Available online at www.sciencedirect.com

Revista Mexicana de Biodiversidad

Revista Mexicana de Biodiversidad 87 (2016) 171–179

Instituto de Biología UNAM
www.ib.unam.mx/revista/

Ecology

Temperature treatments boost subclinical infections of *Batrachochytrium dendrobatidis* in a Mexican salamander (*Pseudoeurycea leprosa*)

Tratamientos de temperatura aumentan las infecciones subclínicas de Batrachochytrium dendrobatidis *en una salamandra mexicana (*Pseudoeurycea leprosa*)*

Cinthya Mendoza-Almeralla [a], Aldo López-Velázquez [a], Ana V. Longo [b], Gabriela Parra-Olea [a,*]

[a] *Departamento de Zoología, Instituto de Biología, Universidad Nacional Autónoma de México, Apartado postal 70-153, 04510 México D.F., Mexico*
[b] *Department of Ecology and Evolutionary Biology, Cornell University, 144 East Ave., 14850 Ithaca, NY, USA*

Received 19 May 2015; accepted 12 October 2015
Available online 28 February 2016

Abstract

The first record about the pathogenic fungus *Batrachochytrium dendrobatidis* in Mexican plethodontid salamanders dates back to the 1970s. However, little is known of the patterns of infection in wild populations and the effects of changes in temperature on the degree of infection. This work quantified *Bd* infection in a population of *Pseudoeurycea leprosa* in La Malinche National Park, Puebla, Mexico from June 2011 to September 2012. A total of 160 adult salamanders were experimentally exposed to temperatures of 10, 15, 20, 23, 25, or 28 °C for 10 weeks. The results of this study revealed that: (1) the population of *P. leprosa* in La Malinche National Park is infected with *Bd* throughout the year at a low prevalence of between 0 and 17%; (2) 20.6% of the salamanders that were *Bd* negative at the time of collection expressed chytridiomycosis after exposure to the experimental temperature treatments; (3) temperature was the cause of death in each treatment, with temperatures of 25 °C and 28 °C affecting the survival of *P. leprosa*; (4) the infection load in certain *P. leprosa* individuals exhibited cycles of increasing and decreasing zoospore genomic equivalents over time.

Keywords: Amphibians; Salamanders; Chytridiomycosis; Plethodontidae; Mexico

Resumen

El primer registro del hongo patógeno *Batrachochytrium dendrobatidis* (*Bd*) en salamandras pletodóntidas de México data de la década de 1970. Sin embargo, se conoce muy poco sobre los patrones de infección por *Bd* en poblaciones silvestres y el efecto de los cambios de temperatura sobre el grado de infección. Este trabajo cuantificó la infección por *Bd* en la población de *Pseudoeurycea leprosa* en el Parque Nacional La Malinche, Puebla, México, de junio de 2011 a septiembre de 2012. Se expusieron 160 individuos adultos a tratamientos de 10, 15, 20, 23, 25 o 28 °C durante 10 semanas. Los resultados de este estudio revelaron que: (1) la población de *P. leprosa* del Parque Nacional La Malinche presenta infección por quitridiomicosis a lo largo del año con prevalencias bajas, entre el 0 y el 17%; (2) el 20.6% de los organismos que resultaron negativos (sin infección) al momento de la recolecta resultaron positivos después de los experimentos de temperatura; (3) la temperatura fue la causa de la muerte en los experimentos realizados a 25 °C y 28 °C, afectando directamente la supervivencia de *P. leprosa*; (4) la carga de infección en algunos individuos de *P. leprosa* presentó ciclos de aumento y disminución de la infección durante los tratamientos.

Palabras clave: Anfibios; Salamandras; Quitridiomicosis; Plethodontidae; México

* Corresponding author.
E-mail address: gparra@ib.unam.mx (G. Parra-Olea).
Peer Review under the responsibility of Universidad Nacional Autónoma de México.

http://dx.doi.org/10.1016/j.rmb.2016.01.020

Introduction

The wild populations of many species are decreasing drastically around the world, which has led scientists to propose that we are in the midst of a sixth mass species extinction event (Medina-Vogel, 2010; Wake & Vredenburg, 2008). This phenomenon has been attributed to a variety of factors, including habitat destruction, pollution, overexploitation, climate change and emerging infectious diseases (Dobson & Foufopoulos, 2001; Medina-Vogel, 2010). Emerging diseases become increasingly important as new cases appear around the world (Medina-Vogel, 2010). To address this threat, scientists have suggested that research on emerging diseases should focus on the causes of animal diseases, transmission of diseases via hosts, physiology and ecology of the pathogens, and impacts of global climate change (Harvell et al., 2002; Medina-Vogel, 2010).

Many pathogens and their hosts are limited by the temperature of the environment (Harvell et al., 2002). Thus, when climate conditions change, the parasite–host relationship can be disrupted, which may increase the virulence of the pathogens and/or the susceptibility of the hosts (Harvell et al., 2002). Examples of diseases that have caused population declines and are dependent on specific temperatures include distemper in seals, parvovirus in lions, white-nose syndrome in bats, and chytridiomycosis in amphibians (Daszak, Cunningham, & Hyatt, 2003; Harvell et al., 2002).

Chytridiomycosis occurs worldwide and has caused the decline of more than 200 amphibian species (Skerratt et al., 2007). The pathogenic fungi that cause the disease are *Batrachochytrium dendrobatidis*, which was discovered in the 1990s and occurs in all of the amphibian orders (Olson et al., 2013), and *Batrachochytrium salamandrivorans*, which was discovered in 2013 in European salamanders (Martel et al., 2014).

Chytridiomycosis is characterized by the colonization of the keratinized layers of amphibian epidermis or larval mouthparts (Berger et al., 1998; Martel et al., 2014; Pessier, Nichols, Longcore, & Fuller, 1999). Motile zoospores develop into sporangia in the keratinized parts of the amphibian skin and release zoospores through a discharge tube. This can result in epidermal hyperplasia and hyperkeratosis. In addition, these changes disrupt the osmoregulatory function of the skin, leading to dehydration, electrolyte imbalances, and death caused by cardiac arrest (Berger et al., 1998; Brutyn et al., 2012; Carver, Bell, & Waldman, 2010; Marcum, St-Hilaire, Murphy, & Rodnick, 2010; Voyles et al., 2007; Voyles, Rosenblum, & Berger, 2011).

To date, more than 500 amphibian species have been infected with *Bd* (Olson et al., 2013). However, the most dramatic examples of the appearance of *Bd* with a consequent population decline occurred in the anurans *Bufo periglenes* and *Atelopus varius* in Costa Rica in the 1980s (Pounds & Crump, 1994). Because of the seasonality of infectious *Bd* outbreaks, Lips, Diffendorfer, Mendelson, and Sears (2008) and Cheng, Rovito, Wake, and Vredenburg (2011) suggested that the pathogen arrived in North America in the early 1970s and moved southward into Central America and South America through introduced species or other vectors as well as by water currents (Cheng et al., 2011; Lips et al., 2008). In Mexico and Central and South America, the epidemiological outbreaks and the prevalence of *Bd* may also have been caused by more suitable climate conditions for the pathogen (Lips et al., 2008; Ron, 2005).

The earliest record of *Bd* infection in Mexico is from plethodontid salamanders collected in 1972 (Cheng et al., 2011). According to the cited study, chytrid infections were present in voucher specimens of several salamander genera collected in the 1970s and 1980s from the states of Veracruz, Oaxaca, and Hidalgo, where severe salamander population declines were reported by Parra-Olea, Garciá-París, and Wake (1999) and Rovito, Parra-Olea, Vásquez-Almazán, Papenfuss, and Wake (2009). These results led Cheng et al. (2011) to propose that an outbreak of chytridiomycosis might have been related to these declines. Thus far, *Bd* has been found in 50 species of Mexican amphibians, including *Pseudoeurycea leprosa* (Mendoza-Almeralla, Burrowes, & Parra-Olea, 2015), and the affected amphibians are mainly distributed in mountainous regions, extending from the north to the south of the country.

Despite the progress made in gathering information about the presence of *Bd* in Mexico, little is known about its infection dynamics in amphibian species distributed in the country. Therefore, this study had the following objectives: (1) determine whether the population of *P. leprosa* in La Malinche National Park is infected with *Bd* year-round; (2) determine if stress caused by temperature treatments affects the *Bd* infection load in *P. leprosa*, and (3) determine the effect of temperature and *Bd* infection on the survival of *P. leprosa*.

Materials and methods

The Malinche National Park is in the Trans-Mexican Volcanic Belt, where 2 of the continent's biogeographic regions converge. The park is located in the Mexican states of Puebla and Tlaxcala, and this region is characterized by high species diversity and endemism (López-Domínguez & Acosta, 2005). The park has a total area of 45,711 ha and a maximum elevation of 4,461 m.a.s.l. (Melo, 1977). Below 2,800 m.a.s.l., the climate is temperate with temperatures ranging between 12 and 18 °C, and above 2800 m.a.s.l., the climate is cold with temperatures from 5 to 12 °C (García, 2004).

We visited La Malinche National Park 15 times from June 2011 to September 2012; we collected specimens according to standard protocols and wore surgical gloves when handling and swabbing the salamanders (Van Rooij et al., 2011). The swab was then placed in a sterile 2 ml vial, and each organism was placed in a plastic bag to prevent cross-contamination. A number of the adult individuals were transported to the laboratory inside a plastic cooler in their individual plastic bags. In the laboratory, the salamanders were placed into a 250 ml plastic container (7.3 cm high, 8 cm upper diameter and 6.4 cm lower diameter), which contained a damp paper substrate. The salamanders were fed 2 flies or 2 crickets every 4 days during the experiments, and they were maintained at room temperature for 5 days before being used in the temperature experiments. After the experiments, 160 salamanders were deposited in the Colección Nacional de Anfibios y Reptiles (CNAR), Universidad Nacional Autónoma de México.

Each container was placed in a cooling system (wine cellar) fixed at a constant temperature of 10, 15, 20, 23, 25, or 28 °C. Humidity was maintained constant between 50 and 60% by spraying bottled water into the containers and changing the substrates every 3 days. The temperature and humidity were checked every 3 days with a thermohydrometer.

The experiment lasted for 10 weeks, and each salamander was swabbed once every week. Thirty salamanders were used for each of the temperature treatments (10, 15, 20, 23, and 25 °C); the first 10 salamanders subjected to 28 °C died within the first few days, so this temperature treatment was discontinued.

Detection and quantification of Bd

DNA was extracted from each swab using the PrepMan protocol (Applied Biosystems, Carlsbad, CA, USA) and analyzed following standard protocols with a real-time PCR assay to quantify *Bd* infection (Boyle, Boyle, Olsen, Morgan, & Hyatt, 2004). The assay uses genetic markers specific to *Bd* and compares each sample to a set of standards to calculate a genomic equivalent. DNA was extracted from each swab and analyzed according to standard protocols with a real-time PCR assay to quantify *Bd* infection (Boyle et al., 2004). The qPCR was performed on a StepOne Real-Time PCR System (Applied Biosystems). The PCR conditions consisted of an initial denaturation at 95 °C for 10 min, followed by 50 cycles of 10 s at 95 °C and 1 min at 60 °C. Each sample was assayed in duplicate together with standards of known *Bd* quantity (0.1, 1, 10, 100, and 1,000 zoospores) and with negative controls (5 μl double distilled water). Following the quantification of the *Bd* zoospore genomic equivalents (ZGEs), each value was multiplied by the dilution factor to obtain the total equivalent zoospores of each sample.

We calculated the *Bd* prevalence by dividing the number of infected salamanders by the total number of specimens swabbed per visit. The differences in the number of salamanders infected before and after the temperature treatments were evaluated using a McNecmar test for each treatment, and the infection load was expressed as the ZGE. To test for differences in the infection load caused by temperature stress, we ran a nonparametric analysis of variance (ANOVA) in SPSS version 21.0.0.0 (IBM Corporation Armonk, NY, USA) using the treatment data, the number of salamanders, and their infection loads before and after treatment.

To determine the effects of temperature on mortality in *P. leprosa*, we compared survivorship curves for each treatment using the survival package in R (Therneau, 2014). We ran an initial analysis that included all of the salamanders regardless of their infection load, and then divided them into 2 groups: salamanders that were *Bd* negative during the entire experiment and salamanders that became *Bd* positive during the experiment. In the survival analysis, greater importance was given to deaths that occurred at the beginning of the experiment (option rho = 1, implemented in the survdiff function) because temperature and humidity are known to affect *Bd*-amphibian dynamics by physiologically limiting vital processes for both the pathogen and the host. In this analysis, the temperature treatment was considered a *level*, and the collection month was considered a *stratum*. We tested for significant differences between all survivorship curves using a Kaplan–Meier analysis, and the probability of death associated with each temperature treatment was determined with Cox models of proportional risk.

To understand the relationship between infection load and survival in *P. leprosa*, the salamanders were classified into 3 groups: (1) the No-Cycle group, which included animals in which the infection load was either maintained or increased over time; (2) the 1–2 Cycles group, which included animals in which the infection load decreased/increased once or twice, and (3) the >3 Cycles group, which included animals in which the infection load decreased/increased 3 or more times. Using these data, we tested the hypothesis that survival depends on the ability to clear or reduce *Bd* infection. For these analyses, we also applied the Kaplan Meier analysis and Cox's proportional risk model. We included the cumulative number of zoospores over the course of the experiment (i.e., the sum of the ZGEs for 10 weeks) as an additional variable to explain the risk of mortality in Cox's proportional risk model.

Results

Over the 15 visits to La Malinche National Park from June 2011 to September 2012, we collected 160 adult *P. leprosa* (Table 1). Information on the microhabitat, specific locality, and ambient temperatures are provided in Appendix.

Our results show that only 12 (7.5%) of the salamanders were infected with *Bd* in the months of July, September, and December, and the prevalence was low in all 3 months, with values of 0.17, 0.09, and 0.16, respectively. The highest infection load in the field was 7812 ZGEs, which was observed in 1 individual in July (Table 1).

In all of the temperature treatments, 45 of the exposed salamanders (24.82%) were positive for *Bd*, although only 12 animals were *Bd* positive in the field, whereas the other 33 were recorded as *Bd* negative at the time of collection and became *Bd* positive at some point during the temperature treatments. These data indicate that in their natural habitat, the salamanders had subclinical levels of infection, and temperature stress caused an increase in *Bd* load. For each temperature treatment, the McNecmar analysis was performed to determine the number of salamanders infected before and after the experiment, and it returned a significant difference ($p \leq 0.05$) between the 10 and 20 °C treatments, indicating that exposing *P. leprosa* to certain temperatures affects pathogen expression.

The number of infected salamanders and the infection loads appeared to differ across treatments (Table 2), although the nonparametric ANOVA did not reveal significant differences ($p \geq 0.05$) in the infection load between the 10, 15, 20, 23 and 25 °C temperature treatments. This result led us to reject the hypothesis that the degree of infection is temperature dependent.

When all of the individuals were included in the analyses, the Kaplan–Meier survival analysis showed significant differences between temperatures (= 59.4, DF = 4, $p < 0.001$) (Table 3) and Cox's proportional risk models using the 10 °C treatment as a reference revealed significant differences in survival with the treatment at 25 °C (LRT = 68.8, DF = 4, $p < 0.001$), which had a risk rate of 63.5 (Table 3, Fig. 1A)

Table 1
Batrachochytrium dendrobatidis (*Bd*) prevalence in the field and after temperature treatments. The ZGE (zoospore genomic equivalent) range represents the infection load range. Prevalence is measured as the number of infected salamanders divided by the total number of organisms collected.

Collection date	No. of collected salamanders	No. of infected salamanders in the field (ZGE range)	Prevalence %	95% CI for prevalence	S.D.
June 2011	4	0	0		
July 2011	35	6 (410–7812)	0.17	(0.047, 0.296)	2937.76
August 2011	21	0	0	–	–
September 2011	21	2 (372.8, 1264)	0.10	(0.000, 0.221)	630.17
December 2011	25	4 (3460–3700)	0.16	(0.016, 0.304)	98.95
January 2012	19	0	0	–	–
February 2012	8	0	0	–	–
March 2012	9	0	0	–	–
April 2012	3	0	0	–	–
September 2012	15	0	0	–	–

Table 2
Results of the temperature treatments. N = number of organisms in the experiment. Prevalence is measured as the number of infected salamanders divided by the total number of organisms in the experiment. The ZGE range is the zoospore genomic equivalent loads or the infection load. Mortality is the percentage of dead salamanders divided by the total number of organisms exposed to each treatment.

Treatment °C	N	*Bd*-Positives after treatment	Prevalence %	95% CI for prevalence	ZGE range	Mortality
10	30	8	26	(0.108, 0.425)	80–7022	63%
15	30	9	30	(0.136, 0.464)	87–9679	40%
20	30	8	26	(0.108, 0.425)	39–41,390.40	73%
23	30	15	4.0	(0.225, 0.575)	8.3–6037	70%
25	30	5	13	(0.012, 0.255)	156–9798	100%
28	10	0				
Total	160	45				

Table 3
Summary of the survivorship analyses that measured the probability of death associated with each temperature treatment. Cox's proportional risk models used the 10 °C treatment as a reference. For the analyses, each temperature treatment was considered a *level*, and the collection month was considered a *stratum*.

Kaplan–Meier test		Cox's proportional risks model			
Analysis model	Test of significance	Analysis model	Test of significance	Comparisons (°C)	Hazard ratio (significance)
All individuals	$\chi^2 = 59.4$	All individuals	LRT = 68.8	15	0.73
	DF = 4		DF = 4	20	1.95
	$P < 0.001$		$p < 0.001$	23	3.29
				25	63.50*
Bd negatives	$\chi^2 = 46.8$	Negatives	LRT = 57.2 DF = 4	15	0.91
	DF = 4		$p < 0.001$	20	3.59
	$p < 0.001$			23	2.79
				25	160.54*
Bd positives	$\chi^2 = 51.1$	Positives	LRT = 32.9 DF = 4	15	0.27
	DF = 4		$p < 0.001$	20	1.91
	$p < 0.001$			23	0.47
				25	43.54*

* Significance level <0.05.

For the *Bd*-negative group, the Kaplan–Meier analysis also showed a difference in the probability of survival ($\chi^2 = 46.8$, DF = 4, $p < 0.001$), Cox's proportional risk models showed significant differences with the treatment at 25 °C (LRT = 57.2, DF = 4, $p < 0.001$), and the hazard ratio for this treatment was 160.54 (Table 3, Fig. 1B). For the *Bd*-positive group, the Kaplan–Meier analysis indicated significant differences (χ^2 test = 51.1, DF = 4, $p < 0.001$), whereas Cox's proportional risk models showed significant differences with the treatment at 25 °C (LRT = 32.9, DF = 4, $p < 0.001$), with a hazard ratio of 43.54 (Table 3, Fig. 1C).

From the first week of temperature treatments to the end of the experiment, 115 salamanders were healthy and 45 were infected (Table 2). Of the 45 infected salamanders, 8 died immediately after manifesting the infection (Fig. 2A); 9 maintained the infection at similar infection loads; 30 exhibited cycles of increasing–decreasing ZGEs once or twice and then presented as *Bd* negative for the remainder of the experiment (Fig. 2B), and

Table 4
Summary of the survivorship analyses that measured the relationship between the infection load and survival in *Pseudoeurycea leprosa*.

Kaplan–Meier test		Cox's proportional risks model			
Formula of the model	Test of significance	Formula of the model	Test of significance	Comparisons	Hazard ratio (significance)
Cycle groups + strata (month)	$\chi^2 = 6.2$	Cycle groups + $\log_{10}$ (total load + 0.1) data = positives	LRT = 27.5	>3 cycle group	0.02***
Rho = 1, data = positives	DF = 2	Reference = no cycle	DF = 3	1–2 Cycle group	0.32*
	$p = 0.046$		$p < 0.001$	$\log_{10}$ (total load + 0.1)	2.55***

Significance level
* <0.05,
*** <0.001.

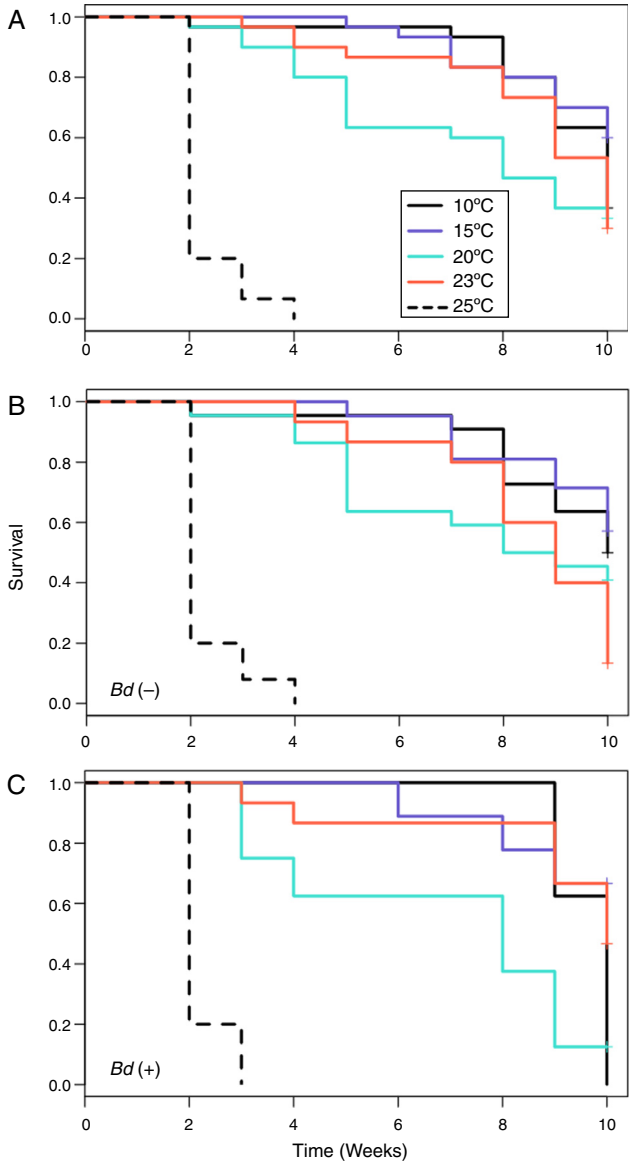

Fig. 1. Probability of survival of 3 groups, including (A) all salamanders, (B) uninfected salamanders, and (C) salamanders infected at some time during the treatment.

6 showed more than 3 cycles of increasing–decreasing ZGEs during the experiment (Fig. 2C). The Kaplan–Meier analysis of infection loads revealed a significant difference between the No-Cycle group and the animals that fell into 1 of the 2 cycle categories. The Cox analysis also detected significant differences between these groups (Table 4, Fig. 3A–C).

Discussion

Reductions in amphibian populations have frequently been associated with chytridiomycosis, a disease caused by the aquatic fungus *Bd*. Globally, forest-associated amphibians that live in or along streams are more likely to present declines compared with species in other assemblages (Ryan, Lips, & Eichholz, 2008; Stuart et al., 2008; Woodhams & Alford, 2005). Differences in habitat characteristics and life history traits as well as taxonomic variations in susceptibility to *Bd* could affect the prevalence of *Bd* in these communities.

Although *Bd* has been detected in numerous salamander species (Byrne, Davie, & Gibbons, 2008; Chatfield, Moler, & Richards-Zawacki, 2012), fully terrestrial plethodontid salamanders appear to be less susceptible to chytridiomycosis compared to anurans (Rothermel et al., 2008; Timpe, Graham, Gagliardo, Hill, & Levy, 2008). We detected *Bd* in *P. leprosa* from La Malinche, although the overall prevalence of the pathogen was relatively low when compared with other reports for Mexican amphibians. Field studies in Mexico have reported higher prevalence rates: for example, Frías-Álvarez et al. (2008) reported *Bd* in 50–100% of the ambystomatid salamanders and anurans analyzed from different parts of the country, Van Rooij et al. (2011) reported a prevalence between 25 and 100% in plethodontid salamanders in central and southern Mexico, and Luja, Rodríguez-Estrella, Ratzlaff, Parra-Olea, and Ramírez-Bautista (2012) also reported a high prevalence of *Bd* in the Baja California tree frog. However, all of these previous studies had small sample sizes, which substantially reduced the probability of an accurate measure for prevalence. Our study provides additional data to the limited number of previous studies that have systematically sampled terrestrial salamander

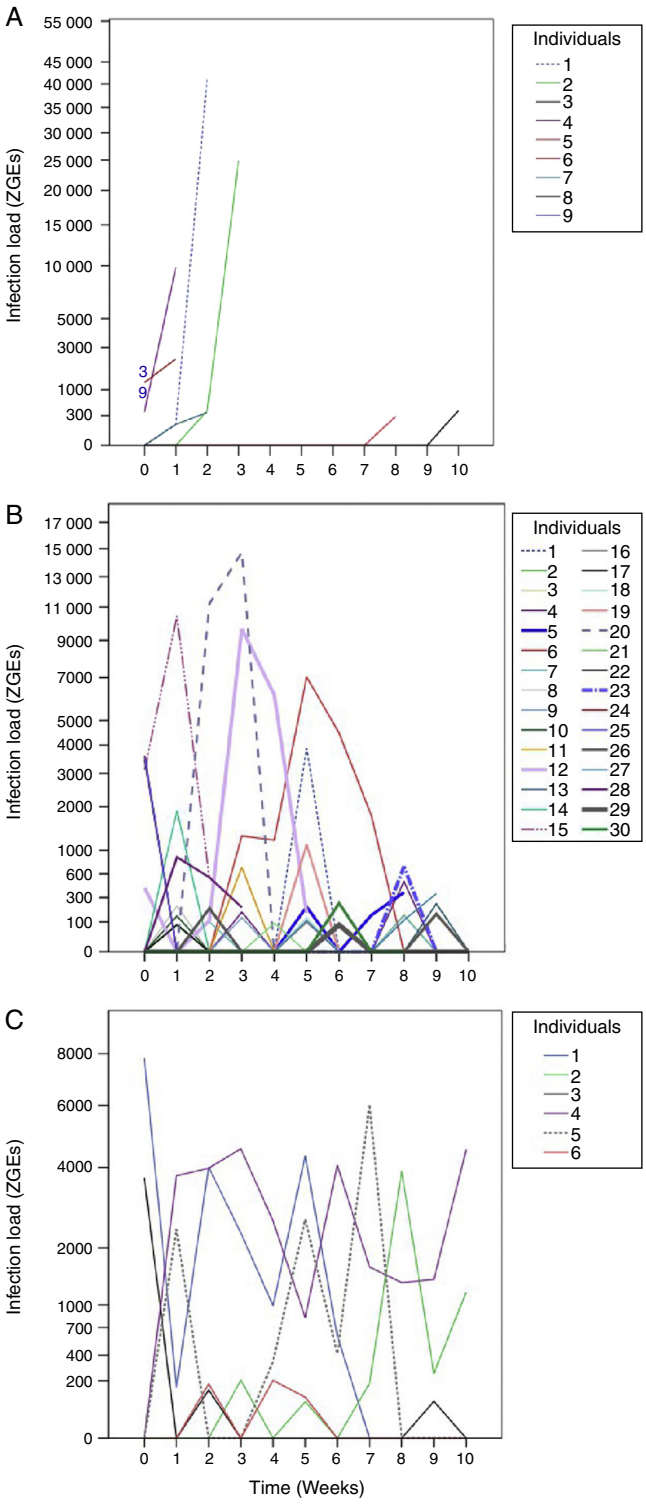

Fig. 2. *Batrachochytrium dendrobatidis* (*Bd*) infection loads. (A) No cycle: animals in which the infection load was either maintained or increased over time. Individuals 3 and 9 died before 1 week of treatment. (B) 1–2 cycles: animals in which the infection load decreased/increased once or twice. (C) >3 cycles: animals in which the infection load decreased/increased 3 or more times.

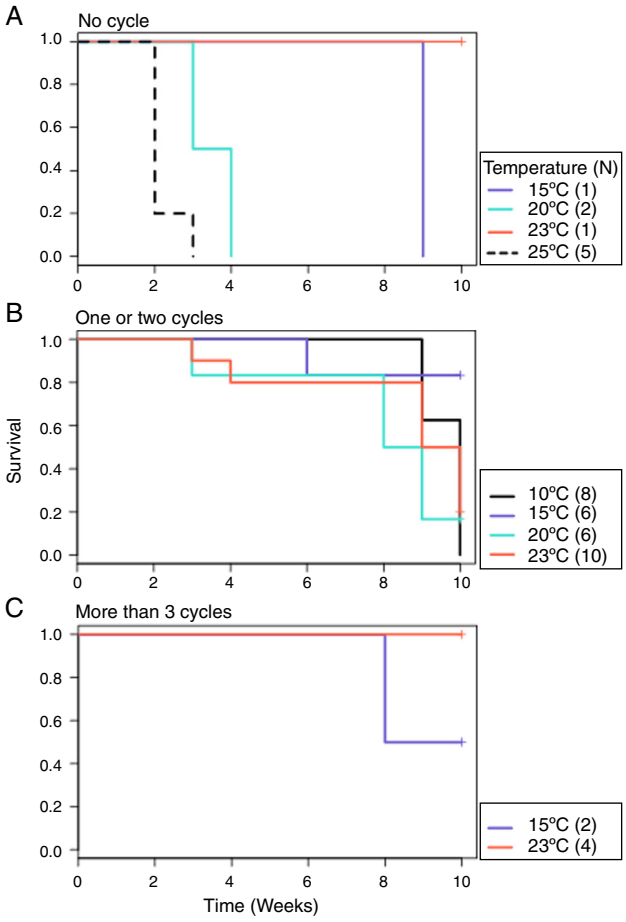

Fig. 3. Probability of survival. (A) No cycle: animals in which the infection load was either maintained or increased over time. Individuals 3 and 9 died before 1 week of treatment. (B) 1–2 cycles: animals in which the infection load decreased/increased once or twice. (C) >3 cycles: animals in which the infection load decreased/increased 3 or more times.

assemblages (Caruso & Lips, 2013; Keitzer, Goforth, Pessier, & Johnson, 2011; Moffitt et al., 2015; Muletz, Caruso, Fleischer, McDiarmid, & Lips, 2014; Rothermel et al., 2008), and the results are consistent with the proposal that salamanders are less affected by chytridiomycosis and may act as an important vector of the disease (Chatfield et al., 2012; Garner et al., 2005).

Ambient environmental conditions, such as temperature and precipitation, are known to affect the vital physiological processes of amphibians, and these conditions have a strong correlation with the outcome of *Bd* infections (population-level infection prevalence and host mortality rates) (James et al., 2015; Rowley & Alford, 2013). In culture, *Bd* grows best between 17 and 25 °C, with low temperatures retarding the pathogen's growth. The air and substrate temperatures observed at the time of collection (Appendix) and those reported for wild populations of *P. leprosa* (Guisado-Rodríguez & García-Vázquez, 2010) are generally lower than optimal for *Bd* to thrive (Piotrowski, Annis, & Longcore, 2004); therefore, these less than optimal temperatures may be the most simple explanation for the observed low prevalence of *Bd* infection and an important factor in the local abundance of this salamander species despite the epidemic

wave of chytridiomycosis that has affected several salamander populations (Rovito et al., 2009).

Although environmental conditions in La Malinche are favorable for the survival of *P. leprosa* with a low prevalence of *Bd*, when the salamanders were exposed to temperature experiments and conditions ideal for *Bd* growth, 33 new cases of infection were detected, indicating that these salamanders had subclinical levels of infection in the field. Over the course of the experiments, we observed variations in the infection loads, and the salamanders exhibited cycles of increasing and decreasing ZGEs over time. A similar pattern was described by Shin, Bataille, Kosch, and Waldman (2014), who found that the ZGEs of individual frogs significantly varied over a 5-day period because they intermittently released zoospores. Our results might also reflect cycles in the release of *Bd* zoospores; however, we cannot rule out the effects of the defense mechanisms of the 19 individuals that expressed *Bd* once and were then *Bd* negative for the remainder of the experiment.

Elevated host body temperatures have been shown to clear frogs of *Bd* infection. Woodhams, Alford, and Marantelli (2003) and Rowley and Alford (2013) showed that individual probabilities of infection by *Bd* decreased strongly with an increasing percentage of body temperatures above 25 °C in tropical species. For *P. leprosa*, the 3 survival analyses revealed that 25 °C is a critical temperature for the survival of these organisms and identified this temperature as the most important factor influencing the survival of the salamanders rather than infection by *Bd*; therefore, both the pathogen and the host are negatively affected by temperatures of 25 °C or higher.

P. leprosa is the most abundant species of plethodontid salamander in Mexico, and its populations currently appear to be stable (García-Vázquez, Gutiérrez-Mayén, Hernández-Jiménez, and Aurioles-López, 2006). However, studies of climate change in mountainous ecosystems have predicted an increase of 2 °C to 3 °C and a decrease in annual precipitation of between 3 and 20% by the year 2050 in the Trans-Mexican Volcanic Belt, where La Malinche National Park is located (Villers-Ruiz & Castañeda-Aguado, 2013). Furthermore, a loss of 75% of the distributional area of *P. leprosa* has also been predicted by the year 2050 because of climate change (Parra-Olea, Martínez-Meyer, & Pérez-Ponce de León, 2005). This study provides evidence that a synergistic effect between temperature stress and infection with *Bd* increases mortality in this species, and the results may provide an example of how temperate species of anurans might be affected by climate change.

Acknowledgments

We thank the Secretaría del Medio Ambiente y Recursos Naturales, Mexico for providing scientific collecting permits (FAUT 0106 and special permit 08657/14 to GP-O). Laura Márquez and Lidia Cabrera helped with the laboratory work. Funding was provided by PAPIIT-UNAM IN209914 and UC-MEXUS CONACyT (CN-13-614) to GPO. Mirna García-Castillo, Rafael Barzalobre and Mariana Meneses helped with the field work. This article fulfills part of the requirements of the first author to obtain the degree of Doctor of Sciences from the Graduate Studies in Biological Sciences Program of the UNAM. The first author expresses her gratitude to the Graduate Studies in Biological Sciences Program, UNAM for support and Conacyt for awarding a graduate studies scholarship (CVU/Becario, No. 205284/202580).

Appendix. Summary of locality parameters at time of collection

Collection data	No. collected individuals	Elevation	Microhabitat	Air temperature (°C)	Substrate temperature (°C)	Humidity (%)
June 2011	**4**					
19°14′32.40 N 98°00′20.4 W	1	2,700	Tree stump	16.5	15.2	58
19°16′36.35 N 98°03′7.4 W	3	3,200	Fallen trunk	17.2–18	21.4	61.4–65.8
July 2011	**35**	2,700–3,200				
19°14′11.00 N 97°59′48.0 W	8	2,745	Tree stump/fallen trunk	16.3–17.2	8.0	56.5–64
19°14′32.40 N 98°0′20.4 W	4	2,700	Tree stump/fallen trunk	15.8–16.6	7.6	54.4–60
19°14′29.40 N 97°59′23.2 W	11	2,709	Tree stump/fallen trunk	17–19.6	8.1	55–63
19°16′20.86 N 98°3′9.02 W	5	3,117	Tree stump/fallen trunk	17.5–18.4	8.9–14.9	62–68
19°16′36.35 N 98°3′7.48 W	7	3,200	Tree stump/fallen trunk	17–18.3	11.2–14	65–73.5
August 2011	**21**	2,700–3,200				
19°14′11.00 N 97°59′48.0 W	3	2,745	Tree stump/fallen trunk	15.5–17.8	7.2	60–66
19°14′32.40 N 98°0′20.4 W	4	2,700	Tree stump/fallen trunk	15.2–17.0	7.8	63.2–69
19°14′29.40 N 97°59′23.2 W	3	2,709	Tree stump/fallen trunk	15.0–17.6	7.5	62.5–77
19°16′20.86 N 98°3′9.02 W	7	3,117	Tree stump/fallen trunk	16.4–18.5	11.3	61–78
19°16′36.35 N 98°3′7.48 W	4	3,200		16.3–18.2	12.2	63–80
September 2011	**21**	2,700–3,200				
19°14′32. 4 N 98°0′20.4 W	8	2,700	Tree stump/fallen trunk	15.8–16.6	8.2–9.5	56–60
19°14′29.40 N 97°59′23.2 W	4	2,709	Tree stump/fallen trunk	15.6–16.9	7.5–9.1	55.3–61
19°16′20.86 N 98°3′9.02 W	4	3,117	Tree stump/fallen trunk	17.4–18	8.8–10.7	63.5–68.4
19°16′36.35 N 98°3′7.48 W	5	3,200	Tree stump/fallen trunk	18.7–19	9.5–12.2	60–69

December 2011	**25**	2,700–3,300				
19°14′32.40 N 98°0′20.4 W	9	2,700	Tree stump/fallen trunk	16.4–18.4	2.2–17.5	32.5–53
19°14′29.40 N 97°59′23.2 W	3	2,709	Tree stump/fallen trunk	16.9–18.5	2.4–8.8	37.9–40.5
19°16′20.86 N 98°3′9.02 W	2	3,117	Tree stump/fallen trunk	18.5	14.9–15.6	39.2
19°16′36.35 N 98°3′7.48 W	3	3,200	Tree stump/fallen trunk	17.2–18.1	5.6–16.7	38.7–39.7
19°15′26.90 N 98°4′1.10 W	8	3,300	Tree stump/fallen trunk	17.5–18.6	5.4–6.9	36.6–37.5
January 2012						
19°18′39.99 N 98°03′18.5 W	**19**	2,818	Tree stump/fallen trunk	17.7–23.1	2.4–13.4	23.9–45.1
February 2012						
19°18′39.99 N 98°03′18.5 W	**8**	2,818	Tree stump/leaf litter	15.3–21.5	7.8–14.8	47.8–65.7
March 2012						
19°18′39.99 N 98°03′18.5 W	**9**	2,818	Tree stump/leaf litter	18.2–23.2	6.8–12.2	22–30.8
April 2012						
19°18′39.99 N 98°03′18.5 W	**3**	2,818	Tree stump/leaf litter	21.3–27.7	8.8–19.2	26.14–33.8
September 2012						
19°18′39.99 N 98°03′18.5 W	**15**	2,818	Tree stump/leaf litter	13–17.8	6.6–12.4	53.3–68.2

References

Berger, L., Speare, R., Daszak, P., Green, D. E., Cunningham, A. A., Goggin, C. L., et al. (1998). Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proceedings of the National Academy of Sciences of the United States of America*, *95*, 9031–9036. http://dx.doi.org/10.1073/pnas.95.15.9031

Boyle, D. G., Boyle, D. B., Olsen, V., Morgan, J. A., & Hyatt, A. D. (2004). Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time TaqMan PCR assay. *Diseases of Aquatic Organisms*, *60*, 141–148. http://dx.doi.org/10.3354/dao060141

Brutyn, M., D'Herde, K., Dhaenens, M., Van Rooij, P. V., Verbrugghe, E., Hyatt, A. D., et al. (2012). *Batrachochytrium dendrobatidis* zoospore secretions rapidly disturb intercellular junctions in frog skin. *Fungal Genetics and Biology*, *49*, 830–837. http://dx.doi.org/10.1016/j.fgb.2012.07.002

Byrne, M. W., Davie, E. P., & Gibbons, J. W. (2008). *Batrachochytrium dendrobatidis* occurrence in *Eurycea cirregera*. *Southeastern Naturalist*, *7*, 551–555.

Caruso, N. M., & Lips, K. R. (2013). Truly enigmatic declines in terrestrial salamander populations in Great Smoky Mountains National Park. *Diversity and Distributions*, *19*, 38–48. http://dx.doi.org/10.1111/j.1472-4642.2012.00938.x

Carver, S., Bell, B. D., & Waldman, B. (2010). Does chytridiomycosis disrupt amphibian skin function? *Copeia*, *2010*, 487–495. http://dx.doi.org/10.1643/CH-09-128

Chatfield, M. W. H., Moler, P., & Richards-Zawacki, C. L. (2012). The amphibian chytrid fungus, *Batrachochytrium dendrobatidis*, in fully aquatic salamanders from southeastern North America. *PLOS ONE*, *7*, e44821. http://dx.doi.org/10.1371/journal.pone.0044821

Cheng, T. L., Rovito, S. M., Wake, D. B., & Vredenburg, V. T. (2011). Coincident mass extirpation of Neotropical amphibians with the emergence of the infectious fungal pathogen *Batrachochytrium dendrobatidis*. *Proceedings of the National Academy of Sciences of the United States of America*, *108*, 9502–9507. http://dx.doi.org/10.1073/pnas.1105538108

Daszak, P., Cunningham, A. A., & Hyatt, A. D. (2003). Infectious disease and amphibian population declines. *Diversity and Distributions*, *9*, 141–150. http://dx.doi.org/10.1046/j.1472-4642.2003.00016.x

Dobson, A., & Foufopoulos, J. (2001). Emerging infectious pathogens of wildlife. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *356*, 1001–1012. http://dx.doi.org/10.1098/rstb.2001.0900

Frías-Álvarez, P., Vredenburg, V. T., Familiar-López, M., Longcore, J. E., González-Bernal, E., Santos-Barrera, G., et al. (2008). Chytridiomycosis survey in wild and captive Mexican amphibians. *Ecohealth*, *5*, 18–26. http://dx.doi.org/10.1007/s10393-008-0155-3

García, E. (2004). *Modificaciones al Sistema de clasificación climático de Köppen* (2nd ed.). México D.F.: Instituto de Geografía, UNAM.

García-Vázquez, U. O., Gutiérrez-Mayén, M. G., Hernández-Jiménez, C. A., & Aurioles-López, V. (2006). Estudio de la densidad poblacional y algunos aspectos ecológicos de *Pseudoeurycea leprosa* en el Parque Nacional la Malinche. Tlaxcala, México. *Boletín de la Sociedad Herpetológica Mexicana*, *14*, 10–17.

Garner, T. W. J., Walker, S., Bosch, J., Hyatt, A. D., Cunningham, A. A., & Fisher, M. C. (2005). Chytrid fungus in Europe. *Emerging Infectious Diseases*, *11*, 1639–1641.

Guisado-Rodríguez, M. A., & García-Vázquez, U. O. (2010). Thermal ecology of *Pseudoeurycea leprosa* (Caudata: Plethodontidae) from Sierra del Ajusco. *The Herpetological Bulletin*, *111*, 15–18.

Harvell, C. D., Mitchell, C. E., Ward, J. R., Altizer, S., Dobson, A. P., Ostfeld, R. S., et al. (2002). Climate warming and disease risks for terrestrial and marine biota. *Science*, *296*, 2158–2162. http://dx.doi.org/10.1126/science.1063699

James, T. Y., Toledo, L. F., Rödder, D., da Silva Leite, D., Belasen, A. M., Betancourt-Román, C. M., et al. (2015). Disentangling host, pathogen, and environmental determinants of a recently emerged wildlife disease: lesson from the first 15 years of amphibian chytridiomycosis research. *Ecology and Evolution*, *5*, 4079–4097. http://dx.doi.org/10.1002/ece3.1672

Keitzer, S. C., Goforth, R., Pessier, A. P., & Johnson, A. J. (2011). Survey for the pathogenic chytrid fungus *Batrachochytrium dendrobatidis* in southwestern North Carolina salamander populations. *Journal of Wildlife Diseases*, *47*, 455–458. http://dx.doi.org/10.7589/0090-3558-47.2.455

Lips, K. R., Diffendorfer, J., Mendelson, J. R., III, & Sears, M. W. (2008). Riding the wave: reconciling the roles of disease and climate change in amphibian declines. *PLOS Biology*, *6*, 441–454. http://dx.doi.org/10.1371/journal.pbio.0060072

López-Domínguez, J. C., & Acosta, R. (2005). Descripción del Parque Nacional La Malinche. In J. A. Fernández, & J. C. López Domínguez (Eds.), *Biodiversidad del Parque Nacional La Malinche Tlaxcala, México* (pp. 3–20). Tlaxcala, México: Coordinación General de Ecología, Gobierno del Estado de Tlaxcala.

Luja, V. H., Rodríguez-Estrella, R., Ratzlaff, K., Parra-Olea, G., & Ramírez-Bautista, A. (2012). The chytrid fungus *Batrachochytrium dendrobatidis* in isolated populations of the Baja California tree frog *Pseudacris hypochondriaca curta* in Baja California Sur, Mexico. *The Southwestern Naturalist*, *57*, 323–327. http://dx.doi.org/10.1894/0038-4909-57.3.323

Marcum, R., St-Hilaire, S., Murphy, P. J., & Rodnick, K. (2010). Effects of *Batrachochytrium dendrobatidis* infection on ion concentrations in the boreal toad *Anaxyrus (Bufo) boreas boreas*. *Diseases of Aquatic Organisms*, *91*, 17–21. http://dx.doi.org/10.3354/dao02235

Martel, A., Blooi, M., Adriaensen, C., Van Rooij, P., Beukema, W., Fisher, M. C., et al. (2014). Recent introduction of a chytrid fungus endangers western Palearctic salamanders. *Science*, *346*, 630–631. http://dx.doi.org/10.1126/science.1258268

Medina-Vogel, G. (2010). Ecología de enfermedades infecciosas emergentes y conservación de especies silvestres. *Archivos de Medicina Veterinaria*, *42*, 11–24.

Melo, G. (1977). *Desarrollo de los parques nacionales mexicanos. Tres estudios sobre el mismo tema*. México, D.F.: Instituto de Geografía, UNAM.

Mendoza-Almeralla, C., Burrowes, P., & Parra-Olea, G. (2015). La quitridiomicosis en los anfibios de México: una revisión. *Revista Mexicana de Biodiversidad*, *86*, 238–248. http://dx.doi.org/10.7550/rmb.42588

Moffitt, D., Williams, L. A., Hastings, A., Pugh, M. W., Gangloff, M. M., & Siefferman, L. (2015). Low prevalence of the amphibian pathogen *Batrachochytrium dendrobatidis* in the Southern Appalachian Mountains. *Herpetological Conservation and Biology*, *10*, 123–136.

Muletz, C., Caruso, N. M., Fleischer, R. C., McDiarmid, R. W., & Lips, K. R. (2014). Unexpected rarity of the pathogen *Batrachochytrium dendrobatidis* in Appalachian *Plethodon* salamanders: 1957–2011. *PLOS ONE*, *9*, e103728. http://dx.doi.org/10.1371/journal.pone.0103728

Olson, D. H., Aanensen, D. M., Ronnenberg, K. L., Powell, C. I., Walker, S. F., Bielby, J., et al. (2013). Mapping the global emergence of *Batrachochytrium dendrobatidis*, the amphibian chytrid fungus. *PLOS ONE*, *8*, e56802. http://dx.doi.org/10.1371/journal.pone.0056802

Parra-Olea, G., Garciá-París, M., & Wake, D. B. (1999). Status of some populations of Mexican salamanders (Amphibia: Plethodontidae). *Revista de Biología Tropical*, *47*, 217–223.

Parra-Olea, G., Martínez-Meyer, E., & Pérez-Ponce de León, G. (2005). Forecasting climate change effects on salamander distribution in the highlands of central México. *Biotropica*, *37*, 202–208. http://dx.doi.org/10.1111/j.1744-7429.2005.00027.x

Pessier, A. P., Nichols, D. K., Longcore, J. E., & Fuller, M. S. (1999). Cutaneous chytridiomycosis in poison dart frogs (*Dendrobates* spp.) and white's tree frogs (*Litoria caerulea*). *Journal of Veterinary Diagnostic Investigation*, *11*, 194–199. http://dx.doi.org/10.1177/104063879901100219

Piotrowski, J. S., Annis, S. L., & Longcore, J. E. (2004). Physiology of *Batrachochytrium dendrobatidis*, a chytrid pathogen of amphibians. *Mycologia*, *96*, 9–15. http://dx.doi.org/10.2307/3761981

Pounds, J. A., & Crump, M. L. (1994). Amphibian declines and climate disturbance: the case of the golden toad and the harlequin frog. *Conservation Biology*, *8*, 72–85. http://dx.doi.org/10.1046/j.1523-1739.1994.08010072.x

Ron, S. R. (2005). Predicting the distribution of the amphibian pathogen *Batrachochytrium dendrobatidis* in the new world. *Biotropica*, *37*, 209–221. http://dx.doi.org/10.1111/j.1744-7429.2005.00028.x

Rothermel, B. B., Walls, S. C., Mitchell, J. C., Dodd, C. K., Irwin, L. K., Green, D. E., et al. (2008). Widespread occurrence of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* in the southeastern USA. *Diseases of Aquatic Organisms*, *82*, 3–18. http://dx.doi.org/10.3354/dao01974

Rovito, S. M., Parra-Olea, G., Vásquez-Almazán, C. R., Papenfuss, T. J., & Wake, D. B. (2009). Dramatic declines in Neotropical salamander populations are an important part of the global amphibian crisis. *Proceedings of the National Academy of Sciences of the United States of America*, *106*, 3231–3236. http://dx.doi.org/10.1073/pnas.0813051106

Rowley, J. J. L., & Alford, R. A. (2013). Hot bodies protect amphibians against chytrid infection in nature. *Scientific Reports*, *3*, 1515. http://dx.doi.org/10.1038/srep01515

Ryan, M. J., Lips, K. R., & Eichholz, M. W. (2008). Decline and extirpation of an endangered Panamanian stream frog population (*Craugastor punctariolus*) due to an outbreak of chytridiomycosis. *Biological Conservation, 141*, 1636–1647. doi:10.1016/j.biocon.2008.04.014.

Shin, J., Bataille, A., Kosch, T. A., & Waldman, B. (2014). Swabbing often fails to detect amphibian chytridiomycosis under conditions of low infection load. *PLOS ONE*, *9*, e111091. http://dx.doi.org/10.1371/journal.pone.0111091

Skerratt, L. F., Berger, L., Speare, R., Cashins, S., Mcdonald, K. R., Phillott, A. D., et al. (2007). Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. *Ecohealth*, *4*, 125–134. http://dx.doi.org/10.1007/s10393-007-0093-5

Stuart, S. N., Hoffman, M., Chanson, J. S., Cox, N. A., Berridge, R. J., Ramani, P., et al. (2008). *Threatened amphibians of the world*. Barcelona/Gland, Switzerland/Arlington, VA: International Union for the Conservation of Nature and Natural Resources, Conservation International, Lynx Editions.

Therneau, T. (2014). *A package for survival analysis in S. R package version 2.37-7.*. Retrieved from http://cran.rproject.org/web/packages/survival/index.html

Timpe, E. K., Graham, S. P., Gagliardo, R. W., Hill, R. L., & Levy, M. G. (2008). Occurrence of the fungal pathogen *Batrachochytrium dendrobatidis* in Georgia's amphibian population. *Herpetological Review*, *39*, 447–449.

Van Rooij, P., Martel, A., Nerz, J., Voitel, S., Van Immerseel, F., Haesebrouck, F., et al. (2011). Detection of *Batrachochytrium dendrobatidis* in Mexican bolitoglossine salamanders using an optimal sampling protocol. *Ecohealth*, *8*, 237–243. http://dx.doi.org/10.1007/s10393-011-0704-z

Villers-Ruiz, L., & Castañeda-Aguado, D. (2013). Species and plant community reorganization in the Trans-Mexican Volcanic Belt under climate change conditions. *Journal of Mountain Science*, *10*, 923–931. http://dx.doi.org/10.1007/s11629-013-2719-0

Voyles, J., Berger, L., Young, S., Speare, R., Webb, R., Warner, J., et al. (2007). Electrolyte depletion and osmotic imbalance in amphibians with chytridiomycosis. *Diseases of Aquatic Organisms*, *77*, 113–118.

Voyles, J., Rosenblum, E. B., & Berger, L. (2011). Interactions between *Batrachochytrium dendrobatidis* and its amphibian hosts: a review of pathogenesis and immunity. *Microbes and Infection*, *13*, 25–32. http://dx.doi.org/10.1016/j.micinf.2010.09.015

Wake, D. B., & Vredenburg, V. T. (2008). Colloquium paper: are we in the midst of the sixth mass extinction? A view from the world of amphibians. *Proceedings of the National Academy of Sciences of the United States of America*, *105*, 11466–11473.

Woodhams, D. C., & Alford, R. A. (2005). Ecology of chytridiomycosis in rainforest stream frog assemblages of tropical Queensland. *Conservation Biology*, *19*, 1449–1459. http://dx.doi.org/10.1111/j.1523-1739.2005.004403.x

Woodhams, D., Alford, R. A., & Marantelli, G. (2003). Emerging disease of amphibians cured by elevated body temperature. *Diseases of Aquatic Organisms*, *55*, 65–67. http://dx.doi.org/10.3354/dao055065