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Realistic heat pulses protect frogs from disease under simulated rainforest frog thermal regimes

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Summary

1. Recent emergences of fungal diseases have caused catastrophic global losses of biodiversity. Temperature is one of the most important factors influencing host-fungus associations but the effects of temperature variability on disease development are rarely examined.
2. The chytrid pathogen *Batrachochytrium dendrobatidis* (Bd) has had severe effects on populations of hundreds of rainforest-endemic amphibian species but we know little about the effects of rainforest-specific host body temperature cycles on infection patterns.
3. To address this challenge, we used body temperature regimes experienced in nature by frogs in the Australian Wet Tropics to guide a controlled experiment investigating the effects of body temperature fluctuations on infection patterns in a model host (*Litoria spenceri*), with emphasis on exposing frogs to realistic 'heat pulses' that only marginally exceed the thermal optimum of the fungus. We then exposed cultured Bd to an expanded array of heat pulse treatments and measured parameters of population growth to help resolve the role of host immunity in our *in vivo* results.
4. Infections developed more slowly in frogs exposed to daily 4-h heat pulses of 26°C or 29°C than in frogs in constant temperature treatments without heat pulses (control). Frogs that experienced heat pulses were also less likely to exceed infection intensities at which morbidity and mortality become likely. Ten of 11 (91%) frogs from the daily 29°C heat pulse treatment even cleared their infections after approximately nine weeks.
5. Cultured Bd also grew more slowly when exposed to heat pulses than in constant-temperature control treatments, suggesting that mild heat pulses have direct negative effects on Bd growth in nature, but precluding us from determining whether there was a concurrent benefit of heat pulses to host immunity.
6. Our results suggest that even in habitats where *average* temperatures may be suitable for fungal growth and reproduction, infection risk or the outcome of existing infections may be heavily influenced by short but frequent exposures to temperatures that only slightly exceed the optimum for the fungus.
7. Our findings provide support for management interventions that promote warm microenvironments for hosts, such as small-scale removal of branches overhanging critical habitat or provision of artificial heat sources.

Introduction

Disease-induced extinctions and population collapses of wildlife have driven concerns about emerging fungal pathogens to the forefront of conservation science worldwide (Fisher et al. 2012, 2016; Bellard et al. 2016). Temperature is one of the most important factors influencing the geographic distribution of fungal pathogens and host-pathogen interactions (Burdon 1987). In nature, ectothermic host body temperatures may range from below to above the optimum for fungal pathogens within the span of a single day (Roznik 2013). This complicates efforts to predict and test the effects of temperature on the host-pathogen relationship in nature. As a result, current information on the temperature-dependent population growth of fungal pathogens on hosts under field conditions is limited to only a few studies. For instance, the virulence of the insect-pathogenic fungus *Beauveria bassiana* decreased under field conditions (Howard et al. 2011), a response attributed to occasional environmental temperature spikes outside the fungus' optimal growth range (Kutywayo et al. 2006; Howard et al. 2011; Santos et al. 2011). This suggests that the physiological processes of fungal pathogens may be highly responsive to temperature fluctuations, especially when peak temperatures exceed optimal conditions, but this has rarely been explicitly tested.

The effects of fluctuating temperature cycles on host immune systems are also poorly known (Martin 2009). Northern bobwhites (*Colinus virginianus*) that experienced daily cold stress (6 h per day at -20°C for 4 d) were more resistant to infection with the bacteria *Pasteurella multocida* than those that experienced daily heat stress (4 h per day at 39°C for 4 d), a response attributed to a cold stress induced increase in phagocytic white blood cell activity (Dabbert et al. 1997). In contrast, the T-lymphocyte mediated immune response of tree swallows (*Tachycineta bicolor*) was suppressed on or immediately following cool days, when insect abundance tended to be low, leading to reduced food intake and subsequent declines in body condition (Lifjeld et al. 2002). Although cool temperature spikes produced opposite changes in immune responses in these studies, they both indicate that there may be little lag time between temperature-influenced changes in the physiological condition of individuals and immune performance. Temperature fluctuations may be a particularly important determinant of the strength of the immune response in ectotherms, in which physiological rates are highly correlated with temperature (Feder 1992).

Batrachochytrium dendrobatidis (Bd) is one of the most pervasive wildlife pathogens and has caused declines or extinctions of nearly 400 amphibian species since the 1970s (Lips 2016). In post-metamorphic amphibians, Bd infects the superficial epidermis. In early-stage infections, zoosporangia (i.e., intracellular stage) are often clustered in the skin, suggesting fungal population growth by repeated local re-infections (Carey et al. 2006). Chytridiomycosis develops when fungal population growth progresses unchecked, eventually reaching a critical density in host epidermis

(Carey et al. 2006). Past this virulence threshold, damage to the skin layers causes loss of water and electrolyte equilibrium and eventual death from cardiac arrest (Voyles et al. 2009).

Like many other biologically and economically important fungi, the performance of Bd under constant temperatures has been well described. In pure culture, optimal short-term growth of Bd occurs at 15-25°C (Longcore et al. 1999; Berger et al. 2004; Piotrowski et al. 2004; Woodhams et al. 2008; Stevenson et al. 2013). Between 4-15°C, the fungus grows slowly but may still possess high virulence potential by trading off slower growth rates for increased fecundity (Woodhams et al. 2008; Stevenson et al. 2013) and zoospore longevity (Voyles et al. 2012). Growth and reproduction cease at isolate-specific thresholds between 26 and 28°C (Longcore et al. 1999; Berger et al. 2004; Piotrowski et al. 2004; Woodhams et al. 2008; Stevenson et al. 2013). The fungus may resume growth when transferred from 26-28°C to lower temperatures, but will generally die or fail to resume growth after extended (≥ one week) exposure to 29°C (Longcore et al. 1999; Piotrowski et al. 2004; Stevenson et al. 2013), moderate (4 d) exposure to 32°C, or brief (4 h) exposure to 37°C (Johnson et al. 2003). The results of these studies of Bd in pure culture align with the results of several experiments in which captive amphibians were exposed to Bd. For example, post-metamorphic amphibians cleared infections after exposure to 30°C for 10 d (Chatfield and Richards-Zawacki 2011), 32°C for 5 d (Retallick and Miera 2007) or 37°C for ~16 h (Woodhams et al. 2003) and larval amphibians cleared infections after exposure to ≥ 26°C for 5 d (Geiger et al. 2011).

A more recent line of research suggests that Bd is also affected by temperature fluctuations. In the laboratory, the fungus can adapt physiologically to optimize growth under predictable daily temperature fluctuations within a suitable temperature range for Bd (25°C during the day and 15°C during the night; Raffel et al. 2012). However, this may not translate into an appreciable advantage for Bd if the host can simultaneously adapt to optimize immunity (Raffel et al. 2012). In the field, host populations in warmer, drier habitats (dry substrate temperatures ≥30°C for at least one hour when frogs are active on land; Daskin et al. 2011) can persist even when infection prevalence is high (Puschendorf et al. 2011). In these 'environmental refuges', host populations do not appear to possess inherent resistance or tolerance mechanisms but rather survive with low infection intensities as a result of environmental checks on pathogen growth (Daskin et al. 2011; Puschendorf et al. 2011). In contrast, the fungus thrives under cool field conditions (15-25°C) and has thus taken a disproportionately high toll on amphibian populations in montane rainforests (Skerratt et al. 2007; Lips 2016).

Even within montane rainforests, probability of infection decreases with increasing frequencies of frog body temperatures above 25°C (Rowley and Alford 2013; Roznik 2013). Frogs may behaviourally raise their body temperatures by selecting warm microhabitats, for instance in

response to pathogen recognition (i.e., behavioural fever) or to aid metabolism or reproduction (Richards-Zawacki 2010; Murphy et al. 2011; Rowley and Alford 2013). Alternatively, changes in frog body temperature may occur passively or by chance, with natural or anthropogenic fluctuations in the micro-environments of individuals or the macro-environments of populations (Rowley and Alford 2013; Roznik et al. 2015). In rainforest ecosystems where environmental conditions are suitable for Bd growth most of the time, these processes may briefly elevate frog body temperatures so that they exceed the thermal optimum of the fungus. At our long-term rainforest field sites in the Australian Wet Tropics where Bd is endemic, frog body temperatures commonly, although briefly, reach 26-29°C and can reach up to 36°C (Roznik 2013). This suggests that in the field Bd can tolerate short periods of exposure to temperatures that only marginally exceed its growth optimum (26-29°C), but the effects of these exposures on rates of population growth of Bd in host tissue are unknown. We used body temperature data collected in nature from frogs in the Australian Wet Tropics to guide a controlled experiment investigating the effects of diurnal heat pulses on Bd infection patterns in a model amphibian host. We then exposed cultured Bd to an expanded array of diurnal heat pulse treatments and measured parameters of population growth to help resolve the role of host immunity in our *in vivo* results.

Materials and Methods

Temperature treatments

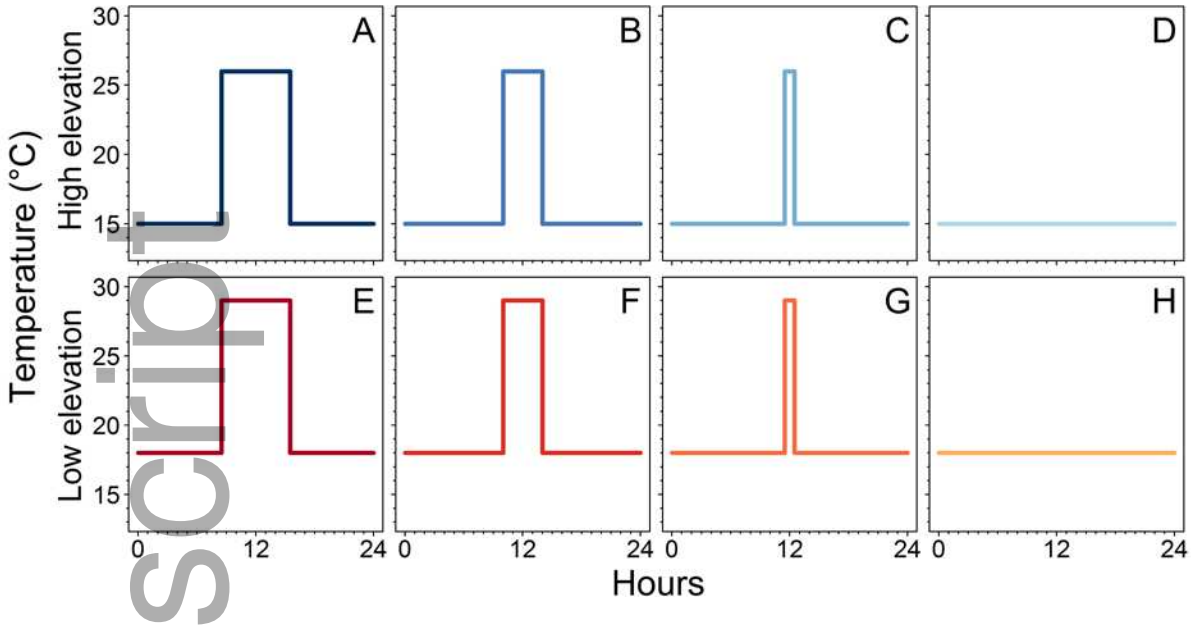
We generated eight temperature treatments with body temperature data from *Litoria serrata* (Fig. 1; Roznik 2013). *Litoria serrata* is a stream-associated frog of the Australian Wet Tropics that experienced declines from chytridiomycosis and now persists in endemic association with Bd (McDonald and Alford 1999; Woodhams and Alford 2005). We recorded the body temperatures of 54 male frogs during winter (the cool/dry season when Bd is most prevalent) at two low- and two high-elevation study sites (Roznik 2013) where Bd occurs. The low-elevation sites were Kirrama Creek #1 in Girramay National Park (4–18 July 2011; 18.203°S, 145.886°E, 100 m; N = 11) and Stoney Creek in Djiru National Park (12–25 August 2011; 17.920°S, 146.069°E, 20 m; N = 14). The high elevation sites were Birthday Creek in Paluma Range National Park (19 July–1 August 2011; 18.980°S, 146.168°E, 800 m; N = 15) and Windin Creek in Wooroonooran National Park (26 August–8 September 2011; 17.365°S, 145.717°E, 750 m; N = 14). We attached a temperature-sensitive radio-transmitter (Model A2414; Advanced Telemetry Systems, Isanti, MN) to each frog with a waistband made of silicone tubing and cotton thread. The pulse rate of the signal emitted by each transmitter varied according to temperature. We recorded the pulse rate of each transmitter every 15 minutes for five to 11 consecutive days with an automated data-logging receiver (Model SRX400A; Lotek

Wireless, Newmarket, ON, Canada; Roznik 2013). We later converted the pulse rates to temperatures with calibration curves provided for each transmitter by the manufacturer.

For each elevation (high and low), we constructed a series of square wave temperature treatments representing simplified frog thermal regimes. We derived the trough temperatures for the square waves from the overall median of individual median body temperatures (high elevation: 15°C; low elevaton: 18°C). We derived the crest (i.e., heat pulse) temperatures for the square waves from the median of individual maximum body temperatures > 25°C (high elevation: 26°C; low elevation: 29°C). We derived the crest lengths for the square waves (heat pulse durations) from the overall maximum length of time that frogs spent with body temperatures >25°C (7 h) and the median of the individual maximum lengths of time that frogs spent with body temperatures >25°C (4 h).

Thus, the four high elevation temperature treatments were: a daily rectangular wave with trough at 15°C and crest at 26°C for (1) 7 h, (2) 4 h, and (3) 1 h (to determine effects of very brief daily heat pulses), as well as (4) a constant 15°C control treatment (Fig. 1). The four low elevation temperature treatments were: a daily rectangular wave with trough at 18°C and crest at 29°C for (1) 7 h, (2) 4 h, and (3) 1 h (to determine effects of very brief daily heat pulses), as well as (4) a constant 18°C control treatment (Fig. 1). The constant temperature control treatments (15°C and 18°C) were suitable for short-term Bd growth (Longcore et al. 1999; Berger et al. 2004; Piotrowski et al. 2004; Woodhams et al. 2008; Stevenson et al. 2013) and thus served as a standard against which to observe effects of heat pulses on fungal growth.

Figure 1. Daily temperature treatments for experiment investigating effects of daily heat pulses on *in vitro* growth of *Batrachochytrium dendrobatidis* (Bd; A–H) and on *in vivo* Bd infection dynamics in a model amphibian host (B, D, F, H). We generated temperature treatments with body temperature data from *Litoria serrata* in high-elevation (blue gradient; A–D) and low-elevation (red-orange gradient; E–H) rainforests of the Australian Wet Tropics.



189
190
191 *Batrachochytrium dendrobatidis* cultures
192 We used the Bd isolate Paluma-Lseratta-2012-RW-1. This isolate is maintained at the College
193 of Public Health, Medical, and Veterinary Sciences, James Cook University. It originated from an
194 adult *L. serrata* that was collected from Birthday Creek (one of our frog tracking sites) and died in
195 captivity. The isolate was cryo-archived after two passages in nutrient broth. We revived aliquots of
196 this isolate and cultured it in tryptone/gelatin hydrolysate/lactose (TGhL) broth in 25-cm^3 tissue
197 culture flasks, passaging it twice before each trial and maintaining cultures at 22°C.
198 To obtain zoospores for each trial, we added cultured broth to Petri dishes containing TGhL
199 broth in 1% agar. Plates were partially dried in a laminar flow cabinet, incubated at 21°C for four
200 days, and then maintained alternatingly at 4°C and 21°C to sustain growth and zoospore production.
201 We then added up to 5 ml of deionized (DI) water to the dishes to form a zoospore suspension. We
202 combined the liquid contents of each dish and calculated the concentration of zoospores with a
203 hemocytometer (Neubauer Improved Bright-line). We prepared a sham (control) inoculant by
204 following the same protocol but with Petri dishes containing only nutrient agar.
205
206 *In vivo* experimental infection trial
207 For the *in vivo* trial, we experimentally infected captive-reared juveniles of the model
208 species *Litoria spenceri*, the spotted tree frog (sourced from a captive breeding facility at the
209 Amphibian Research Centre, Victoria, Australia). Use of captive-reared frogs ensured no previous
210 exposure to Bd, which can influence the progression of subsequent infections (McMahon et al.
211 2014); captive-reared *L. serrata* were unavailable. *Litoria serrata* and *L. spenceri* are both found on
212 vegetation and rocks along forested streams at a range of elevations in eastern Australia (Gillespie

and Hollis 1996; Williams 2006). *Litoria spenceri* is susceptible to Bd in the wild and its tendency to bask on exposed rock stream banks suggests that it could experience large temperature fluctuations in its native habitat (Gillespie and Hollis 1996; Gillespie and Hines 1999).

To ensure infection, we inoculated frogs on three consecutive days. On each day, we added DI water to the zoospore suspension to produce a final concentration of 1×10^6 zoospores per ml. To inoculate, we placed each frog into an individual 70-ml plastic container and added 3 ml (enough to cover the bottom of the container) of zoospore inoculant (n = 72 frogs) or sham inoculant (n = 24 frogs) to each container. We left frogs in inoculant baths for 8 h per day. To ensure regular contact of frogs with the inoculant, we monitored frogs every 15 minutes during each inoculation period. If a frog had climbed out of the inoculant onto the wall of the container, we gently tilted the container to bathe the frog in the inoculant. After each inoculation period, we returned frogs with their inoculant to individual permanent enclosures comprising 70 x 120 x 170 mm plastic containers lined with tap water-saturated paper towels.

Sixteen to 18 inoculated frogs (four frogs died during the inoculation for unknown reasons) and six uninfected control frogs were assigned to each of four temperature treatments (both 4-h heat pulse treatments [Fig. 1B, F] and both constant-temperature control treatments [Fig. 1D, H]). Six replicate temperature-controlled chambers (Greenspan et al. 2016) were programmed to perform each treatment, for a total of 24 chambers, each containing two or three inoculated frogs and one sham-inoculated control frog in separate enclosures. The chambers were arranged in a blocked design, such that there were six spatial blocks, each containing one chamber following each of the four temperature treatments. The location of each temperature treatment within each block was determined randomly. The actual temperature of each chamber (measured with a digital temperature sensor [DTH22; accurate to ±0.1°C] mounted inside each chamber) was recorded every minute and confirmed with Thermochron iButton temperature loggers (Maxim Integrated Products, Sunnyvale, California, USA; accurate to ±0.5°C) that recorded chamber temperatures every 15 minutes. Actual chamber temperatures remained within 0.5°C of target temperatures during the experiment. We reduced effects of frog history and body size on disease development by assigning frogs to experimental treatments proportionally by clutch (reported by the captive breeding facility) and snout-urostyle length (measured prior to inoculation). We divided frogs into the 24 chambers on the day after the last inoculation. We systematically rotated the placement of the three or four frog enclosures within each chamber every other day to ensure that they were evenly exposed to any small differences in local temperatures that might exist within the chamber. Temperature-controlled chambers were programmed to maintain a 12 hr: 12 hr photoperiod. Every other day, we moistened paper towels with tap water to maintain a consistent moisture level (paper towel saturated but no

247 standing water) and fed frogs pinhead crickets *ad libitum*. We changed paper towels at every other
248 feeding. We refrained from performing husbandry duties at times of day coinciding with heat pulses.
249 To monitor Bd infection status and intensity over the course of the experiment, we swabbed
250 frogs upon arrival from the captive breeding facility (all tested Bd-negative) and every eight days
251 thereafter, following a standard protocol (Stockwell et al. 2015). We determined the number of Bd
252 zoospore genome equivalents per swab using a real-time quantitative PCR protocol modified from
253 Boyle et al. (2004) with standards prepared from the Bd isolate CW34. We checked frogs daily for
254 signs of chytridiomycosis (e.g., lethargy, reddening of feet) and first observed these signs on day 36;
255 frogs exhibiting signs of disease were immediately removed from the experiment. A receiver
256 operating characteristic (ROC) analysis (Stockwell et al. 2016) for the first 43 days of the experiment
257 (1 frog died and 14 frogs had shown signs of chytridiomycosis by this time) indicated that frogs with
258 infection loads >13,700 zoospore genome equivalents (ZGE) had a 63% chance of dying or showing
259 signs of chytridiomycosis. Therefore, to prevent subsequent morbidity and mortality, we removed
260 sub-clinically infected frogs from temperature treatments gradually, as swabs from frogs exceeded
261 the threshold infection intensity of 13,700 ZGE. We treated frogs for chytridiomycosis with the
262 antifungal Itraconazole following removal from the experiment (Brannelly et al. 2012). Our
263 experiment was carried out under James Cook University Animal Ethics permit A2234.
264
265 *In vitro* Bd population growth trial
266 We conducted the *in vitro* trial in tissue culture-treated 96-well plates comprising 12
267 columns of eight wells. We added TGhL broth to the zoospore suspension to produce a final
268 zoospore concentration of 5×10^5 zoospores per ml. We pipetted 100 µl of the zoospore suspension
269 into even-numbered columns and 100 µl of TGhL broth into odd-numbered columns in each plate.
270 We excluded 36 peripheral wells from analyses to avoid well position effects, leaving 30 wells
271 containing the zoospore suspension and 30 wells containing TGhL broth that were included in
272 analyses.
273 Three replicate temperature-controlled chambers (Greenspan et al. 2016) were
274 programmed to perform each of the eight temperature treatments, for a total of 24 chambers, each
275 containing one inoculated 96-well plate. Actual chamber temperatures were recorded and verified in
276 the same way as for the *in vivo* trial and remained within 0.5°C of target chamber temperatures
277 during the experiment. We rotated plates 180° daily to account for any small temperature gradients
278 in the chambers. We quantified Bd growth by measuring the optical density of each well daily for 7 d
279 (1–2 Bd generations) with a Multiskan Ascent 96/384 plate reader (MTX Lab Systems Inc., Vienna,
280 Virginia, USA) at an absorbance of 492 nm. We then used the daily measurements of optical density

to construct population growth curves for each well. We calculated daily optical density values by subtracting the average optical density of the wells containing only TGhL broth in the corresponding plate from the optical density of each well containing Bd. For each growth curve, we used the grofit package in Program R to fit the curve to a set of conventional, parametric growth functions (logistic, Gompertz, modified Gompertz, and Richards) and a model free spline function, select the best-fitting function according to Akaike's information criterion, and estimate parameters of the best-fitting growth curve (Kahm et al. 2010; R core Team 2015). The parameters were lag duration (time preceding the exponential growth phase), maximum slope of the curve (representative of the maximum rate of exponential growth), and two measures of total growth: maximum height of the curve and area under the curve (Kahm et al. 2010).

Predicting Bd growth potential

We used published population growth curves for three Bd isolates from Australia (Stevenson et al. 2013) to predict patterns of Bd growth under our temperature treatments. The isolates were collected from infected frogs in Queensland (QLD, northeastern Australia, isolate Paluma-Lgenimaculata #2 [tadpole]-2010-CO), New South Wales (NSW, east-central Australia, isolate AbercrombieR-Lbooroolongensis-09-LB1), and Tasmania (TAS, southeastern Australia, isolate MtWellington- Lewingii [tadpole]-2012-RW1), and thus represent a wide range of temperature conditions to which Bd may be adapted. For each isolate, Stevenson et al. (2013) documented population growth at constant 13°C, 15°C, 17°C, 19°C, 21°C, 23°C, 25°C, 26°C, 27°C, and 28°C.

We constructed thermal performance curves for each isolate by plotting the maximum slope of each constant-temperature growth curve (see grofit methods above) and smoothing the curve with the loess function in Program R (Cleveland et al. 1992; Fig. 2A). We used maximum slope as the growth curve parameter with which to construct thermal performance curves because this parameter represents *exponential* Bd growth potential, an important determinant of disease outcome (Briggs et al. 2010).

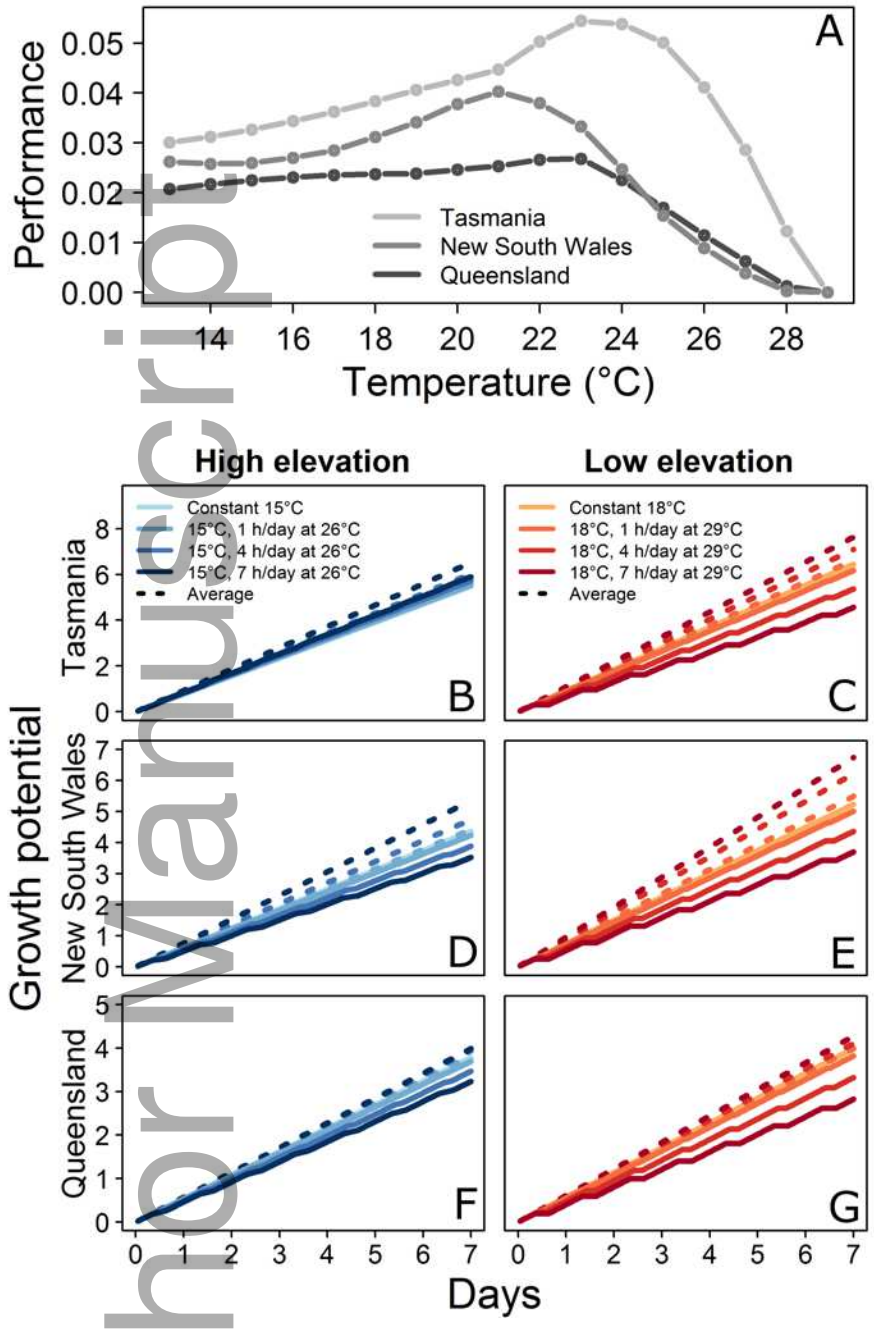
We then predicted exponential growth potential over 7 d (the length of our *in vitro* experiment) based on the hourly temperatures of our treatments, by plotting cumulative thermal performance for each hourly temperature (solid lines in Fig. 2B–G). We also predicted exponential growth potential over 7 d for hypothetical constant-temperature treatments representing the average daily temperature of each heat pulse treatment (dashed lines in Fig. 2B–G).

In general, as the duration of heat pulses increased, growth potential for the heat pulse treatments decreased but growth potential for the hypothetical average-temperature treatments increased. The magnitude of these relationships was greater for the low elevation treatments (red-

orange gradient) compared to the high elevation treatments (blue gradient). An exception to this pattern was the unusually high growth potential of the Tasmanian isolate under the high-elevation heat pulse treatments (Fig. 2B). This isolate initially grew well at 26°C but stopped growing after the first generation (Stevenson et al. 2013), indicating that our prediction may have overestimated the fitness of this isolate at 26°C.

Based on these predictions, if exposure to 26°C and 29°C disrupt Bd growth on the scale of 1–7 h at a time, we would expect decreases in Bd growth in our experiment as the duration of heat pulses increases. Alternatively, if 26°C and 29°C are too close to optimal temperatures to appreciably disrupt Bd growth on the scale of 1–7 h at a time, and growth is instead more heavily influenced by average daily temperatures, we would expect increases in Bd growth in our experiment as the duration of heat pulses increases.

Figure 2. *In vitro* population growth potential for *Batrachochytrium dendrobatidis* (Bd) under experimental temperature treatments. We generated thermal performance curves for three Bd isolates (A) based on published constant-temperature population growth curves (Stevenson et al. 2013). Solid lines (B–G) indicate growth potential based on the hourly temperatures of our treatments (cumulative thermal performance for each hourly temperature). Dashed lines (B–G) indicate growth potential for hypothetical constant-temperature treatments representing the average daily temperature of each heat pulse treatment.



Statistical analysis

We performed all statistical analyses with Program R software (R core Team 2015). We estimated differences in survival probabilities between groups of frogs with Cox Proportional Hazards survival analysis (Cox 1972). In this analysis, time until death is the typical response. Instead, we used number of days until frogs reached the threshold infection intensity (13,700 ZGE) as a proxy for death, as this level of infection was linked to death in our ROC analysis and has been linked to development of lethal chytridiomycosis in other species (Briggs et al. 2010; Vredenburg et al. 2010; Kinney et al. 2011). This analysis also accommodates 'censored' individuals (i.e., those with

incomplete survival records), allowing for inclusion of 10 frogs that never reached the threshold infection intensity and 24 frogs that were removed on day 36 for a concurrent study (six of the most heavily but sub-clinically infected frogs from each of the four temperature treatments).

We then analyzed our data using linear mixed effects models (lmer function in lme4 package; Bates et al. 2015). First, we tested for effects of elevation (high or low), heat exposure (daily 4-h heat pulses or constant cool temperature), and their interactions on log-transformed *in vivo* infection loads for the first 36 days of the experiment (before any frogs were removed from the experiment). We included day of swabbing event (4 d, 12 d, 20 d, 28 d, 36 d) as an additional interactive fixed effect and replicate (1—6) as well as frog (1—3) within chamber (1—4) as random effects.

Second, we tested for effects of elevation (high or low), heat pulse duration (0 h, 1 h, 4 h, 7 h), and their interactions on each of the four *in vitro* population growth parameters, including replicate (1—3) as a random effect. For each combination of elevation and heat exposure/duration in each model, we calculated the least square mean of the response variable (population growth parameter) and performed pairwise comparisons of least square means with general linear hypothesis tests (glht function).

Results

In our *in vivo* experiment, all inoculated frogs became infected with Bd. Our analysis of infection levels during the first five weeks of the eleven-week study (before any frogs were removed from the experiment) revealed that frog infection intensities increased more slowly and peaked at a lower level in the heat pulse treatments than in the constant temperature treatments ($P < 0.001$), especially under the hotter pulses of the low elevation heat pulse treatment ($P = 0.005$; Figure 3; Table 1). Among the low elevation treatments, frogs that experienced heat pulses had markedly lower infection intensities than frogs in the constant 18°C treatment starting at the very first infection monitoring event on day 4 and on each subsequent monitoring event (Fig. 3). Among the high elevation treatments, infection levels were similar in the first two weeks of the experiment but began to diverge by day 20, with lower levels in the heat pulse treatment than in the constant temperature treatment (Fig. 3). In both the low and high elevation heat pulse treatments, the median infection level did not reach our estimated chytridiomycosis threshold by day 36 (Fig. 3). In contrast, in both the low and high elevation constant temperature treatments, infection levels began to exceed our estimated chytridiomycosis threshold on day 20 and median infection levels reached (low elevation) or exceeded (high elevation) the threshold on day 28 (Fig. 3). By day 36, 50% of frogs in the low elevation constant temperature treatment had exceeded the threshold infection level and

the average infection intensity was 69,091 ZGE greater than in the low elevation heat pulse treatment, in which only 6% of frogs had exceeded the threshold infection intensity (Table 2; Fig. 3). Similarly, 50% of frogs from the high elevation constant temperature treatment had exceeded the threshold infection level by day 36 and the average infection intensity was 72,519 ZGE greater than in the high elevation heat pulse treatment, in which only 41% of frogs had exceeded the threshold infection intensity (Table 2; Fig. 3).

Table 1. Summary of the results of linear mixed effects models. We modelled the effects of elevation (high [15°C] vs. low [18°C] elevation treatments), heat exposure (heat pulse temperature treatments [26°C or 29°C for four hours per day] vs. constant cool temperature treatments [15°C or 18°C]), and their interactions on log-transformed *Batrachochytrium dendrobatidis* infection loads (number of zoospore genome equivalents detected on swabs) in the model host *Litoria spenceri*. We included day of swabbing event (4 d, 12 d, 20 d, 28 d, 36 d) as an additional interactive fixed effect and replicate (1—6) as well as frog (1—3) within temperature-controlled chamber (1—4) as random effects. We also modelled the effects of elevation, duration of daily heat exposure (0 h, 1 h, 4 h, 7 h), and their interactions on four *in vitro* population growth parameters for *Batrachochytrium dendrobatidis*, including replicate (1—3) as a random effect. The four population growth parameters were lag duration (time preceding the exponential growth phase), maximum slope of the exponential growth phase, maximum height of the growth curve, and area under the curve.

Experiment	Response	Predictor	Chi Square	DF	P-value
In vivo	Bd infection load	Elevation	7.141	1	0.008
		Heat	61.123	1	<0.001
		Day	92.439	4	<0.001
		Elevation x heat	7.731	1	0.005
		Elevation x day	3.528	4	0.474
		Heat x day	15.171	4	0.004
		Elevation x heat x day	2.307	4	0.679
In vitro	Lag duration	Elevation	8.5823	1	0.003
		Heat pulse duration	54.6165	3	<0.001
		Elevation x heat pulse duration	14.2298	3	0.003
	Max. slope	Elevation	209.70	1	<0.001
		Heat pulse duration	202.19	3	<0.001
		Elevation x heat pulse duration	124.30	3	<0.001

	Max. height	Elevation	0.6705	1	0.413
		Heat pulse duration	394.3840	3	<0.001
		Elevation x heat pulse duration	44.9060	3	<0.001
	Area under curve	Elevation	66.552	1	<0.001
		Heat pulse duration	213.985	3	<0.001
		Elevation x heat pulse duration	146.934	3	<0.001

Figure 3. *Batrachochytrium dendrobatidis* infection loads (log-transformed zoospore genome equivalents detected on swabs) on the model host *Litoria spenceri* over 36 d of exposure to four temperature treatments. Heavy lines in each box indicate the median value, boxes indicate the 1st and 3rd quartiles, and whiskers indicate the range of the data. The dashed line marks an infection level at which odds predicted morbidity or mortality from chytridiomycosis. Note that over time most frogs gradually exceeded the threshold for morbidity or mortality except those from the low elevation heat pulse treatment, and both low and high elevation heat pulse treatments took longer to approach or exceed this threshold.

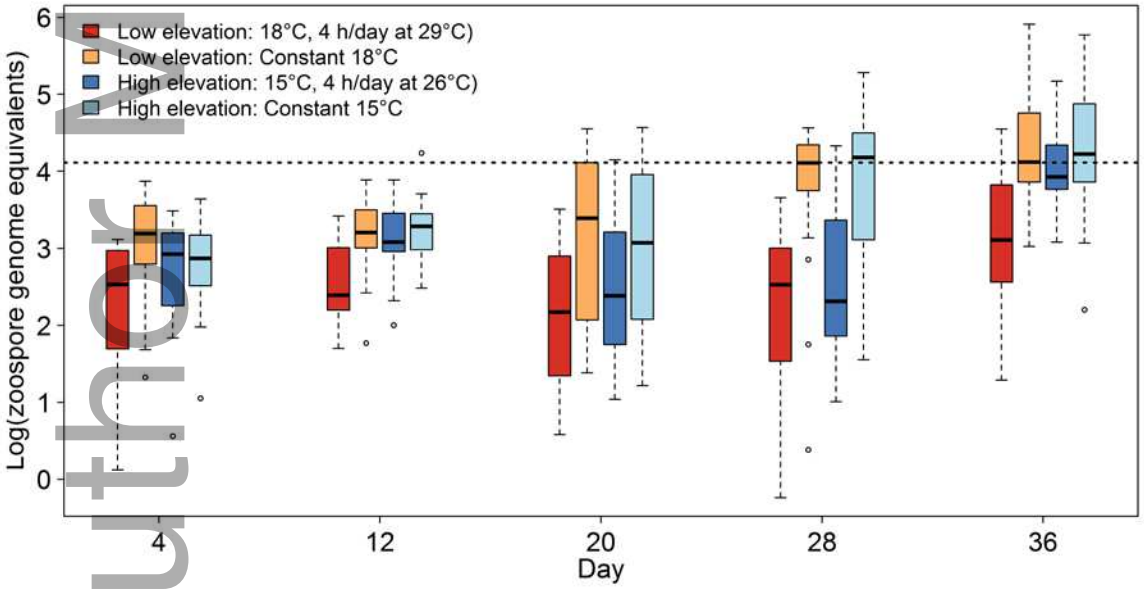


Table 2. Mean (± SD) and maximum *Batrachochytrium dendrobatidis* infection loads (zoospore genome equivalents detected on swabs) on individuals of the model host *Litoria spenceri* after 36 days of exposure to four temperature treatments: low elevation constant (18°C), low elevation heat pulse (18°C with daily 4-h heat pulses of 29°C), high elevation constant (15°C), and high elevation heat pulse (15°C with daily 4-h heat pulses of 26°C).

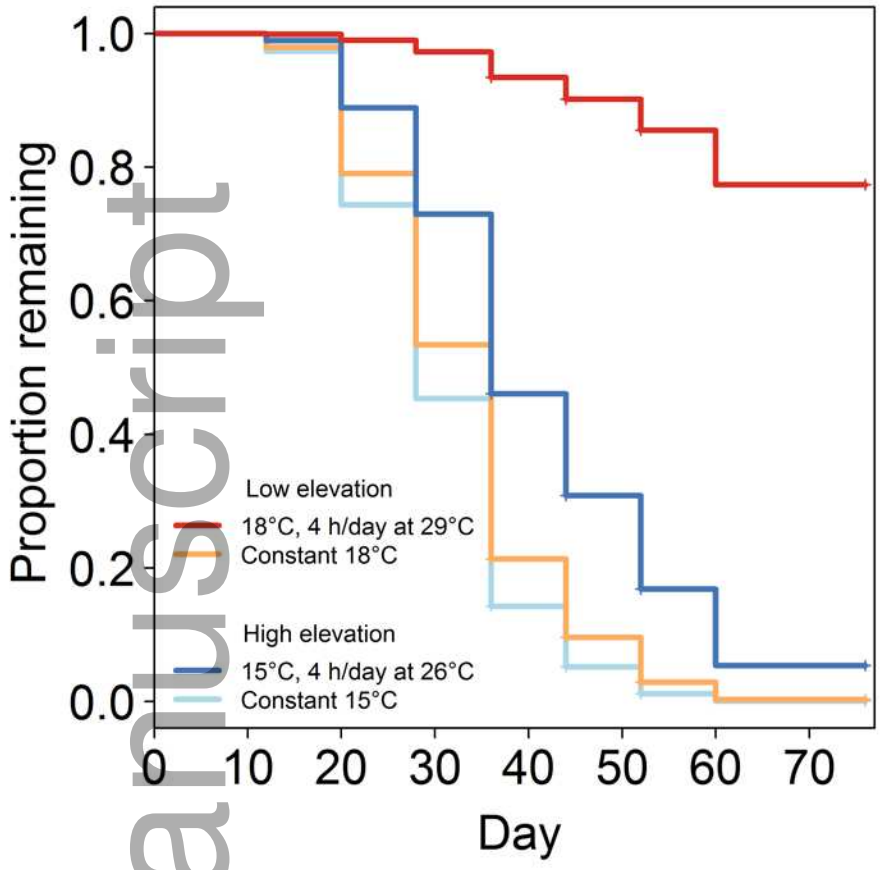
Elevation	Heat exposure	Mean	SD	Max
Low	Constant	73,740	186,941	811,333
	Heat pulse	4,649	8,585	35,233
High	Constant	99,415	176,727	590,667
	Heat pulse	26,896	43,223	148,333

414

415 Our survival analysis corroborated these patterns. The pooled risk of exceeding the
416 threshold infection intensity was lower in both heat pulse treatments than in either of the constant-
417 temperature treatments (P = 0.02), with the strength of this relationship significantly greater under
418 hotter pulses (P = 0.01; Fig. 4). All inoculated frogs from the constant temperature treatments that
419 were followed for the duration of the study had exceeded the threshold infection intensity by day
420 44, followed by all frogs from the high elevation heat pulse treatment by day 52. Ten of 11 (91%)
421 inoculated frogs from the low elevation heat pulse treatment that were followed for the duration of
422 the study maintained infection loads < 13,700 ZGE for the first nine weeks of the study and
423 eventually cleared their infections, as indicated by consecutive swabs that tested negative for Bd on
424 days 68 and 76.

425

426 Figure 4. Survival probabilities of groups of *Batrachochytrium dendrobatidis*-infected *Litoria spenceri*
427 exposed to four temperature treatments, estimated with a Cox Proportional Hazards analysis. In
428 this analysis, time until death is the typical response. Instead, we used number of days until frogs
429 reached a threshold infection intensity of 13,700 ZGE as a proxy for death. A receiver operating
430 characteristic (ROC) analysis (Stockwell et al. 2016) for the first 43 days of the experiment (1 frog
431 died and 14 frogs had shown signs of chytridiomycosis by this time) indicated that frogs with
432 infection loads >13,700 zoospore genome equivalents (ZGE) had a 63% chance of dying or showing
433 signs of chytridiomycosis. This level of infection has also been linked to development of lethal
434 chytridiomycosis in other species (Briggs et al. 2010; Vredenburg et al. 2010; Kinney et al. 2011).



In our *in vitro* experiment, Bd growth generally decreased as the duration of heat pulses increased (Fig. 5), corresponding with our estimates of growth potential based on the hourly temperatures of our treatments (solid lines in Fig. 2B–G) and contrasting with our estimates of growth potential based on the average daily temperatures of our treatments (dashed lines in Fig. 2B–G). Specifically, the lag period increased ($P < 0.001$) and the maximum slope ($P < 0.001$), maximum height ($P < 0.001$), and area under the growth curves ($P < 0.001$) decreased with increasing heat pulse duration (Fig. 6; Table 1). The negative effect of heat on fungal growth parameters was greatest under the hotter, low-elevation (29°C) pulses (lag: $P = 0.003$; slope: $P < 0.001$; height: $P < 0.001$; area: $P < 0.001$; Table 1) – the magnitude of differences in measures of growth among low elevation treatments (red-orange gradients in Fig. 6) was greater than that among high elevation treatments (blue gradients in Fig. 6). However, for the low elevation treatments, a minimum of 4 h of heat daily was required to detect a statistically significant reduction in maximum slope, maximum height, and area under the curve, and 7 h of heat daily was required to detect a statistically significant increase in lag time (Fig. 6). In contrast, for the high elevation treatments, we detected statistically significant reductions in growth parameters after only 1 h of heat exposure (Fig. 6).

Figure 5. Average population growth over 7 d for *Batrachochytrium dendrobatidis* exposed to experimental temperature treatments. Treatments represent body temperature regimes of *Litoria serrata* in high-elevation rainforests (blue gradient) and low-elevation rainforests (red-orange gradient) of the Australian Wet Tropics.

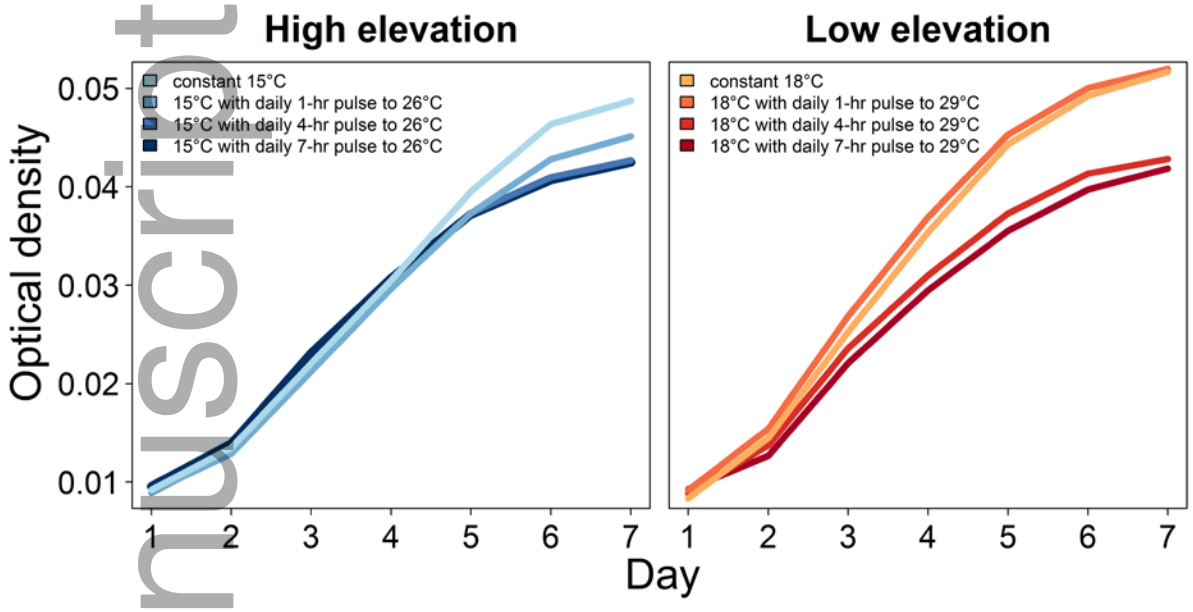
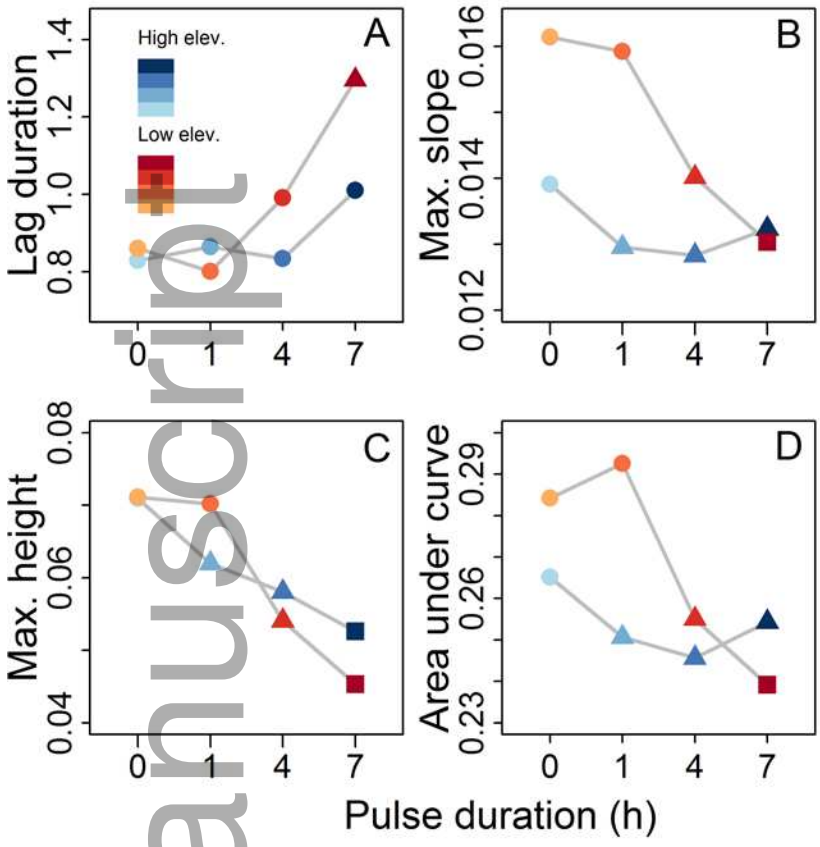


Figure 6. Characteristics of population growth curves for *Batrachochytrium dendrobatidis* exposed to experimental temperature treatments. Treatments represent body temperature regimes of *Litoria serrata* in high-elevation rainforests (blue gradient) and low-elevation rainforests (red-orange gradient) of the Australian Wet Tropics and differ in the duration of heat pulses (0 h, 1 h, 4 h, 7 h; darker colours correspond to longer heat pulses). The growth curve parameters were lag duration (time preceding the exponential growth phase), maximum slope of curve, maximum height of curve, and area under curve. Each symbol represents the least square mean of the population growth parameter calculated with linear mixed effects models. Different symbols indicate statistically significant pairwise differences determined with general linear hypothesis tests (glht function in Program R).



Discussion

In our study, experimentally inoculated frogs remained infected with Bd for at least several weeks, even when they experienced daily 26°C or 29°C heat spikes, temperatures that induce dormancy or mortality in the fungus after longer-term exposure (Piotrowski et al. 2004; Stevenson et al. 2013). This finding indicates that Bd can persist in hosts despite short exposures to temperatures above its thermal optimum, and helps explain the previous observation that warm, dry, 'marginal' habitats support frog communities with high pathogen prevalences (up to 100%; Puschendorf et al. 2013). However, infection loads were markedly lower under daily 4-h temperature spikes of 29°C, representing conditions at our low elevation rainforest sites, compared to the corresponding low elevation constant temperature treatment that lacked heat pulses. After five weeks, the mean infection load in frogs in the low elevation heat pulse treatment was more than 9,000 ZGE below a critical threshold at which odds predicted morbidity or mortality from chytridiomycosis, whereas the mean infection load in the low elevation constant temperature treatment was 60,000 ZGE above the threshold. Ten of 11 (91%) frogs from the low elevation heat pulse treatment whose infections were tracked for the duration of the 11-week study even cleared their infections, although complete infection clearance took at least nine weeks. In contrast, 4-h temperature spikes of 26°C, which are representative of conditions at our high elevation rainforest sites, failed to promote infection

clearance by hosts in our study, consistent with previous findings that Bd-related declines are more severe in upland amphibian populations in comparison to lowland populations (Richards et al. 1993; Lips 1999; Stuart et al. 2004). However, the high elevation heat pulse treatment was effective in reducing the rate at which infections exceeded the threshold for morbidity/mortality. Although we did not allow infections to progress to clinical levels, our modified Cox Proportional Hazards analysis suggests a clear survival benefit of these heat pulse treatments, especially the warmer pulses. These results offer new insight into disease responses in our model species *L. spenceri*, an endangered species, but may also be generalized to other species with similar behaviours and habitat preferences.

There are several plausible mechanisms that could explain the differences in infection patterns that we observed between paired constant temperature and heat pulse treatments. First, heat pulses could have had direct negative effects on the growth rate of Bd in host tissue, in accordance with the previous finding that 33°C heat spikes lowered exponential growth of Bd *in vitro* either by reducing rates of zoospore survival or encystment or by reducing production of zoospores within zoosporangia (Daskin et al. 2011). The results of our *in vitro* study (Fig. 5) were consistent with the prediction that mild heat pulses directly interfere with Bd proliferation on the scale of hours (solid lines in Fig. 2B–G) and were opposite to the prediction that Bd growth is more heavily influenced by average daily temperatures (dashed lines in Fig. 2B–G). Four-hour heat spikes to 26°C and 29°C substantially delayed the exponential growth phase (lag duration), slowed the apparent rate of exponential growth (max. slope) and reduced overall population growth of Bd (max. height and area under curve) and these effects were most extreme under hotter pulses. That heat pulses had a considerable moderating effect on Bd growth *in vitro* after only 7 d (1–2 Bd generations) suggests that heat pulses could dramatically influence the trajectory of infections over the course of weeks or months in nature. Our work could thus help to explain the previous findings that infection risk among wild frogs decreased with increasing frequency of body temperatures above 25°C (Rowley and Alford 2013; Roznik 2013) and that frog populations in dry forest survived a chytridiomycosis outbreak by perching on sun-warmed rocks upon nightly emergence from streams while neighbouring rainforest populations succumbed to the disease (Puschendorf et al. 2011). Caveats to our study, however, are that we tested only a single Bd isolate and the full heat tolerance spectrum of all known Bd strains has not been established.

A second, non-mutually-exclusive mechanism that could explain the effects of our temperature treatments on infection levels is that heat pulses could have promoted host immunity, either directly because of temperature-dependent rates of physiological processes in ectotherms (Feder 1992; Berger et al. 2004; Carey et al. 1999), or indirectly by preventing infections from

reaching intensities that otherwise would have overwhelmed the host immune system. However, because we observed direct effects of heat on Bd performance *in vitro*, we could not determine whether there might have been a concurrent effect of heat pulses on host immune function in the *in vivo* trial. The hypothesis that heat pulses simultaneously hindered Bd population growth while facilitating host immunity would help explain our finding that most frogs from the low elevation heat pulse treatment eventually cleared infections despite the relatively rapid generation time of the pathogen. The direct and indirect effects of heat pulses on amphibian immunity would be a useful avenue for future study since temperature strongly influences immune responses in ectotherms (Feder 1992; Berger et al. 2004; Carey et al. 1999), yet previous research has rarely addressed the effects of realistic diurnal temperature patterns on immune parameters.

In our infection experiment, the low elevation heat pulse treatment apparently exceeded a threshold at which the rate of Bd growth was either insufficient to maintain fungal populations within hosts, the host immune system outpaced the population growth of the fungus, resulting in eventual elimination of infections, or both. The high elevation heat pulse treatment failed to exceed this threshold but strongly reduced infection load, which is an important determinant of disease outcome (i.e., whether an individual develops chytridiomycosis) in this system (Briggs et al. 2010). Together, our results highlight the importance of incorporating realistic, fluctuating temperatures into experiments investigating host-pathogen interactions. Specifically, our results indicate that even in habitats in which *average* environmental temperatures may be suitable for pathogen growth and reproduction, infection risk or the outcome of an existing infection may be heavily influenced by host behaviours, such as microhabitat selection and thermoregulation, that briefly increase body temperatures to those that are detrimental to the parasite. Thus, management interventions incorporating environmental manipulation could aid in protecting some frog populations from chytridiomycosis-related declines (Scheele et al. 2014; Roznik et al. 2015; Garner et al. 2016), although variability in the heat tolerance of different Bd strains could limit the applicability of this type of intervention to only some of the regions where Bd occurs. Providing canopy openings that facilitate frog basking, *via* small-scale removal of trees or large branches overhanging critical habitat (e.g., streams) has been proposed as a simple, non-invasive management strategy for riverine and other host species (Scheele et al. 2014; Roznik et al. 2015). Artificial heat sources have also been recommended as an alternative to habitat modification where other types of environmental manipulation are not possible (Scheele et al. 2014). Our results, combined with evidence that shade reduction is linked to decreased infection risk (Raffel et al. 2010; Becker and Zamudio 2011; Becker et al. 2012; Roznik et al. 2015; Scheele et al. 2015) suggest that these interventions could be highly effective at reducing burdens of Bd and could therefore improve survival in populations with

555 endemic infection. This management approach could be particularly applicable to translocation or
556 reintroduction sites for critically endangered species (Garner et al. 2016).
557
558 Authors' Contributions
559 SEG, DSB, EAR, GM, DAP, LS and RAA conceived the ideas and designed methodology; SEG, DSB, LAS,
560 and RJW collected the data; SEG and RAA analysed the data; SEG and DSB led the writing of the
561 manuscript. All authors contributed critically to the drafts and gave final approval for publication.
562
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569
570 Data Accessibility
571 Data deposited in the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.989r4,
572 (Greenspan et al., 2017)
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