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Synergistic effects of warming and disease linked to high mortality in cool-adapted terrestrial frogs

Wesley J. Neely[a,*], Sasha E. Greenspan[a], Luisa P. Ribeiro[b], Tamilie Carvalho[b], Renato A. Martins[c], David Rodriguez[d], Jason R. Rohr[e], Célio F.B. Haddad[f], Luís Felipe Toledo[b], C. Guilherme Becker[a]

[a] Department of Biological Sciences, The University of Alabama, Tuscaloosa, AL 35487, USA
[b] Departamento de Biologia Animal, Universidade Estadual de Campinas (UNICAMP), Campinas, SP 13083-862, Brazil
[c] Centro de Ciências Biológicas e da Saúde, Universidade Federal de São Carlos, São Carlos, SP 13565-905, Brazil
[d] Department of Biology, Texas State University, San Marcos, TX 78666, USA
[e] Department of Biological Sciences, University of Notre Dame, Notre Dame, IL 46556, USA
[f] Departamento de Biodiversidade, Instituto de Biociências and Centro de Aquicultura (CAUNESP), Universidade Estadual Paulista (UNESP), Rio Claro, SP 13506-900, Brazil

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ABSTRACT

Cool-adapted species inhabiting montane tropical forests are vulnerable to heat stress associated with climate warming. According to the thermal mismatch hypothesis, pathogens are predicted to have broader thermal tolerances than their hosts, which can lead to increased pathogen loads and host mortality when temperatures are outside hosts' thermal optima. We tested the thermal mismatch hypothesis in two habitat generalist species expected to have wide thermal tolerance ranges: *Dendropsophus minutus* captured at a high elevation and *D. elegans* captured at a warmer low elevation. We also tested high-elevation individuals of the Brazilian direct-developing species *Ischnocnema parva*, which are expected to have a narrow thermal tolerance range based on life history. We exposed all frogs to the pathogenic fungus *Batrachochytrium dendrobatidis* (*Bd*) under three temperatures ranging from the average nighttime temperature where frogs were collected (16 and 21 °C for high and low elevations, respectively) to a simulated warming event (26 °C). *Bd* loads across all three host species were negatively associated with temperature. However, the interaction between *Bd* infection and warming led to increased mortality in cool-adapted *I. parva* at higher temperatures, despite lower infection loads. Our results indicate that *Bd* may lead to declines of cool-adapted montane frogs under the combined pressures of pathogen infection and warming, even at temperatures approaching the pathogen's upper thermal limit. Thus, climatic warming at a level that lowers fitness of heat-sensitive pathogens may not uniformly reduce host disease risk.

1. Introduction

Anthropogenic climate change, including higher and more variable temperatures, threatens species adapted to narrow thermal ranges (Chen et al., 2011a; Cordier et al., 2019; Kissel et al., 2018; Parmesan, 2006; Walther et al., 2002). Rising cases of emerging infectious diseases are additional contemporary threats to wildlife that may occur in combination with climate change (Catenazzi et al., 2011; Harvell et al., 2002; Lips et al., 2008; Rohr et al., 2011; Shukla, 2019). For instance, recent emergences of fungal diseases, such as snake fungal disease (Lorch et al., 2016), coral aspergillosis (Ward et al., 2007), amphibian chytridiomycosis (Lips et al., 2008; Longcore et al., 1999), and white nose syndrome in bats (Blehert et al., 2009; Gargas et al., 2009), have been linked to climatic shifts. Species inhabiting montane forests are particularly vulnerable to climate change because cold-adapted populations at high elevations are unable to migrate to avoid the physiological stress of rising temperatures (Beniston et al., 1997; Chen et al., 2011b; Clavel et al., 2011; Ohlemüller et al., 2008). Thus, predicting the synergistic effects of climate change and disease on cold-adapted, high-elevation species is a conservation priority and will advance our understanding of disease dynamics in understudied systems.

Several recent amphibian population declines have been attributed to interactions between emerging pathogens and climatic anomalies (Burrowes et al., 2018; Carvalho et al., 2017; Lips, 2016; Puschendorf et al., 2005; Wake and Vredenburg, 2008). The amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*), is an aquatic-associated

* Corresponding author at: 1325 Science and Engineering Complex, Department of Biological Sciences, The University of Alabama, 300 Hackberry Lane, Tuscaloosa, AL 35487, USA.
E-mail address: wjneely@crimson.ua.edu (W.J. Neely).

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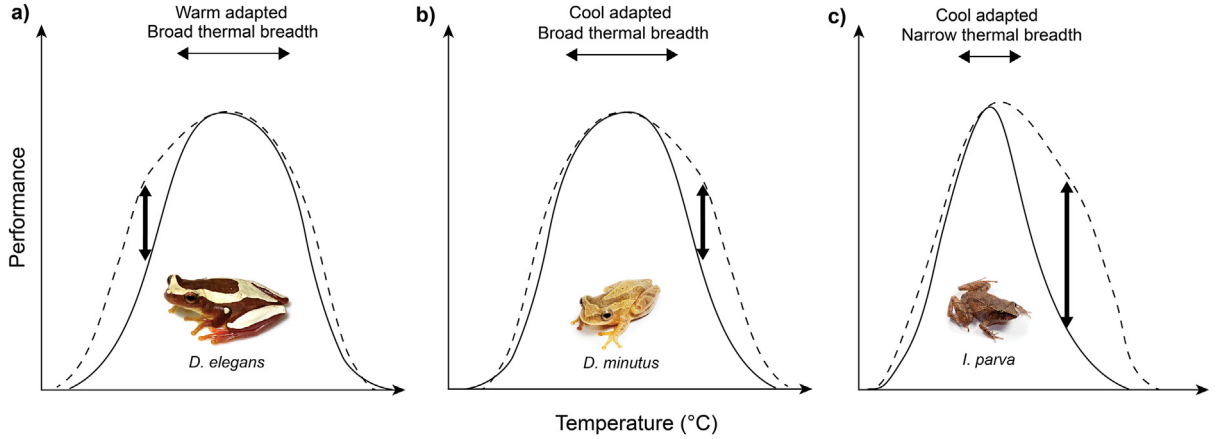

Fig. 1. Conceptual diagram demonstrating the expected differences in thermal adaptation between pathogen and host for the three species in this study. Pathogen thermal adaptation is represented by a dashed line for each species.

pathogen that causes the amphibian disease chytridiomycosis (Longcore et al., 1999). *Bd* is heat-sensitive, with optimal growth between 15 and 25 °C in culture across different isolates (Piotrowski et al., 2004; Stevenson et al., 2013), corresponding to temperatures in tropical montane forests where severe outbreaks of chytridiomycosis have occurred (Berger et al., 2006; Catenazzi et al., 2011; Hirschfeld et al., 2016; Lips, 1999; Muletz-Wolz et al., 2017; Piotrowski et al., 2004; Stevenson et al., 2013). However, outbreaks have also been linked to warmer periods (Rohr et al., 2008; Rohr and Raffel, 2010), some of which have ranged outside the optimal temperatures of the pathogen in culture (Bosch et al., 2007), which suggests that the outcome of *Bd* infection depends not only on the thermal performance of the pathogen alone, but also on interactions between the thermal performance of the host and pathogen (Cohen et al., 2017).

Previous contradictory findings regarding links between temperature and *Bd* outbreaks can, in many cases, be explained by the thermal mismatch hypothesis (Cohen et al., 2017). Hosts and parasites physiologically adapt to environmental temperatures and are thus limited by variable or extreme temperatures, but parasites tend to have broader thermal breadths than hosts (Rohr et al., 2018). This may give parasites a fitness advantage when cold-adapted hosts and parasites experience warming or when warm-adapted hosts and parasites experience cooling (Cohen et al., 2017). The thermal mismatch hypothesis predicts that hosts will exhibit greatest disease susceptibility at temperatures more mismatched from the temperatures to which hosts are adapted (Cohen et al., 2017). For example, cold-adapted *Atelopus zeteki* from Panama developed high *Bd* loads at 26 °C, a temperature above the optimal range for both the host and the *Bd* isolate in culture, and warm-adapted frogs developed high *Bd* loads at temperatures below optimal for the host and the *Bd* isolate in culture (Cohen et al., 2017).

Previous work on direct-developing frogs suggests that this biologically diverse group may be particularly vulnerable to aquatic pathogens as well as climatic warming. Recent studies demonstrate that direct-developing frogs acquire higher and more lethal *Bd* infections than sympatric aquatic-breeding species when experimentally exposed to the fungus (Becker et al., 2019; Greenspan et al., 2018; Mesquita et al., 2017). This could be due to their terrestrial breeding behavior which offers fewer opportunities to develop adaptive immunity against aquatic pathogens, compared to aquatic-breeding host species that may regularly encounter the pathogen in water bodies. In addition, most direct-developing frogs have specialized habitat requirements, completing their life cycle under the mild microclimates of forest leaf litter (Haddad et al., 2013). This could explain why these species appear to have exceptionally narrow thermal breadths, with relatively low upper thermal tolerances, which could be especially low among high-elevation populations (Catenazzi et al., 2014; Nowakowski et al., 2016; von May et al., 2019). In contrast, more generalist aquatic-breeding species that experience higher temperature variability across different habitat types appear to have broader thermal breadths, with relatively high upper thermal tolerances (Nowakowski et al., 2016; von May et al., 2019).

The Brazilian Atlantic Forest (BAF) is an amphibian biodiversity hotspot comprised of both montane and lowland habitats that are home to endemic frog species living across diverse habitat gradients (Toledo et al., 2014). In light of the thermal mismatch hypothesis, we performed a laboratory experiment investigating the interactive effects of *Bd* and warming in BAF frog populations from warm lowlands and cool uplands. We first compared responses to *Bd* under warming between populations of habitat-generalist aquatic-breeding *Dendropsophus* spp. adapted to cool uplands and warm lowlands and predicted that the relatively cool-adapted frogs would be more vulnerable to *Bd* under warming than the relatively warm-adapted frogs. We then compared responses to *Bd* under warming between high elevation populations of *Dendropsophus minutus* and direct-developing *Ischnocnema parva*. These frog species were both relatively cool-adapted; however, *I. parva* were expected to have narrower thermal breadths and lower heat tolerance based on their microhabitat use. Because we expected the thermal breadth of the direct developing frogs to be more mismatched from the warming treatment than the thermal breadth of the habitat-generalist aquatic-breeding frogs (Catenazzi et al., 2014), we predicted that *I. parva* would be more vulnerable to *Bd* under warming (Fig. 1). Our study reveals strong synergistic effects of warming and disease on the health of direct-developing frogs, highlighting the critical need for safeguards against climate change and emerging disease.

2. Methods

2.1. Study species and sampling localities

Our study species were *I. parva*, a direct-developing forest-floor specialist, and two habitat generalist treefrogs with aquatic larval development, *D. elegans* and *D. minutus* (Haddad et al., 2013). *Dendropsophus* species tolerate anthropogenic alterations, thriving in both natural forests and disturbed, open environments with high temperature variability, while *I. parva* is more sensitive to landscape alteration, occurring at higher densities in moist, closed-canopy forests with milder microclimates (Haddad et al., 2013; Toledo et al., 2014). In November 2016, we collected frogs from three relatively undisturbed forested sites in Ubatuba and São Luiz do Paraitinga (São Paulo, Brazil). In total, we captured 35 *I. parva* from a high-elevation population (906 m above sea level), 35 *D. minutus* from a high-elevation population (998 m above sea level), and 35 *D. elegans* from a low-elevation

population (5 m above sea level). All three host species seek cooler refugia in burrows or tree cavities when temperatures are high during the day and forage and breed when temperatures are cooler at night (Haddad et al., 2013; Martins et al., 2010). Thus, we used the historical average monthly nighttime temperature of the high-elevation (16 °C) and low-elevation (21 °C) sites as a proxy for the temperatures to which high-elevation and low-elevation frogs were adapted, respectively. While 16 °C represented the adapted temperature for both species that were captured at high elevation, we expected that *D. minutus* experienced a greater breadth of temperatures in the wild compared to *I. parva* due to differences in habitat use.

2.2. Experimental design

Upon capture of frogs in the field, we collected skin swabs following standard protocols (Hyatt et al., 2007). We then placed frogs individually in plastic bags and transported them to the laboratory at State University of Campinas. We randomly allocated 11–12 frogs of each species to each of three temperature treatments: 16 °C (representative of temperature to which high-elevation frogs were adapted), 21 °C (representative of temperatures to which low-elevation frogs were adapted), and 26 °C (warming treatment). We then inoculated half of each group with a *Bd* zoospore suspension or water (control).

To obtain *Bd* zoospores for experimental inoculations, we used a *Bd* Global Panzootic Lineage isolate (GPL-2/CLFT 181), isolated from an American bullfrog tadpole (*Lithobates catesbeianus*) collected in Pindamonhangaba, São Paulo, Brazil in April 2016. We cultured *Bd* in Petri dishes containing 1% tryptone agar at 17 °C for 7 days. To inoculate frogs with *Bd* (day zero), we filled each Petri dish with 5 ml of distilled water for 30 min and scraped the substrate with a sterile scalpel to facilitate zoospore release. We then transferred the liquid contents of each dish to a sterile beaker, sampled 1 ml of the solution to quantify the zoospore concentration with a hemocytometer, and diluted the solution with distilled water to obtain the desired zoospore concentration for experimental inoculations. We inoculated frogs individually in Petri dishes each containing 2.1×10^6 zoospores in 2.5 ml of distilled water for 45 min. We exposed control frogs to the same volume of distilled water. We did not clear natural *Bd* infections in frogs prior to the experiment in order to avoid the adverse effects of antimicrobial drugs or heat stress. Instead, we controlled for natural infection loads in our statistical analyses, which is appropriate given that the distributions of field loads for the three species overlapped (Fig. S1).

After inoculations, we placed frogs individually in plastic containers (15 × 15 × 5 cm) with sterilized, moist *Sphagnum* moss substrate which we then stored in incubators set at each treatment temperature. The experiment lasted 57 days, during which frogs were fed pinhead crickets daily *ad libitum* and *Sphagnum* substrate was changed every 10 days. We collected skin swabs on day 30 and monitored mortality daily. We extracted DNA from skin swabs using 50 ml PrepMan Ultra and quantified *Bd* loads using Taqman qPCR assays (Boyle et al., 2004) with *Bd* standard curves ranging from 0.1 to 1,000 zoospore genome equivalents (g.e.).

2.3. Statistical analysis

We used general linear models (GLM; normal probability distribution and identity link function) to determine how *Bd* loads are influenced by two different forms of thermal mismatch: mismatch in the average temperature based on host capture location and mismatch in expected thermal breadth based on host life history. To understand how *Bd* loads are influenced by mismatches in the average temperature at the host capture location, we used data from the two habitat-generalist frog species. We fit a GLM with *Bd* loads of inoculated frogs as the response and average monthly nighttime temperature (16 °C for cool-adapted *D. minutus* and 21 °C for warm-adapted *D. elegans*), experimental temperature (16, 21, or 26 °C), and their interaction as predictors. Natural *Bd* loads (from field swabs) were also included as a predictor to account for prior *Bd* infection. To understand how *Bd* loads are influenced by mismatches in the thermal breadth of hosts, we used data from the two species captured at high elevation. We fit a GLM with *Bd* loads of inoculated frogs as the response and expected thermal breadth (narrow for *I. parva* and broad for *D. minutus*), experimental temperature (16, 21, or 26 °C), their interaction, and natural *Bd* load as predictors.

To test for independent and interactive effects of *Bd* infection and temperature on host survival, we fit a censored Cox proportional hazards survival model (coxph function in survival package in Program R; Therneau, 2015; R Core Team, 2018) with *I. parva* survival status as the response and experimental temperature, infection treatment (*Bd*-inoculated vs. control), and their interaction as predictors. To test for independent and interactive effects of *Bd* load and temperature on host survival, we fit a similar model using only *Bd*-inoculated frogs, with experimental temperature, *Bd* load, their interaction, and natural *Bd* load as predictors. We were not able to record *Bd* loads at death for the 3 frogs that died before day 30, so they were excluded from this model. All *Bd* loads were log10-transformed to satisfy the assumptions of normality based on residual distributions. Statistical analyses were performed in R v. 3.1.4 and JMP v. 14 (JMP®, 2019; R Core Team, 2018).

3. Results

All three host species developed relatively higher *Bd* infection loads at lower experimental temperatures (Fig. 2; Table 1). Experimental *Bd* loads averaged 549.7, 392.9, and 4.3 for *D. elegans*, 140.4, 19.2, and 6.8 for *D. minutus*, and 15137.5, 6923.2, and 14.4 for *I. parva*, at 16 °C, 21 °C, and 26 °C, respectively. Warm-adapted *D. elegans* developed higher *Bd* infection loads than cool-adapted *D. minutus* at lower experimental temperatures (interaction term: $\beta = -0.060 \pm 0.025$; $t = -2.36$, $P = 0.025$; Table 1). Direct-developing *I. parva*, which were expected to have relatively narrow thermal breadths based on microhabitat use, developed higher *Bd* infection loads across all experimental temperature treatments compared to *D. minutus*, which were expected to have broader thermal breadths ($\beta = -1.008 \pm 0.191$, $t = -5.28$, $P < 0.001$). Patterns in *Bd* loads were not a function of body size as *I. parva*, the smallest frog, developed the highest pathogen loads.

We observed *Bd*-induced mortality in cool-adapted *I. parva*, with the highest mortality rate at 26 °C, the highest experimental temperature (interaction term: $\chi^2 = 4.29$, $P = 0.038$; Table 2; Fig. 2d). Additionally, we found that mortality was driven by the interaction between experimental temperature and *Bd* loads ($\chi^2 = 6.30$, $P = 0.012$; Table 2), where mortality was highest for highly infected frogs exposed to 26 °C. Mean survival time (± SE) for *Bd*-inoculated *I. parva* at 26 °C was 26.6 ± 10.14 days, compared to 45.4 ± 4.84 and 50.8 ± 3.95 days for those at 21 °C and 16 °C, respectively. Of nine *Bd*-inoculated frogs that died during the experiment, four were from the 26 °C group, three were from the 21 °C group, and two were from the 16 °C group. In contrast, only one control (not inoculated with *Bd*) individual of *I. parva* died across all temperature treatments. All *D. minutus* survived to the end of the experiment and one *D. elegans* died during the experiment.

Bd infection prevalence upon capture in the field was lowest in *I. parva* (14.3%), intermediate in *D. elegans* (20.0%) and highest in *D. minutus* (48.6%; Table 3). Mean infection intensity at capture was highest for *I. parva* (75.2 ± 68.2 g.e.) and lowest for *D. elegans* (19.1 ± 9.4 g.e.; Table 3). In all species, infection intensities in the field were consistently lower than infection intensities in the experiment. We did not detect effects of natural *Bd* loads on experimental *Bd* loads in either host comparison (narrow vs. broad thermal breadth: $\beta = 0.028 \pm 0.368$, $t = 0.08$, $P = 0.939$; cool vs. warm adapted: $\beta = -0.245 \pm 0.182$, $t = -1.35$, $P = 0.187$; Table 1). Natural *Bd*

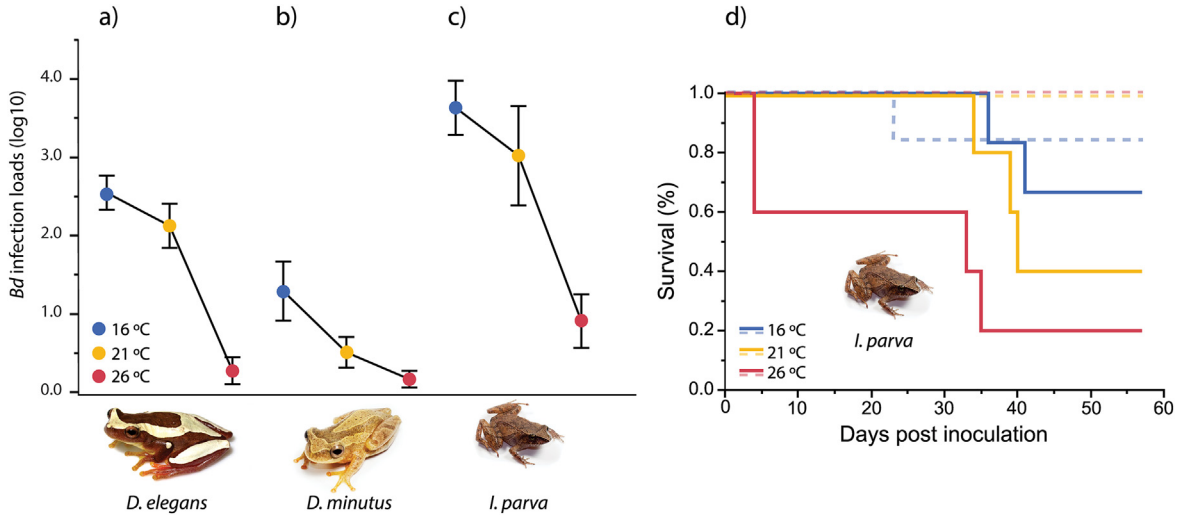

Fig. 2. Plots of infection loads on day 30 after experimental *Bd* inoculation for *D. elegans* (a), *D. minutus* (b), and *I. parva* (c). *Bd* loads are split by temperature trial and standard error bars are included. The survival plot (d) shows percent of *Batrachochytrium dendrobatidis* (*Bd*)-inoculated *Ischnocnema parva* surviving for 57 days across three temperature treatments. Control (not inoculated) groups are represented by a lighter dashed line. Colors relate to the temperature treatment: Blue = 16 °C, yellow = 21 °C, and red = 26 °C. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Result of general linear models (GLM) comparing *Bd* loads on day 30 for thermally constrained vs. thermally flexible species (*Ischnocnema parva* vs. *Dendropsophus minutus*, respectively) and cool- vs. warm-adapted species (*D. minutus* vs. *D. elegans*, respectively). For each model, variables included thermal adaptation (using host species as a proxy for adapted temperature or adapted thermal breadth), experimental temperature, thermal adaptation/temperature interaction, and natural *Bd* infection loads. Values in bold type represent significant correlations at alpha = 0.05.

Response: *Bd* load at day 30.

Predictors	β	SE	t	*P*
Cool- vs. warm-adapted				
Average nighttime temp. (cool vs. warm)	0.47	0.11	4.50	< **0.001**
Experimental temperature	−0.19	0.03	−6.48	< **0.001**
Average nighttime × experiment temperature	−0.06	0.03	−2.36	**0.025**
Natural *Bd* load	−0.25	0.18	−1.35	0.187
Narrow vs. broad thermal breadth				
Thermal breadth (narrow vs. broad)	−1.01	0.19	−5.28	< **0.001**
Experimental temperature	−0.18	0.05	−4.06	< **0.001**
Thermal breadth × experimental temperature	0.07	0.04	1.63	0.115
Natural *Bd* load	0.03	0.37	0.08	0.939

Whole model tests – *Narrow* vs. *broad thermal breadth*: $F_{(4,28)} = 16.24$, $R^2 = 0.70$, $P <$ **0.001**; *Cool-* vs. *warm-adapted*: $F_{(4,31)} = 18.70$, $R^2 = 0.71$, $P <$ **0.001**.

loads were positively correlated with survival time ($\chi^2 = 8.27$, $P = 0.004$; Table 2).

Table 2
Censored cox proportional hazards survival models comparing survival time of *Ischnocnema parva* by *Bd* infection treatment (*Bd*-inoculated or control) and *Bd* infection loads (*Bd*-inoculated frogs only). Variables in each model include measure of infection (treatment or *Bd* loads), experimental temperature (16, 21, or 26 °C), and the interaction between these two variables. Natural *Bd* infection loads were included for the model comparing *Bd* infection loads to control for prior infection. Values in bold type represent significant correlations at alpha = 0.05.

Response: Survival time.

Predictors	β	χ^2	*P*
Model 1			
Temperature	−1.89	0.98	0.322
Treatment	10.32	12.28	< **0.001**
Temperature × Treatment	2.07	4.29	**0.038**
Model 2			
Temperature	6.40	11.72	< **0.001**
Bd loads	0.01	8.70	**0.003**
Temperature × *Bd* loads	0.01	6.30	**0.012**
Natural loads	5.80	8.27	**0.004**

Whole model tests – Model 1: $\chi^2 = 17.22$, DF = 3, $P <$ **0.001**; Model 2: $\chi^2 = 14.45$, DF = 4, $P =$ **0.006**.

Table 3
Natural *Bd* infection loads from frogs at the time of capture in the field. Standard error (SE) is included for mean infection intensity and 95% confidence intervals (CI) are included for prevalence. Mean intensity is given as genomic equivalents (g.e.).

Host	n	n infected	Prevalence % (95% CI)	Mean intensity ± SE
D. elegans	35	7	20.0 (12.5–43.3)	19.1 ± 9.4
D. minutus	35	17	48.6 (31.4–66)	42.9 ± 28.8
I. parva	35	5	14.3 (6.6–33.6)	75.2 ± 68.2

4. Discussion

The thermal mismatch hypothesis predicts that climatic warming will threaten cool-adapted hosts facing heightened parasite pressure during warmer periods (Cohen et al., 2017). Specifically, parasites are expected to cope with warming better than hosts because they tend to have broader thermal breadths (Cohen et al., 2018; Rohr et al., 2018). Thus, host susceptibility to disease is predicted to be highest at temperatures where parasite performance most exceeds that of the host, rather than the optimal growth temperature of the pathogen in isolation (Cohen et al., 2017). For instance, Raffel et al. (2013) found that *Bd* grew faster in culture at higher temperatures (25 °C) but grew faster at cooler temperatures on warm-adapted Cuban treefrogs (*Osteopilus septentrionalis*), causing higher host mortality at 15 °C. In keeping with these findings, we predicted that cool-adapted *D. minutus* would be more sensitive to *Bd* under warming than warm-adapted *D. elegans*. We also predicted that direct-developing *I. parva* with thermal breadths spanning a relatively narrow range of cool temperatures would be more sensitive to warming than *D. minutus* with thermal breadths that overlapped with *I. parva* but spanned a broader range of temperatures.

Contrary to our predictions, individuals of all three species developed higher *Bd* infection loads at lower temperatures regardless of the environment to which they were adapted. However, disease-associated mortality rates of cool-adapted and thermally constrained *I. parva* were consistent with thermal mismatch. While warming slowed *Bd* growth, the synergistic effects of pathogen infection and heat stress were sufficient to increase mortality rates. We did not record *Bd* loads for the frogs that died prior to day 30 because of poor condition of the carcasses, so these frogs may have had higher infection loads at death than frogs that survived to day 30, but in general, we observed a relatively high rate of mortality coupled with low *Bd* infection loads in *I. parva* under warming. The high survival of *I. parva* at high temperatures in the absence of *Bd* and the high survival of *Bd*-infected *I. parva* at low temperatures indicate that mortality was linked to a synergism between *Bd* and high temperatures rather than disease or heat sensitivity of the host alone.

We found that interactive effects of heat and infection can cause host mortality in cool-adapted hosts, even near the upper thermal limit of *Bd*, when pathogen growth is relatively slow and infection loads are relatively low. Our results align with previous studies reporting that high parasite thermal breadth may give *Bd* a fitness advantage over more thermally constrained hosts (Cohen et al., 2018, 2017; Rohr et al., 2018). There are two possible mechanisms by which synergistic interactions between heat and infection could cause host mortality: (i) heat stress may have lowered host tolerance to *Bd* infection or (ii) *Bd* infection may have reduced host tolerance of warming. While we cannot determine the mechanism driving our results, infection loads were relatively low in our warming treatment, which is more consistent with indirect effects of mild *Bd* infections on host fitness through lowered host tolerance to heat. Our findings are in line with previous results showing that *Bd* lowers the upper thermal tolerance of hosts (Greenspan et al., 2017c), potentially by damaging tissues or stressing the biochemical processes involved in temperature responses. Climatic warming, to an extent that lowers pathogen fitness, may be expected to uniformly reduce host vulnerability to heat-sensitive pathogens like *Bd* if host immune function is not concomitantly reduced by increasing temperatures. Conversely, our findings indicate that interactions between *Bd* and heat can have negative fitness consequences for thermally constrained hosts even under temperatures that inhibit *Bd* growth and reproduction. Some studies suggest that behavioral fever, in which hosts seek out higher temperatures to improve immune function, could be a mechanism by which frogs clear *Bd* infection (Richards-Zawacki, 2010; Rowley and Alford, 2013; Woodhams et al., 2003). However, other recent data indicate that this might be driven mainly by prior host thermal preference and not a change in behavior in response to infection (Sauer et al., 2018). Regardless of whether this mechanism operates in nature, it is unlikely to benefit *I. parva* as increased temperatures could be fatal for infected frogs.

Previous studies suggest that direct-developing species as a group are particularly sensitive to climate warming even in the absence of disease (Catenazzi et al., 2014; Nowakowski et al., 2016). In a study of thermal tolerance of Costa Rican frogs, including two species in the genus *Dendropsophus* and six direct-developing species (four leaf-litter specialists in the genus *Craugastor*), direct-developers had lower critical thermal maxima (CTmax) than aquatic-breeding species (Supplement 1 in Nowakowski et al., 2016). The CTmax for direct developers ranged from 27.7 to 28.4 °C while the two species of *Dendropsophus* ranged from 35.1 to 38.3 °C. In another study, 11 direct-developers from Peru in the family Strabomantidae had similarly low CTmax, ranging from 24.9 to 29.8 °C (Catenazzi et al., 2014). Our results show that *Bd* infection can lower the thermal tolerance of direct-developing hosts even further.

Direct-developing amphibians were initially determined to be at low risk in the context of the *Bd* epidemic because their terrestrial life cycle was expected to limit exposure to this aquatic pathogen (Bustamante et al., 2005; Lips et al., 2003; Olson et al., 2013). However, recent studies indicate that direct-developing species, including *I. parva*, may develop lethal *Bd* infections and may have weak immune defenses against *Bd* (Becker et al., 2019; Greenspan et al., 2018; Mesquita et al., 2017). Moreover, extreme weather events like drought may increase *Bd* exposure and transmission by forcing direct-developing frogs to congregate in moist areas (Burrowes et al., 2018; Longo et al., 2010), which highlights the vulnerability of direct-developing species considering that warming may co-occur with other weather anomalies such as drought. In addition, direct-developers often occur within diverse amphibian communities at high population densities and may thus acquire *Bd* infections from heterospecifics that use water bodies more frequently (Becker et al., 2019) or from contaminated substrates (Johnson and Speare, 2005; Kolby et al., 2015; Raffel et al., 2015; Rowley and Alford, 2007). Further, direct-developing frogs represent ~20% of global anuran diversity, and over 1100 species are in the superfamily Brachycephaloidea, a group composed entirely of direct-developers (Frost et al., 2006; IUCN, 2019). Our study indicates interactive effects of warming and disease as yet another threat to direct-developing frogs and underscores the need for increased population and disease monitoring of this diverse group.

Promisingly, we found a positive effect of natural *Bd* infection loads on survival of *I. parva* (Table 2), suggesting that frogs exposed to *Bd* in the field acquired immune defenses. This is consistent with previous studies showing that prior exposure to *Bd* results in lower experimental *Bd* loads (Becker et al., 2016) and induces immune defenses which lower *Bd*-induced mortality (McMahon et al., 2014). However, we found no correlation between natural and experimental *Bd* loads (Table 1), indicating that this is an effect of acquired tolerance to *Bd* infection and not resistance to infection. These findings suggest that if frogs are able to survive initial infection, they may develop immune defenses that could possibly make them more robust to the interactive effects of warming and disease.

In contrast to *I. parva*, both *D. minutus* and *D. elegans* have large geographic range distributions and homogeneous spatial distributions, occurring across the Brazilian Atlantic Forest, Savanna and the Amazon (IUCN, 2019). Higher likelihood of gene flow among populations across large areas (Simon et al., 2015), coupled with wider habitat suitability, indicate that these species are more thermally flexible and thus less likely to succumb to infections as they would not experience thermal mismatch under warming scenarios. Alternatively, evolutionary adaptations to cooler high-elevation habitats may have influenced the lower thermal limits of our *D. minutus* population while not affecting the upper thermal limits (Hoffmann et al., 2013; Simon et al., 2015), thus enabling this species to effectively respond to *Bd* at warm temperatures which are associated with higher metabolic rates and increases in immune functions in ectotherms (Greenspan et al., 2017a; Rollins-Smith and Woodhams, 2012). Slight increases in temperature could benefit these species through a reduction in pathogen loads (Greenspan et al., 2017b; Rowley and Alford, 2013); although we found no changes in mortality even when frogs hosted high infection intensities. In contrast to our cool-adapted study populations of *I. parva* and *D. minutus*, we predicted that warm-adapted lowland *D. elegans* would develop lower infection loads at warm temperatures and higher infection loads at cool temperatures. While *Bd* loads of cool-adapted *D. minutus* and warm-adapted *D. elegans* were similarly low at warm temperatures, infection loads were higher at cool temperatures for *D. elegans*, as predicted by the thermal mismatch hypothesis. Alternatively, this result could be explained by species-specific differences in innate or acquired immunity (e.g., field prevalence of *Bd* was higher in *D. minutus*; Table 2). Differences in genetic resistance to infection may exist between species but are unlikely to have played a role in this experiment as we found higher natural *Bd* loads in *D. minutus* and higher experimental loads in *D. elegans*.

According to current global warming trends, disease risk is expected to disproportionately increase for cool-adapted organisms. *Bd* infection risk is generally lower for frogs that use warm microhabitats (Becker

et al., 2012; Becker and Zamudio, 2011; Richards-Zawacki, 2010; Rowley and Alford, 2013; Roznik et al., 2015) or frogs exposed to daily heat pulses (Greenspan et al., 2017b; Rohr et al., 2018). Our results suggest that even if the thermal tolerance envelope of healthy frogs includes temperatures offering thermal refuge from *Bd*, interactions between warming and infection may still lead to population declines. A *Bd* mitigation strategy recently proposed by Hettyey et al. (2019) is minor habitat modification to increase thermoregulatory opportunities, but our study indicates that this strategy would be ineffective for cool-adapted direct-developers that avoid disturbed environments and experience increased heat sensitivity associated with *Bd* infection. Our study highlights challenges in conserving montane biodiversity based on the combined effects of disease and climate change, emphasizing the importance of investigating interactive effects of multiple stressors when considering risks to jeopardized species.

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CRediT authorship contribution statement

Wesley J. Neely: Methodology, Validation, Formal analysis, Data curation, Writing - original draft, Writing - review & editing, Visualization.**Sasha E. Greenspan:**Validation, Formal analysis, Writing - review & editing.**Luisa P. Ribeiro:**Investigation.**Tamilie Carvalho:**Investigation.**Renato A. Martins:**Investigation.**David Rodriguez:**Investigation, Writing - review & editing.**Jason R. Rohr:**Conceptualization, Writing - review & editing.**Célio F.B. Haddad:**Methodology, Data curation, Resources, Supervision, Funding acquisition.**Luís Felipe Toledo:**Conceptualization, Methodology, Investigation, Data curation, Writing - review & editing, Supervision, Funding acquisition.**C. Guilherme Becker:**Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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