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In vitro thermal tolerance of a hypervirulent lineage of *Batrachochytrium dendrobatidis*: Growth arrestment by elevated temperature and recovery following thermal treatment

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ABSTRACT

Chytridiomycosis, an emerging infectious disease caused by *Batrachochytrium dendrobatidis* (Bd), poses a serious threat to amphibians. The thermal optimum of Bd is lower than that of most amphibians, providing an opportunity to cure infected individuals with elevated temperature. However, this approach 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 needed to characterize thermal tolerance of the fungus without complexities introduced by the species-specific characteristics of hosts' immune systems. The aim of our study was to evaluate the thermal tolerance of a hypervirulent isolate of Bd, considering the limits of its thermal tolerance and its capacity to rebound following heat treatment. 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 of the maximum values observed in the lower temperature treatment. At temperatures of 27 C and above, the fungus showed no measurable growth. However, when we moved the cultures back 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 a treatment duration of 5 days was needed to achieve the same result, revealing that these moderately elevated temperatures applied for only a few days have merely a fungistatic rather than a fungicidal effect under in vitro conditions.

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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 (GPL) 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 (Lips 2016; O'Hanlon et al. 2018; Scheele et al. 2019; Skerratt et al. 2007). 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 (Garner et al. 2016; Scheele et al. 2014, 2019; Woodhams et al. 2011). Thus, detailed research is needed into the biology of this pathogen to inform 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 treatments in captive populations of amphibians. However, these approaches could negatively affect the treated amphibians themselves, or would be impractical or ineffective, in natural settings (Bosch et al. 2015; Garner et al. 2016; Scheele et al. 2014).

One potential approach to mitigating the impact of chytridiomycosis is to capitalize on the thermal optimum mismatch between Bd and its hosts (Chatfield

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and Richards-Zawacki 2011; Heard et al. 2014; Hettyey et al. 2019; Scheele et al. 2015; Woodhams et al. 2003). Previous in vitro studies have shown 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 (Cohen et al. 2017; Johnson et al. 2003; Piotrowski et al. 2004; Stevenson et al. 2013; 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 and 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 (Hettyey et al. 2019; Scheele et al. 2014; Woodhams et al. 2011).

For the in vivo application of localized heating, we need detailed knowledge about the thermal tolerance of Bd. In vitro studies provide the practical first step toward 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 the so-called "3Rs" (replacement, reduction, refinement) of animal experimentation (Russell and Burch 1959).

We examined the effect of elevated temperature on Bd growth in liquid cell cultures, using 30 different combinations of treatment temperatures and durations. We focused on an isolate of Bd representing the GPL. Because recovery of the fungus after heat treatments was observed previously (Longcore et al. 1999; Stevenson et al. 2013; Voyles et al. 2017), we 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).

MATERIALS AND METHODS

Isolate and culture.— We used the isolate Bd IA042 of the hypervirulent GPL (Farrer et al. 2011), provided by T. W. J. Garner (Institute of Zoology, Zoological Society of London, London, UK). The isolate originated from a dead *Alytes obstetricans* (Alytidae) found in Spain during a mass mortality event in 2004. The cell culture was maintained at 4 C in cell culture flasks (25 cm^2, closed cap; Orange Scientific, Braine-l'Alleud, Belgium) using mTGhL liquid medium (8 g tryptone, 2 g gelatin hydrolyzate, and 4 g lactose in 1000 mL distilled water).

Testing Bd IA042 growth at elevated temperatures.— Four days before the start of the experiment, we started 10 new cell culture lines by inoculating 2.5 mL Bd IA042 culture into 175-cm^2 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.

We inoculated 1.2 mL of cell culture lines into 25-cm^2 cell culture flasks containing 9 mL of mTGhL. We exposed all eight 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 (25-cm^2 cell culture flasks). 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 controlled 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; Blacksburg, Virginia) served as incubators. We recorded the temperature in incubators every 10 min using data loggers (HOBO Pendant Temperature/Light 8 K; Bourne, Massachusetts). Actual temperatures (mean ± 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 (Grogan et al. 2020; Voyles et al. 2012), 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 blindly 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. We calculated a mean number of zoospores for each sample. 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.

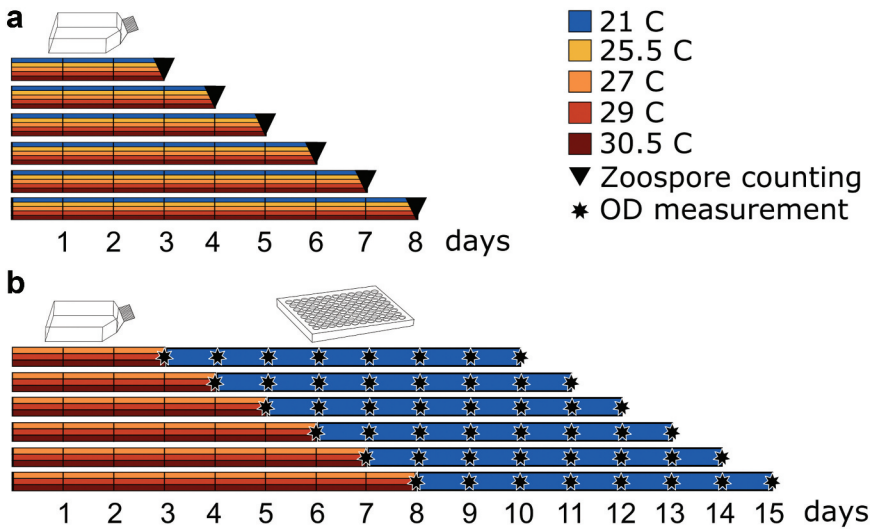

Figure 1. Experimental design of the two experiments. A. In the first experiment, we estimated the effects of the 30 combinations of the five temperatures and six treatment durations on Bd growth in culture flasks by counting zoospore numbers. B. In the second experiment, we assessed the recovery of Bd IA042 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 1 week.

We 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.

Recovery of Bd IA042 growth following elevated temperature treatments.—In a second experiment, we investigated how Bd IA042 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 (Terrassa, Spain). During the heat treatments, we recorded the temperature every 10 min using data loggers as above. Actual temperatures (mean ± 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 IA042 culture into 175-cm^2 culture flasks containing 100 mL mTGhL. After incubating them at 21 C for 1 week, we blended the three culture lines and diluted the mixture to ca. 1.8×10^6 zoospores/mL. From this mixture, we started 10 cell culture lines by inoculating 2 mL into 25-cm^2 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 10 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, Braine-l'Alleud, Belgium). We inoculated five samples from each of the 10 culture lines on two plates. Each well contained 50 µL Bd IA042 culture and 50 µL 1% tryptone broth (10 g tryptone in 1000 mL distilled water). We also had three wells on all plates containing 50 µL 1% tryptone and 50 µL sterile mTGhL as blanks.

After inoculation, plates were stored at 21 C, with daily estimation of Bd growth for 1 week (starting on the day when cultures were moved back to 21 C, i.e., day 0). Growth was evaluated by measuring optical density at an absorbance of 492 nm (OD_{492}, FIG. 1B) using a Multiskan MS Microplate Reader (4.0; LabSystems, Waltham, Massachusetts). To validate the viability of our original culture lines, we inoculated five replicate subsamples from each of the 10 culture lines (50 µL Bd IA042 and 50 µL 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 1 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 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 with 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% confidence intervals (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 OD_{492} values by entering well ID nested within plate ID and Bd culture line ID as random factors. To correct for plate-specific differences, we subtracted the mean OD_{492} 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% CIs of the first and the eighth measures 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

At 21 C, Bd IA042 showed significant growth from the initial zoospore concentration until reaching a plateau after 6 days (FIG. 2; SUPPLEMENTARY TABLE 1). 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 on day 5. At 27 C and above, the fungus did not show considerable growth on any of the 8 days of elevated temperature treatment, as indicated by overlapping 84% CIs (see FIG. 2).

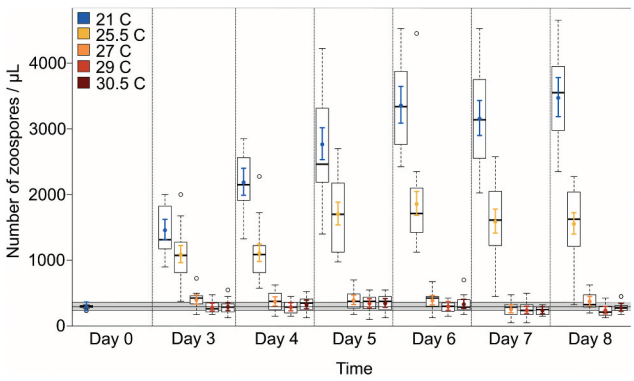

Figure 2. Concentration of Bd IA042 zoospores as a function of time and incubation temperature. Boxes show interquartile ranges and medians, whiskers represent minimum and maximum values, and open circles represent outliers (deviating from the boundary of the interquartile range [IQR] by more than 1.5× 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.

The number of moving zoospores was highest in control cell cultures incubated at 21 C (SUPPLEMENTARY FIG. 1; SUPPLEMENTARY TABLE 2). With 595 zoospores/µL, it was highest on day 4 and then gradually decreased to 320 zoospores/µL on day 8. In cell cultures maintained at 25.5 C, the number of moving zoospores was 170/µL on day 3, around 100/µL between days 4 and 7, and further decreased to 60/µL by day 8. In cell cultures maintained at 27 C, the mean number of moving zoospores never exceeded 5/µL, 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: on average 33% of zoospores were moving on day 3, which gradually decreased to 9% by day 8 (SUPPLEMENTARY FIG. 2). 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 remained below 2% throughout the sampling period.

Recovery of Bd IA042 growth following elevated temperature treatments.—As in the first experiment, Bd IA042 kept at 21 C showed extensive growth (SUPPLEMENTARY FIG. 3). 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; SUPPLEMENTARY TABLE 3). After exposure to 29 C for 3 or 4 days, OD_{492} values indicated similar growth to that in the unheated group. However, Bd IA042 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 1 week. In the Bd IA042 cultures exposed to 30.5 C for 3 days, we detected significant growth after 1 week, which was still apparent, although diminished, following a 4-day thermal treatment. Treating Bd IA042 cultures at 30.5 C for at least 5 days prevented detectable recovery.

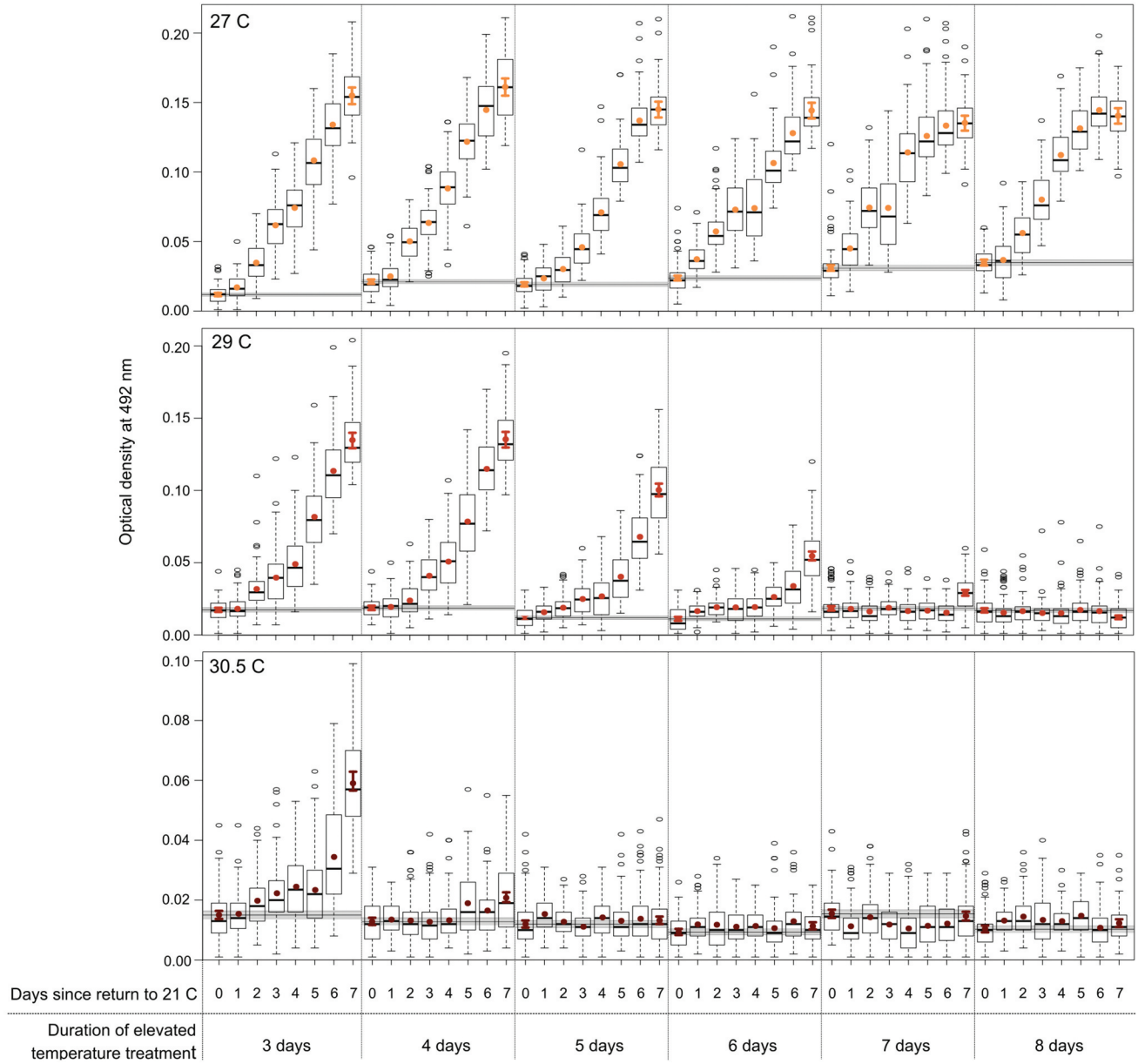

Figure 3. Optical density values at 492 nm (OD_{492}) of Bd IA042 cultures measured daily for 1 week after treatment with high temperatures (27, 29, or 30.5 C for 3, 4, 5, 6, 7, or 8 days). OD_{492} values were corrected for blanks. Boxes show interquartile ranges and medians, whiskers represent minimum and maximum values, and open circles represent outliers (deviating from the boundary of the interquartile range [IQR] by more than 1.5× 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. Note that the scales are different for the three temperature treatments. For OD_{492} values of the control plates kept at 21 C, see SUPPLEMENTARY FIG. 3.

DISCUSSION

We documented extensive Bd IA042 growth at 21 C until day 6, after which no further increase in zoospore numbers could be observed. Compared with this ideal temperature, zoospore numbers plateaued at about half height in cell cultures incubated at 25.5 C and growth of the isolate was essentially halted already at 27 C. By moving back Bd IA042 cultures to 21 C after elevated temperature treatments and measuring growth repeatedly, we observed that the fungus has a surprisingly high ability to recover: even after 8 days of completely suppressed replication at 27 C, growth recovered and was similar to that observed in milder treatments lasting for just 3 days. Further, the isolate showed considerable growth even after incubation at 29 C for 5 days, or at 30.5 C for 3 days, whereas recovery of growth was only

prevented entirely by exposure to 29 C for 8 days or to 30.5 C for at least 5 days.

Our results regarding the temperature-dependent arrestment of the growth of Bd IA042 align with those of previous experiments performed in vitro. 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 IA042 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 and 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, whereas 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, and even different isolates of a given strain, can somewhat differ in their heat tolerance depending on their geographic origin: upper thermal limit of isolates varies between 26 and 28 C (Stevenson et al. 2013; Voyles et al. 2017). Also, when cultured on media in the laboratory, Bd strains can undergo evolutionary change, so that the time passed since isolation and the number of laboratory passages may result in lowered heat tolerance (Brem et al. 2013). Finally, different maintenance conditions can also differentially influence reproductive life history patterns of cultured Bd (Voyles et al. 2012). These factors may all have contributed to the somewhat lower temperature, resulting in halted growth in our experiment as compared with some previous experiments and call for caution regarding the generalization of findings of any single study.

The plateauing of the growth curve 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 days 6 and 7. The observed decreasing tendency of the relative number of moving zoospores was likely due to the accumulation of nonliving or inactive, thus nonmoving 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 life span of Bd cells at higher temperatures (Stevenson et al. 2013; Woodhams et al. 2008), a shift in life history strategies (Muletz-Wolz et al. 2019; Voyles et al. 2012; Woodhams et al. 2008), 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 the ability of the fungus to recover once temperature returns within its optimal range (Longcore et al. 1999; Piotrowski et al. 2004). Bd IA042 started to grow again after termination of thermal treatments, even in treatment groups where temperatures were high enough to block its growth entirely for more than a week. Treatment with 27 C for as long as 8 days did not kill Bd IA042 cells, which 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. Our study revealed that exposure to 29 C for at least 8 days, or exposure to 30.5 C for at least 5 days, can be necessary for a definite clearance of Bd IA042.

The observation that the growth of Bd IA042 can be lowered by temperature as low as 25.5 C and can at least temporarily be inhibited by ca. 30 C supports the idea that elevating temperature in natural habitats by decreasing canopy cover around water bodies (Geiger et al. 2011; Heard et al. 2014; Raffel et al. 2010) 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; Cohen et al. 2017; Geiger et al. 2011; Greenspan et al. 2017; Ribas et al. 2009; Robak and Richards-Zawacki 2018; Sonn et al. 2017). 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 the growth of a Bd isolate from the GPL 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 IA042 (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 (Gutiérrez-Pesquera et al. 2016; Sunday et al. 2011; Ultsch et al. 1999) 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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DISCLOSURE STATEMENT

No potential conflict of interest was reported by the author(s).

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