Coincident mass extirpation of neotropical amphibians with the emergence of the infectious fungal pathogen *Batrachochytrium dendrobatidis*

Tina L. Cheng, Sean M. Rovito, David B. Wake, and Vance T. Vredenburg

Amphibians highlight the global biodiversity crisis because ~40% of all amphibian species are currently in decline. Species have disappeared even in protected habitats (e.g., the enigmatic extinction of the golden toad, *Bufo periglenes*, from Costa Rica). The emergence of a fungal pathogen, *Batrachochytrium dendrobatidis* (Bd), has been implicated in a number of declines that have occurred in the last decade, but few studies have been able to test retroactively whether Bd emergence was linked to earlier declines and extinctions. We describe a noninvasive PCR sampling technique that detects Bd in formalin-preserved museum specimens. We detected Bd by PCR in 83–90% (n=38) of samples that were identified as positive by histology. We examined specimens collected before, during, and after major amphibian decline events at established study sites in southern Mexico, Guatemala, and Costa Rica. A pattern of Bd emergence coincident with declines at these localities is revealed—the absence of Bd over multiple years at all localities followed by the concurrent emergence of Bd in various species at each locality during a period of population decline. The geographical and chronological emergence of Bd at these localities also indicates a southbound spread from southern Mexico in the early 1970s to western Guatemala in the 1980s/1990s and to Monteverde, Costa Rica by 1987. We find evidence of a historical “Bd epidemic wave” that began in Mexico and subsequently spread to Central America. We describe a technique that can be used to screen museum specimens from other amphibian decline sites around the world.

chytridiomycosis | emerging infectious disease | epizootic

The global biodiversity crisis, which predicts the sixth mass extinction in Earth’s history (1), is often illustrated with examples from class Amphibia because ~40% of all amphibian species are currently in decline (2). Habitat destruction, over-exploitation for food and the pet trade, pollution and climate change all have been implicated, but an emerging infectious fungal disease, chytridiomycosis, has raised alarm because it has spanned taxonomic and geographical barriers, reaching areas of protected habitat and further compounding the effects on biodiversity loss attributed to other factors (3). This disease is caused by the chytridiomycete fungus *Batrachochytrium dendrobatidis* (Bd). Bd has a flagellated infective life stage called the zoospore that imbeds itself into the keratinized skin of amphibians causing hyperkeratosis, loss of skin function, osmoregulatory failure, and death (4–6). The emergence of Bd, described in 1999, has been definitively tied with collapse of amphibian populations in Australia (5), Panama (7), California (8), and Peru (9) and has been implicated in many declines that occurred decades ago (10, 11). Chytridiomycosis is unusual because multiple host species in at least one region have disappeared (7), apparently before density-dependent factors could slow the spread of disease (8, 12). Establishing the presence of Bd in museum specimens from vanished populations could be the key to uncovering the historical and geographical spread of this pathogen and would provide objective evidence of Bd emergence and subsequent Bd-driven amphibian decline. In this study, we use noninvasive sampling and molecular techniques to detect Bd in formalin-preserved specimens to investigate the role of Bd in two well-studied cases of enigmatic amphibian decline in Mesamerica (i): the decline and disappearance of anurans from Costa Rica’s Monteverde Reserve in the late 1980s (13, 14), and (ii) the decline and disappearance of plethodontid salamanders from the mountains of southern Mexico and western Guatemala in the 1970s and 1980s (15).

The sudden extinction of the golden toad (*Bufo periglenes*) and harlequin frog (*Atelopus varius*) from Costa Rica’s Monteverde Reserve in the late 1980s (13, 14) are among the earliest and best-documented cases of enigmatic declines that have come to characterize the global amphibian crisis. The subsequent disappearance of 40% (20/49 species) of anurans from Monteverde’s cloud forest (16) places the Monteverde declines among the most extreme cases of documented biodiversity loss in amphibian faunas. Various hypotheses have arisen regarding the cause of this decline, including the arrival of Bd to naïve amphibian populations in Monteverde as part of a southward-moving Bd wave (7) and climate-linked Bd emergence (13, 16, 17), with implications for worldwide Bd emergence. Remarkably, despite the central role that Bd has been hypothesized to play in these declines, no direct evidence has been reported of Bd emergence in Monteverde coincident with declines.

The declines of bolitoglossine salamanders (family: Plethodontidae) from the neotropics of southern Mexico and Guatemala (15, 17) are among the few records of enigmatic decline occurring in salamanders. As in Monteverde and in other cases of enigmatic decline from around the world, these salamander populations seem to have disappeared from montane environments despite the availability of suitable, protected habitat (11, 15). Although preliminary data from Rovito et al. (15) show that Bd currently exists in San Marcos, Guatemala, where plethodontid salamanders are known to have declined, interpretation of these data is difficult because many species have been extirpated.

In this study, we introduce a reliable noninvasive molecular technique that enables us to detect Bd in formalin-preserved amphibian specimens. Furthermore, we use this technique to examine amphibian specimens collected in areas of documented decline in Mexico, Guatemala, and Costa Rica. In addition, we present laboratory studies that investigate the susceptibility of two neotropical plethodontid species to Bd and demonstrate that Bd can cause mortality in these species.

Author contributions: T.L.C., D.B.W., and V.T.V. designed research; T.L.C. performed research; S.M.R. and D.B.W. collected specimens; T.L.C. and V.T.V. analyzed data; and T.L.C., S.M.R., D.B.W., and V.T.V. wrote the paper.

The authors declare no conflict of interest.

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Results

Noninvasive sampling methods (swab/Qiagen and swab/Prepman) of formalin-preserved amphibian specimens collected and preserved at the Museum of Vertebrate Zoology (MVZ) as far back as 1972 found Bd in 90 and 83% of samples, respectively, that were identified as positive by histology (Table 1). We measured the sensitivity of the recovery of DNA between replicate runs on real-time PCR (singlicate, duplicate, triplicate, and quadruplicate) and found that resulting Bd prevalences were 61.8, 78.1, 84.2, and 89.5% accurate in relation to histology, respectively (Table S1). Bd prevalence resulting from a single sample qPCR run was found to be significantly different from histology results (χ² test, P = 0.00987), but prevalence resulting from duplicate or higher qPCR runs did not differ significantly from histology results (Table S1). We also found that estimated infection intensities (measured in zoospore equivalents) detected in museum specimens were not consistent with actual infection intensities (taken before animals were killed and fixed in formalin), and thus we only relied on positive versus negative results instead of reporting actual infection intensities from qPCR results (Table S2).

Using the swab/Prepman and swab/Qiagen techniques, we surveyed amphibian specimens collected in five neotropical amphibian communities from Mexico, Guatemala, and Costa Rica where amphibian declines occurred. A strong pattern of Bd emergence was found for all countries—Bd is absent over multiple years and is followed by the concurrent emergence in several species from each locality (Tables 2 and 3 and Tables S3 and S4). Furthermore, we found Bd emergence to coincide directly during the year of decline (1987) in Monteverde, Costa Rica (Table 3) and the absence of Bd in healthy populations (with exception in Cerro San Felipe, Mexico where specimens were not available for sampling before the earliest date of detection, 1974) followed by the emergence and presence of Bd in declining and impacted populations in Mexico (Fig. 1) and Guatemala. We further examined the strength of detection if Bd had been present at a prevalence of 5% (probability of a false negative). The emergence of Bd in Mexico, Guatemala, and Costa Rica, which is predicated on the verification of its absence before the earliest date of detection, was found to have low probability of a false negative for all countries: Mexico (1964–1971), 0.019; Guatemala (1969–1979), 8.1 × 10⁻¹¹; and Costa Rica (1967–1984), 0.01. In Mexico, Bd was eventually detected in all species sampled except Pseudoeurycea unguidentis and Parmidobatrachus townsendi, despite relatively large sample sizes for those two species (probability of a false negative: 0.081 and 0.12, respectively). In Guatemala, Bd was found in 7 out of 10 species sampled, with no Bd detected in Bolitoglossa franklini franklini, Pseudoeurycea rex, and Bolitoglossa moto; however, collections after 1994 (earliest date of detection in Guatemala) for all three species were small (probability of a false negative: 0.95, 0.77, and 0.40, respectively).

From 20 anuran species that have disappeared from Monteverde Reserve, we tested 4 of these species during the year of decline and found 3 to be positive with Bd: Craugastor andi, Craugastor angelicus, and Isthmohyla rivularis. Three other species reported as disappeared were found to be Bd negative predecline; however, these specimens were not collected in 1987 (B. periglenes, A. varius, and Duellmanohyla uranochrous) and thus were not sampled during decline. Agalychnis lemur is reported to have disappeared from Monteverde, but is only represented by one specimen that did not test positive for Bd.

In the laboratory, we observed the effects of Bd in two species of wild-caught salamanders and one frog species from Mexico. All 10 frogs, Plectrohyla matutaii, and 5 of 10 Pseudoeurycea leprosa contained Bd infections when collected in the field; all 6 Bolitoglossa rufescens were uninfected from the field and 3 were experimentally infected with Bd in the laboratory. Both species of salamanders, P. leprosa and B. rufescens, revealed high susceptibility to Bd infection, but the frog, P. matutaii, was resistant to Bd infection (Fig. 2). All infected salamanders (five P. leprosa and three B. rufescens) rapidly increased in infection intensity and suffered mortality when average level of infection intensity of ~10,000 ZE (zoospore equivalents) was reached (x ± SE) = 37,841 ± 7,111 zoospore equivalents × swab⁻¹. All uninfected salamanders (negative controls; 10 P. leprosa) remained uninfected during the infection trial. In contrast to infected salamanders, all infected frogs (n = 10) maintained Bd infection at levels well below the lethal zone (<1,000 ZE) and also remained healthy for the duration of the trial.

Discussion

Our results indicate that the chytrid pathogen emerged in plethodontid salamanders in Mexico and Guatemala, and in salamanders and frogs at Monteverde, Costa Rica, is coincident with the amphibian community collapse and extinctions that occurred at these localities, providing direct evidence for the hypothesis that Bd played a major role in these declines. Furthermore, our data corroborate a pattern of temporal and spatial spread of Bd previously posited by Lips et al. (7, 18), which we now are able to extend much further northwest, from southern Mexico in the early 1970s, reaching Guatemala by the 1980s/1990s, and spreading to Monteverde, Costa Rica by 1987 (Fig. 3). The absence of Bd in all countries before first detection, despite multiyear sampling, runs counter to the idea that the pathogen was present in these environments and emerged in response to climatic changes (16).

The pattern elucidated here of the arrival and emergence of the chytrid pathogen in concert with amphibian declines was best documented in two other cases of Bd-driven amphibian decline (7, 8). In these studies, the absence of Bd in healthy populations was well documented through multiyear sampling periods followed by documentation of mass mortality and population decline directly caused by Bd epizootic outbreak. The spatial-temporal spread of Bd we document from Mexico to Costa Rica is further supported by previously published accounts of Bd-linked amphibian decline in Mexico and Costa Rica. In southern Mexico, the decline of 19–45% of anuran fauna is documented to have occurred in the mid- to late-1970s and early 1980s, and these declines were posited to be Bd driven because Bd was found during the most recent surveys (18). These results are consistent with the decline of plethodontids in southern Mexico (15), which was previously unexplained, and the emergence of Bd in the early 1970s that we report in this study. Furthermore, histological examination of amphibian specimens documented Bd in amphibians collected in 1986 from a location about 75 km east-southeast of Monteverde; Bd thus had emerged close to Monteverde just 1 y before we find Bd in amphibians at Monteverde when the declines there began (19). We detected the earliest record of Bd in Monteverde from a small but extremely valuable collection of specimens collected from this reserve in 1987 and obtained only through extraordinary permission.

Table 1. Comparison of recovery rates between histology and three methods using noninvasive sampling and molecular tools to detect Bd in formalin-preserved specimens (Genus: Batrachoseps)

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>1971</td>
<td>7</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>1973</td>
<td>15</td>
<td>15</td>
<td>13</td>
<td>15</td>
<td>9</td>
</tr>
<tr>
<td>1974</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>1993</td>
<td>7</td>
<td>7</td>
<td>3</td>
<td>4</td>
<td>7</td>
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<tr>
<td>1995</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
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<tr>
<td>2007</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>38</td>
<td>29</td>
<td>26</td>
<td>24</td>
<td>22</td>
</tr>
<tr>
<td>Recovery rate, no. positives/histology positives</td>
<td>100%</td>
<td>89.7%</td>
<td>82.8%</td>
<td>75.9%</td>
<td></td>
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</table>
Janthinobacterium lividum and Pseudoeurycea unguidentis (0.5 which describes mortality in individuals (27)).

In our laboratory trials, interpretation of P. leprosa and B. rufescens Bolitoglossa species, such as the P. matudai we report here, persist with low density between terrestrial specialists and arboreal specialists, even if experienced rare cases of mortality in individuals due to Bd infection, could persist on a population level due to low population densities and low transmission rates. In contrast, terrestrial specialists oc-

Table 2. Bd prevalence detected in museum specimens collected from Guatemala

<table>
<thead>
<tr>
<th>Species</th>
<th>% Bd prevalence and 95% Bayesian Credible Interval</th>
<th>n</th>
<th>% Bd prevalence and 95% Bayesian Credible Interval</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bolitoglossa engelhardti</td>
<td>0 (0–6)</td>
<td>61</td>
<td>10 (3–30)</td>
<td>20</td>
</tr>
<tr>
<td>Bolitoglossa flavimembris</td>
<td>0 (0–6)</td>
<td>60</td>
<td>19 (4–30)</td>
<td>15</td>
</tr>
<tr>
<td>Bolitoglossa franklini franklini</td>
<td>0 (0–12)</td>
<td>29</td>
<td>0 (0–11)</td>
<td>6</td>
</tr>
<tr>
<td>Bolitoglossa franklini x Bolitoglossa lincolni</td>
<td>0 (0–6)</td>
<td>61</td>
<td>100 (16–99)</td>
<td>1</td>
</tr>
<tr>
<td>Bolitoglossa lincolni</td>
<td>0 (0–12)</td>
<td>27</td>
<td>20 (4–64)</td>
<td>5</td>
</tr>
<tr>
<td>Bolitoglossa morio</td>
<td>0 (0–3)</td>
<td>13</td>
<td>0 (0–18)</td>
<td>18</td>
</tr>
<tr>
<td>Bolitoglossa occidentalis</td>
<td>0 (0–6)</td>
<td>56</td>
<td>31 (13–58)</td>
<td>13</td>
</tr>
<tr>
<td>Dendrotriton bromeliacius</td>
<td>0 (0–8)</td>
<td>43</td>
<td>3 (1–15)</td>
<td>35</td>
</tr>
<tr>
<td>Eleutherodactylus greggi</td>
<td>0 (0–6)</td>
<td>61</td>
<td>10 (2–41)</td>
<td>10</td>
</tr>
<tr>
<td>Pseudoeurycea rex</td>
<td>0 (0–8)</td>
<td>43</td>
<td>0 (0–46)</td>
<td>5</td>
</tr>
<tr>
<td>Totals</td>
<td>0 (0–1)</td>
<td>454</td>
<td>9 (5–16)</td>
<td>128</td>
</tr>
</tbody>
</table>

Red and bold font indicate positive Bd prevalence.

We also examined the question of whether Bd, described as an aquatic fungus (6), could cause lethality in neotropical plethodontids, which are terrestrial and direct-developing amphibians. Previous studies have reported mortality from Bd infection in plethodontids through laboratory investigation of Batrachoseps from California (25) and in the field, with reports of dead Oedipina and Bolitoglossa (8, 26). The presence of Bd we report in field-collected animals as well as in historical specimens provides additional evidence that neotropical plethodontids are hosts of the chytrid pathogen. Results from our infection trials demonstrate that under laboratory conditions, the disease, chytridiomycosis, quickly develops in individuals harboring Bd infection and is highly lethal in at least two species of neotropical plethodontids—P. leprosa and B. rufescens. Additionally, our infection trials reveal a pattern of mortality in infected individuals that agrees with “Vredenburg’s 10,000 Zoospore Rule,” which describes mortality in individuals (27) and population extinctions in the wild (8) when average infection intensities reached ~10,000 ZE. In our laboratory study, all infected salamanders died at high infection intensities ([x ± SE] = 37,841 ± 7,111 zoospore equivalents x swab−1). Resistant species, such as the P. matudai we report here, persist with low infection levels (<1,000 ZE) in the laboratory. These infection intensities are close to those reported in nonsusceptible (enzy- otic) wild populations of Rana sierrae infected with Bd (12). Further investigation of other suspected susceptible and non-
susceptible species is needed to determine whether Bd infection intensity can predict susceptibility of amphibian hosts to Bd. Despite the high susceptibility to Bd infection found for P. leprosa and B. rufescens in our laboratory trials, interpretation of these results should be done cautiously, because responses to Bd infection may differ under field conditions. In the laboratory, manipulation of climatic variables significantly affects host responses to Bd infection and survivability (25, 28).

In both Mexico and Guatemala, Bd was not detected in three species despite (i) good sample sizes (n ≥ 15, probability of a false negative = 0.46)–Pseudoeurycea unguidentis, Parvimolge townsendi, and B. morio and (ii) presence of Bd in heterospecifics at the site. The absence of Bd in these species may indicate vari-
ability in susceptibility to Bd between species; variation in Bd prevalence between species has also been reported in Panama (29). Additionally, Rovito et al. (15) reported a disproportionate decline in terrestrial specialists over arboreal specialists and microhabitat generalists in Guatemalan salamander populations. A possible explanation for the discrepancy in the decline of terres-
trial specialists may be explained by differences in population density between terrestrial specialists and arboreal specialists/ microhabitat generalists. In the 1970s, terrestrial species in both Mexico and Guatemala were found in high abundance and high densities, and arboreal and microhabitat generalists were found at lower abundances and densities (15, 30). Thus, the pattern seen among habitat specialists may be following a density-dependent host–pathogen dynamic where Bd outbreak operates independ-ently from differences in host susceptibility or virulence in Bd strains (12). From this model, an epizootic outbreak occurs in a population when host densities permit Bd transmission rates to surpass the critical threshold leading to an outbreak (12). Thus, arboreal specialists and microhabitat generalists, even if experi-
enced rare cases of mortality in individuals due to Bd infection, could persist on a population level due to low population densities and low transmission rates. In contrast, terrestrial specialists oc-
Climate change has been invoked as the trigger for amphibian declines in Monteverde in a number of studies (34–36). In Europe, a study suggested a link between climate change and Bd emergence (37). Whereas our results do not rule out a role for climate change, we have found no necessary connection between climate change and the particular extinction events at our study sites. We suggest that the emergence and spread of a pathogen into naïve host populations can explain amphibian declines in the neotropics as other studies have also posited (7, 38, 39). The spatial-temporal pattern of Bd spread we describe, using a Bd PCR assay on museum specimens coincident with larger Bd-related amphibian declines occurring throughout Central America, indicates that Bd played a major role in these declines, regardless of other contributing factors. Our Bd PCR assay could be used to determine whether Bd was associated with other enigmatic amphibian declines that occurred historically and may help delineate the spread of Bd throughout the world’s amphibians.

**Materials and Methods**

**Noninvasive Sampling of Formalin-Preserved Specimens.** We investigated the effectiveness of noninvasive sampling techniques in combination with molecular methods to detect Bd in formalin-preserved amphibian specimens. We sampled a total of 38 specimens from six species of the genus *Batrachoseps*...
examined formalin-preserved × 7,111 zoospore represents μ when possible
Bolitoglossa rufescens P. leprosa and three
Map of the spatial-temporal spread of Bd southward from Mexico
Results from laboratory trials monitoring Bd infection for one neo-
and three TE Plectrohyla matudai and (B. rufescens Pseudoeurycea leprosa S
swab were stroked 30 times over the ventral surface of salamanders from
sampling and gloves were rinsed or changed between animals. Brushes and
collected between 1971 and 2007 in Northern California. All specimens had
been previously examined for Bd using histology (25), which determined
that 29 specimens were Bd positive and 9 were Bd negative.
We tested two noninvasive sampling methods on formalin-preserved
museum specimens used previously but unsuccessfully (40): skin swab (41)
and brush (Oral-b interdental refill brush) (40). To decrease chances of
contamination by erant skin pieces or other floating zoospores in preser-
vation jars, each individual specimen was rinsed with 70% ETOH before
sampling and gloves were rinsed or changed between animals. Brushes and
swabs were stroked 30 times over the ventral surface of salamanders from
neck to vent, stored in 1.5-ML microcentrifuge tubes, and kept at 4 °C
until processing.
We extracted swabs using two comparative extraction methods: Prepman
Ultra (41) and Qiagen DNeasy blood and tissue kit. Qiagen DNeasy extra-
tions were used according to their tissue extraction protocol, with a varia-
tion in final elution volume to 40 μL of AE buffer. Brush samples were
extracted using Qiagen DNeasy blood and tissue kit, also with a final elution
volume of 40 μL AE buffer. All extractions were diluted 1:10 in 0.25x TE
buffer and run in triplicate on real-time PCR following Boyle et al. (41) along
with positive controls at dilution levels of 0.1, 1.0, 10, and 100 ZE. Raw ge-
nomic output from real-time PCR was multiplied by 80 to account for di-
lution during extraction, resulting in a relative infection intensity measured
in terms of ZE. Samples were regarded as being Bd positive if one out of
three replicates returned a positive result (>0.1 ZE). False positives are rare
when working with Bd and real-time PCR (42), but negative controls were
run to ensure against false positives. Real-time PCR results were compared
with results from histological examination to determine accuracy in Bd de-
tection. Recovery rate was calculated as the percentage of Bd positives
resulting from our noninvasive sampling technique out of a total of 29
histology-confirmed Bd positives.

**Fig. 1.** Timeline of mean relative salamander abundance (line) and mean
Bd prevalence (bars) for all sites and species in Mexico. Asterisk indicates
zero Bd prevalence for which sample sizes were large and probability of a
false negative was low (<10%). Arrow indicates earliest year of Bd de-
tection in Mexico (1972). The absence of Bd occurs during high abundance
years and is followed by Bd emergence and increasing Bd prevalence that
coincides with the marked decline of salamanders (15) at all sites in Mexico.

**Sampling Specimens from Mexico, Guatemala, and Costa Rica.** For specimen
sampling from Monteverde, Costa Rica, we used the swab/Qiagen method to
take a total of 26 specimens from 9 different species collected predede-
(1967, 1976, and 1977) and 48 specimens from 16 different species collected
during the first reported year of decline (1987). We sampled all specimens
using synthetic cotton swabs (41). To decrease chances of contamination by
erant skin pieces or other floating zoospores in preservation jars, each in-
dividual specimen was rinsed with 70% ETOH before sampling and gloves
were rinsed or changed between animals. Frogs were swabbed on the ven-
tral surface, including the inner thighs, abdomen, and between toes. Sala-
manders were swabbed on the ventral surface from neck to vent. We
averaged 30 strokes per individual to standardize sampling. Swabs were
extracted using a Qiagen DNeasy blood and tissue kit, and run in triplicate on
Taqman real-time PCR using the same methods as described above (41, 42).
Using the swab/Prepman method, we examined formalin-preserved
specimens that were part of reported declines in Mexico and Guatemala (15)
for the presence of Bd. We tested 615 specimens from Guatemala collected
between 1969 and 2010 and 537 specimens from Mexico collected between
1964 and 2009 for the presence of Bd. No field studies were conducted in
Guatemala between 1979 and 1990, so no samples exist for this time period.
We attempted to achieve a sample size of 30 individuals per species, per
locality, per year—when possible—to achieve a minimum detection preva-
ence of 5% (8); for later sampling years where species were rare, we sam-
ped all individuals available.

Bd prevalence was calculated for each locality by species and year along
with posterior distributions calculated using Bayesian probability for 95% credible intervals (43). For instances of zero Bd prevalence, we also calcu-
lated the probability of a false negative on the basis of a true Bd prevalence
of 5% in the population using the formula (1 − 0.05²), where S represents
sample size. Mean relative salamander abundance (Fig. 1) was based on data
from Rovito et al. (15) (1969 estimates were calculated using the number of
collectors listed in the MVZ database) and was calculated by first nor-
malizing the data relative to each species’ highest abundance (relative sal-

**Fig. 2.** Results from laboratory trials monitoring Bd infection for one neo-
tropical frog, (A) Plectrohyla matudai, and two neotropical plethodontid
salamanders, (B) Pseudoeurycea leprosa and (C) Bolitoglossa rufescens.
Dotted lines with square points represent Bd-infected individuals and solid
lines with circle points represent Bd-uninfected individuals. All infected sal-
amanders (five P. leprosa and three B. rufescens) increased infection in-
tensity rapidly over time and suffered mortality. The average infection
intensity of animals that died was (S ± 1 SE) = 37,841 ± 7,111 zoospore
equivalents × swab⁻¹. Uninfected salamanders (10 P. leprosa and three B.
rufescens) remained healthy and Bd negative over time. For P. matudai, all
individuals (n = 10) were infected but persisted with low levels of infection.
In 2008, six uninfected *B. rufescens* and 10 infected *P. matudai* were collected from Chipas, Mexico. In 2009, 15 *P. leprosa* were collected from Puebla, Mexico. All animals were individually housed and imported live to the animal care facility at San Francisco State University (SFSU), where they were individually housed in plastic 5-L containers with lids and moist paper towels. Each animal was checked daily by animal care staff; containers where changed and animals were fed live crickets once a week during the entire length of the experiment (7 wk). During the 7-wk infection trial, all frogs and salamanders were swabbed once a week and monitored for health. For the infection trial, three *B. rufescens* were infected with Bd, whereas the remaining three served as negative controls. Infection with Bd was achieved by housing each *B. rufescens* together with one Bd-positive *P. matudai* in a small Tupperware with 0.5-inches of double distilled H2O for 1 h a day for 5 consecutive days. All frogs (*P. matudai*) were housed in 10-L plastic rat containers that were lined with moist paper towels and also included a small water dish (100 mL). Frogs were fed crickets once a week, and cages were misted daily and changed every 2 wk. Swabbing of salamanders consisted of 30 strokes on the ventral side of the animal, from neck to vent. Swabbing of frogs consisted of a total of 30 strokes with 20 strokes across the ventral side of the body including down each thigh, with 10 strokes distributed among toe webbing (30 strokes total). Swabs were stored in microcentrifuge tubes and kept at 4 °C until processing. Swabs were extracted using PrepMan Ultra protocol and run in singlicate on Taqman real-time PCR (41). Identical methods for calculating infection intensity and determining positive versus negative individuals used for formalin-preserved animals (described above) were also used for live animals in laboratory infection trials.

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