Infection increases vulnerability to climate change via effects on host thermal tolerance

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Unprecedented global climate change and increasing rates of infectious disease emergence are occurring simultaneously. Infection with emerging pathogens may alter the thermal thresholds of hosts. However, the effects of fungal infection on host thermal limits have not been examined. Moreover, the influence of infections on the heat tolerance of hosts has rarely been investigated within the context of realistic thermal acclimation regimes and potential anthropogenic climate change. We tested for effects of fungal infection on host thermal tolerance in a model system: frogs infected with the chytrid Batrachochytrium dendrobatidis. Infection reduced the critical thermal maxima (CT_{max}) of hosts by up to ~4 °C. Acclimation to realistic daily heat pulses enhanced thermal tolerance among infected individuals, but the magnitude of the parasitism effect usually exceeded the magnitude of the acclimation effect. In ectotherms, behaviors that elevate body temperature may decrease parasite performance or increase immune function, thereby reducing infection risk or the intensity of existing infections. However, increased heat sensitivity from infections may discourage these protective behaviors, even at temperatures below critical maxima, tipping the balance in favor of the parasite. We conclude that infectious disease could lead to increased uncertainty in estimates of species’ vulnerability to climate change.

Projections of the future global climate indicate that temperature means, variances, and extremes will change1–6. These changes may be hazardous for some animals by shifting daily, seasonal, or intermittent temperature cycles away from optimal conditions or closer to lethal extremes7. Risks to populations due to climate change can be estimated using warming tolerance, which is the difference between the species’ maximum heat tolerance (critical thermal maximum [CT_{max}]) and maximum environmental temperature8–11. When this value is large, individuals theoretically have a high thermal safety margin in the context of rising environmental temperatures11. In contrast, when this value is small, risk is high because even slight increases in environmental temperatures may cause the body temperatures of individuals to reach lethal limits12. This is further compounded when temperatures approaching critical thermal maxima lead to behaviors or ecological interactions that reduce fitness. For example, heat stress may cause individuals to seek refuge at the expense of activities that promote fitness (e.g., foraging or reproduction)13. Similarly, altered temperature patterns may lead to changes in phenology, resource availability, or predator interactions that threaten individual and population survival14.

Thermal stress and fitness costs associated with global climate change are likely to occur in combination with other natural and anthropogenic stressors such as land use change, environmental contaminants, and disease15–17. Fungal diseases are currently emerging at record rates, posing a direct threat to global biodiversity in the face of climate change18. Reduced maximum thermal tolerance can be a major side effect of infections in amphibians19, fish20,21, and mollusks22–28. For example, ill newts Notophthalmus viridescens infected with a mesomycetozoon parasite had lower CT_{max} than uninfected newts (by 0.6–1.7 °C)19. Similarly, resistance to high temperature (hours at 25 °C until 50% mortality) was lower in brook trout Salvelinus fontinalis infested with gill lice Salmincola edwardsi, and was inversely correlated with extent of secondary bacterial infection, a measure of fish health29.

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Here we investigate interactions between fungal disease and upper thermal tolerance in a model host-pathogen system: frogs infected with the chytrid *Batrachochytrium dendrobatidis* (*Bd*). We experimentally infected frogs with *Bd* and acclimated them to constant cool temperatures or daily heat pulses mimicking the body temperature regimes of frogs in nature. We then examined the effects of *Bd* infection status, infection intensity and acclimation on their critical thermal maxima and considered the implications of our findings in light of current and projected global change.

### Results

The critical thermal maxima of our model frog species *Litoria spenceri*, measured as temperature at onset of spasms and temperature at loss of righting ability, were significantly lower for *Bd*-infected frogs than for uninfected frogs (spasms: *p* < 0.001; righting: *p* = 0.009; Table 1; Fig. 1), after controlling for a positive relationship between frog snout-urostyle length and critical thermal maxima (Table 1). Across acclimation temperature treatments, mean temperature at onset of spasms (± SD) ranged from 34.2 °C ± 2.1 °C to 35.6 °C ± 3.1 °C in infected frogs and 36.2 °C ± 1.4 °C to 38.5 °C ± 1.2 °C in uninfected frogs (Table 2). Likewise, mean temperature at loss of righting ability ranged from 37.4 °C ± 2.2 °C to 39.9 °C ± 1.3 °C in infected frogs and 39.6 °C ± 0.5 °C to 40.5 °C ± 1.0 °C in uninfected frogs (Table 2; Fig. 1).

![Table 1. Summary of analyses of covariance on the effects of Batrachochytrium dendrobatidis infection status, infection intensity, elevation (high [15 °C] vs. low [18 °C] acclimation treatments), heat exposure (pulse [26 °C or 29 °C for four hours per day] vs. constant acclimation treatments) and the interactions between infection and acclimation on two metrics of the critical thermal maximum (temperature at onset of spasms and temperature at loss of righting response) for the model amphibian host Litoria spenceri, with frog snout-urostyle length as a covariate.](image)

The magnitude of the effect of infection status on temperature at loss of righting ability depended on acclimation to heat pulses (*p* = 0.014; Table 1); compared to uninfected individuals, the temperature at loss of righting for infected individuals under constant acclimation regimes was reduced by an average of up to 2.7 °C, whereas the temperature at loss of righting for infected individuals under pulsed acclimation regimes was only reduced by an average of up to 1 °C (Table 2; Fig. 1). A similar pattern emerged for the magnitude of the effect of infection status on temperature at onset of spasms, although this was not statistically significant. Specifically, the temperature at onset of spasms for infected individuals under constant acclimation regimes was reduced by an average of up to 2.7 °C, whereas the temperature at onset of spasms for infected individuals under pulsed acclimation regimes was only reduced by an average of up to 2 °C (Table 2; Fig. 1).

Infection intensity at the time of CT<sub>max</sub> measurement varied widely among temperature treatments (Fig. 2). By day 36, the day that we measured CT<sub>max</sub> in six of the most highly infected frogs from each temperature treatment, the mean infection load exceeded our established threshold for disease development (13,700 ZGE) in all treatments except the low elevation heat pulse treatment (Table 2). After day 36, all frogs from both high elevation treatments and the low elevation constant treatment eventually exceeded the threshold infection intensity. In contrast, only one frog from the low elevation heat pulse treatment exceeded the threshold infection intensity after day 36; the other 10 of 17 frogs in this treatment (59%) maintained low infection loads, eventually cleared their infections, and were therefore excluded from the study. Although infection status had a significant effect on CT<sub>max</sub>, we were unable to detect a statistically significant effect of infection intensity on CT<sub>max</sub> (Table 1). However, low elevation heat pulse was the only treatment in which (1) the negative effect of infection on CT<sub>max</sub> was greatly

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**Table 1. Summary of analyses of covariance on the effects of *Batrachochytrium dendrobatidis* infection status, infection intensity, elevation (high [15 °C] vs. low [18 °C] acclimation treatments), heat exposure (pulse [26 °C or 29 °C for four hours per day] vs. constant acclimation treatments) and the interactions between infection and acclimation on two metrics of the critical thermal maximum (temperature at onset of spasms and temperature at loss of righting response) for the model amphibian host *Litoria spenceri*, with frog snout-urostyle length as a covariate.**

| Response          | Predictor       | Infection status | Infection intensity | Squares | DF | F-value | P-value | Squares | DF | F-value | P-value |
|-------------------|-----------------|------------------|---------------------|---------|----|---------|---------|---------|----|---------|---------|
| Onset of spasms   |                 |                   |                     |         |    |         |         |         |    |         |         |
|                   | Snout-urostyle length | 20.59 | 4.382 | 0.041 | 8.523 | 1   | 1.309 | 0.261 |
|                   | Infection       | 70.08 | 14.912 | <0.001 | 0.349 | 1   | 0.0536 | 0.818 |
|                   | Elevation       | 1.08 | 0.2291 | 0.634 | 0.042 | 1   | 0.0065 | 0.936 |
|                   | Heat            | 3.05 | 0.6482 | 0.424 | 8.649 | 1   | 1.3286 | 0.258 |
|                   | Infection × elevation | 1.88 | 0.4000 | 0.530 | 0.077 | 1   | 0.0119 | 0.914 |
|                   | Infection × heat | 16.27 | 3.461 | 0.068 | 7.074 | 1   | 1.0866 | 0.305 |
|                   | Residuals       | 244.38 | 52 | 32 | 208.312 | 32 |         |         |
| Loss of righting  |                 |                   |                     |         |    |         |         |         |    |         |         |
|                   | Snout-urostyle length | 6.46 | 3.689 | 0.060 | 6.874 | 1   | 3.2534 | 0.081 |
|                   | Infection       | 12.75 | 7.2832 | 0.009 | 1.089 | 1   | 0.5153 | 0.478 |
|                   | Elevation       | 0.02 | 0.0118 | 0.914 | 3.257 | 1   | 1.5415 | 0.223 |
|                   | Heat            | 4.25 | 2.4268 | 0.125 | 1.227 | 1   | 0.5807 | 0.452 |
|                   | Infection × elevation | 4.51 | 2.5762 | 0.115 | 4.754 | 1   | 2.2500 | 0.143 |
|                   | Infection × heat | 11.29 | 6.446 | 0.014 | 3.173 | 1   | 1.5015 | 0.229 |
|                   | Residuals       | 91.06 | 52 | 32 | 67.614 | 32 |         |         |
reduced (standard errors of mean CT_max temperatures for infected and uninfected frogs overlap (Fig 1) and (2) the average infection loads on day 36 and for the entire duration of the experiment did not exceed the threshold level of 13,700 ZGE (Table 2; Fig. 2).

Discussion
The global climate and the microclimates experienced by animals are becoming warmer and more extreme1, 3, 30, 31, increasing risk of population losses and even species extinctions by decreasing the margin of safety between the maximum heat thresholds of organisms and the maximum ambient temperatures they encounter. For example, recent extirpations of Sceloporus lizards were linked to elevated maximum temperatures during the breeding season, which restricted individuals to cool refuges at the expense of foraging and reproduction and in turn caused declining population growth rates since the 1970s13. On a shorter-term timescale, record high temperatures on a single day in 2002 were associated with deaths of thousands of flying foxes (Pteropus spp.) in eastern Australia 12. Three years later, and in a neighboring Australian state, another heat wave nearly drove the upland endemic white lemuroid possum (Hemibelideus lemuroides) to extinction 32.

The thermal safety margins of organisms may be compressed not only by rises in environmental temperatures but also reductions in maximum heat tolerance. In our study, heavy fungal infections lowered the critical thermal maxima of juvenile frogs by up to ~4 °C. The maximum heat tolerance of organisms can be determined by temperature effects on the molecules, cells, and biochemical reactions of organ systems, including the circulatory, respiratory, and nervous systems 33–35. The temperature resistance of these systems could plausibly decrease if already weakened by Bd infection or stress, especially since Bd causes tissue damage36 and blocks oxygen, water, and electrolyte balance through the skin37. Alternatively, lowered thermal tolerance could indicate manipulation of host physiology by the fungus to promote movement of the host to microhabitats that favor the fungus, which has a low tolerance for elevated temperatures38, 39. Further studies are needed to determine the physiological and

Figure 1. Average critical thermal maxima (± SE) for the model amphibian host Litoria spenceri acclimated to four temperature treatments, with and without infections by the fungus Batrachochytrium dendrobatidis. Metrics of the critical thermal maximum were (A) body temperature at onset of spasms and (B) body temperature at loss of righting ability.

Table 2. Average critical thermal maxima for the model amphibian host Litoria spenceri with and without infections by the fungus Batrachochytrium dendrobatidis and average infection intensities of the infected individuals.
evolutionary causes of reduced thermal tolerance from infection and the synergistic effects of infection and temperature on fitness at non-critical temperatures.

Ours is the first study to directly test for the effects of a fungal parasite on the upper thermal tolerance of its hosts. Existing literature on the interactions between infection and the upper thermal tolerance of animals is limited to six marine mollusk host species, one freshwater mollusk host species, eight freshwater fish host species, and one amphibian host species (a newt), with trematodes as the dominant parasite (Table 3). Whereas upper thermal tolerance of hosts was enhanced in only 10.5% (2/19) of these host-parasite systems and did not change in 31.5% (6/19) of these systems, our finding that Bd infections lowered host thermal tolerance is consistent with 58% (11/19) of host thermal responses to parasites (Table 3), including the only previous study of thermal thresholds in parasitized amphibians19 and similar studies of parasitized fish20, 21 and mollusks22–28. What does this mean for the present and coming decades, during which animals will face unprecedented changes in the global climate and in rates of infectious disease emergence? Our results suggest that infections by parasites and pathogens may profoundly alter the thermal physiology of hosts, often eliciting significantly reduced heat tolerance. We argue that a diminished upper temperature threshold may not only increase risk of population losses in accordance with the warming tolerance hypothesis8–11, but also to perpetuate infections by altering host thermoregulatory behavior, with added implications for host survival.

In ectothermic hosts, including frogs, behaviors that elevate body temperature may decrease heat-intolerant parasite performance or increase immune function, thereby reducing infection risk or the intensity of existing infections40–42. Our study demonstrates the infection-limiting benefits of thermoregulation – for most frogs, four hours of daily exposure to 29 °C (in our low elevation heat pulse treatment) was sufficient to prevent infection levels from exceeding the threshold marking increased risk for morbidity and/or mortality from infection. A recently proposed conceptual model that expands on the relationship between CTmax and infection risk predicts that infection risk will increase as the difference between the CTmax of the host and parasite decreases (tolerance mismatch hypothesis; Fig. 3)43 because infection risk is higher when the host occupies microenvironments that are also favorable for the parasite. Species’ CTmax are highly variable even within genera and can be overestimated using laboratory techniques44, 45. While our model host species performed at the high end of the CTmax spectrum46, our study suggests that in ecological systems in which tolerance mismatch is precariously small, high parasite burdens can shrink the gap between host and pathogen thermal tolerances even further (Fig. 3), potentially discouraging protective thermoregulatory behaviors, even at temperatures below upper maxima, and tipping the balance in favor of the parasite.

In contrast to heavy infections, mild infections may not significantly lower host thermal tolerance. The low elevation heat pulse treatment was the only group in which (1) most individuals had infection loads below the threshold for disease development, and (2) the CTmax of infected and uninfected individuals was similar, suggesting that any effects of light infection levels on the thermal tolerance of frogs were minimal. However, this warrants further study, especially because we did not detect a statistically significant effect of infection intensity on CTmax (Table 1).

We observed higher upper thermal tolerances in infected frogs that were acclimated to realistic daily heat pulses than in infected frogs that were acclimated to constant cool temperatures. These results highlight the importance of incorporating biologically meaningful acclimation temperature regimes into the design of experiments and support the recent finding that small-bodied hosts may be more capable of temperature acclimation than previously thought (Rohr et al., in review). Whereas Rohr et al. (in review) found that the magnitude of
### Table 3. Review of studies on the effects of infections on upper thermal tolerance in animal hosts.

| Agent phylum | Agent species | Host taxon | Host species | Effect on thermal tolerance | Reference |
|--------------|---------------|------------|--------------|-----------------------------|-----------|
| Artropoda    | Lernaea cyprinacea | freshwater fish | Pimephales promelas | no effect | Vaughan and Cole 2016 |
| Artropoda    | Salmiscola edwardsii | freshwater fish | Salvelinus fontinalis | decreased | Vaughan and Cole 2016 |
| Choanozoa    | Ichthyophonus-like sp. | newt | Notophthalmus viridescens | decreased | Sherman19 |
| Chytridiomycota | Batrachochytrium dendrobatidis | frog | Litoria sp. | decreased | Greenspan et al. this study |
| Platyhelminthes | Crassipila bulbosus | freshwater fish | Perca fluviatilis | no effect | Vaughan and Cole 2016 |
| Platyhelminthes | Cryptocotyle lingua | marine snail | Littorina littorea | decreased | McDaniel13 |
| Platyhelminthes | Himasthla elongata, Renicola roscoyvi | marine clam | Cardium edule | decreased | Lauckner27 |
| Platyhelminthes | Lepocreadium ovalis, Zoogonus rubellus | marine snail | Nassarius obsoletus | decreased | Vernberg and Vernberg26 |
| Platyhelminthes | Maritrena sp. | marine snail | Zeacumantis subcarinatus | increased | Bates et al.28 |
| Platyhelminthes | Philothelmins | marine snail | Zeacumantis subcarinatus | decreased | Bates et al.28 |
| Platyhelminthes | Schistosoma mansoni | freshwater snail | Biomphalaria glabrata | decreased | Lee and Cheng44 |
| Platyhelminthes | Schistosoma ondatrae | freshwater snail | Notropis chryscephalus | no effect | Hocket and Mundahl65 |
| Platyhelminthes | Schistosoma mansoni | freshwater snail | Notropis sp. | no effect | Hocket and Mundahl65 |
| Platyhelminthes | Schistosoma mansoni | freshwater snail | Pimephales notatus | no effect | Hocket and Mundahl65 |
| Platyhelminthes | 10 species* | marine snail | Cerithidea californica | no effect | Sousa and Gleason66 |
| Platyhelminthes | 3 species** | marine snail | Nassarius obsoletus | increased | Rie26 |
| Platyhelminthes (dominant); Acanthocephales, Nematoda | 6 species*** | freshwater fish | Lepomis macrochirus | decreased | Lutterschmidt et al.28 |
| Platyhelminthes (dominant); Acanthocephales, Nematoda | 7 species**** | freshwater fish | Lepomis megalotis | decreased | Lutterschmidt et al.28 |
| Platyhelminthes | unknown | marine snail | Littorina littorea | decreased | Lauckner26 |
| Platyhelminthes | unknown | marine snail | Nassarius reticulatus | decreased | Tallmark and Norrgren25 |

acclimation plasticity may be underestimated in laboratory experiments due to its dependence on acclimation duration and body mass, our use of an atypically long acclimation duration (≥36 days) and small-bodied hosts suggests that our study is robust to these common experimental artifacts. Importantly, however, under most of our acclimation treatments, the magnitude of the parasitism effect exceeded the magnitude of the acclimation effect. This suggests that for populations of some species, even as thermal tolerances are adjusted to long-term increases in temperature from climate change, any benefit this provides to warming tolerance may not be sufficient to protect animals from the thermal consequences of parasitism.

In contrast to infected frogs, which exhibited enhanced thermal tolerances when acclimated to daily heat pulses, we did not detect this acclimation effect in uninfected frogs (i.e., uninfected frogs exhibited similar [or lower, in the case of onset of spasms in the low elevation heat pulse treatment] thermal tolerances when exposed to daily heat pulses compared to constant cool temperatures). It is unclear why the temperature at onset of spasms was reduced in uninfected frogs from the low elevation heat pulse treatment. Lack of an acclimation effect in the other paired constant temperature vs. heat pulse treatments could be attributed to inherent physiological limits (i.e., a ceiling effect) on thermal tolerance or tradeoffs between thermal tolerance and acclimation plasticity46. A related avenue for future research is the capacity for heat hardening and resistance adaptation in common parasites.

While gradual increases in average temperatures could favor the hosts of some parasites, such as cool-loving fungi, our study illustrates that we may currently be unable to predict the combined effects of infections and...
climate change on host populations. Of particular concern are unpredictable heat waves that are long enough to impose thermal stress on hosts but are too short to be therapeutic, for example by ridding hosts of heat-intolerant parasites, or to allow for thermal acclimation. We conclude that infectious disease could lead to increased uncertainty in estimates of species’ vulnerability to climate change.

**Methods**

**Acclimation temperature treatments.** To generate realistic acclimation temperature treatments, we used body temperature data from *Litoria serrata*, a stream-associated frog of the Australian Wet Tropics. We used temperature-sensitive radio-transmitters (Model A2414; Advanced Telemetry Systems, Isanti, MN) to record the body temperatures of 54 male frogs in rainforests during the dry season (when *Bd* is typically most prevalent in this region). The radio-transmitters recorded frog body temperatures every 15 min for 5–11 d. We created simplified, rectangular-wave acclimation temperature treatments to approximate the patterns we found in the field data. We derived the trough temperatures of the rectangular waves from the overall medians of individual median body temperatures at the two high elevation sites (750–800 m elevation; 15 °C) and two low elevation sites (20–100 m elevation; 18 °C) where tracking occurred. We derived the crest temperatures of the rectangular waves from the median of individual maximum body temperatures >25 °C at the same sites (high elevation: 26 °C; low elevation: 29 °C). We derived the crest length of the rectangular waves from the median of the individual maximum lengths of time that frogs spent with body temperatures >25 °C for all sites combined (4 h).

Thus, our two high elevation treatments were (1) a daily rectangular wave with trough at 15 °C for 20 h per day and crest at 26 °C for four hours per day (hereafter high elevation heat pulse; inoculated: n = 11; control: n = 5) and (2) a constant 15 °C control treatment (hereafter high elevation constant; inoculated: n = 11; control: n = 5; Fig. 4). Our two low elevation treatments were (1) a daily rectangular wave with trough at 18 °C for 20 hours per day and crest at 29 °C for four hours per day (hereafter low elevation heat pulse; inoculated: n = 17; control: n = 5) and (2) a constant 18 °C control treatment (hereafter low elevation constant; inoculated: n = 10; control: n = 5; Fig. 4). The constant temperature control treatments (15 °C and 18 °C) served as a standard against which to observe effects of acclimation to realistic heat pulses on host thermal tolerance. Our temperature treatments are also pertinent to *Bd* physiology as this fungus shows optimal short-term growth at 15–25 °C, and ceases growth and reproduction at 26–29 °C.

**Batrachochytrium dendrobatidis cultures and inoculations.** We used the *Bd* isolate Paluma-Lseratta-2012-RW-1. This isolate is part of the collection maintained at the College of Public Health, Medical, and Veterinary Sciences, James Cook University. This isolate originated from an adult *L. serrata* that was collected from Birthday Creek, a site in the Wet Tropics region of Queensland, Australia (18°58′54″ S, 146°10′02″ E), and died in captivity. The isolate had been cryo-archived after two passages in nutrient broth. We revived an aliquot of the isolate and cultured it in tryptone/gelatin hydrolysate/lactose (TGHl) broth in 25-cm³ tissue culture flasks, passaging it twice before the experiment and maintaining cultures at 22 °C.

To obtain zoospores for inoculations, we inoculated Petri dishes containing TGHl broth in 1% agar with ~1/3 ml of cultured broth. Plates were partially dried in a laminar flow cabinet, incubated at 21 °C for four days, and then maintained alternatingly at 4 °C and 21 °C to sustain growth and zoospore production. For each
experiment with the same cohort of *L. spenceri* native risk of morbidity and mortality using a receiver operating characteristic (ROC) analysis for a concurrent treatment) as well as for all control frogs (n = 10–17 per treatment) to maximize our samples of infected individuals and capture natural variability in infection levels.

We inoculated frogs on three consecutive days. To inoculate, we placed each frog into an individual 70-ml plastic container and added 3 ml of zoospore inoculant or sham inoculant (enough to cover the bottom of the container) to each container using a syringe. We left frogs in inoculant baths for eight hours 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 × 120 × 170 mm plastic containers lined with tap water-saturated paper towel.

We allocated frogs in their individual enclosures to 24 temperature-controlled chambers on the day after the last inoculation. Six replicate chambers were programmed to execute each of the four acclimation temperature treatments. 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. We distributed inoculated and control frogs into the chambers as evenly as possible and reduced effects of frog history and body size by assigning frogs to temperature treatments proportionally by clutch of origin (reported by captive breeding facility) and snout-urostyle length (measured prior to inoculation). We systematically rotated the placement of the frog enclosures within each chamber every other day to ensure that they were evenly exposed to any local differences in temperature that might exist within the chamber.

**Frog disease monitoring and husbandry.** To monitor Bd infection status and intensity, we swabbed frogs upon delivery from the captive breeding facility (all frogs tested negative for Bd before the experiment) and every eight days thereafter following a standard protocol. We determined the number of Bd zoospore genome equivalents (ZGE) per swab with a real-time quantitative PCR protocol modified from Boyle et al.

The temperature-controlled chambers were programmed to maintain a 12 hr: 12 hr light: dark cycle. Every other day, we moistened the paper towels in frog containers with tap water as needed to maintain a consistent moisture level (paper towels were saturated but there was no standing water) and fed frogs pinhead crickets *ad libitum*. We changed paper towels at every other feeding and measured CT_max on days on which feeding did not occur.

**CT_max measurement and statistical analysis.** Our goal was to measure CT_max when frogs had well-developed infections but before they displayed clinical signs of infection. By day 36, infection loads in most inoculated frogs were relatively high; in an effort to avoid morbidity and mortality from infection, we measured CT_max for a subset of inoculated frogs (n = 6 of the most heavily infected frogs in each acclimation temperature treatment) as well as for all control frogs (n = 5 per temperature treatment) on day 36. We then determined relative risk of morbidity and mortality using a receiver operating characteristic (ROC) analysis for a concurrent experiment with the same cohort of *L. spenceri*. This analysis indicated that frogs with infection loads >13,700...
ZGE had a 63% chance of dying or showing signs of chytridiomycosis. Subsequently, we measured CT$_{\text{max}}$ for the remaining inoculated frogs gradually over time, as swab results indicated that frogs were approaching or had exceeded the threshold infection intensity of 13,700 ZGE. We measured CT$_{\text{max}}$ within 48 hours of swabbing. All frogs were processed by day 56 except for 10 frogs from the low elevation heat pulse treatment that were excluded from analyses because they never reached the threshold infection intensity and eventually cleared their infections, possibly due to their temperature treatments. All inoculated frogs had sub-clinical Bd infections when we measured CT$_{\text{max}}$.

To measure CT$_{\text{max}}$, we placed individual frogs into a perforated container containing a suspended thermocouple. Each frog was brought to room temperature in its permanent enclosure and then transferred to the perforated container and placed in a temperature-controlled chamber programmed to increase from room temperature at a rate of ~1 °C per minute. This rate of temperature increase allows the body temperature of small ectotherms to follow ambient temperature without an appreciable time lag, and is routinely used for measuring CT$_{\text{max}}$.

We used two measures of CT$_{\text{max}}$: onset of spasms and loss of righting ability. Onset of spasms, when frogs began displaying erratic movements such as increased jumping and leg twitches, was the first sign of thermal discomfort. We considered this metric to be a conservative estimate of the temperature at which a frog will seek refuge from high temperatures in the wild. After onset of spasms, at each 1 °C increase in chamber temperature, we quickly opened the chamber, gently moved the container until the frog jumped, and closed the chamber. Loss of righting ability, an animal’s upper heat threshold, was determined when animals were unable to right themselves for three seconds after this manipulation. To minimize stress to the frogs, we elected to record the ambient (i.e., thermocouple) temperature at each behavioral indicator of CT$_{\text{max}}$ for each frog. Frogs were then immediately placed in room-temperature water to recuperate (all frogs survived). After a recovery period following CT$_{\text{max}}$ measurement, we treated Bd infections with Itraconazole.

To determine frog body temperatures at CT$_{\text{max}}$, we later exposed four haphazardly selected _L. spenceri_ of average age sizes to the same program of gradually increasing temperature in the same chamber, following Itraconazole treatment. For each frog, we recorded body temperature at 25 °C, 30 °C, 35 °C, and 40 °C ambient temperature. We measured body temperature with a non-contact infrared thermometer (OS425-LS, Omega Engineering Ltd, Irlam, Manchester, UK; emissivity 0.95)$.^55$ We then modeled the relationship between ambient and body temperatures using linear regression and used this analysis to convert ambient CT$_{\text{max}}$ temperatures to body temperatures for all experimental frogs (y = 0.7985x + 4.0675; R$^2$ = 0.9886; 95% confidence interval for slope = 0.751, 0.846; 95% confidence interval for intercept = 2.503, 5.632).

We used R software for all statistical analyses.$^54$ We used analyses of covariance (ANCOVAs; Anova function in car package; Fox and Weisberg 2011) to test for effects of Bd infection status (infected or uninfected), elevation (high [15 °C] vs. low [18 °C] acclimation treatments), heat exposure (pulse [26 °C or 29 °C] vs. constant acclimation treatments) and interactions between infection status and acclimation on our metrics of CT$_{\text{max}}$ with log-nutritional length as a covariate (n = 69; a = 0.05). To determine whether infection intensity might affect thermal tolerance, we performed separate ANCOVAs using data for infected frogs only, with log-transformed ZGE values as the infection variable (n = 39; a = 0.05).

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B.R.S. conceived of the study. S.E.G., D.S.B., G.M. and B.R.S. designed and coordinated the study. S.E.G., D.S.B., E.A.R., D.A.P. and B.R.S. collected data for the study. S.E.G., R.A.A. and B.R.S. carried out the statistical analyses. S.E.G. wrote the manuscript. D.S.B., E.A.R., G.M., D.A.P., R.A.A., L.S. and B.R.S. critically revised the manuscript. All authors gave final approval for publication.

Additional Information
Competing Interests: The authors declare that they have no competing interests.

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