

Hotspot shelters stimulate frog resistance to chytridiomycosis

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Many threats to biodiversity cannot be eliminated; for example, invasive pathogens may be ubiquitous. Chytridiomycosis is a fungal disease that has spread worldwide, driving at least 90 amphibian species to extinction, and severely affecting hundreds of others^{1–4}. Once the disease spreads to a new environment, it is likely to become a permanent part of that ecosystem. To enable coexistence with chytridiomycosis in the field, we devised an intervention that exploits host defences and pathogen vulnerabilities. Here we show that sunlight-heated artificial refugia attract endangered frogs and enable body temperatures high enough to clear infections, and that having recovered in this way, frogs are subsequently resistant to chytridiomycosis even under cool conditions that are optimal for fungal growth. Our results provide a simple, inexpensive and widely applicable strategy to buffer frogs against chytridiomycosis in nature. The refugia are immediately useful for the endangered species we tested and will have broader utility for amphibian species with similar ecologies. Furthermore, our concept could be applied to other wildlife diseases in which differences in host and pathogen physiologies can be exploited. The refugia are made from cheap and readily available materials and therefore could be rapidly adopted by wildlife managers and the public. In summary, habitat protection alone cannot protect species that are affected by invasive diseases, but simple manipulations to microhabitat structure could spell the difference between the extinction and the persistence of endangered amphibians.

Global biodiversity is decreasing rapidly¹, owing, in large part, to intractable threats such as emerging infectious diseases. Amphibians are the worst-affected vertebrate lineage, and are experiencing devastating declines as a result of the fungal disease chytridiomycosis (caused mainly by *Batrachochytrium dendrobatidis*; hereafter, *Bd*)^{2–4}. Despite a global research effort, we still lack methods for improving the survival and return of species to the wild^{5,6}. A possible solution could lie in exploiting vulnerabilities of the disease; the severity of chytridiomycosis declines with increasing temperatures in many host species⁷ and some hosts can develop resistance after infection with low-virulence *Bd* strains or after infection clearance⁸. *Bd* is an aquatic psychrophile that thrives at low temperatures⁴ but cannot tolerate warmer conditions—temperatures higher than 30.0 °C are lethal in vitro^{9,10}. Moderate increases (from 17.0 to 22.0 °C) or pulses (4 h at 29.0 °C daily) in temperature can reduce *Bd* infections^{11,12}. For some species that are adapted to cooler climates, the benefits of increasing environmental temperature might be limited¹³ (but see also ref. 14), but this strategy should work for warm-adapted species¹³. Seasonal outbreaks of chytridiomycosis in temperate regions typically occur through winter and early spring,

and these periods of high mortality limit natural population recovery and sabotage reintroduction programmes^{5,7,15}.

Artificial hotspot refugia

The risk of disease is heterogeneous across landscapes, and thermal refugia (warmer sites) can enable the persistence and eventual adaptation of the host population^{16,17}. Given the scarcity of natural ‘hotspot’ refugia, one possibility would be to provide artificial shelters that are thermally favourable to amphibians but inhospitable to *Bd*. Such shelters might enable frogs to control and even cure themselves of infection. This approach has the added benefit of stimulating long-term host resistance to the disease, as occurs in some frog species after initial clearance of the infection⁸. By both decreasing disease severity and stimulating long-term resistance, artificial hotspot shelters could enable amphibian populations to persist in areas in which they have been extirped (made locally extinct) by chytridiomycosis.

To develop a management tool against chytridiomycosis, we performed a multifaceted study on temperature, thermoregulation and

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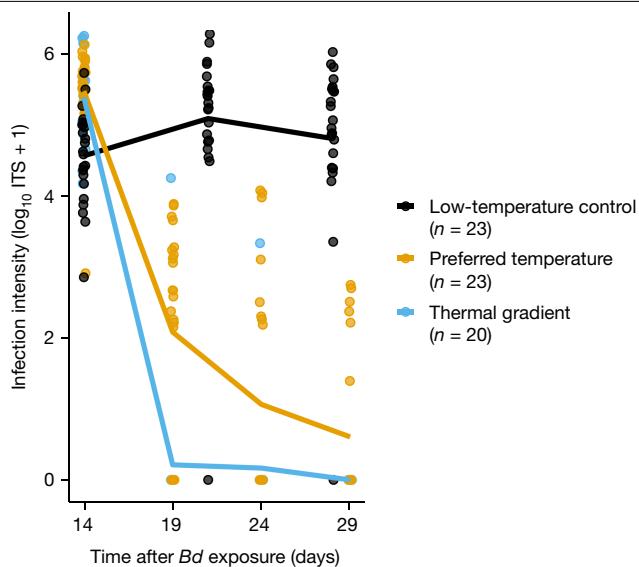


Fig. 1 | Effects of various thermal treatments on chytrid infection. Infection intensities of chytrid fungus (*Bd*) in green and golden bell frogs (*L. aurea*) held under laboratory conditions. Infections are quantified using quantitative real-time PCR of *Bd*-specific internal transcribed spacer (ITS) region DNA counts obtained from skin swab samples. The first 14 days indicate infection intensities during the period when all frogs were held at 19.0 °C to allow infections to develop. After 14 days, ‘thermal gradient’ frogs ($n = 20$ individuals) were placed into thermal gradients, ‘preferred temperature’ frogs ($n = 23$ individuals) were moved to their average preferred temperature (26.4 °C, 29.1 °C or 31.0 °C) and ‘low-temperature control’ frogs ($n = 23$ individuals) were maintained at 19.0 °C. Statistical significance was determined using a linear mixed model (time \times treatment $F_{2,33} = 4.732, P = 0.013$) and post-hoc Tukey tests (maximum mean difference = $-1.9, P < 0.001$, *Bd* gradient versus *Bd* constant temperature on day 19). All data points are shown, and lines are drawn through the mean infection intensities of the respective groups at each time period. The n number denotes biological replicates. This experiment was performed once.

reinfection to test the effect of acquired immunity on frog survival and whether providing access to hotspot shelters can improve *Bd* infection outcomes. Our study builds on more than two decades of research to test the idea that we can manage chytridiomycosis by deploying artificial thermal refugia. This concept has been suggested in the literature (for example, building actively heated microclimates¹⁸), but empirical studies assessing the utility of such refugia are lacking. We performed experiments in the laboratory and in semi-natural outdoor mesocosms, using the endangered green and golden bell frog (*Litoria aurea*). This Australian species has disappeared from more than 90% of its former native range since the arrival of *Bd*⁴. Bell frogs shelter in terrestrial habitats during cooler months and select high body temperature by basking. Hence, bell frogs provide an ideal model with which to evaluate the potential anti-pathogen benefits of thermal refugia for endangered amphibians.

Frogs select high temperatures

We first determined whether bell frogs select temperatures that reduce or eliminate *Bd* infections. The mean \pm standard deviation (s.d.) selected temperature (T_{sel}) of frogs ($n = 73$) using thermal gradients^{19–21} was 29.0 ± 3.9 °C, which is unfavourably high for *Bd*. To evaluate, first, whether frogs shifted their T_{sel} after *Bd* infection, and, second, how temperature affected disease progression, we placed frogs into four treatment groups after determining their baseline T_{sel} : no *Bd* thermal gradient; *Bd* thermal gradient; *Bd* constant temperature (similar to individual T_{sel}); and a *Bd*-exposed low-temperature control held at 19.0 °C

(same as the *Bd* control for ‘prior exposure’ experiments, described below). We determined whether T_{sel} remained consistent across trials to validate the repeatability of T_{sel} measurements using the no *Bd* gradient group. We placed uninfected frogs back into the thermal gradient a second time and found that they did not differ significantly across trials (estimate = -0.13 , standard error (s.e.) = $0.41, t = -0.31, P = 0.76$). We then determined whether infected frogs in the *Bd* gradient group shifted their T_{sel} after *Bd* infection (that is, evidence of behavioural fever). We exposed frogs to *Bd*, allowed their infections to increase over two weeks and placed them back into the thermal gradient. There was no evidence of behavioural fever, with the T_{sel} of frogs infected with *Bd* being similar to their baseline uninfected T_{sel} (estimate = 0.30 , s.e. = $0.51, t = 0.59, P = 0.56$). We next determined how individual variation in thermal preference can affect a frog’s susceptibility to *Bd* infection, as has been seen in other systems²⁰. We infected frogs, allowed their infections to grow over two weeks and placed them either at constant temperatures similar to their individual T_{sel} (*Bd* constant temperature: held at 26.4 °C, 29.1 °C or 31.0 °C) or in thermal gradients in which they could freely thermoregulate (*Bd* thermal gradient). We compared these two groups of frogs to another group that were infected and held at 19.0 °C (cold temperature control). Frogs at a constant temperature of 19.0 °C had the highest infection intensities, whereas frogs held at 26.4 °C, 29.1 °C or 31.0 °C all had lower and similar infection intensities (and were thus grouped together for further analysis; Fig. 1 and Extended Data Fig. 1). Finally, infection intensities reduced more rapidly in frogs in the *Bd* gradient group than they did in frogs in the *Bd* constant temperature group (time \times treatment $F_{2,33} = 4.732, P = 0.013$; Tukey test mean difference = $-1.9, P < 0.001$, *Bd* gradient versus *Bd* constant temperature on day 19; Fig. 1).

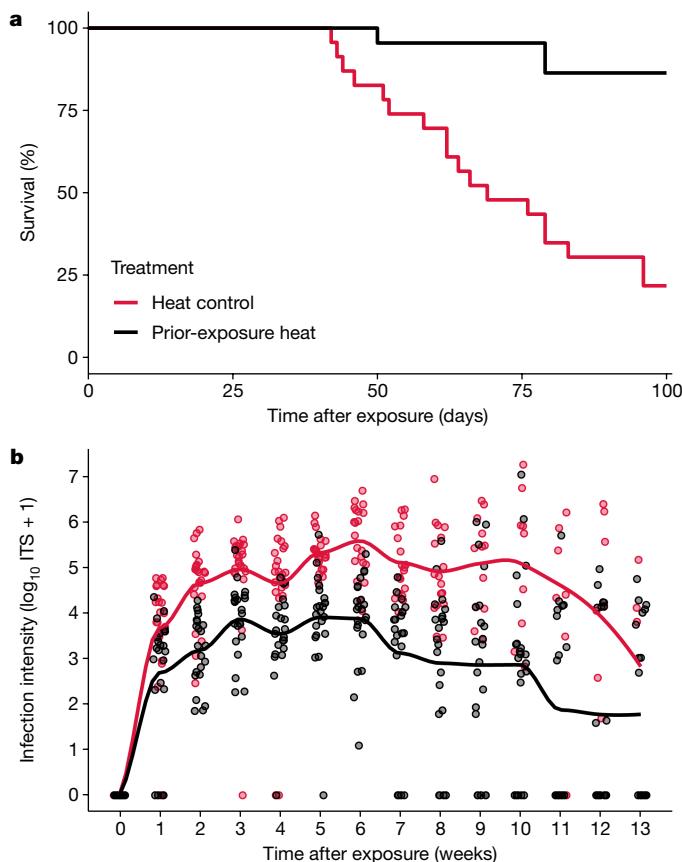
Pathogen-experienced frogs are resistant

We infected frogs with *Bd*, then cleared their infections after six weeks (32.0 °C exposure for 14 days; referred to as prior exposure) to test whether heat-cleared frogs developed resistance to chytridiomycosis. For comparison, we sham-exposed frogs to a sterile inoculum, monitored them for six weeks and treated them with heat (referred to as heat control). Heat treatment cleared all infections, and all except one frog (22/23) survived until the next *Bd* exposure in the prior-exposure group. All (23/23) frogs in the heat control group survived heat treatments. We found that prior infection with *Bd* and heat clearance led to improved survivorship ($\chi^2 = 19.69$, degrees of freedom (df) = 1, $P < 0.0001$; Fig. 2a) and reduced infection burdens over time after re-exposure (treatment \times time $F_{13,459} = 3.92, P < 0.0001$; Fig. 2b). Previously infected frogs were 23 times more likely to survive the second exposure, compared with conspecifics that had been heat-treated but had no prior infection (19/22 or 86% survivorship compared with 5/23 or 22% survivorship; odds ratio = 22.8, 95% confidence interval (CI) 4.74 to 109, $P = 0.0001$). All unexposed controls remained *Bd* negative and survived until the end of the experiment.

We also determined the long-term effects of heat treatments on *Bd* susceptibility by comparing the heat control group described above with a group of frogs that were sham-exposed, not heat-treated, and then exposed to *Bd* (referred to as *Bd* control). Heat treatments did not significantly affect infections or survivorship (Supplementary Results, Extended Data Fig. 2). Thus, heat treatment alone appears safe and did not influence subsequent *Bd* infection. Laboratory *Bd* exposure studies were run at 19.0 °C, which is environmentally relevant for *L. aurea* but favourable for *Bd*.

Hotspot shelters prevent disease

To test whether access to high temperatures and prior pathogen exposure could reduce the effects of chytridiomycosis in a semi-wild setting, we used artificial refugia in outdoor mesocosms. Each mesocosm was



identically set up with water, gravel, artificial plants, terracotta pots and thermal refugia (black, ten-hole masonry bricks placed inside a greenhouse; see Extended Data Fig. 3); bricks are a preferred shelter for these frogs²¹. To assess the ability of greenhouses to act as hotspot shelters, they were left unshaded in half of the mesocosms and were covered in green 90% shade cloth in the other half as a control (Extended Data Fig. 3). To determine the effects of prior pathogen exposure, half of the frogs in each treatment mesocosm ($n = 4$ unshaded greenhouse mesocosms; $n = 4$ shaded greenhouse mesocosms) had prior *Bd* infection, and the remaining half did not (naive). Half of the prior-exposure (5/10) and half of the naive (5/10) frogs in each mesocosm were infected with *Bd* seven days before being placed inside; the remaining frogs were *Bd* negative. We also maintained four control mesocosms in which all frogs were *Bd* negative ($n = 2$ unshaded greenhouse mesocosms; $n = 2$ shaded greenhouse mesocosms). We recaptured frogs at 1, 2, 4, 6, 8, 10 and 15 weeks after release into mesocosms, and we collected data on

body temperature, habitat choice and body mass and took skin swabs to measure infection prevalence and intensity. Because *L. aurea* is an endangered species²², we ran this study from mid-winter into spring (July–November in Australia) so that rising ambient temperatures over this period minimized mortality (high temperatures curtail *Bd* infections). Because mortality from chytridiomycosis is directly correlated with infection burden⁸, we used infection load as a proxy for disease impacts.

Inside thermal shelters, mean maximum daily temperatures were approximately 4.5 °C hotter in unshaded than in shaded greenhouses (Extended Data Table 1). Frogs were captured in the thermal shelters 74% of the time—four times more than would be expected by chance on the basis of the proportion of the ground surface covered by the shelters within each enclosure ($\chi^2 = 2941.6$, $df = 1$, $P < 0.0001$, Fig. 3e). At the times of capture, frogs in mesocosms with unshaded greenhouses were 3.1 °C warmer (mean = 20.0 ± 5.5 °C) than frogs in mesocosms with shaded greenhouses (mean = 16.9 ± 3.7 °C, estimate = -2.69 , s.e. = 0.25 , $t = -10.7$, $P < 0.0001$; Fig. 3a), with similar body temperatures in prior-exposure and naive frogs (estimate = 0.15 , s.e. = 0.28 , $t = 0.55$, $P = 0.58$). Infection intensities of frogs were higher in mesocosms with shaded greenhouses than in those with unshaded greenhouses (estimate = 1.96 , s.e. = 0.53 , $t = 3.7$, $P < 0.01$; Fig. 3b). Prior exposure reduced infections in both unshaded and shaded greenhouse mesocosms (estimate = -1.2 , s.e. = 0.51 , $t = -2.4$, $P = 0.02$), but especially in the shaded greenhouse mesocosms (Fig. 3c,d). At week 15, we recaptured similar numbers of frogs from unshaded greenhouse and shaded greenhouse mesocosms (59/80 and 58/80 frogs, respectively) and from prior-exposure and naive groups (62/80 and 55/80, respectively). Recapture rates were also similar between control mesocosms (50/80 = 63%) and chytrid-infected mesocosms (117/160 = 73%). Frogs are soft bodied and decay very quickly, therefore the exact causes of mortality among the treatments could be a variety of factors, including chytridiomycosis, cannibalism (very common in this species) or a failure to thrive.

Discussion

Both in the laboratory and in outdoor mesocosms, endangered bell frogs chose high-temperature hotspots, enabling them to cure themselves of otherwise fatal chytridiomycosis and generate resistance to subsequent exposures. Specifically, frogs that had cleared themselves of *Bd* infection were more resistant when they were exposed to the pathogen six weeks later, under thermal conditions that cause lethal infection in frogs with no prior *Bd* infection. In combination, these results suggest that the provision of artificial hotspot shelters, passively heated by sunlight, can substantially increase the survival of endangered frogs in both the short and the long term. The implications for the management of endangered amphibians in the field are clear: in situ thermal refugia could limit the effects of *Bd* by reducing individual infection load and population-level prevalence, generating frogs that are resistant to subsequent chytridiomycosis. Reducing annual winter die-offs that limit many populations²³ could increase the abundance of frogs and enable the recolonization of habitats.

In our laboratory experiments, bell frogs that could behaviourally thermoregulate, or that were kept at temperatures similar to their T_{sel} , carried lower *Bd* infection loads than did frogs held at 19.0 °C. Notably, the opportunity to thermoregulate led to reduced infection, even compared with frogs that were held constantly above the thermal maximum of *Bd* in vitro (30.0 °C) (ref. 9). Therefore, a frog that can modulate its body temperature in a heterogeneous environment may be better able to fight the pathogen than would be expected from a simple ‘thermal threshold’ model. Consistent with this idea, experiments with the congeneric *Litoria spenceri* found that daily thermal fluctuations improved disease outcomes in the laboratory¹².

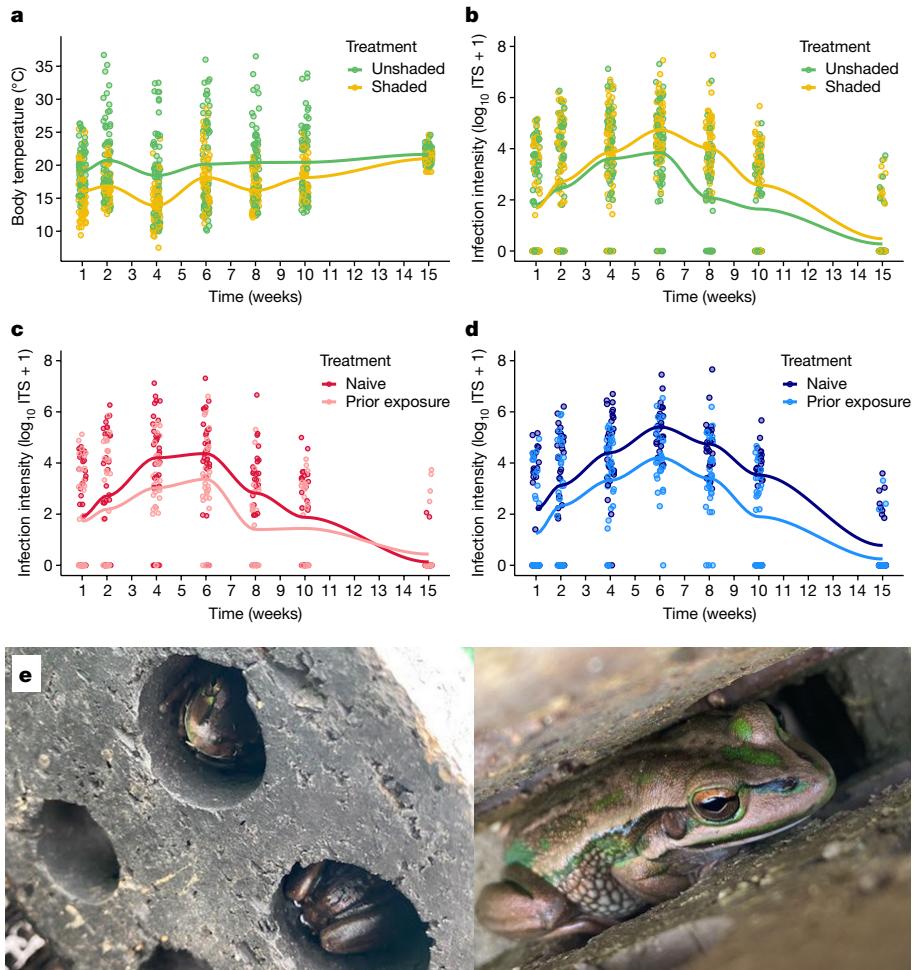


Fig. 3 | Effects of higher operative temperatures and prior pathogen exposure on frog body temperature and chytrid infection. **a**, Body temperatures of green and golden bell frogs (*L. aurea*) in outdoor mesocosms at the time of sampling. The graph shows data for *L. aurea* in mesocosms containing unshaded greenhouses versus those containing shaded greenhouses ($n = 80$ individuals per treatment). Differences in body temperature were determined using a generalized additive mixed effect model (GAMM, treatment estimate = -2.69 , s.e. = 0.25 , $t = -10.7$, $P < 0.0001$). Body temperatures were 3.1°C higher on average in unshaded versus shaded greenhouse hotspot shelters ($20.0 \pm 5.5^\circ\text{C}$ versus $16.9 \pm 3.7^\circ\text{C}$ (mean \pm s.d.)). **b–d**, Infection intensities of green and golden bell frogs *L. aurea* after exposure to the chytrid fungus *Bd*. All data points are shown, and lines are drawn through each respective group's mean weekly infection intensity. Data points have been jittered along the x axis by adding random error to weekly values for clarity. We used GAMMs

to determine significant differences in infection intensities between groups. **b**, Differences in infection intensities between unshaded and shaded greenhouse treatments ($n = 80$ individuals per treatment). Infections were substantially higher in shaded compared with unshaded greenhouse treatments (estimate = 1.96 , s.e. = 0.53 , $t = 3.7$, $P < 0.01$). **c**, Infection intensities between naive and prior-exposure frogs in unshaded treatments ($n = 40$ naive; $n = 40$ prior exposure). **d**, Infection intensities between naive and prior-exposure frogs in shaded greenhouse treatments ($n = 40$ naive; $n = 40$ prior exposure). Significant differences in infection intensity between naive and prior-exposure frogs across greenhouse treatments were determined using a GAMM. Prior exposure led to lower infection intensities compared with naive frogs (estimate = -1.2 , s.e. = 0.51 , $t = -2.4$, $P = 0.02$). These experiments were done once. **e**, *L. aurea* individuals using thermal refugia in the mesocosm experiment. The n number denotes biological replicates. Photo credit: A.W.W.

We found that heat-enabled recovery from a prior *Bd* infection conferred subsequent resistance to chytridiomycosis, as reflected in survival rates as well as infection loads. In this respect, bell frogs resemble leopard frogs from North America (*Rana onca* and *Rana pipiens*). Laboratory studies reported that prior exposure to a hypovirulent *Bd* strain or infection followed by clearance with an antifungal agent conferred long-term resistance to chytridiomycosis (up to 34 weeks)⁸. In another set of experiments, Cuban tree frogs (*Osteopilus septentrionalis*) with multiple *Bd* infections that were cleared with heat²⁴ showed resistance to *Bd* infection 11 days after reinfection.

Encouragingly, our laboratory results directly translated to our outdoor mesocosms: frogs exploited the availability of higher temperatures to reduce their pathogen burdens, and prior exposure to *Bd* markedly reduced vulnerability to infections. An interaction between these two effects suggests that the benefits of prior exposure to *Bd* were

greatest for frogs with less opportunity to thermoregulate. This result is consistent with reports of temperature independence in components of the innate and adaptive immune system that comprise an inflammatory response²⁵. In essence, the protective effect of prior infection accrues even under cool conditions, but is partially masked when a frog is able to select high temperatures and can more rapidly recover from chytridiomycosis. Indeed, higher operative temperatures were a major driver of lower infection intensities in unshaded versus shaded greenhouses (Fig. 3b), possibly reflecting thermal tolerances of the pathogen⁹ and/or more effective innate immune responses in hotter frogs, as seen in red-spotted toads (*Anaxyrus punctatus*)²⁵. Further research on the immune mechanisms that drive these trends is needed.

Because our shaded (control) shelters also reached temperatures that inhibit *Bd* (Extended Data Table 1), our results are likely to underestimate the benefits of thermal shelters. The average difference in

body temperatures measured at capture between the two treatments was only 3 °C, but the effects on infection intensities were still considerable. The shaded shelters were used to generate identical structures with lower operative temperatures, without the confounding effects of habitat differences. We would expect hotspot refugia to have even greater effects *in situ* if the only alternative shelters were in cooler habitats that favour disease progression, such as in aquatic vegetation²⁶. Such habitats reached an average daily high temperature of only around 20–21 °C (see Extended Data Table 1). Our experiments test the idea that increasing operative temperatures with artificial refugia can reduce the severity of infection in a field setting. Our structures worked well, but any design that is favoured by frogs and provides high operative temperatures should be similarly effective.

Plausibly, an ectotherm could enhance its ability to fight thermally dependent pathogens by modifying its body temperature (behavioural fever²⁷). However, this response is far from universal. In the laboratory, southern toads (*Anaxyrus terrestris*) reduce their ranaviral infections through behavioural fever²⁷. Field research on Panamanian golden frogs (*Atelopus zeteki*) reported increased body temperatures after the arrival of *Bd* in the environment, suggesting behavioural fever²⁸, but laboratory experiments with a diversity of species, including *A. zeteki*, found no evidence for behavioural fever²⁰. Consistent with the latter study, we found no evidence for behavioural fever either in the laboratory or in mesocosms. Instead, bell frogs are thermophilic regardless of infection status, preferring temperatures that exceed the thermal optimum for *Bd*. Similarly, thermophilic species probably persist in *Bd*-infected areas only if they have access to high temperatures. For example, armoured mist frogs (*Litoria lorica*) persist in sites where large boulders absorb heat from the sun all day and provide warm basking sites at night, whereas conspecific populations in more densely forested areas without such basking sites were extirpated by *Bd*²⁹. By contrast, in the cool-adapted Panamanian golden frog (*A. zeteki*), moderate temperatures higher than the thermal optimum of the species seem to facilitate *Bd* growth in the laboratory¹³ (but see also ref. 14).

For thermophilic and *Bd*-susceptible amphibians in areas with limited access to warm sites, our artificial hotspot shelter design offers a simple, inexpensive, low-risk conservation intervention. Although we have only tested this intervention in one species, translation to other species with similar ecologies should be possible. Worldwide, at least 501 species of amphibians have declined owing to *Bd*, and many of these taxa are thermophilic⁴. Research into spatio-temporal heterogeneity in thermal pathogen refugia, and into the role of such refugia in enabling population persistence, could identify candidate systems in which artificial hotspot shelters could be trialled²⁹. Our results indicate that thermal refugia can help an endangered species that has been devastated by chytridiomycosis—an unusually positive outcome in contemporary conservation research. Because frogs that are heat-cured of their infections have greater resistance to future exposures (even at cooler temperatures), such thermal refugia can act as ‘self-propagating resistance engines’ without the need for intensive management intervention after initial establishment. Infected frogs that use the shelters will be more likely to recover from their infections and will be more resistant to subsequent infections. An increase in the number of *Bd*-resistant individuals could facilitate population persistence and enhance the opportunity for ‘evolutionary rescue’, whereby alleles for *Bd* resistance can arise and spread. The combination of hotspot shelters with infection-then-cure could also increase the success of captive breeding for release programmes to re-establish extirpated populations.

Thermal refugia could have broader applicability to other wildlife diseases, especially other fungal pathogens that increase in severity in cooler months, such as bat white nose syndrome and snake fungal disease^{30,31}. Therefore, providing thermal refugia could mitigate the worst impacts of disease during seasonally low temperatures. One potential disadvantage of thermal refugia is an increased risk of desiccation,

but optimized designs could maintain hydric as well as thermal regimes that are favourable to frogs. Hotter shelters might also increase metabolic demands, although we found no difference in mass change between frogs in our two temperature treatments. Changing thermal regimes could also alter breeding phenology, but again we saw no such shift in our mesocosm study (breeding occurred simultaneously in shaded and unshaded experimental enclosures after the experiment ended). Further investigations into how changing winter thermal regimes affect growth, breeding, immune competency and offspring fitness are warranted.

Amphibians are such a diverse group that no single management approach will be suitable for all species; a silver bullet is not a sensible approach for holistically addressing the monumental challenge of chytridiomycosis³². Nonetheless, a useful tool for even one threatened or endangered species is a cause for optimism, especially given the current paucity of options for species affected by chytridiomycosis in nature. As for most wildlife diseases, we will probably need a variety of approaches to buffer affected species against chytridiomycosis. For example, targeted genetic interventions might be a more sensible approach for some critically endangered species that are affected by *Bd*³³. Our results indicate that thermal refugia can provide a strategy with immediate usefulness to conserve an endangered species that has been devastated by chytridiomycosis. Moreover, the methods that we use can readily be modified for other host-pathogen systems in which host thermal biology can be exploited to give hosts the edge over disease.

Research into chytridiomycosis provides a framework for progress from the discovery phase through to application. Early epidemiological and ecological studies identified temperature as a key determinant of chytridiomycosis^{7,29}, and researchers’ understanding has been refined by field models of population recovery³⁴ and laboratory experiments¹¹. Two decades on, we have exploited this knowledge, along with new insights into host resistance, to devise an intervention ready to be used and assessed for local populations. Emerging diseases are increasingly affecting biodiversity, and new interventions are needed because habitat conservation alone cannot shield endangered species against invasive pathogens.

Online content

Any methods, additional references, Nature Portfolio reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at <https://doi.org/10.1038/s41586-024-07582-y>.

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Article

Methods

Husbandry in the laboratory

Frogs were housed individually in plastic aquaria ($21 \times 6 \times 12$ cm (length \times width \times height)) with 500 ml aged tap water that was replaced weekly. Each frog was provided with a plastic storage container ($12 \times 6 \times 10$ cm, size 10 plastic storage tote, Bunnings Warehouse) that provided a shelter and a surface to escape the water and bask. We fed frogs three times weekly with one or two large crickets each time. We maintained frogs at 19.0°C and with a 12-h photoperiod under UV lights (Reptisun 5.0 UVB). The temperature of 19.0°C represents the average monthly high in the Austral winter (Sydney, New South Wales) and is within the range of ambient temperatures in which the effects of chytridiomycosis are severe⁷. We focused our efforts on post-metamorphic life stages because immune function in amphibians can vary markedly between tadpole, metamorphic and post-metamorphic life stages³⁵.

Outdoor mesocosms

Mesocosms ($n = 12$) were constructed from 10,000-l polyethylene aquaculture tubs (1 m deep, 3.5 m diameter) that were set up in the Macquarie University Fauna Park ($33.7968^\circ\text{S}, 151.1244^\circ\text{E}$). Each mesocosm was partially filled with gravel and water to a depth of 30 cm to simulate an aquatic–terrestrial interface in which *L. aurea* are typically found³⁶. All mesocosms contained a symmetrically arranged set of artificial plants and terracotta pots to provide a variety of shelter types. We placed a pile of black-painted bricks (three layers, each of three ‘common’ bricks that contained ten holes per brick) in the middle of each mesocosm adjacent to the water, to create a preferred shelter site for *L. aurea*³⁴. We placed a greenhouse (Greenlife Square Drop Over Greenhouse with PE Cover, $900 \times 900 \times 1,020$ mm; Extended Data Fig. 3) over the top of the brickpile in each mesocosm, with an opening on the lower edges of the greenhouse (by lifting and securing the greenhouse covers with clips, creating a 5-cm gap between the ground and the bottom edge of the cover). We stretched bird netting over the top of each mesocosm (Diamond EcoNetting white $10 \text{ m} \times 10 \text{ m} \times 5 \text{ mm}$) to contain frogs and prevent predator ingress. Frogs were housed in groups (19 or 20) in each mesocosm and were fed (3 crickets per frog) once weekly in the Austral winter (August) and twice weekly in spring (September–November).

Bd exposures and detection of infection

We used two *Bd* strains for our experiments. On the basis of the most recent genetic data, all *Bd* isolates in Australia descend from a single global panzootic lineage (GPL) strain that may have arrived via the Port of Brisbane in 1978 (refs. 37,38). The strains we used, however, have yet to be sequenced. The first *Bd* strain (Yançep ‘Yançep-Litoria moorei-2019-RW’, AUS 79) was isolated by R. Webb (James Cook University) from an apparently healthy *Litoria moorei* in Yançep National Park, Western Australia, using previously described methods³⁹. We isolated the second *Bd* strain (LFMU ‘Macquarie University-Litoria fallax-2020-AW’, AUS 86) from an apparently healthy wild juvenile *Litoria fallax* caught in the Macquarie University Fauna Park ($33.7968^\circ\text{S}, 151.1244^\circ\text{E}$) using previously described methods⁴⁰. Both isolates were on their third passage at the time of use. We inoculated frogs with one million *Bd* zoospores once a day for three days to achieve high infection rates⁴¹. We grew *Bd* in 1% tryptone broth medium until conspicuous clumps of *Bd* growth formed. We then pipetted 0.5 ml of the liquid medium onto 9-cm Petri dishes containing 1% tryptone and 1% agarose. We allowed *Bd* to grow on Petri dishes at $20.0\text{--}22.0^\circ\text{C}$ until zoospores were observed swimming on plates. The dishes were flooded with 1–2 ml sterile 1% tryptone broth and decanted into the final volume. We counted zoospores using a haemocytometer and calculated the volume needed to achieve a 1,000,000 *Bd* zoospore inoculation. We diluted the zoospore inoculum to a concentration

of 1,000,000 per ml using 1% tryptone broth. We exposed frogs to zoospores by pipetting inoculum over each frog’s ventral surface and into housing containers. We then allowed frogs to remain in their containers without water changes for four days. Unexposed controls were given a sham exposure of sterile 1% tryptone and 1% agar plates. We took skin swabs of frogs^{41,42}, extracted DNA from swabs using PrepMan Ultra Sample Preparation Reagent⁴³ and used an optimized quantitative PCR assay to detect and quantify *Bd* infections⁴⁴. To prevent infection transmission and contamination we used a new, clean pair of nitrile gloves when swabbing each frog.

Infection clearance

We heat-cleared frogs of their *Bd* infections by placing them into plant growth chambers (Conviron A1000) held at 32.0°C for 14 days. Thermal maxima for *Bd* vary among isolates, but prolonged exposure to temperatures higher than 30.0°C in vitro is generally lethal for the pathogen¹⁰.

Selected body temperatures of non-infected frogs

Individual frogs were placed inside thermal gradients (range $10\text{--}38.0^\circ\text{C}$; Extended Data Fig. 4), using an experimental set-up similar to that described in a previous study¹⁹. Sphagnum moss on the floor of each channel was kept moist by spraying with water. We used an infrared dot laser thermometer (HCJYET HT-830) to measure the temperature at the centre of each frog’s dorsal surface and from the substrate beside the frog at four-hour intervals, four times daily over four days. We refer to these measurements of body temperatures as the selected temperature (T_{sel}).

Evaluating changes in selected body temperatures after pathogen challenge. After baseline T_{sel} measurements, we returned individuals to their containers and divided them into four treatment groups. These comprised: (1) ‘no *Bd* then gradient’—a non-exposed *Bd*-naïve control group in which frogs were given a sham inoculum and then allowed to thermoregulate inside the gradients; (2) ‘*Bd* then gradient’—a *Bd*-exposed group in which frogs were given the opportunity to thermoregulate after infection; (3) ‘*Bd* then constant temperature’—a *Bd*-exposed group in which infected frogs were held at a constant temperature inside the plant growth chambers on the basis of their individual average baseline non-infected T_{sel} ($26.4^\circ\text{C}, 29.1^\circ\text{C}$ or 31.0°C); and (4) ‘cold temperature control’—a *Bd*-exposed group of frogs that was held at 19°C . Constant temperature treatments were limited by the number of incubators (plant growth chambers), so we divided frogs into three groups and used the average of their T_{sel} within those three groups for each of the treatments. We used *Bd* isolate LFMU for this set of experiments. Frogs were held at 19°C for two weeks after infection before being placed into their experimental thermal treatments. For the no *Bd* then gradient group, frogs were given a sham exposure two weeks before being placed into *Bd* gradients. After placement into thermal treatments, frogs were swabbed every 5 days (that is, 3 swabbing periods over 15 days) to quantify *Bd*.

Increasing operative temperatures in refuge sites

On the basis of laboratory work showing that high individual selected body temperatures enable frogs to survive *Bd* infection²⁰, we designed retreat sites to attract frogs and to create thermal regimes unfavourable to *Bd*. In pilot studies, we found that piles of black-painted bricks exposed to sunlight exhibited high temperatures. Because most potential refuges nonetheless remained at temperatures lower than 30.0°C on cool days, we further increased ambient temperatures inside brickpiles by placing small greenhouses (Greenlife Large Drop Over Greenhouse with PE Cover, $1,850 \times 950 \times 1,020$ mm) over them. This resulted in temperatures within brickpiles averaging more than 3.0°C higher than adjacent microhabitats on land or in water.

Prior inoculation with *Bd* to increase resistance to chytridiomycosis

We conducted a laboratory challenge study to explore the effects of prior exposure to *Bd* on a frog's resistance to chytridiomycosis. We divided frogs randomly into four groups ($n = 23$ per group): prior exposure, heat control, *Bd* control and negative control. We exposed frogs in the prior-exposure group to the Yanchep isolate of *Bd*, allowed their infections to grow for six weeks, cleared those infections using heat, allowed the frogs to re-acclimatize to 19.0 °C for six weeks and then exposed them to the *Bd* isolate LFMU. We used this isolate for the second exposure because (1) it is the strain of *Bd* present in a future translocation site for *L. aurea*, (2), the Yanchep strain appeared to have low virulence against *L. aurea* and (3) cross-isolate protection is likely⁸. Heat control frogs were given a sham exposure, heat-treated six weeks later, kept at 19.0 °C for six weeks and then exposed to LFMU. We controlled for heat effects by including a *Bd* control group that was sham-exposed, not heat-treated (that is, was kept at 19.0 °C), and then was exposed to LFMU. The negative control group consisted of frogs that were sham-exposed to *Bd*, not heat-treated and then given a second sham exposure. After the final *Bd* or sham exposure, we monitored frogs for a further 14 weeks, conducting weekly skin swabbing to quantify *Bd* infections. We also monitored frogs daily for mortality and clinical signs of chytridiomycosis. We ran these experiments at 19.0 °C because this is the average daily high temperature in spring in Sydney, New South Wales. Therefore, that temperature is environmentally relevant, but would favour *Bd*.

Assessing the effects of prior *Bd* exposure and thermal refugia on disease dynamics

We evaluated the effects of, first, prior pathogen exposure followed by clearance, and, second, increased operative temperatures on *Bd* infection dynamics in *L. aurea* in a semi-wild setting. Because *L. aurea* is an endangered species and is highly susceptible to chytridiomycosis³², we ran this study from mid-winter into spring (July–November). The rising ambient temperatures over this period allowed us to minimize mortality (high temperatures curtail *Bd* infections) while still revealing how our treatments affected infection dynamics. We used 12 large outdoor mesocosms (see above) divided into 2 thermal treatments: unshaded and shaded. Unshaded greenhouses in mesocosms were set up as described above (see Extended Data Fig. 3a,b) with black-painted brick shelters (see Extended Data Fig. 3c) inside greenhouses. Shaded mesocosms were identical except that the greenhouses were covered with 90% green shade cloth. We placed thermal dataloggers (iButton Thermocron, Maxim Integrated) in replicated microhabitats in each mesocosm to measure ambient temperature regimes. We placed one datalogger in the deepest part of the water, one under a mat of artificial (plastic) vegetation on land and two in bricks (within each three-layer stack of bricks, one in the lowest level and one in the highest level). We set up eight treatment mesocosms (four with unshaded greenhouses and four with shaded greenhouses) and four control mesocosms. Within each treatment mesocosm we randomly placed ten frogs with no prior infection (*Bd*-naive) and ten previously exposed frogs. Because we were interested in how *Bd* infections and infection status changed over time, we only infected ten (half) of the frogs in each mesocosm (five previously exposed and five naive) and left ten others free of *Bd* (five previously exposed and five *Bd*-naive) at the start of the experiment. For frogs with prior *Bd* exposure, we used previously exposed frogs from the laboratory experiments that had either one ($n = 63$) or two ($n = 37$) previous *Bd* exposures before heat clearance. Because previously exposed frogs were infected and cleared in different ways (see Supplementary Table 1), we stratified prior treatments across experimental groups and mesocosms. Our 4 control mesocosms (2 unshaded and 2 shaded) contained 19 or 20 *Bd*-negative frogs. Before being released into mesocosms, frogs were individually microchipped

(Trovan ID-100A/1.4 Mini Transponder) and acclimatized to 19.0 °C for two weeks. *Bd*-infected frogs were exposed to *Bd* strain LFMU a week before release into mesocosms and *Bd*-negative frogs were given a sham exposure at the same time. We recaptured frogs at 1, 2, 4, 6, 8, 10 and 15 weeks after release into mesocosms, recording their microhabitat use (where in the mesocosm the frogs were captured) and temperatures of their bodies and of the adjacent substrate. We took *Bd* skin swabs at each capture event, determined mass using a digital scale and noted overall health and any clinical signs of chytridiomycosis.

Statistical analyses

We performed our analyses using R v.4.1.0 (ref. 45). We used GAMMs to assess shifts in T_{sel} in frogs in the laboratory. Treatment (*Bd* infection or sham pathogen exposure), time and their interactions were used as variables with individual frog ID set as a random effect. To assess changes in infection intensities of frogs in the various temperatures (constant or thermal gradient) we used linear mixed models. Treatment, time and their interactions were set as fixed effects and individual frog ID was used as a random effect. We used the same type of analysis to assess changes in infection intensities in frogs in the prior-exposure experiments. We used Mantel–Cox tests to assess differences in survivorship. For the mesocosm study, we used GAMMs to assess differences in body temperature and infection intensities. Our variables for those analyses included: treatment mesocosm (shaded or not), infection treatment (infected or not), prior-exposure treatment (had a prior exposure or not) and overall treatment (uninfected control or chytrid-positive mesocosm). Individual frog ID was used as a random effect.

Ethics and inclusion

The research included Australian and international researchers. The findings of the research are immediately relevant for endangered Australian frogs. Roles and responsibilities were discussed when initiating the research and were discussed throughout experiments. The research was possible in Australia with appropriate ethics approvals (Macquarie University Animal Ethics ARA 2019/041). We carefully chose our study species and strains of the fungal pathogen to reduce biosafety risks. All researchers were required to meet Macquarie University standards (for example, training and risk assessments). We have cited relevant literature including research conducted on amphibians and chytridiomycosis in Australia.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

Our data are available through figshare at <https://doi.org/10.6084/m9.figshare.23672805> (ref. 46). Source data are provided with this paper.

Code availability

The R code used for our GAMMs is available through GitHub: <https://github.com/erinsauer/Waddle-et-al.-Hotspot-shelters>.

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Author contributions Conceptualization: A.W.W., S.C., E.L.S. and R.S. Methodology: A.W.W., S.C., R.S. and E.L.S. Investigation: A.W.W., A.A., H.G., I.D., R.S. and S.W.K. Validation: A.W.W., Y.L., E.L.S. and R.S. Visualization: A.W.W., E.L.S. and Y.L. Funding acquisition: A.W.W., R.S. and S.C. Data curation: A.W.W., C.M., J.A.F., P.T.C., R.S., Y.L. and E.L.S. Writing (original draft): A.W.W. Writing (review and editing): A.W.W., L.F.S., L.B., R.S. and Y.L.

Competing interests The authors declare no competing financial interests.

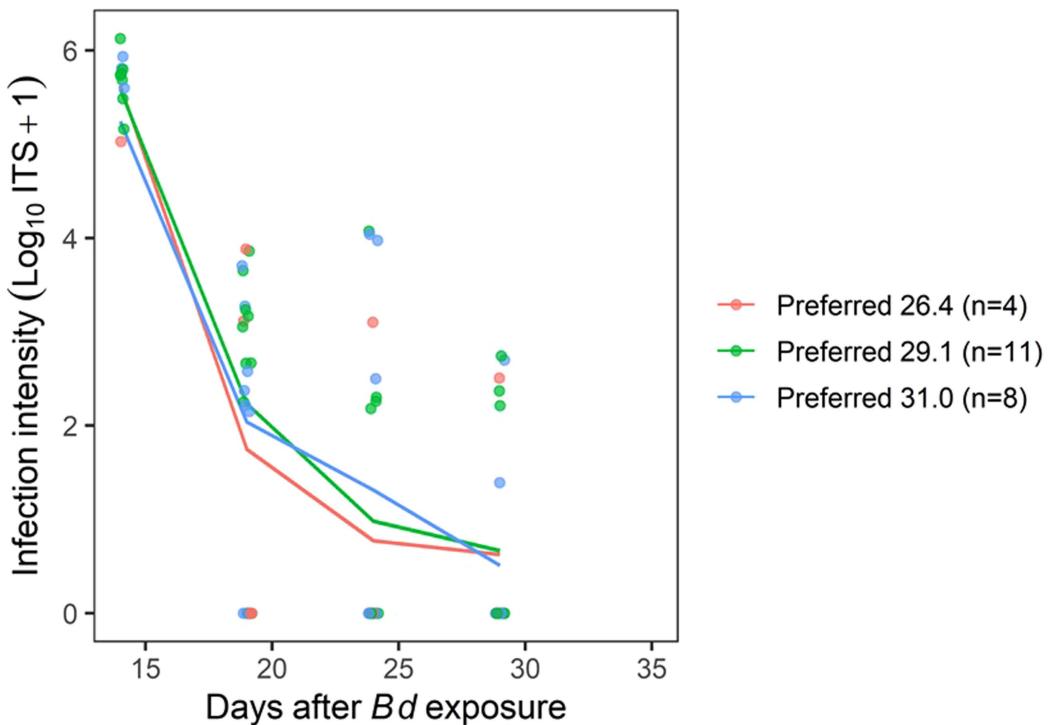
Additional information

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s41586-024-07582-y>.

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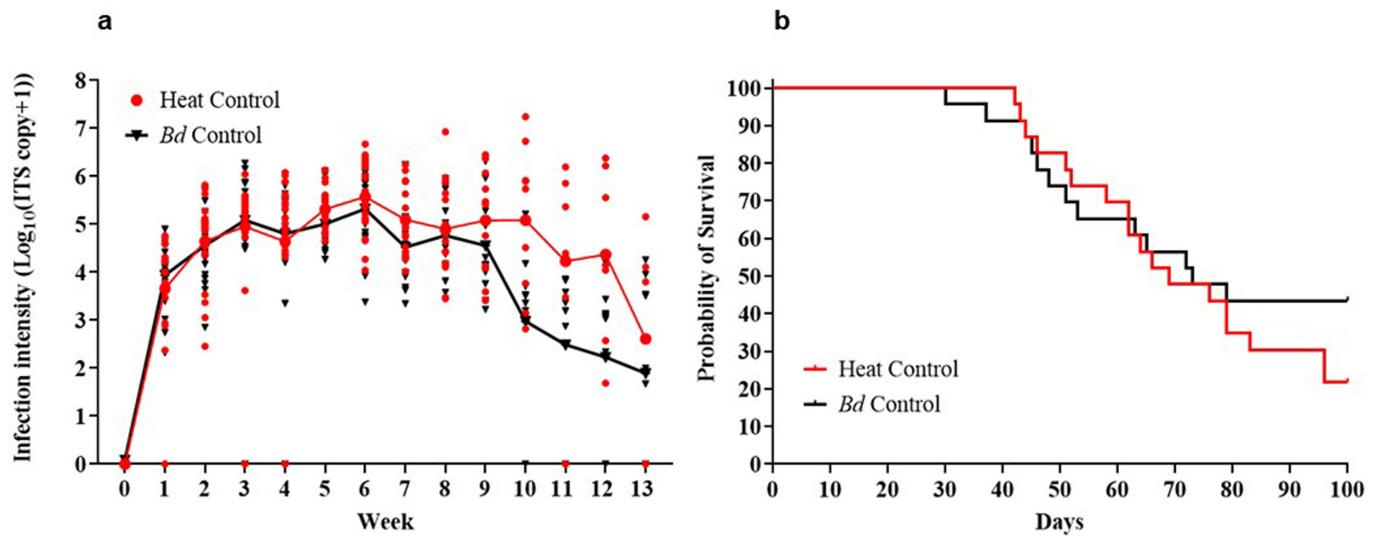


Extended Data Fig. 1 | Effects of various preferred temperature regimes

on chytrid infection. Infection intensity data for green and golden bell frogs (*L. aurea*) that were exposed to *Bd*. Frogs were infected with *Bd*, held at 19.0 °C

for 14 days and then placed at one of three temperatures (26.4 °C, 29.1 °C or 31.0 °C), on the basis of the temperatures selected by those individuals in a previous study.

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Extended Data Fig. 2 | Effects of heat treatments on chytrid infection intensity and survivorship. a, b, Infection intensity (a) and survivorship data (b) for green and golden bell frogs (*L. aurea*) that were exposed to *Bd*.

Heat control frogs ($n = 23$) were treated with heat and then exposed to *Bd*, whereas *Bd* control frogs ($n = 23$) had no heat treatment before *Bd* exposure.



Extended Data Fig. 3 | Experimental treatments and design of hothouse thermal shelters for the mesocosm study. **a,b**, Mesocosm set-up for unshaded greenhouse (**a**) and shaded greenhouse (**b**) treatments. **c**, Brick configuration inside greenhouses. Photo credit A.W.W.

Article



Extended Data Fig. 4 | Design of thermal gradients for thermal selection experiments. Green and golden bell frogs (*L. aurea*) in thermal gradients. Photo credit A.W.W.

Extended Data Table 1 | Daily temperature extremes in thermal shelters and the surrounding environment

Treatment	Mean daily maximum temperature (°C)	Standard deviation (°C)	Unshaded–Shaded difference (°C)
Unshaded Upper Brick	39.84	8.76	4.49
Shaded Upper Brick	35.35	7.83	-
Unshaded Lower Brick	27.62	5.98	4.60
Shaded Lower Brick	23.02	3.67	-
Unshaded Under Vegetation	24.77	6.64	1.08
Shaded Under Vegetation	23.70	5.25	-
Unshaded Water	20.90	4.57	0.34
Shaded Water	20.57	4.41	-

Mean maximum daily temperatures in unshaded greenhouse ($n=6$) or shaded greenhouse ($n=6$) treatments by location.

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Software and code

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Data collection **no software was used to collect data**

Data analysis **Data was analyzed using R version 4.1.0**

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Research involving human participants, their data, or biological material

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Reporting on sex and gender	n/a
Reporting on race, ethnicity, or other socially relevant groupings	n/a
Population characteristics	n/a
Recruitment	n/a
Ethics oversight	n/a

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	Sample sizes were determined using proportional changes expected from published literature and a power analysis to detect differences at 95% power.
Data exclusions	No data were excluded
Replication	Each experiment was conducted once.
Randomization	We used a random number generator to assign frogs to each group unless otherwise stated.
Blinding	Blinding was not possible due to biosafety concerns. We needed to know which frogs were infected or potentially infected at what times in order to achieve a high standard of hygiene procedures.

Behavioural & social sciences study design

All studies must disclose on these points even when the disclosure is negative.

Study description	n/a
Research sample	n/a
Sampling strategy	n/a
Data collection	n/a
Timing	n/a
Data exclusions	n/a
Non-participation	n/a
Randomization	n/a

Ecological, evolutionary & environmental sciences study design

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Study description	n/a
Research sample	n/a
Sampling strategy	n/a
Data collection	n/a
Timing and spatial scale	n/a
Data exclusions	n/a
Reproducibility	n/a
Randomization	n/a
Blinding	n/a

Did the study involve field work? Yes No

Field work, collection and transport

Field conditions	n/a
Location	n/a
Access & import/export	n/a
Disturbance	n/a

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems		Methods	
n/a	Involved in the study	n/a	Involved in the study
<input checked="" type="checkbox"/>	Antibodies	<input type="checkbox"/>	ChIP-seq
<input checked="" type="checkbox"/>	Eukaryotic cell lines	<input type="checkbox"/>	Flow cytometry
<input checked="" type="checkbox"/>	Palaeontology and archaeology	<input type="checkbox"/>	MRI-based neuroimaging
<input type="checkbox"/>	Animals and other organisms		
<input checked="" type="checkbox"/>	Clinical data		
<input checked="" type="checkbox"/>	Dual use research of concern		
<input checked="" type="checkbox"/>	Plants		

Antibodies

Antibodies used	n/a
Validation	n/a

Eukaryotic cell lines

Policy information about [cell lines](#) and [Sex and Gender in Research](#)

Cell line source(s)	n/a
Authentication	n/a
Mycoplasma contamination	
Commonly misidentified lines (See ICLAC register)	n/a

Palaeontology and Archaeology

Specimen provenance	n/a
Specimen deposition	n/a
Dating methods	n/a
<input type="checkbox"/> Tick this box to confirm that the raw and calibrated dates are available in the paper or in Supplementary Information.	
Ethics oversight	n/a

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Animals and other research organisms

Policy information about [studies involving animals](#); [ARRIVE guidelines](#) recommended for reporting animal research, and [Sex and Gender in Research](#)

Laboratory animals	Each animal (frog) was at least 3 months post-metamorphosis and all were born in the same breeding season
Wild animals	No wild animals were involved in this project
Reporting on sex	Frogs were juveniles or subadults, so sexing was not done
Field-collected samples	No samples were collected from the field
Ethics oversight	All experiments involving frogs were approved by Macquarie University Animal Research ethics: ARA 2019/041

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Clinical data

Policy information about [clinical studies](#)

All manuscripts should comply with the ICMJE [guidelines for publication of clinical research](#) and a completed [CONSORT checklist](#) must be included with all submissions.

Clinical trial registration	n/a
Study protocol	n/a
Data collection	n/a
Outcomes	n/a

Dual use research of concern

Policy information about [dual use research of concern](#)

Hazards

Could the accidental, deliberate or reckless misuse of agents or technologies generated in the work, or the application of information presented in the manuscript, pose a threat to:

No	Yes
<input checked="" type="checkbox"/>	<input type="checkbox"/> Public health
<input checked="" type="checkbox"/>	<input type="checkbox"/> National security
<input checked="" type="checkbox"/>	<input type="checkbox"/> Crops and/or livestock
<input checked="" type="checkbox"/>	<input type="checkbox"/> Ecosystems
<input checked="" type="checkbox"/>	<input type="checkbox"/> Any other significant area

Experiments of concern

Does the work involve any of these experiments of concern:

No	Yes
<input checked="" type="checkbox"/>	<input type="checkbox"/> Demonstrate how to render a vaccine ineffective
<input checked="" type="checkbox"/>	<input type="checkbox"/> Confer resistance to therapeutically useful antibiotics or antiviral agents
<input checked="" type="checkbox"/>	<input type="checkbox"/> Enhance the virulence of a pathogen or render a nonpathogen virulent
<input checked="" type="checkbox"/>	<input type="checkbox"/> Increase transmissibility of a pathogen
<input checked="" type="checkbox"/>	<input type="checkbox"/> Alter the host range of a pathogen
<input checked="" type="checkbox"/>	<input type="checkbox"/> Enable evasion of diagnostic/detection modalities
<input checked="" type="checkbox"/>	<input type="checkbox"/> Enable the weaponization of a biological agent or toxin
<input checked="" type="checkbox"/>	<input type="checkbox"/> Any other potentially harmful combination of experiments and agents

Plants

Seed stocks

n/a

Novel plant genotypes

n/a

Authentication

n/a

ChIP-seq

Data deposition

- Confirm that both raw and final processed data have been deposited in a public database such as [GEO](#).
- Confirm that you have deposited or provided access to graph files (e.g. BED files) for the called peaks.

Data access links

May remain private before publication.

n/a

Files in database submission

n/a

Genome browser session
(e.g. [UCSC](#))

n/a

Methodology

Replicates

n/a

Sequencing depth

n/a

Antibodies

n/a

Peak calling parameters

n/a

Data quality

n/a

Software

n/a

Flow Cytometry

Plots

Confirm that:

- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

n/a

Instrument

n/a

Software

n/a

Cell population abundance

n/a

Gating strategy

n/a

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.

Magnetic resonance imaging

Experimental design

Design type

n/a

Design specifications

n/a

Behavioral performance measures

n/a

Imaging type(s)

n/a

Field strength

n/a

Sequence & imaging parameters

n/a

Area of acquisition

n/a

Diffusion MRI

 Used Not used

Preprocessing

Preprocessing software

n/a

Normalization

n/a

Normalization template

n/a

Noise and artifact removal

n/a

Volume censoring

n/a

Statistical modeling & inference

Model type and settings

n/a

Effect(s) tested

n/a

Specify type of analysis: Whole brain ROI-based Both

Statistic type for inference

n/a

(See [Eklund et al. 2016](#))

Correction

n/a

Models & analysis

n/a Involved in the study

Functional and/or effective connectivity

Graph analysis

Multivariate modeling or predictive analysis

Functional and/or effective connectivity

n/a

Graph analysis

n/a

Multivariate modeling and predictive analysis

n/a

