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In vitro thermal tolerance of a hypervirulent lineage of the chytrid fungus, *Batrachochytrium dendrobatidis*: growth arrestment by elevated temperature and recovery following thermal treatment --Manuscript Draft--

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Full Title:	In vitro thermal tolerance of a hypervirulent lineage of the chytrid fungus, <i>Batrachochytrium dendrobatidis</i> : growth arrestment by elevated temperature and recovery following thermal treatment
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Abstract:	Chytridiomycosis, an emerging infectious disease caused by the chytrid fungus <i>Batrachochytrium dendrobatidis</i> (Bd) poses a serious threat to amphibian biodiversity. The thermal optimum of Bd is lower than that of most amphibians, which provides an opportunity to cure infected individuals with elevated temperature. The application of elevated temperature, however, presupposes detailed knowledge about the thermal tolerance of the fungus. To determine the temperature that may effectively reduce infection burdens <i>in vivo</i> , detailed <i>in vitro</i> studies are invaluable because their outcomes are characteristic for the fungus and are not complicated by species-specific temperature-dependence of the hosts' immune system. The aim of our study was to gather accurate information about the thermal tolerance of a hypervirulent isolate of Bd. We incubated Bd cell cultures at five different temperatures (21, 25.5, 27, 29 or 30.5 C) for one of six exposure durations (3, 4, 5, 6, 7 or 8 days) and subsequently counted the number of zoospores to assess the temperature dependence of Bd growth. We observed intensive Bd growth at 21 C. At 25.5 C the number of zoospores also increased over time, but the curve plateaued at about half height. At temperatures of 27 C and above, the fungus showed no considerable growth. However, when we moved back Bd cultures to 21 C after the elevated temperature treatments, we observed recovery of Bd growth in all cultures previously treated at 27 C. At 29 C a treatment duration of 8 days was necessary to prevent recovery of Bd growth and at 30.5 C 5 days of thermal treatment were required for total Bd-wipeout, revealing that these moderately elevated temperatures applied for only a few days have merely a fungistatic rather than a fungicidal effect, at least <i>in vitro</i> .
Keywords:	Amphibia; disinfection; growth inhibition; pathogen; temperature dependence; thermal optimum mismatch
Author Comments:	Dear Editor, Please find enclosed a manuscript entitled "In vitro thermal tolerance of a hypervirulent lineage of the chytrid fungus, <i>Batrachochytrium dendrobatidis</i> : growth arrestment by elevated temperature and recovery following thermal treatment" submitted for consideration of publication as an Original Research Article in <i>Mycologia</i> . This is the resubmission of a manuscript entitled "In vitro thermal tolerance of a hypervirulent lineage of the chytrid fungus, <i>Batrachochytrium dendrobatidis</i> ", Ms. No. UMYC-2020-0175, which has been undertaken major revisions. Yours sincerely, Andrea Kásler, M.S.

Kásler et al.: Heat tolerance of a chytrid pathogen

In vitro thermal tolerance of a hypervirulent lineage of the chytrid fungus, *Batrachochytrium dendrobatidis*: growth arrestment by elevated temperature and recovery following thermal treatment

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ABSTRACT

Chytridiomycosis, an emerging infectious disease caused by the chytrid fungus *Batrachochytrium dendrobatidis* (Bd) poses a serious threat to amphibian biodiversity. The thermal optimum of Bd is lower than that of most amphibians, which provides an opportunity to cure infected individuals with elevated temperature. However, it presupposes detailed knowledge about the thermal tolerance of the fungus. To determine the temperature that may effectively reduce infection burdens *in vivo*, detailed *in vitro* studies are invaluable because their outcomes are characteristic for the fungus and are not complicated by species-specific

temperature-dependence of the hosts' immune system. The aim of our study was to gather accurate information about the thermal tolerance of a hypervirulent isolate of Bd. We incubated Bd cell cultures at five different temperatures (21, 25.5, 27, 29 or 30.5 C) for one of six exposure durations (3, 4, 5, 6, 7 or 8 days) and subsequently counted the number of zoospores to assess the temperature dependence of Bd growth. We observed intensive Bd growth at 21 C. At 25.5 C the number of zoospores also increased over time, but the curve plateaued at about half height. At temperatures of 27 C and above, the fungus showed no considerable growth. However, when we moved back Bd cultures to 21 C after the elevated temperature treatments, we observed recovery of Bd growth in all cultures previously treated at 27 C. At 29 C a treatment duration of 8 days was necessary to prevent recovery of Bd growth and at 30.5 C 5 days of thermal treatment were required for total Bd-wipeout, revealing that these moderately elevated temperatures applied for only a few days have merely a fungistatic rather than a fungicidal effect, at least *in vitro*.

KEY WORDS: Amphibia, disinfection, growth inhibition, pathogen, temperature dependence, thermal optimum mismatch

INTRODUCTION

Batrachochytrium dendrobatidis (Bd) is the causative agent of chytridiomycosis, an emerging infectious fungal disease of amphibians (Longcore et al. 1999). Although several lineages of Bd have been described so far (Farrer et al. 2011, James et al. 2015, Lips 2016, O'Hanlon et al. 2018, Scheele et al. 2019), and most of them coexist with local amphibian species, the Global Pandemic Lineage (BdGPL) has caused dramatic population declines in hundreds of amphibian species and led to the extinction of dozens of species over the last few decades, since its rapid spread across the globe (Skerratt et al. 2007, Lips 2016, O'Hanlon et al. 2018, Scheele et al. 2019). This is especially worrying because 41 % of amphibian species are

already listed as threatened (IUCN 2020), and a widely applicable cure for chytridiomycosis is not available (Woodhams et al., 2011; Scheele et al., 2014, 2019; Garner et al., 2016). Thus, detailed research is needed into the biology of this pathogen to provide new insights that could form the basis of novel ways of mitigating impacts of the disease.

Some chemical disinfection methods, such as the application of salt (Stockwell et al. 2012), general disinfectants (Parker et al. 2002) and antibiotics (Bishop et al. 2009) or antifungals are effective in captive populations, but if applied to populations under natural conditions, these would cause vast collateral damage, possibly also negatively affect the treated amphibians themselves, or would be impractical or ineffective (Scheele et al., 2014; Bosch et al., 2015; Garner et al., 2016). One potential approach to mitigating the impact of chytridiomycosis is to capitalize on the thermal optimum mismatch between Bd and its hosts (Woodhams et al. 2003, Chatfield and Richards-Zawacki 2011, Heard et al. 2014, Scheele et al. 2015, Hettyey et al. 2019). Previous *in vitro* studies have found, that the thermal optimum of Bd is around 20 C, it grows well below 25 C, and its critical thermal maximum (CT_{max}) is somewhere around 28 C (Johnson et al., 2003; Piotrowski et al., 2004; Stevenson et al., 2013; Cohen et al., 2017; Voyles et al., 2017). By contrast, ca. 80 % of amphibian species' CT_{max} exceeds 32 C (Sunday et al. 2011, 2014). This difference in the thermal tolerance of the pathogen and its hosts has been successfully used to treat infected individuals in captivity (Berger et al., 2010; Chatfield & Richards-Zawacki, 2011; McMahon et al., 2014). Consequently, in combination with the thermoregulatory behavior of amphibians, elevating temperatures to ca. 28 C locally may be a safe and effective way of fighting chytridiomycosis in free-living populations as well (Woodhams et al. 2011, Scheele et al. 2014, Hettyey et al. 2019).

For the *in vivo* application of localized heating we need detailed knowledge about the thermal tolerance of Bd in order to prevent malign effects on treated amphibians that could

arise from excessive use of heat. Also, if applied under field conditions where the availability of energy, generally in the form of electric power, will often be a limiting factor, we have to know the minimum effective heat to minimize the energy requirement of the temperature treatment. *In vitro* studies provide the practical first step towards achieving this goal because their results are not confounded by species-specific thermal dependence of the immune function of the hosts (Wright & Cooper, 1981; Raffel et al., 2006; Andre et al., 2008; Cohen et al., 2017, 2019a, 2019b; Sauer et al., 2018; Bradley et al., 2019). Also, they allow for restricting the temperature range which is worthy of further *in vivo* investigations, and thereby contribute to meeting all principles of the 3Rs (replacement, reduction, refinement) of animal experimentation (Russell & Burch, 1959).

Therefore, we performed a comprehensive investigation on the effect of elevated temperature on Bd-growth in liquid cell cultures, using 30 different combinations of treatment temperatures and durations. We predicted intensive growth at temperatures that are within the thermal optimum of the fungus, reduced Bd growth above 25 C and no growth above 28 C. Because recovery of the fungus after heat-treatments was observed previously (Longcore et al. 1999, Stevenson et al. 2013, Voyles et al. 2017), we also investigated growth in Bd cultures after removing them from thermal treatments and keeping them for another 7 days at 21 C. We thereby aimed to clarify which thermal treatments are merely fungistatic (i.e. suppress the growth of the fungus temporarily) and which ones are truly fungicidal (i.e. exterminate the fungus irrecoverably). We predicted that after incubation at around 28 C Bd would show recovery, but after incubation above 30 C for around 7 days the fungus would show no growth when placed back to an optimal temperature.

MATERIALS AND METHODS

Bd cell culture history.—

We used the isolate IA042 of the hypervirulent BdGPL strain, provided by T. W. J. Garner (Institute of Zoology, Zoological Society of London, London, UK) originating from a dead *Alytes obstetricans* found in Spain during a mass mortality event in 2004. The cell culture was maintained at 4 C in cell culture flasks (25 cm², closed cap; Orange Scientific, Belgium) using mTGhL liquid medium (8 g tryptone, 2 g gelatin hydrolysate, 4 g lactose in 1000 mL distilled water).

Arrestment of Bd growth at elevated temperatures.—

Four days before the start of the experiment we started ten new cell culture lines by inoculating 2.5 mL Bd culture into 175 cm² cell culture flasks containing 50 mL mTGhL and incubating them at 20 C. At the start of the experiment (day 0), we chose the eight best-growing Bd cell culture lines for testing. To obtain equal initial zoospore concentrations across cell cultures that were sufficiently high to allow for reliable estimation, but were low enough to provide plenty of space for growth, we assessed zoospore concentrations using a standard hemocytometer and subsequently diluted the selected cell culture lines to ca. 2.9×10^5 zoospores / mL. Subsequently, we inoculated 1.2 mL of cell culture lines into 25 cm² cell culture flasks containing 9 mL mTGhL. We exposed cell culture lines to all possible combinations of five temperatures (21, 25.5, 27, 29 or 30.5 C) and six exposure durations (3, 4, 5, 6, 7 or 8 days), resulting in a total of 240 experimental units. Cell cultures maintained at 21 C served as positive controls. Culture flasks were kept in the dark during heat-treatments. Large boxes covered with lids and filled with tap water heated with thermostated aquarium heaters (Tetra HT 200 in the 25.5 and 27 C treatments and Tetra HT 300 in the 29 and 30.5 C treatments) served as incubators. We recorded the temperature in incubators every 10 minutes using data loggers (HOBO Pendant Temperature/Light 8K). Actual temperatures (mean \pm SE)

in the five heat-treatments were 21.055 ± 0.242 , 25.406 ± 0.094 , 26.860 ± 0.123 , 28.906 ± 0.212 and 30.424 ± 0.261 C.

To assess the temperature dependence of the growth curve from day 3 on, when the first reproductive cycle of Bd was presumably completed (Voyles et al. 2012, Grogan et al. 2020), we took four samples from each cell culture flask assigned to the given day (FIG. 1A). Flasks were sorted in a random order and were intensively shaken before sampling. We assessed samples blind with respect to treatment, and counted zoospores in five squares of a hemocytometer for each sample, resulting in 20 concentration estimates per cell culture flask. From these estimates, we calculated the mean and used these values henceforth. Besides the total number, we also assessed the number of moving zoospores as proxies of the number of live cells. Zoospores with an undulating flagellum were counted as moving even if actual dislocation was not observable. We also calculated ratios of moving zoospores by dividing the number of moving zoospores by the total number of zoospores. From the analyses we excluded five cell cultures that were contaminated by bacteria, as evidenced by an opaque appearance of the culture, the presence of bacterial cells visible under the microscope, and low Bd growth. **[Figure 1 should be placed shortly after this paragraph]**

Recovery of Bd growth following elevated temperature treatments.—

The second experiment investigated how Bd cultures recovered after exposure to high temperatures. We applied the three highest temperature treatments of the previous experiment (27, 29 and 30.5 C) in combination with the same six exposure durations (3 to 8 days) using a Raypa ID-90 natural convection incubator. During the heat-treatments we recorded the temperature every 10 minutes using data loggers (HOBO MX Pendant Temperature). Actual temperatures (mean \pm SE) in the thermal treatments were 27.113 ± 0.002 , 29.122 ± 0.002 and 30.527 ± 0.002 C. For each of the three temperatures we started three new cell culture lines by

inoculating 2 mL Bd culture into 175 cm² culture flasks containing 100 mL mTGhL. After
 incubating them at 21 C for one week we blended the three culture lines and diluted the
 mixture to ca. 1.8×10^6 zoospores / mL. From this mixture we started ten cell culture lines by
 inoculating 2 mL into 25 cm² cell culture flasks containing 10 mL mTGhL. We thereby
 obtained a concentration of ca. 2.9×10^5 zoospores / mL in the culture flasks, as in the
 previous experiment. We incubated the ten culture lines at each of the three temperatures. At
 the end of each exposure duration we inoculated subsamples in 96-well flat bottom culture
 plates (Orange Scientific, Belgium). We inoculated five replicate samples from each of the ten
 culture lines on two plates. Each well contained 50 μ L Bd culture and 50 μ L 1 % tryptone
 broth (10 g tryptone in 1000 mL distilled water). We also had three wells on all plates
 containing 50 μ L 1 % tryptone and 50 μ L sterile mTGhL as blanks. After inoculation, we
 moved plates back to 21 C and estimated Bd growth daily for a week (starting on the day
 when cultures were moved back to 21 C, i. e. day 0) by measuring optical density at an
 absorbance of 492 nm (OD₄₉₂, FIG. 1B) using a Multiskan MS Microplate Reader
 (LabSystems, Version 4.0). To validate the viability of our original culture lines we
 inoculated five replicate subsamples from each of the ten culture lines (50 μ L Bd and 50 μ L 1
 % tryptone broth) on two replicate plates before the start of the three heat-treatments,
 incubated plates at 21 C and assessed Bd growth each day for one week, using the same
 spectrophotometry-based method of estimation as described above.

Statistical analysis.—

We analyzed the effects of temperature and exposure time on the number of zoospores by
 implementing generalized linear mixed models in R version 3.6.1 (function: ‘glmmPQL’,
 package: ‘MASS’), where we entered temperature and day as fixed factors and cell culture ID
 as a random factor. For deviance table analysis we used the ‘Anova’ function in the ‘CAR’

package. To compare zoospore concentrations in various temperature treatments to the control group incubated at 21 C within each day, we used the same generalized mixed model. For multiple comparisons, we used the ‘lsmeans’ function with Bonferroni correction. We considered the means of two groups to differ when the relevant 84 % CIs did not overlap (Julious 2004).

In the analysis of data obtained in the second experiment we used a similar statistical approach as before: we analyzed the effect of temperature and exposure time on the OD₄₉₂ values by entering plate ID and Bd culture line ID as random factors. To correct for plate-specific differences, we subtracted the mean OD₄₉₂ value of the three blank wells on the particular plate from values obtained for each well on the plate. For multiple comparisons, we used the same method as above. We considered the fungus to grow when the 84 % CI of the first and the eighth measure did not overlap and the eighth measure had higher values than the first measure. We confirmed that our data fit the assumptions of analyses by inspecting diagnostic plots.

RESULTS

Arrestment of Bd growth at elevated temperatures.—

At 21 C Bd showed significant growth from the initial 290 zoospores / μ L until reaching a plateau at ca. 3300 zoospores / μ L after 6 days (Fig. 2; TABLE S1). On each sampling day, the number of zoospores was significantly lower in all cell cultures incubated at higher temperatures than in the control incubated at 21 C. In the cell cultures incubated at 25.5 C the number of zoospores also increased over time, but the curve was lower by 25 % on day 3 and plateaued at about half height (ca. 1700 zoospores / μ L) on day 5. At 27 C and above the fungus did not show considerable growth on any of the eight days of elevated temperature treatment as indicated by overlapping 84 % CIs (see FIG. 2). At 27 C mean zoospore numbers

were 72 % lower on day 3 as compared to zoospore numbers in the control on the respective day, and this difference gradually reached 90 % by day 8. In cell cultures incubated at 29 and 30.5 C, we did not observe significant Bd growth as compared to the initial value on day 0, and zoospore numbers were lower than in the control maintained at 21 C by 80 % on day 3, and by 90 % on day 6. **[Figure 2 should be placed shortly after this paragraph]**

The number of moving zoospores was highest in control cell cultures incubated at 21 C (FIG. S2, TABLE S2). With 595 zoospores / μ L it was highest on day 4, and then gradually decreased to 320 zoospores / μ L on day 8. In cell cultures maintained at 25.5 C the number of moving zoospores was 170 / μ L on day 3, around 100 / μ L between days 4 to 7 and further decreased to 60 / μ L by day 8. In cell cultures maintained at 27 C the mean number of moving zoospores never exceeded 5 / μ L and in cell cultures incubated at higher temperatures (29 and 30.5 C) we did not find moving zoospores on any sampling occasion.

The ratio of moving zoospores was highest in cell cultures incubated at 21 C on all sampling occasions: ca. 33 % of zoospores were moving on day 3, which gradually decreased to 9 % by day 8 (FIG. S2, TABLE S3). In cell cultures incubated at 25.5 C 17 % of zoospores were moving on day 3, this dropped to around 7 % on days 4 to 7 and decreased again to 4 % on day 8. In cell cultures incubated at 27 C the percentage of moving zoospores was very low throughout the sampling period, with the highest value of 2 % observed on day 7.

Recovery of Bd growth following elevated temperature treatments.—

As in the first experiment, Bd kept at 21 C showed intensive growth (FIG. S3). Bd cultures incubated at 27 C for any of the six durations (3 to 8 days) also showed significant growth after they were moved back to 21 C (FIG. 3) **[Figure 3 should be placed close to this paragraph]**: One week after placing Bd cultures back to 21 C the OD₄₉₂ values were at least four times higher than at the first measurement immediately after the end of the thermal

treatment (TABLE S4). After exposure to 29 C for 3 or 4 days the OD₄₉₂ values indicated similar growth as before. Bd growth diminished gradually following 5, 6 and 7 days of 29 C treatments, and treatment at 29 C for 8 days resulted in no detectable recovery. In the Bd cultures exposed to 30.5 C for 3 days we detected significant growth after one week, which was still apparent, although diminished following a 4-day thermal treatment (OD₄₉₂ values were ca. 4 and 1.5 times higher after one week of recovery, respectively, than at the beginning). Treating Bd cultures at 30.5 C for at least 5 days prevented detectable recovery.

DISCUSSION

We documented intensive Bd growth at 21 C until day 6, after which no further increase in zoospore numbers could be observed. Compared to this ideal temperature, zoospore numbers plateaued at about half height in cell cultures incubated at 25.5 C and Bd growth was essentially halted already at 27 C. By moving back Bd cultures to 21 C after elevated temperature treatments and measuring Bd growth repeatedly, we observed that the fungus has a surprisingly high ability to recover: even after eight days of completely suppressed replication at 27 C, growth recovered and was similar to that observed in milder treatments lasting for just three days. Further, Bd showed considerable growth even after incubation at 29 C for five days, or at 30.5 C for three days, while recovery of Bd growth was only prevented entirely by exposure to 29 C for eight days or to 30.5 C for at least five days. Our findings thereby refine our understanding about the lower end of elevated temperatures that may arrest Bd growth temporarily or lead to its eradication, and could potentially be used to fight chytridiomycosis.

Our results regarding the arrestment of Bd growth are in tight agreement with those of previous experiments performed *in vitro* on the temperature-dependence of Bd growth. Temperatures around 20 C have been known to be ideal for Bd, manifesting in intensive

growth (Stevenson et al. 2013, Voyles et al. 2017). Stevenson et al. (2013) also reported zoospore numbers to plateau at a lower density in cultures incubated at 25 C than in cultures kept at 21 and 23 C. Our observation that Bd did not grow at 27 C and higher temperatures partially agrees with Stevenson et al. (2013), who found that at this temperature one Bd isolate did not grow, while two other isolates showed moderate growth. Voyles et al. (2017) and Cohen et al. (2017) detected limited Bd growth at 27 C and no growth at 28 C. Piotrowski et al. (2004) also did not observe Bd growth at 28 C, while Longcore et al. (1999) reported low levels of Bd growth at 28 C which only ceased at 29 C. The explanation for the somewhat varying critical thermal maxima of Bd may be that strains can somewhat differ in their heat-tolerance depending on their geographic origin (Stevenson et al., 2013; Voyles et al., 2017). Also, when cultured on media in the laboratory, Bd strains can go through rapid evolutionary change, so that the time passed since isolation and the number of laboratory passages may also lower heat-tolerance (Brem et al. 2013), which may also have contributed to the somewhat lower temperature resulting in halted growth in our experiment as compared to some previous experiments.

The fact that the growth curve of the fungus plateaued on day 6 in the cell cultures incubated at 21 C was likely due to the depletion of nutrients in the liquid medium. This explanation is supported by the observation that the number of moving zoospores, which was rather steady up to that point, also dropped by 30 % between day 6 and 7. The observed decreasing tendency of the relative number of moving zoospores was likely due to the accumulation of non-living or inactive, thus non-moving zoospores in the cultures. The observation that maximum zoospore concentrations reached at 25.5 C was about half the value reached at 21 C corresponds well with the results previously obtained by Voyles and colleagues (2017). A shorter lifespan of Bd cells at higher temperatures (Woodhams et al. 2008, Stevenson et al. 2013), a shift in life history strategies (Woodhams et al. 2008, Voyles

et al. 2012, Muletz-Wolz et al. 2019) and, perhaps, costly heat-endurance may all have contributed to the observed lower apex in zoospore concentrations at higher temperatures.

Importantly, results of our second experiment stressed that suppression of Bd growth does not equal clearance of Bd, which is due to its high ability to recover after temperature returns to the optimal range (Longcore et al. 1999, Piotrowski et al. 2004): Bd started to grow again after termination of thermal treatments, even in treatment groups exposed to temperatures that were high enough to block Bd growth entirely for more than a week of incubation. Treatment with 27 C for as long as eight days did not kill Bd cells, because cultures grew substantially during the recovery period following the elevated temperature treatment. This corresponds well with a result of Stevenson and colleagues (2013), who found that Bd cultures recovered and grew again after they were moved to 23 C following a treatment period of 14 days at 27 C. Further, our study revealed that exposing Bd cultures to 29 C can lead to the total clearance of Bd, but even at this temperature, an exposure duration of eight days was necessary for total wipeout. Finally, treatment of Bd cultures with 30.5 C for four days proved to be insufficient for total Bd-wipeout, because the fungus still showed some level of growth by the seventh day after returning cultures to 21 C, but exposure to 30.5 C for at least five days resulted in a definite clearance of the fungus.

The observation that the growth of Bd can be lowered by temperature as low as 25.5 C and can at least temporarily be inhibited by c.a. 30 C supports the idea that elevating temperature in natural habitats by decreasing canopy cover around water bodies (Raffel et al., 2010; Geiger et al., 2011; Heard et al., 2014) or by employing localized heating (Hettyey et al. 2019) is a promising mitigation strategy. Besides the evidence delivered by *in vitro* experiments, including this one, studies performed *in vivo* also document lowered infection prevalence and intensity at temperatures approaching or surpassing the CT_{max} of Bd (i.e. 26–28 C; Berger et al., 2004; Ribas et al., 2009; Geiger et al., 2011; Cohen et al., 2017;

Greenspan et al., 2017; Sonn et al., 2017; Robak & Richards-Zawacki, 2018). Finally, correlative studies performed in natural habitats also support the hypothesis that increased ambient temperature can have beneficial effects on chytridiomycosis-stricken populations (Forrest and Schlaepfer 2011, Heard et al. 2014, Scheele et al. 2015). Nonetheless, it is important to note that elevating temperature may not always be beneficial to all amphibian species threatened by chytridiomycosis: if high enough temperatures (i.e. >29 C) cannot be applied, some amphibians may actually do better at lower temperatures because of a better functioning of their cold-adapted immune system (see the ‘thermal mismatch hypothesis’; Cohen et al., 2017; Sauer et al., 2018).

In summary, the results of this study refine our understanding of the effects of elevated temperature on Bd growth while decoupled from the species-specific and temperature-dependent immune function of hosts. By simultaneously identifying both the temperature that temporarily inhibits growth in Bd (ca. 27 C), and the CT_{max} at which the fungus dies (ca. 30 C, but strongly depending on treatment duration), this study provides an upgraded starting point for *in vivo* studies targeting the development of new mitigation approaches that rely on elevated temperature. Raising body temperature to around 28 C for therapeutic purposes is probably a safe approach in case of most amphibians, especially if applied for a few days only (Ultsch et al. 1999, Sunday et al. 2011, Gutiérrez-Pesquera et al. 2016) and if elevated temperature treatment can be employed outside the most thermosensitive periods (i.e., onset of sexual differentiation and metamorphosis; Chardard et al. 2004, Wells 2007). To what extent and for how long the curative effects of temperature treatment persist beyond the treatment period and how much these effects can be generalized across amphibian taxa and Bd strains will have to be scrutinized by future *in vivo* studies.

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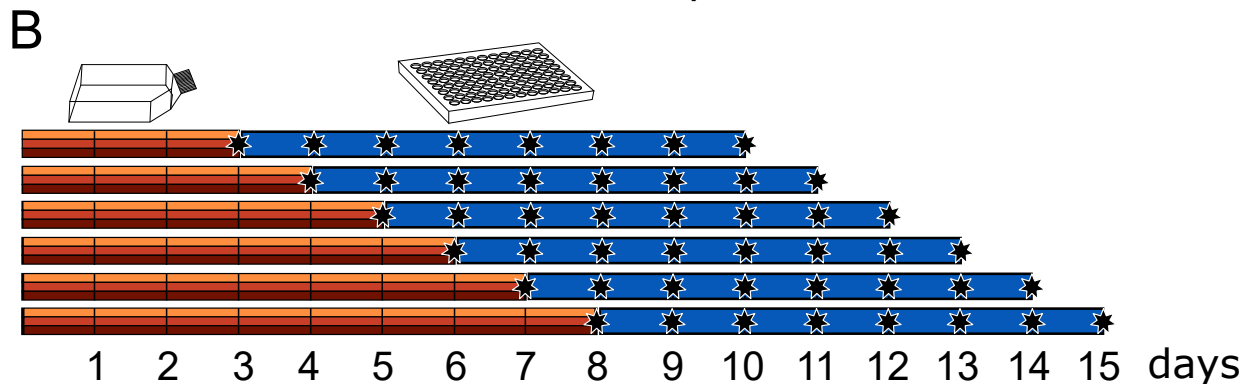
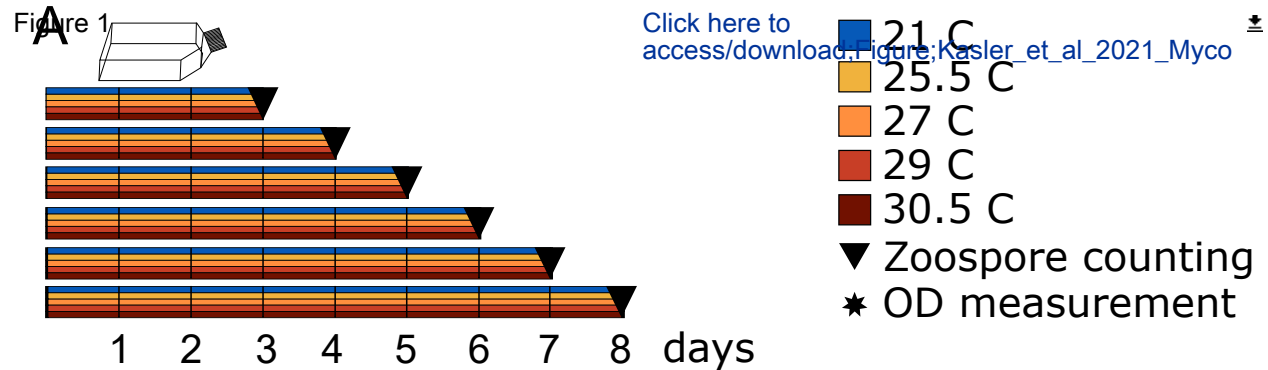
LEGENDS

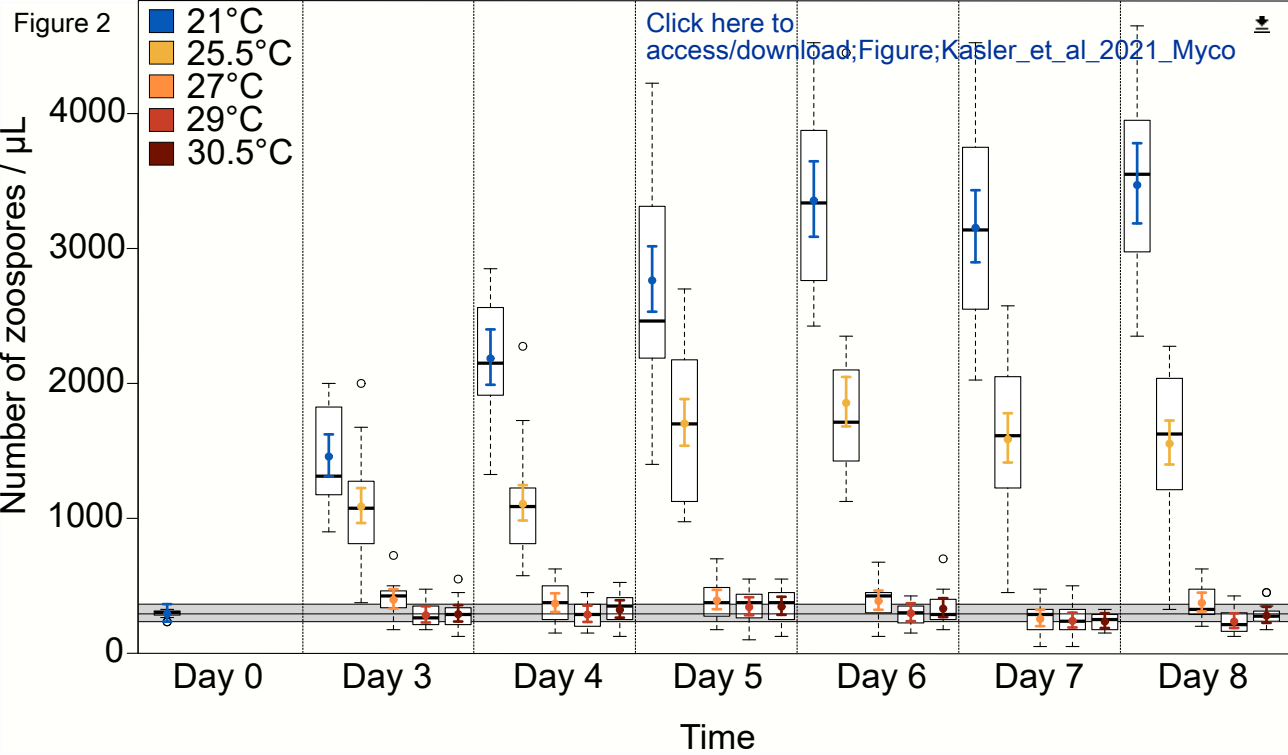
Figure 1. Experimental design of the two experiments. A. In the first experiment we estimated the effects of the 30 combinations of the five temperatures and six treatment durations on Bd-growth in culture flasks by counting zoospore numbers. B. In the second experiment we assessed the recovery of Bd cultures after thermal treatments performed in culture flasks by subsequently incubating cultures at 21 C on microplates and measuring changes in the optical density daily for one week.

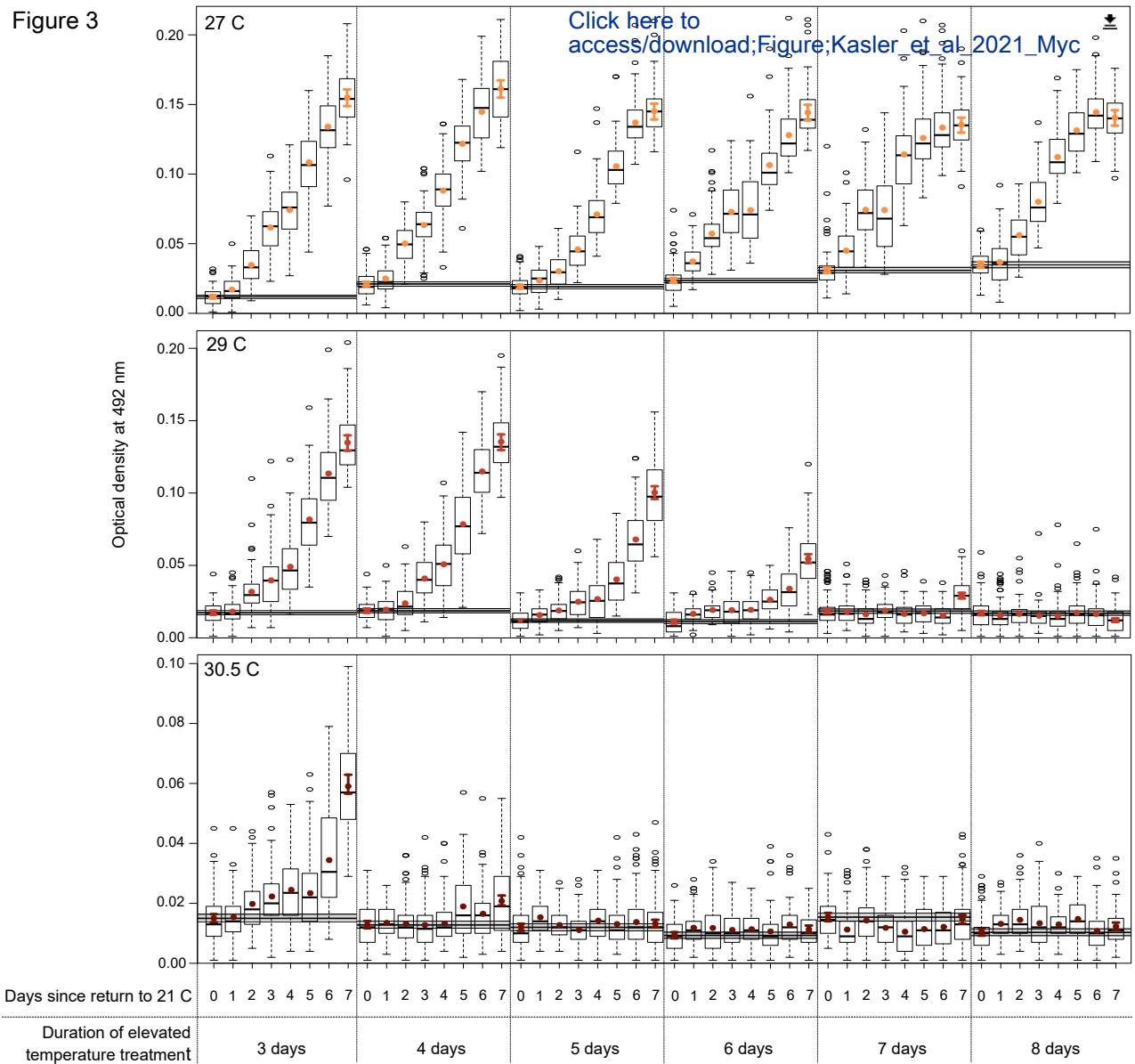
Figure 2. Concentration of Bd zoospores as a function of time and incubation temperature. Boxes show interquartile ranges and medians, whiskers represent minimum and maximum values, open circles represent outliers (deviating from the boundary of the interquartile range (IQR) by more than $1.5 \times \text{IQR}$). Colored error bars depict means and 84 % confidence intervals. The horizontal solid line shows the initial zoospore concentration, with the 84% confidence interval in gray.

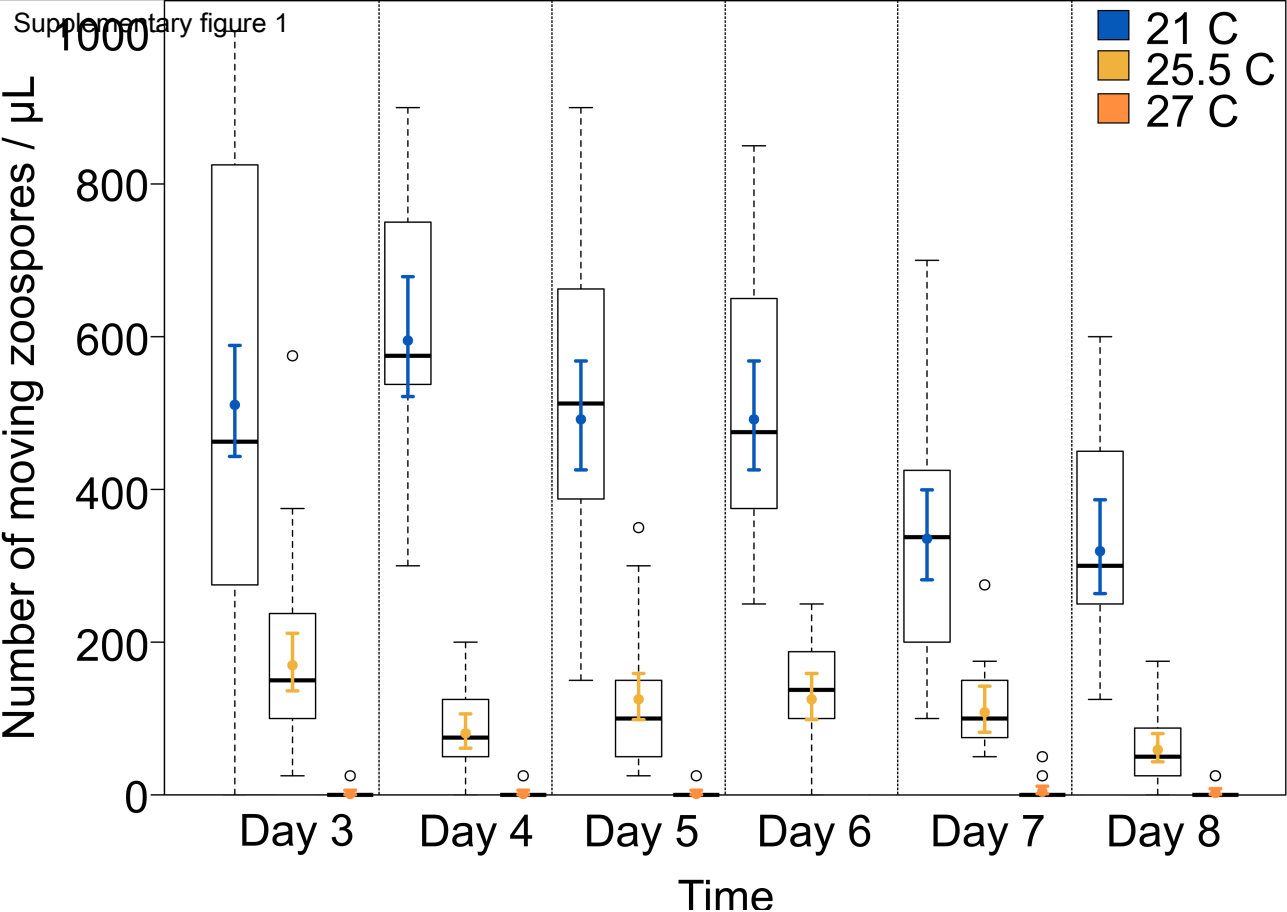
Figure 3. Optical density values at 492 nm (OD_{492}) of Bd cultures measured for one week daily after treatment with high temperatures (27, 29 or 30.5 C for 3, 4, 5, 6, 7 or 8 days). OD_{492} values were corrected for blanks. Boxes show interquartile ranges and medians, whiskers represent minimum and maximum values, open circles represent outliers (deviating from the boundary of the interquartile range (IQR) by more than $1.5 \times \text{IQR}$). Colored error bars depict means and 84 % confidence intervals. The horizontal solid lines show the initial OD_{492} value, with the 84% confidence interval in gray for every treatment combination separately. Please note, that the scales are different for the three temperature treatments. For OD_{492} values of the control plates kept at 21 C see FIG. EA4.

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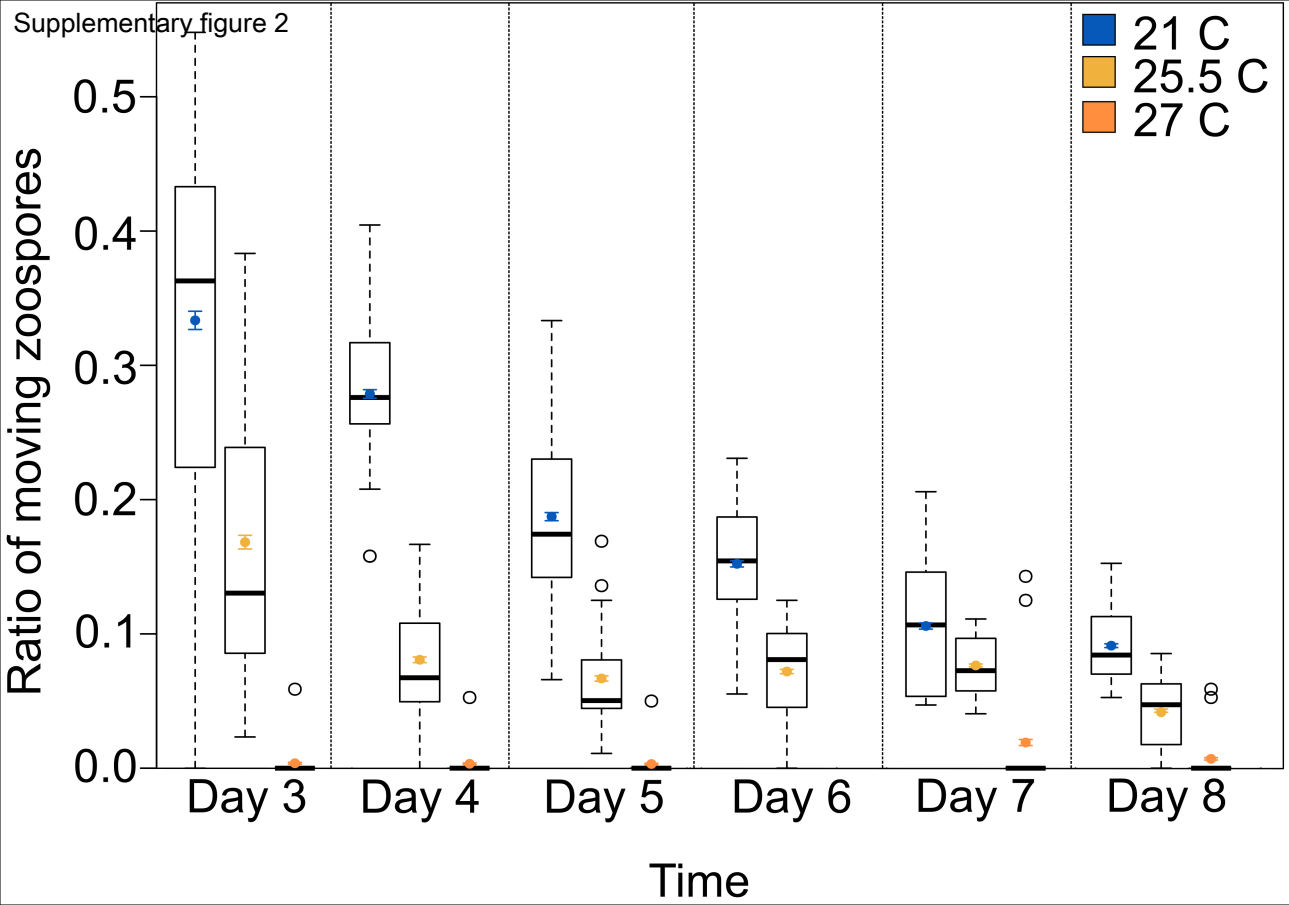








Supplementary figure 2



Optical density at 492 nm

Days since start

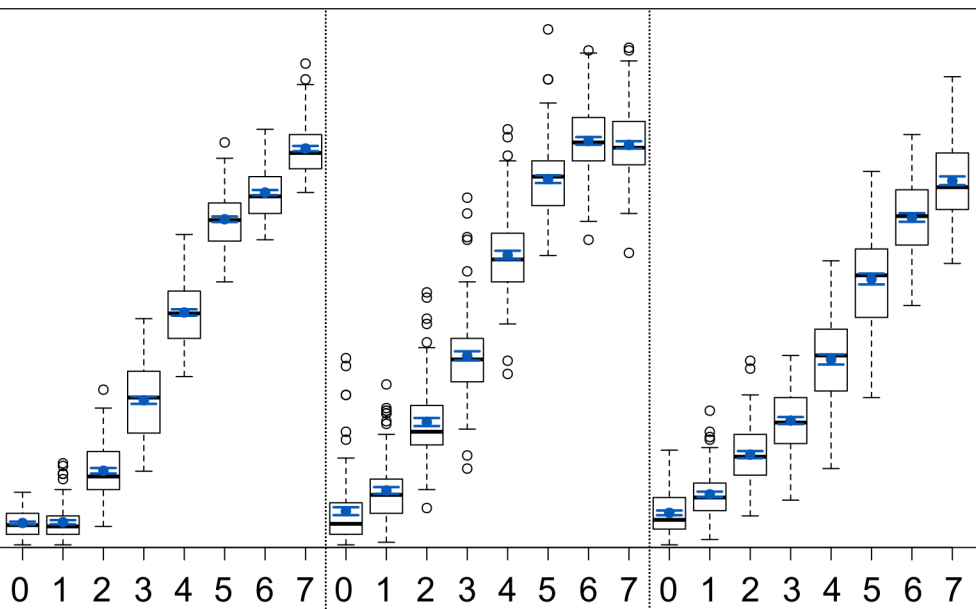
Plate

27 control

29 control

30.5 control

0.20
0.15
0.10
0.05
0.00



1

Supplementary tables for

2

***In vitro* thermal tolerance of a hypervirulent lineage of the chytrid fungus,**

3

***Batrachochytrium dendrobatidis*: growth arrestment by elevated temperature and**

4

recovery following thermal treatment

5

Andrea Kásler, János Ujszegi, Dóra Holly, Boglárka Jaloveczki, Zoltán Gál, Attila Hettyey

Table S1. Concentration of Bd zoospores in 1 μ L of cell culture as a function of time and incubation temperature. We regarded two groups to differ when the relevant 84 % CIs did not overlap. Groups with no significant growth from day 0 are with bold.

Time	Temperature (C)	Mean \pm SE	df	84 % CI	
				Lower	Upper
day 0	21	292.219 \pm 40.911	7	234.500	364.144
day 3	21	1457.896 \pm 98.893	7	1310.056	1622.394
	25.5	1087.323 \pm 82.048	7	965.401	1224.623
	27	409.080 \pm 46.322	7	330.897	475.142
	29	281.899 \pm 37.797	7	228.219	348.198
	30.5	289.643 \pm 38.354	7	235.104	356.828
day 4	21	2185.489 \pm 129.917	7	1989.963	2400.188
	25.5	1107.459 \pm 82.992	7	984.084	1246.281
	27	368.637 \pm 43.736	7	305.786	444.398
	29	286.545 \pm 38.132	7	232.349	353.377
	30.5	322.170 \pm 40.630	7	264.115	392.979
day 5	21	2763.226 \pm 153.515	7	2531.457	3016.166
	25.5	1702.234 \pm 109.539	7	1538.023	1883.947
	27	391.870 \pm 45.234	7	326.707	470.023
	29	342.305 \pm 41.995	7	282.142	415.291
	30.5	345.403 \pm 42.203	7	284.920	418.719
day 6	21	3354.903 \pm 177.148	7	3086.852	3646.173
	25.5	1855.574 \pm 116.089	7	1681.296	2047.885
	27	387.223 \pm 44.937	7	322.518	464.902
	29	296.984 \pm 41.831	7	237.899	370.792
	30.5	330.868 \pm 44.168	7	268.132	408.333
day 7	21	3153.547 \pm 169.152	7	2897.770	3431.846
	25.5	1586.521 \pm 115.616	7	1413.815	1778.910
	27	254.136 \pm 37.889	7	200.948	321.430
	29	240.078 \pm 34.680	7	191.214	301.426
	30.5	235.432 \pm 34.320	7	187.122	296.209
day 8	21	3470.277 \pm 188.220	7	3185.968	3780.281
	25.5	1553.540 \pm 103.095	7	1399.237	1724.831
	27	373.283 \pm 44.038	7	309.966	449.528
	29	235.432 \pm 34.320	7	187.122	296.209
	30.5	278.801 \pm 37.573	7	225.468	344.744

Table S2. Concentration of moving Bd zoospores in 1 μ L of cell culture as a function of time and incubation temperature. We regarded two groups to differ when the relevant 84 % CIs did not overlap. Treatments of 29 and 30.5 C are not shown because we did not observe moving zoospores at these temperatures.

Time	Temperature (C)	Mean \pm SE	df	84% CI	
				Lower	Upper
day 3	21	510.719 \pm 46.090	7	443.185	588.545
	25.5	169.778 \pm 23.776	7	136.235	211.580
	27	1.263 \pm 1.260	7	0.261	6.120
day 4	21	594.953 \pm 49.788	7	521.627	678.588
	25.5	80.573 \pm 14.110	7	61.185	106.104
	27	1.263 \pm 1.260	7	0.261	6.120
day 5	21	505.407 \pm 45.843	7	438.253	582.851
	25.5	109.349 \pm 17.335	7	85.232	140.290
	27	1.263 \pm 1.260	7	0.261	6.120
day 6	21	491.747 \pm 45.213	7	425.579	568.203
	25.5	125.175 \pm 19.054	7	98.540	159.010
	27	0	N.A.	0	0
day 7	21	335.420 \pm 37.277	7	281.661	399.440
	25.5	108.075 \pm 18.956	7	82.035	142.382
	27	4.182 \pm 2.668	7	1.535	11.397
day 8	21	319.159 \pm 38.849	7	263.583	386.453
	25.5	58.991 \pm 11.555	7	43.359	80.259
	27	2.526 \pm 1.877	7	0.786	8.122

Table S3. Ratio of moving zoospores (number of moving zoospores / total number of zoospores) as a function of time and incubation temperature. Treatments of 29 and 30.5 C are not shown because we did not observe moving zoospores at these temperatures.

Time	Temperature (C)	Mean ratio \pm SE
day 3	21	0.334 ± 0.007
	25.5	0.168 ± 0.006
	27	0.004 ± 0.001
day 4	21	0.279 ± 0.003
	25.5	0.080 ± 0.002
	27	0.003 ± 0.001
day 5	21	0.187 ± 0.003
	25.5	0.067 ± 0.002
	27	0.003 ± 0.001
day 6	21	0.152 ± 0.002
	25.5	0.072 ± 0.002
	27	$0 \pm \text{N.A.}$
day 7	21	0.106 ± 0.002
	25.5	0.077 ± 0.001
	27	0.019 ± 0.002
day 8	21	0.091 ± 0.001
	25.5	0.043 ± 0.001
	27	0.007 ± 0.001

Table S4. Mean optical density (OD₄₉₂) values of Bd cultures 0 or 7 days after treating with high temperatures. OD₄₉₂ values were corrected by subtracting the mean OD₄₉₂ value of broth controls assigned to the particular plate. We regarded the two measures to differ when the relevant 84 % CIs did not overlap.

Temperature (C)	Time (days)	Days after treatment	Mean ± SE	df	84 % CI	
					Lower	Upper
27	3	0	0.0118 ± 0.0008	9	0.0107	0.0130
		7	0.1547 ± 0.0039	9	0.1488	0.1608
	4	0	0.0211 ± 0.0010	9	0.0196	0.0227
		7	0.1610 ± 0.0040	9	0.1549	0.1673
	5	0	0.0191 ± 0.0010	9	0.0177	0.0207
		7	0.1448 ± 0.0037	9	0.1392	0.1506
	6	0	0.0236 ± 0.0011	9	0.0220	0.0254
		7	0.1440 ± 0.0037	9	0.1385	0.1498
	7	0	0.0309 ± 0.0013	9	0.0290	0.0330
		7	0.1350 ± 0.0035	9	0.1298	0.1405
	8	0	0.0348 ± 0.0014	9	0.0327	0.0370
		7	0.1402 ± 0.0036	9	0.1348	0.1459
29	3	0	0.0173 ± 0.0009	9	0.0159	0.0188
		7	0.1345 ± 0.0035	9	0.1292	0.1399
	4	0	0.0186 ± 0.0010	9	0.0172	0.0202
		7	0.1350 ± 0.0035	9	0.1297	0.1405
	5	0	0.0118 ± 0.0008	9	0.0107	0.0130
		7	0.1002 ± 0.0029	9	0.0959	0.1047
	6	0	0.0111 ± 0.0009	9	0.0098	0.0125
		7	0.0546 ± 0.0020	9	0.0516	0.0577
	7	0	0.0184 ± 0.0010	9	0.0169	0.0199
		7	0.0291 ± 0.0013	9	0.0271	0.0311
	8	0	0.0169 ± 0.0010	9	0.0155	0.0185
		7	0.0121 ± 0.0008	9	0.0109	0.0134
30.5	3	0	0.0150 ± 0.0009	9	0.0137	0.0164
		7	0.0597 ± 0.0021	9	0.0566	0.0629
	4	0	0.0128 ± 0.0008	9	0.0117	0.0141
		7	0.0209 ± 0.0011	9	0.0193	0.0226
	5	0	0.0120 ± 0.0008	9	0.0108	0.0132
		7	0.0132 ± 0.0008	9	0.0120	0.0145
	6	0	0.0093 ± 0.0007	9	0.0083	0.0104
		7	0.0114 ± 0.0008	9	0.0102	0.0126
	7	0	0.0154 ± 0.0009	9	0.0140	0.0168
		7	0.0146 ± 0.0009	9	0.0132	0.0161
	8	0	0.0103 ± 0.0007	9	0.0092	0.0115
		7	0.0122 ± 0.0008	9	0.0110	0.0136

***In vitro* thermal tolerance of a hypervirulent lineage of the chytrid fungus,
Batrachochytrium dendrobatidis: growth arrestment by elevated temperature and
recovery following thermal treatment**

Andrea Kásler, János Ujszegi, Dóra Holly, Boglárka Jaloveczki, Zoltán Gál, Attila Hettyey

LEGENDS FOR SUPPLEMENTARY FIGURES

Figure S1. Concentration of moving Bd zoospores as a function of time and incubation temperature. Boxes show interquartile ranges and medians, whiskers represent minimum and maximum values, open circles represent outliers (deviating from the boundary of the interquartile range (IQR) by more than $1.5 \times \text{IQR}$). Colored error bars depict means and 84 % confidence intervals.

Figure S2. Ratio of moving zoospores (number of moving zoospores / total number of zoospores) in treatment groups where moving zoospores were present. Boxes show interquartile ranges and medians, whiskers represent minimum and maximum values, open circles represent outliers (deviating from the boundary of the interquartile range (IQR) by more than $1.5 \times \text{IQR}$). Error bars represent means ± 1 SE.

Figure S3. Optical density (OD_{492}) values measured on control plates. Plates were inoculated before exposing Bd cultures to heat-treatments, and were kept at 21 C and measured daily for one week to validate the viability of our original culture lines. Boxes show interquartile ranges and medians, whiskers represent minimum and maximum values, open circles represent outliers (deviating from the boundary of the interquartile range (IQR) by more than $1.5 \times \text{IQR}$). Error bars represent means ± 1 SE.