tadpoles of the locally endangered midwife toad (*Alytes obstetricans*) (Schmidt and Zumbach, 2005) in our experiment because the species is known to be highly susceptible to chytridiomycosis and carries *Bd* infections in many parts of its range, often at high infection prevalence and infection loads (Bosch, Martínez-Solano and Garca-París, 2001; Garner et al., 2005; Tobler and Schmidt, 2010).

*Alytes obstetricans* tadpoles where caught in a pond near Zunzgen (canton Baselland, Switzerland) with dip nets. The site was selected because we knew that *Bd* prevalence was high (Tobler and Schmidt, 2010). Standard hygiene recommendations were followed during field work (Schmidt et al., 2009a, 2009b). For transport to the University of Zürich, tadpoles were individually packed in small plastic containers (1.5 l) filled up to two thirds with pond water. Before the experiment began, they were kept indoors at 19°C (1.5 l) filled up to two thirds with pond water. Before the experiment began, they were kept indoors at 19°C. Water was changed twice a week, followed by feeding (*Spirulina* suspension in tap water).

After capture, and again 6-10 days after the experimental treatments, each tadpole was swabbed with a sterile rayon tipped plain swab with a plastic applicator (Copan (Italy)) in the mouthpart to test for infection with the fungus *Batrachochytrium dendrobatidis* (*Bd*). Metamorphs (post Gosner (1960) stage 42) were swabbed on each foot and on the belly. *Bd* DNA extraction and subsequent real-time PCR followed standard protocols (Boyle et al., 2004; Tobler and Schmidt, 2010). We ran each sample twice according to Tobler and Schmidt (2010). If the two results of one sample were different, the analysis was repeated. For the experiment, we only used tadpoles that tested positive for *Bd*. One tadpole metamorphosed during on the penultimate day of the experiment.

To test if warm water cures *Bd* infection in tadpoles of *Alytes obstetricans*, we conducted a factorial experiment in which we manipulated temperature. Tadpoles were exposed to one of three different treatments: a “low” temperature treatment with a constant water temperature of 21.4°C, a “medium” temperature treatment with a constant water temperature of 26.2°C, and a “high” temperature treatment. While the cold and warm treatment were kept at a constant temperature over the whole time of the experiment (5 days or 120 hours), tadpoles in the high treatment were treated differently. The containers were kept at approximately 30°C twice for 8 hours, followed by room temperature for 16 hours. Afterwards they were kept at approximately 30°C for 43 hours. Then they were kept at room temperature until the end of the experiment. Thus, in the “high” treatment, tadpoles were exposed to a mean of 29.7°C for 59 hours, while the mean temperature across the 5 days was 27.1°C. Heating was done by placing experimental containers on two different heating mats (a 25 cm by 35 cm 15 watt Thermolux heat basis and a 51.6 cm by 27.4 cm 28 watt Lucky Reptile thermo mat). The “low” treatment was kept at room temperature without heating or cooling. Temperatures in each experimental unit were measured daily.

For the experiment, we used small round plastic containers. Each contained 500 ml tap water and one tadpole each. Tadpoles were fed 2.5 ml of *Spirulina* suspension (= 120 mg dry food) at the beginning of the experiment. Water was changed twice a week, followed by feeding (*Spirulina* suspended in tap water).

After capture, and again 6-10 days after the experimental treatments, each tadpole was swabbed with a sterile rayon tipped plain swab with a plastic applicator (Copan (Italy)) in the mouthpart to test for infection with the fungus *Batrachochytrium dendrobatidis* (*Bd*). Metamorphs (post Gosner (1960) stage 42) were swabbed on each foot and on the belly. *Bd* DNA extraction and subsequent real-time PCR followed standard protocols (Boyle et al., 2004; Tobler and Schmidt, 2010). We ran each sample twice according to Tobler and Schmidt (2010). If the two results of one sample were different, the analysis was repeated. For the experiment, we only used tadpoles that tested positive for *Bd*. One tadpole metamorphosed during on the penultimate day of the experiment.

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The response variable for the statistical analyses was the infection status after the experimental treatment (all tadpoles were initially infected). We conducted two tests. First, we used the experimental treatment levels (“low”, “medium”, “high”) as categorical explanatory variables and a Fisher’s exact test to test the null hypothesis of no difference among treatment levels. Second, because there was some variation in temperature among experimental units within treatment levels, we used the mean temperature that a tadpole experienced as a continuous explanatory variable in a logistic regression (Dalgaard, 2008). Statistical analyses were done using the program R (R Development Core Team, 2009). Type I error probability was 5%.

Three tadpoles died in the “medium” and “high” treatments (one and two, respectively). Loss of *Bd* infection depended on the temperature treatment. At low, medium and high temperature 2, 5 and 7 out of 10, 8 and 8 tadpoles,
respectively, lost the *Bd* infection (proportions: 0.2, 0.625, 0.875, respectively). The difference among treatments was significant (Fisher’s exact test: $p = 0.0262$). The logistic regression based on the mean temperature experienced by tadpoles also showed a highly significant decline of the probability to remain infected with increasing temperature ($p = 0.00825$; fig. 1).

Growth of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* is maximal at 17-25°C (Piotrowski et al., 2004). Based on this knowledge, Woodhams et al. (2003) and Retallick and Miera (2007) showed that adult frogs can clear *Bd* infection when kept for some time at high temperature. Our results (fig. 1) show that tadpoles also clear infection when kept at temperatures that are above the fungus’ optimal temperature. It may be possible that tadpoles did not clear infection but rather that infection level was below the detection threshold after the treatments. However, Briggs, Knapp and Vredenburg (2010) showed that fungal load dynamics probably explain mortality associated with *Bd* infection. Hence, even if our treatments only reduced infection loads (as opposed to clearing infection), then this would probably lead to reduced *Bd*-associated mortality because mortality depends on *Bd* infection loads (Tobler and Schmidt, 2010).

While there was a clear effect of temperature on the rate of loss of the *Bd* infection, 2 out of 10 tadpoles that were kept at a mean temperature of 20-21°C also lost the infection. Such a loss of infection was not observed in *Alytes obstetricans* tadpoles in another laboratory experiment where tadpoles were kept at $\sim 19$°C (Tobler and Schmidt, 2010). A spontaneous loss of infection (and re-infection) was observed, however, in other species (Briggs, Knapp and Vredenburg, 2010). Hence, not all losses of infection at higher temperatures observed in our experiment may be due to temperature. Nevertheless, the rate of loss is much higher at elevated temperatures.

Because prolonged exposure to high temperatures cleared most *Bd* infections, exposure to high temperature may be a simple and inexpensive way to treat large numbers of tadpoles against *Bd* infections in relatively short time (as it is possible in adult frogs: Woodhams et al., 2003). We did not measure costs associated with exposure to elevated temperature but we believe that the benefits of the treatment outweigh costs due to side effects. Temperature does not seem to be as efficient (in terms of clearing rates) as antifungal drugs. For instance, Tobler and Schmidt (2010) reported that 100% of the tadpoles were free of *Bd* after treatment with Itraconazole. Yet, while antifungal drugs may have unwanted side effects (e.g., Garner et al., 2009), temperature probably has no such strong side effect because the temperatures that we used are well below the critical thermal maxima reported for tadpoles (usually $>38$°C; Ultsch, Bradford and Freda, 1999). Hence, one may test the efficacy of higher temperatures or longer exposure times such that *Bd* infection is eliminated from all tadpoles. Based on the logistic regression equation, less than 1% of the tadpoles is predicted to remain infected at 34°C. When temperature...
perature is used to treat tadpoles against *Bd* infection, we recommend some acclimatization of the tadpoles before the treatment. When novel species are treated, then a pilot study with a small number of tadpoles would be important.

Exposure to temperatures that tadpoles occasionally experience in natural ponds (Thiesmeier, 1992; Indermaur et al., 2010; S. Böll, pers. comm.) cleared most, but not all, *Bd* infections. It may be possible to use this piece of information to design a mitigation strategy. In some habitats, it may be possible to reduce canopy cover, especially when there are no closed-canopy specialists and when canopy closure is the results of shrub and tree encroachment in the wetland. In a natural experiment where beaver reduced canopy cover, the entire amphibian community benefited (Dalbeck, Lüscher and Ohlhoff, 2007). As a result of reduced canopy cover, temperatures would rise (Skelly, Freidenburg and Kiesecker, 2002) and perhaps prevalence may drop. It may drop to a level that the population may be able to compensate, for example through density dependent processes (Boyce, Sinclair and White, 1999). Such a strategy may have additional benefits in a species with overwintering larvae, such as *Alytes obstetricans*. If warmer water speeds up development such that fewer tadpoles hibernate (Thiesmeier, 1992), then population growth rate is predicted to increase (Govindarajulu, Altwegg and Anholt, 2005). If this reasoning is correct, then warmer ponds may be beneficial for amphibians because *Bd* prevalence may be lower and population growth rate higher.

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### References


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