Detection of *Batrachochytrium dendrobatidis* in amphibians inhabiting cloud forests and coffee agroecosystems in central Veracruz, Mexico

Rene Murrieta-Galindo · Gabriela Parra-Olea ·
Alberto González-Romero · Fabiola López-Barrera ·
Vance T. Vredenburg

Abstract The chytrid fungus *Batrachochytrium dendrobatidis* (Bd) is a threat to the survival of amphibians worldwide, a situation that is compounded by several other factors. In this study, we determined the prevalence of Bd and its relationship to biotic and abiotic variables for six amphibian communities in two cloud forest fragments and four coffee agroecosystems in central Veracruz, Mexico. A sampling effort of 768 person-hours and 109 skin swabs resulted in the detection of *B. dendrobatidis* in four amphibian species belonging to three families. The co-inertia model showed the following as the most important variables: tree density, fern species, temperature and elevation, fragment or site size, and structural index. Conversely, we did not find a clear relationship between Bd prevalence and the habitat management gradient. The highest prevalence was found in the second cloud forest, but a very similar result was found in one of the traditional agroecosystems; the lowest levels of prevalence were found in another second traditional agroecosystem and the first cloud forest. The degree of infection was highest in the cloud forests where the diversity of trees, orchids, and elevation was higher. *Ecnomiohyla miotympanum* was the most abundant species and was found to be infected in four of the five sites, presenting the highest degree of infection.

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R. Murrieta-Galindo (✉) · A. González-Romero
Red de Biología y Conservación de Vertebrados, Instituto de Ecología, Km. 2.5 Carretera Antigua a Coatepec 351, Congregación El Haya AP 63, 91070 Xalapa, Veracruz, Mexico
e-mail: murrieta13@gmail.com

F. López-Barrera
Red de Ecología Funcional, Instituto de Ecología, Km. 2.5 Carretera Antigua a Coatepec 351, Congregación El Haya, 91070 Xalapa, Veracruz, Mexico

G. Parra-Olea
Departamento de Zoología, Instituto de Biología, Universidad Nacional Autónoma de México, A.P. 70-153, 04510 México City, Distrito Federal, Mexico

V. T. Vredenburg
Department of Biology, San Francisco State University, San Francisco, CA 94132, USA

Introduction

Chytridiomycosis is caused by infection with the chytrid fungus *Batrachochytrium dendrobatidis* (Bd). This disease is considered novel because Bd is the only member of the phylum Chytridiomycota that infects vertebrates and has caused mass deaths in amphibian populations (Daszak et al. 2001; Young et al. 2005; Blaustein and Dobson 2006; Lips et al. 2006; Longcore et al. 2007).

Some authors argue that the impact of this fungus has been so strong because it interacts synergistically with other factors, including the following: climate change (Corn 2005; Pounds et al. 2006); habitat transformation and loss (Parra-Olea et al. 1999; Wake 2007); disturbances in the vegetation structure, quality of breeding habitats, and presence of predators (Semlitsch 2003); shifts in the type of land use (Rovito et al. 2009), river length or associated ponds (Woodhams et al. 2011); patches of vegetation cover and their configuration; and large-scale elements built or caused by humans (e.g., roads, urbanization, pollution, construction of transportation infrastructure) that decrease the access to rivers (Hartel et al. 2010). Therefore, the complex scenario of habitat disturbance

has negative effects on amphibian communities in their native habitat and on *B. dendrobatidis* propagation (Fisher et al. 2009).

In Mexico, this pathogen has been detected in several localities from the northern states of Baja California (Luja et al. 2012) and Sonora (Hale et al. 2005), across the Trans-Mexican Volcanic Belt (Frias-Alvarez et al. 2008; Van Rooij et al. 2011) to the southern forests of Oaxaca (Cheng et al. 2011) and Chiapas (Muñoz-Alonso 2010). Specifically, with regard to the state of Veracruz, Cheng et al. (2011) reported the presence of Bd in museum specimens collected in the 1970s, and more recently, Van Rooij et al. (2011) found two infected salamander species in the cities of Cordoba and Orizaba.

With 96 species of amphibians, Veracruz is the third most speciose state after Oaxaca and Chiapas, and more than 50 % of this diversity is endemic to the state (Challenger and Dirzo 2009). One of the factors that has most likely contributed to the high diversity of amphibians in Veracruz is the abundance of cloud forests (López-Barrera and Landgrave 2008; Challenger and Dirzo 2009), the most species-diverse biome per unit area (Williams-Linera 2007). Unfortunately, cloud forests are also the most threatened ecosystem in Mexico (Torres et al. 2008; Toledo-Aceves et al. 2011), and a considerable proportion of this biome has been transformed into such agricultural areas as coffee agroecosystems (Manson et al. 2008; Jose 2009).

Coffee production systems involve a degree of management, where by shade is provided by nonnative trees, and agrochemicals are used at certain stages of production (Perfecto and Armbrecht 2003; Moguel and Toledo 2004). Within this context, the most important aspect regarding coffee management is whether there is a canopy cover of differing structural degrees that creates a habitat management gradient (Vandermeer and Perfecto 2007).

Habitat loss and chytridiomycosis have been identified as two of the main causes for amphibian decline worldwide (Wake and Vredenburg 2008). Paradoxically, it has recently been documented that infection by and the occurrence of Bd is inversely associated with habitat loss in the American tropics and Australia (Becker and Zamudio 2011); with higher rates of infection by Bd in amphibians from pristine habitats than in anthropic environments (St-Amour et al. 2008).

We have a long-term monitoring project underway at the Instituto de Ecología A.C. (INECOL), called BIOCAFE, with the broad objective of evaluating the impact on amphibian communities of transforming land from cloud forest to coffee agroecosystems. To precisely define the importance of Bd as an aspect of species detectability in the dynamics of this transformation, the detailed knowledge of the complex amphibian life histories suggests that numerous variables and multivariate analyses are the best method to address such a multifaceted scenario (Woodhams et al. 2011). The main objecti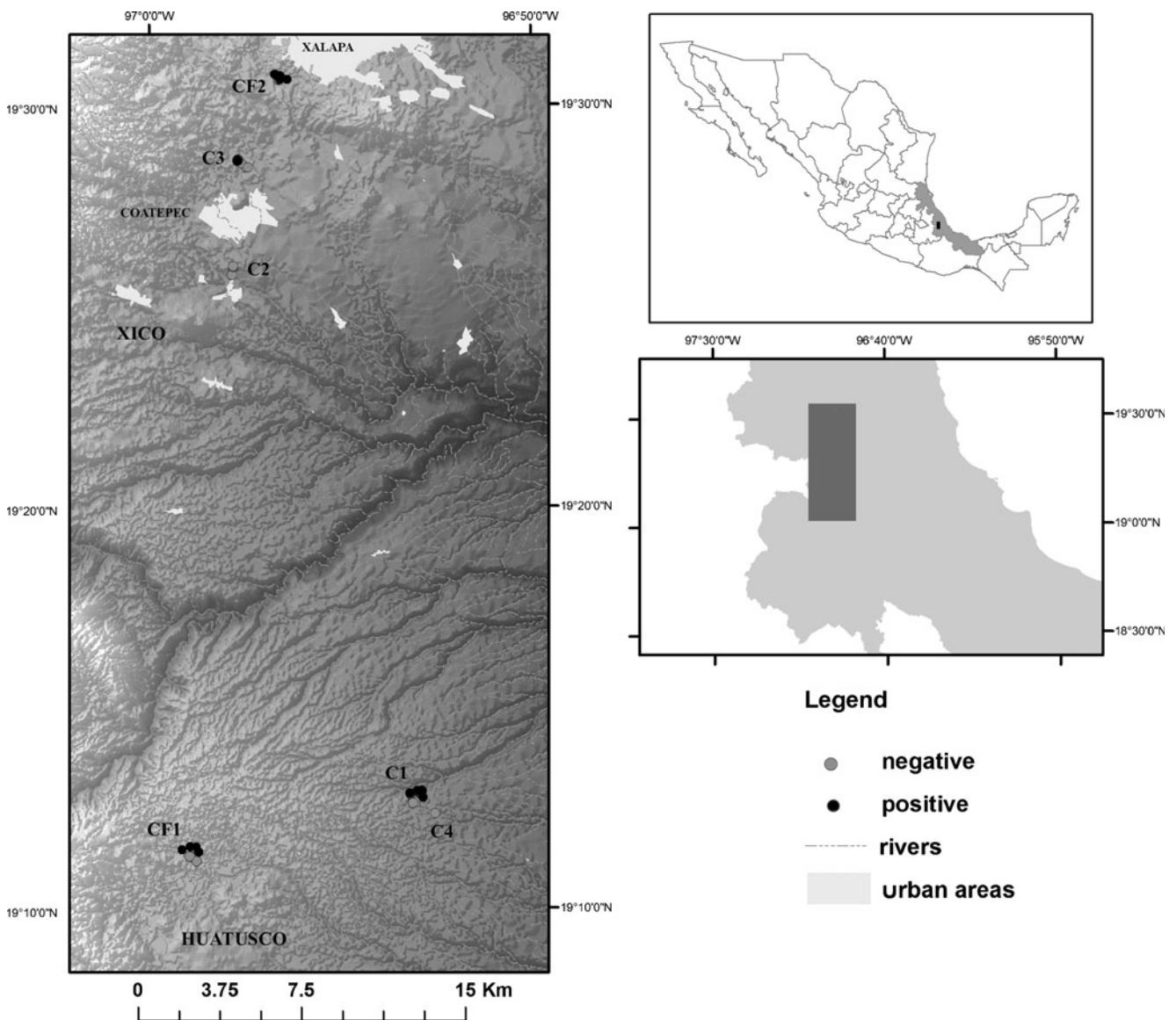ves of this study were as follows: (a) to determine if Bd is present in amphibians from six communities that form a habitat management gradient (four coffee agroecosystems and two fragments of cloud forest); (b) to establish whether the presence of Bd is determined by a set of synergistic explanatory variables, abiotic and biotic variables, through the application of a multivariate mathematical co-inertia model (Co-IA); and (c) to determine whether Bd infects amphibians differentially depending on their reproductive mode along a habitat management gradient in central Veracruz, Mexico.

Materials and methods

The study was performed in the central region of Veracruz in the municipalities of Coatepec, Xico, Xalapa, and Huatusco (Fig. 1 and Table 1). We selected four coffee agroecosystems that represent a gradient of increasing management: two traditional systems (C1 and C2) characterized by native elements (i.e., vegetation endemic to cloud forests) along with cultivated species (e.g., orange and banana trees) and coffee plants growing beneath them; two specialized shade coffee agroecosystems (C3 and C4) where cover is provided by nitrogen-fixing *Inga* sp. trees with the coffee plants, *Coffea arabica*, in the lower stratum; and two fragments of cloud forest (CF1 and CF2) with a high diversity of trees, epiphytes, and vines (Fig. 1 and Table 1). The sites had permanent rivers or wetlands and were separated by at least 3 km; however, sites C1 and C4 did not comply with this rule because they were previously selected for the project BIOCAFE (Fig. 1). Data were collected from June 2008 to January 2009 during the rainy season (Soto and Gómez 1990).

The amphibian survey was performed over 4 h (1900–2300 h) at night, each time using the visual encounter survey technique and taking into account its assumptions (see Crump and Scott 1994, p. 85). The sampling effort was measured and standardized in person-hours (Crump and Scott 1994). Based on this technique, the collection points for each amphibian species were recorded using a Garmin Etrex Legend global positioning device.

Adult amphibians were captured and handled using unpowdered latex gloves to prevent sample cross-contamination. Each specimen was swabbed using sterile cotton swabs in a standardized fashion (Van Rooij et al. 2011), and the swabs were preserved in Eppendorf tubes containing 100 % ethanol. To determine Bd infection, intensity for each infected amphibian was generated using standard techniques for the real-time polymerase chain reaction quantification of Bd (qPCR) (Boyle et al. 2004; Hyatt et al. 2007) that includes comparisons to universal Bd standards (provided to the authors by A.S. Hyatt). The ethanol in the vials was evaporated in a vacuum centrifuge at room temperature, and the resulting DNA from the swabs was extracted using 160 µl of the PrepMan Ultra reagent following the Applied

Fig. 1 Study area with the different coffee agroecosystems and cloud forest fragments where the presence of *Batrachochytrium dendrobatidis* was determined. Traditional coffee (C1, C2), shade coffee (C3, C4), and cloud forest (CF1 and CF2)

Biosystems manufacturer's protocols. The extract was then used directly as the template in Taqman qPCR assays for the detection of *B. dendrobatidis* (Boyle et al. 2004). This assay uses the species-specific primers ITS1-3 Chytr and 5.8 S Chytr in addition to the fluorescently labeled probe Chytr MGB2 to amplify a fragment of the *B. dendrobatidis* genome at the junction of the ITS-1 and 5.8 S regions. The Taqman PCR assays were conducted using an Applied Biosystems Prism 7700 Sequence Detection System. The PCR reactions were performed in a total volume of 25 μl containing 12.5 μl of the Taqman Master Mix (Applied Biosystems), the PCR primers (900 nM), the MGB probe (250 nM), and 5 μl of the DNA template. The DNA standards (provided by Vance Vredenburg) were diluted to produce 100, 10, and 1 zoospore genome equivalents for use in the Taqman assay (Boyle et al.

2004). The amplification conditions were 2 min at 50 °C, 10 min at 95 °C, followed by 15 s at 95 °C and 1 min at 60 °C for a total of 50 cycles.

As the DNA extracts were diluted by a factor of 1/40 for PCR, we corrected the scores by the same factor and considered a genomic equivalent (Ge) of 0.1 as evidence of the presence of *B. dendrobatidis*. The assay results are presented quantitatively as the number of zoospore genomic equivalents (derived from standard curves) recovered from the skin swab assay for each specimen. We estimated prevalence as the proportion of individuals per species and per locality that tested positive for *B. dendrobatidis* and derived the 95 % confidence interval (CI) using a two-tailed CI for the proportions or a one-tailed CI when the prevalence was zero (Garner et al. 2006).

Table 1 Chytridiomycosis (*Batrachochytrium dendrobatidis*) in the amphibian communities of different coffee agroecosystems and cloud forest fragments in central Veracruz, Mexico

RM	IC	Amphibian species	Cloud forest CF1 No. of individuals [positive for Bd]	Infection load	Cloud forest CF2 No. of individuals [positive for Bd]	Infection load	Traditional agrosystem C1 No. of individuals [positive for Bd]	Infection load	Traditional agrosystem C2 No. of individuals [positive for Bd]	Infection load	Specialized agrosystem C3 No. of individuals [positive for Bd]	Infection load	No. of specimens examined [no. positive]	Prevalence of Bd per species (%)	IUCN red list category
		Family Bufonidae													
Tadpole	Iv	*Incilius valliceps*					1	0	2	0		0	3 [0]	0	LC
		Family Craugastoridae													
Direct	Cb	*Craugastor berkenbuschii*	1	0									1 [0]	0	NT
Direct	Cl	*Craugastor loki*	5	0	6 [4]	0.1–75					2 [1]	1.2	13 [5]	38	LC
Direct	Cm	*Craugastor mexicanus*	6	0							1	0	7 [0]	0	LC
Direct	Cr	*Craugastor rhodopis*	1	0	1 [1]	0.2					1	0	3 [1]	33.30	VU
Direct	Cp	*Craugastor pygmaeus*									1	0	1 [0]	0	VU
Direct	C9	*Craugastor* sp. 9	4	0							4 [0]		4 [0]	0	Nd
		Family Centrolenidae													
Tadpole	Hf	*Hyalinobatrachium fleischmanni*					1	0	6	0	1	0	8 [0]	0	LC
		Family Hylidae													
Tadpole	Ct	*Charadrahyla taeniopus*	4	0									4 [0]	0	VU
		Family Ranidae													
Tadpole	Em	*Ecnomiohyla miotympanum*	25 [2]	13–175	8 [2]	0.1–0.2	18 [8]	0.1–94	1	0	7 [1]	0.1	59 [13]	22	NT
Tadpole	Lb	*Lithobates berlandieri*			3	0					1 [1]	7.5	4 [1]	25	LC
Tadpole	Lv	*Lithobates vaillanti*					1				1		1	0	LC
		Family Plethodontidae													
Direct	Bp	*Bolitoglossa platydactyla*							1				1 [0]	0	NT
		No. of individuals	46 [2]		18 [7]		21 [8]		10		14 [3]		109 [21]	19.20	
		Average infection load		94.5		12.0		21.9		0.0		3.0			
		Prevalence of Bd per locality	4.30 %		38.80 %		38.00 %		0 %		21.40 %				

RM reproductive mode, *IC* identification code, *Nd* no data, *DD* data deficient, *LC* least concern, *NT* near threatened, *VU* vulnerable, *EN* endangered, *CR* critically endangered, *IUCN* International Union for Conservation of Nature

Biotic and abiotic variables

The relative temperature and humidity were measured every 10 min with a Kestrel© portable climatic station. All of the data including precipitation, substrate pH, elevation, tree density, number of native trees, tree height, tree biomass, number of orchid, bromeliad and fern species, plant structure management, and biological impact indices were obtained from the data base BIOCAFE II (Mehltreter and González-Gálvez 2006; S. Table 1).

The plant structure index was considered using the following parameters: the cover provided by epiphytes, the height, the basal area, the number of trees, the proportion of native tree species, the canopy cover, and the distribution of trees in the vertical strata. For the coffee agroecosystems, we also added the total coffee plant (*C. arabica*) density per hectare. The biological impact index was calculated to show the degree of management applied, which is the total amount of organic and inorganic fertilizer, weed control, and pest control (pesticides). The indices were calculated based on the mean at each cluster of ten plots (20×20 m) in each study site.

Landscape variables were measured within a one-and-a-half-kilometer-wide buffer zone from each georeferenced amphibian species at every study site during our nocturnal surveys using the ArcView 3.2 program (spatial analyst tools); classified and georeferenced LANDSAT-7 and IKONOS images from 2004, taking into account the displacement area for a semiaquatic amphibian as the criterion, were also used (Ficetola et al. 2009). We obtained the river length, fragment or site size (correspond to forests and cultivated lands), and human population density from the national 2005 census (Instituto Nacional de Estadística y Geografía 2006) within the buffer zones. Landscape quality data were obtained from López-Barrera and Landgrave (2008) using their green vegetation index: open areas and cover (open, dense, and moderate) over a 2-km band around the sites (S. Table 1).

Statistical analysis

The data sets were transformed according to their distribution (Zar 1999) and tested a posteriori using Levene's test to identify the possible effect of multiple colinearity using the package lawstat (Hui et al. 2008), but no combined effects were detected ($P > 0.6$).

We used a mathematical Co-IA to determine whether there was a synergistic effect between the sets of explanatory variables (abiotic and biotic) and, as the response variable, the presence of amphibians infected by Bd for each amphibian community at each study site (Legendre and Legendre 1998; Dray et al. 2003; Dray and Dufour 2007). A Monte Carlo test was performed with 999 permutations to test the model and determine its statistical significance. That is, we compared the total inertia of the data set matrices ([explanatory] variables

and communities with Bd [response variable]) when the co-structure of both is destroyed (Dray et al. 2003). To test the Co-IA model and determine its statistical significance, we performed a Monte Carlo test with 999 permutations (Dray et al. 2003) using the ADE4 package (Dray and Dufour 2007).

After generating the best co-inertia explanatory model, the canonical weights of the variables with the greatest importance were selected and tested in a univariate manner using Pearson correlations. These data were then fitted to a straight line (linear regression) to detect in a post hoc analysis any significant tendencies of Bd infection (Quinn and Keough 2002) that could not be observed in the multifactorial scenario.

We evaluated the effect of the presence of Bd on the different amphibian reproductive modes by habitat gradient (coffee agroecosystems and cloud forest fragments) using a generalized linear model (GLM) with a structured binomial error and the logit link function (Warnes 2009) at a probability of 0.05. All of the analyses were performed using the program R 2.15.3 (R Core Team 2013).
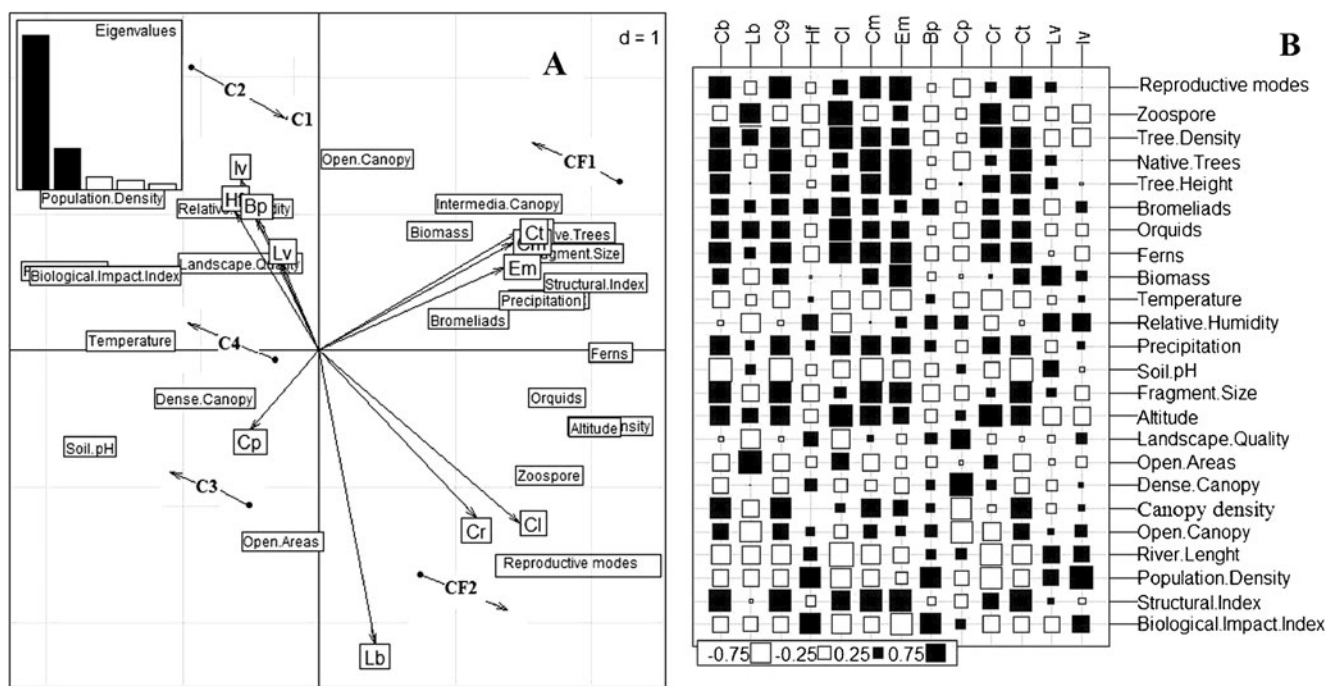
Results

With a sampling effort of 768 person-hours and 192 effective sampling days, we collected a total of 109 amphibians from 13 species (six families). We did not find any specimens at site C4 (Table 1). *Ecnomiohyla miotympanum* was the most abundant species at all sites where the specimens were found.

A total of 109 skin swabs were collected and analyzed using the qPCR technique. Bd was detected on four species (Table 1) in two cloud forest fragments (CF1 and CF2), in one traditional coffee agroecosystem (C1), and in one specialized shade coffee agroecosystem (C3) (Table 1 and Fig. 1).

The species infected were *Craugastor loki*, *Craugastor rhodopis*, *E. miotympanum*, and *Lithobates berlandieri*. *E. miotympanum* was the only species that was infected in four of the five localities and also had the highest degree of infection, with up to 175 zoospores detected.

The co-inertia model revealed a significant co-structure (Monte Carlo test, $P < 0.005$) and explained 83 % of the variation and importance of the covariance in the six variables that contributed the most to the modeling of the five communities. These variables were the number of zoospores, tree density, fern species, temperature and elevation, fragment size, and structural index (Fig. 2).

The species infected with Bd and those not infected, along with their positive and negative covariation, were explained as positive and negative correlations with respect to each variable (Fig. 2). The species presence was correlated as follows: *Craugastor berkenbuschii*, *L. berlandieri*, *Craugastor* sp. 9, *C. loki*, *Craugastor mexicanus*, *C. rhodopis*, *E. miotympanum*, and *Charadrahyla taeniopus*. They were positively correlated ($r^2 = 0.83$; Monte Carlo test: $P < 0.005$)

Fig. 2 **a** Factorial co-inertia biplot map. **b** Positive (*black square*) and negative (*white square*) correlations are shown where the size of the square is proportional to the magnitude of the relationship of the response by the amphibian community to the biotic and abiotic variables. Site abbreviations: Traditional coffee (*C1*, *C2*), shade coffee (*C3*, *C4*), and cloud forest (*CF1*, *CF2*). Amphibian species: *Iv, Incilius valliceps*; *Cb, Craugastor berkenbuschii*; *Cl, C. loki*; *Cm, C. mexicanus*; *Cp, C. pygmaeus*; *Cr, C. rhodopis*; *C9, C.* sp. 9; *Hf, Hyalinobatrachium*

fleischmanni; *Em, Ecnomiohyla miotympanum*; *Ct, Charadrahyla taeniopus*; *Lb, Lithobates berlandieri*; *Lv, L. vaillanti*; *Bp, Bolitoglossa platydactyla*. Variables: Reproductive modes, zoospores, tree density, native trees, tree height, bromeliads, orchids, ferns, biomass, temperature, relative humidity, precipitation, soil pH, fragment size, elevation, landscape quality, open areas, dense canopy, canopy density, open canopy, river length, population density, structural index, and biological impact index

with abiotic variables (low temperature, high relative humidity, high precipitation, and high elevation) and biotic variables (high tree density, tall trees, native trees and highest biomass per hectare, highest number of bromeliad species, orchids, and ferns, and a high structural index) (Fig. 1). In contrast, *Hyalinobatrachium fleischmanni*, *Bolitoglossa platydactyla*, *Craugastor pygmaeus*, *Lithobates vaillanti*, and *Incilius valliceps* were positively correlated (r^2=0.83; Monte Carlo test: P<0.005) with the abiotic variables of high temperature, low relative humidity, low temperature, low precipitation, low landscape quality, and a high proportion of rivers and the biotic variables of low dense cover, low open areas, low human population density, and high biological impact index (Fig. 2).

The post hoc linear fit of the regression after the co-inertia analysis was negative and significantly associated with Bd infection versus the biological impact index (r^2=−0.85; P<0.003) and the length of the rivers that run through the study area (r^2=−0.93; P<0.007). However, there were three positively significant results: tree density (r^2=0.95; P<0.001), the number of orchid species (r^2=0.93; P<0.03), and elevation (r^2=0.93; P<0.006).

The analysis of infection according to the amphibian reproductive mode along the habitat management gradient

revealed that the cloud forest fragments and aquatic reproductive modes were most affected by Bd (Z=2.952, df=105, P<0.003).

Discussion

According to Cheng et al. (2011), chytridiomycosis has been present in Veracruz since the 1970s and corresponds to the dramatic decline in salamanders reported by Rovito et al. (2009). More recently, Van Rooij et al. (2011) reported Bd in two species of salamander from the cities of Cordoba and Orizaba. Although it is known that chytridiomycosis is widespread in Mexico (Lips et al. 2004; Hale et al. 2005; Frias-Alvarez et al. 2008; Muñoz-Alonso 2010; Cheng et al. 2011; Luja et al. 2012), this is the first study designed to analyze the presence of Bd in terms of the habitat management gradient.

Amphibians infected with Bd were found at four of our sites, in the cloud forests and traditional and specialized coffee agroecosystems. C2, one of the traditionally managed agroecosystems, was the only site that was free of Bd, but it was also the site with the lowest abundance of amphibians, which may be due to the quality of the water and the human-

modified landscape that surrounds this site. C2 has a permanent river that serves as drainage from nearby towns; the water is clearly polluted and has a dark foam on its surface (personal observations). The species found in this site might be resistant to some degree of pollution, but the water quality, including whether there are pesticides or fertilizers in it, might affect the Bd zoospores (Johnson et al. 2003).

We found Bd infection loads from a low of 0.1 zoospores to highs of 94 and 175 zoospores in *E. miotympanum* in one of the cloud forest fragments (CF1) and one of the traditional agroecosystems (C1). Interestingly, *E. miotympanum* was also the most abundant species in this study. Although this species has been classified by the IUCN as near threatened because much of its habitat has been transformed, it has a wide range of distribution from the Atlantic slopes of the Sierra Madre Oriental mountain range to southern Chiapas. Muñoz-Alonso (2010) suggested that the conservation status of this species be reevaluated because the populations from Chiapas have apparently become extinct. In the present study, *E. miotympanum* was infected in four of the five sites where it was present and exhibited the highest infection load (175 zoospores). This species is found in all types of microhabitats, even those with direct sunlight. It appears that populations of *E. miotympanum* from Veracruz are resistant to habitat transformation and to some degree of pollution (site C2) and are capable of surviving with low loads of Bd infection.

The presence of *H. fleischmanni*, *B. platydactyla*, *C. pygmaeus*, *L. vaillanti*, and *I. valliceps* was positively correlated with variables that are not favorable for Bd, such as high temperature, low relative humidity, low precipitation, low landscape quality, low cover density, open areas, high human population density (Hyatt et al. 2007; Woodhams et al. 2011), and such management variables as intensive coffee crop management (r^2=0.83; Monte Carlo test: P<0.005). Indeed, these species were only present at transformed sites, and all were negative for Bd. In contrast, *L. berlandieri*, *C. loki*, and *C. rhodopis* were positively correlated (r^2=0.83; Monte Carlo test: P<0.005) with variables that favor Bd (low temperature, high relative humidity, high precipitation, high elevation, high tree density, tall trees, native trees and the highest biomass per hectare, highest number of bromeliad species, orchids, and ferns, and a high structural index) and were positive for Bd, though their infection loads were generally low.

It has recently been documented that infection by and the occurrence of Bd is inversely associated with habitat loss in the American tropics and Australia (Becker and Zamudio 2011), with higher rates of Bd infection in amphibians from pristine habitats than in anthropic environments (St-Amour et al. 2008). However, in the present study, we did not find this to be the case: an almost equal prevalence (high and low) of Bd infection was found in the cloud forests and traditional agroecosystems.

Most of the amphibian species were negative for Bd; however, it is interesting that *L. berlandieri* was positive and positively correlated with open canopy. This may result from the fact that members of *Lithobates* (formerly *Rana*) prefer open sites (Stebbins and Cohen 1997) and can endure both a low and high Bd prevalence (Briggs et al. 2010). We propose that sunlight may also have an effect on the degree of infection (Bancroft et al. 2008). A study in Australia with results similar to ours reported that a hylid (*Litoria wilcoxii*) exhibited a lower degree of Bd infection in open urban areas (Van Sluys and Hero 2009); Bd was not found to grow well in highly anthropized locations but was instead associated with sites having little or no disturbance, which offer ideal survival conditions (St-Amour et al. 2008).

With respect to the structural management index, we found that the correlation between the degree of management at each site and the presence, prevalence, or infection load of Bd was most intense at C4 where a wide array of agrochemicals, such as pesticides, are used (Moguel and Toledo 1999, 2004; Hernandez-Martinez et al. 2009), resulting in the complete eradication of amphibians at the site. The other specialized agroecosystem (C3) had the lowest average infection load in comparison to the traditional agroecosystems and cloud forests, but the site with the lowest prevalence of Bd was CF1. Traditional agroecosystem C1 had a higher average Bd infection load and the same Bd prevalence as CF2.

Amphibian species with direct or aquatic development reproductive modes, such as the Craugastoridae and Hylidae families, were differentially affected by the habitat management gradient. Regardless, our results indicate that the aquatic reproductive mode was most affected by a higher load of Bd at CF1, implying that spread likely occurs when these species move to the river to reproduce and share a pool of water with Bd-infected species that have the same reproductive mode (Fisher et al. 2009) or take water into their body, such as the Craugastoridae family (Gascon et al. 2007).

Compared to other regions of the world, we did not find the same magnitude of Bd infection in the present study. Indeed, we found the infection loads to be generally low. Thus, to protect amphibians from chytridiomycosis in central Veracruz, it is necessary to take into account all of the relevant characteristics of the habitat and to apply the current knowledge of the variables associated with the disease and its requirements along with the life history of the amphibians (Gascon et al. 2007). Within this context, such management strategies as subterranean viaducts and road tunnels might be a recommended solution to facilitate the amphibian's movement among patches (Smit et al. 2006; Clevenger and Huijser 2011). In addition, an attempt to estimate why the river complex scenario could have an important effect on Bd and the threshold levels at which these populations begin to decline is fundamental for future studies. Accordingly, more extensive sampling should be performed across a larger

number of sites while making an effort to accurately document the degree of habitat transformation.

The complexity of coffee production, as well as of protecting cloud forests and the amphibian species that live there, necessitates short- and medium-term studies to understand in greater detail, and preferably at the species level, how Bd spreads through the mosaic of coffee agroecosystems that surround cloud forests.

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