AMPHIBIAN DISEASES

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Geographic Variation in *Batrachochytrium dendrobatidis* Occurrence Among Populations of *Acris crepitans blanchardi* in Texas, USA

Differences in the susceptibility of amphibian species to infection by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*), which can cause the disease chytridiomycosis, have been documented in the laboratory (Daszak et al. 2004) and among wild populations (Carey 2000; Lips 1999; Retallick et al. 2004). The severity of *Bd* infection has also been linked to climatic conditions, with temperature as a major determinant strongly affecting the outcome of infection at specific locations or seasonally (Berger et al. 2004; Bosch et al. 2007; Drew et al. 2006; Kriger and Hero 2007a, b). In addition to climatic conditions such as temperature, variability in site-specific environmental conditions also might affect the probability of infection, as indicated by large differences in abundance of *Bd* in replicate samples from the same site (Kirshtein et al. 2007), or large differences in infection of amphibians at different sites from the same area (Walker et al. 2007).

The goal of our study was to investigate the relationships of site-specific environmental conditions with the presence and the infection levels of *Bd* in amphibians. Blanchard's Cricket Frogs (*Acris crepitans blanchardi*) were selected as the focal species because it is the most abundant and conspicuous member of anuran breeding assemblages in our study area in eastern and central Texas, USA. Previously, we had detected *Bd* in *A. c. blanchardi* in central Texas, with seasonal variation in its prevalence and not accompanied by any detectable mortality or signs of chytridiomycosis (Gaertner et al. 2009). In south-central Texas, *A. c. blanchardi* occurs sympatrically with the endemic endangered Houston Toad (*Anaxyrus* [*Bufo*] *houstonensis*) at a few locations that include breeding ponds on the Griffith League Ranch (GLR) and the adjacent Welsh (WEL) property in Bastrop County. These properties are located in the Bastrop Lost Pines ecoregion and are characterized by deep sandy soils, with forest patches dominated by Loblolly Pine (*Pinus taeda*), Post Oak (*Quercus stellata*), and Eastern Red Cedar (*Juniperus virginianus*), with interspersed grassland patches and land cleared for grazing (Gaertner et al. 2010). We selected six ephemeral to semi-permanent ponds on these properties that ranged in size from ~300 to 2,400 m^2 and differed by their use in cattle ranching operations (cattle ponds C-1 GLR and D-5 GLR), overuse by feral hogs (feral hog ponds E-11 GLR and F-WEL), or lack of these practices (low use ponds A-12 GLR and B-16 GLR). The maximum distance between ponds was about 3 km.

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At these six ponds, *A. c. blanchardi* were collected by hand or net once a month over a one-year period starting February 2009. At least 20 adults were collected per pond from February to August (February to May for ponds C-1 and D-5), however, lower numbers or no captures were obtained at ponds from September to January, with drought conditions resulting in periodic drying of ponds. Animals were swabbed with sterile cotton tips with a wood handle following the method outlined in (Kriger et al. 2006b). To avoid potential cross-contamination, nets were treated with commercial bleach (final conc. 1% NaOCl) for 10 min and gloves were changed between captures. Swabs were placed in sterile 2 ml cryotubes and stored at -80°C until further processing. DNA was extracted from swabs with the Wizard Genomic DNA Purification kit (Promega Corporation, Madison, WI) following the protocol for extraction from animal tissue. DNA extracts were then tested for the presence of *Bd* using a TaqMan quantitative real time PCR (*q*PCR) assay (Boyle et al. 2004).

Temperature, total phosphorus (TP), and pH were analyzed in unfiltered water samples, while concentrations of chlorophyll *a* (Chl *a*), non-volatile suspended solids (NVSS), organic matter (OM), dissolved organic carbon (DOC), soluble reactive phosphorus (SRP), nitrate (NO_3^-), and ammonium (NH_4^+) were analyzed from filters or in filtrates of water samples. Water samples were analyzed in duplicate. In addition to water samples, a sediment core (approximately 5 cm deep) was taken at the shore-line of each pond. Sediment samples were dried, and subsequently combusted at 550°C for 4 h to determine percent sediment organic matter (SOM). Principal components analysis (PCA; SAS v.9.3) was then used to assess differences in environmental parameters among pond types and months. Environmental parameters were z-scored transformed (Krebs 1999) and

TABLE 1. Average temperature and cumulative precipitation data for the 30 days preceding sampling events at six ponds in Bastrop County, Texas, USA, during spring and summer of 2009. Values were obtained from the National Climatic Data Center and represent averages for the three closest surrounding stations (Elgin-412820, Smithville-418415, and Cedar Creek-411541). No frogs were obtained in September, October, December, and January.

Months	Feb	Mar	Apr	May	Jun	Jul	Aug	Nov
Temperature (°C)	12.1	15.4	17.1	23.8	25.4	31.2	31.2	17.9
Precipitation (mm)	21	55	35	82	61	3	42	14

TABLE 2. Percentage of Blanchard's Cricket Frogs (*Acris crepitans blanchardi*) infected with *Batrachochytrium dendrobatidis* during the spring and summer of 2009 (± 95% confidence interval) at six ponds in Bastrop County, Texas, USA. No frogs were collected in September, October, December, and January, and all frogs collected in November tested positive (pond A-12, N = 10 and pond B-16, N = 7; pond C-1, N = 1; pond D-5, N = 1). Nd represents times when no frogs were obtained.

Pond	February	March	April	May	June	July	August	Average
A-12	58 (± 22) N = 19	100 N = 20	65 (± 21) N = 20	100 N = 20	80 (± 18) N = 20	95 (± 10) N = 20	75 (± 19) N = 20	88
B-16	100 N = 20	85 (± 16) N = 20	85 (± 16) N = 20	90 (± 13) N = 20	65 (± 21) N = 20	Nd	94 (± 11) N = 17	89
C-1	90 (± 13) N = 20	100 N = 4	88 (± 23) N = 7	100 N = 6	Nd	Nd	Nd	90
D-5	100 N = 20	100 N = 8	85 (± 16) N = 20	100 N = 20	Nd	Nd	Nd	89
E-11	Nd	92 (± 16) N = 12	86 (± 26) N = 7	85 (± 16) N = 20	80 (± 18) N = 20	100 N = 20	100 N = 20	88
F-WEL	95 (± 10) N = 19	93 (± 13) N = 14	75 (± 42) N = 4	100 N = 13	100 N = 7	100 N = 20	88 (± 16) N = 16	73
Average	89	94	80	95	78	98	89	89

the appropriate number of principal components to retain in the model was determined using a scree plot as a guide.

Further analyses used a linear mixed-effects model (Program R v.2.9.1) to test for differences ($\alpha = 0.05$) among pond types and months in the number of *Bd* genome equivalents (GE) per individual. Number of *Bd* GE per individual were $\log_{10}$ (N+1) transformed to improve assumptions of the linear model. Interaction between pond type and months lacked sufficient replication and was therefore not tested. To further explore the factors associated with *Bd*-GE differences among months and pond type, the cumulative precipitation and mean temperature of the 30 d preceding sampling dates were downloaded from the National Climatic Data Center (Table 1). Because no station is available for Bastrop, daily values from the three surrounding stations (Elgin-412820, Smithville-418415, and Cedar Creek-411541) were averaged. Months were converted to day of collection (e.g., Julian date) and diagnostic plots were used to assess interactions between time and pond type on *Bd* GE per individual. In the absence of any apparent interaction, regression models were used to predict $\log_{10}$ (N+1) transformed *Bd* GE per individual from Julian date, air temperature, and precipitation across pond type.

Bd was detected on 89% (N = 572) of *A. c. blanchardi* collected. The overall monthly percentage of infected *A. c. blanchardi* ranged from 78% (N = 67) in June to 100% (N = 19) in November (Table 2). Although the percent of infected *A. c. blanchardi* were the same for each pond during November (100% of individuals tested were positive at ponds A-12, B-16, C-1, and D-5), percentages varied for different ponds within the same month by as little as 15% (pond C-1 [100%] and pond B-16 [85%] in March) to as much as 42% (pond D-5 [100%] and pond A-12 [58%] in February) (Table 2). These results confirm the occurrence of *Bd* in central Texas where it has been detected in different *Eurycea* species (Gaertner et al. 2008) and in an urban population of *A. c. blanchardi* (Gaertner et al. 2009). In the latter study, 83% of individuals tested positive for *Bd* in one sampling event, with none of the infected individuals showing clinical signs of infection during handling (e.g., lethargy, lack of righting reflex, excessive sloughing of skin), as was also the case in this study.

The number of *Bd* genome equivalents (GE) detected on *A. c. blanchardi* ranged from 0 to up to 3×10^5 GE per individual with an overall mean of 2,400 GE per individual. The highest values exceeded lethal levels reported for other species (Vredenburg et al. 2010), however, most values were within ranges obtained in other studies (Kriger and Hero 2007a, b). Although small variations in numbers of *Bd* GE were noted on *A. c. blanchardi* collected within ponds with a minimum of 15 individuals during some sampling events (e.g., 0–54 GE for *A. c. blanchardi* from pond A-12 in February and 0–153 GE for *A. c. blanchardi* from pond E-11 in June), differences as large as four orders of magnitude were not uncommon (e.g., $0–3 \times 10^4$ GE for *A. c. blanchardi* from pond F-WEL in March) (Table 3). Large seasonal variation in numbers of *Bd* GE were detected on *A. c. blanchardi* throughout

TABLE 3. Average number of genomic equivalents of *Batrachochytrium dendrobatidis* on individuals of Blanchard's Cricket Frogs (*Acris crepitans blanchardi*) from six ponds located in Bastrop County, Texas, USA during the spring and summer of 2009 (± 95% confidence interval). No frogs were obtained in September, October, December, and January, and average number of genomic equivalents of *Bd* (± 95% confidence interval) on individuals collected in November were: pond A-12, 370 (± 570); pond B-16, 71(± 24); pond C-1, 18 (N = 1); and pond D-5, 2,500 (N = 1). Nd represents times when no frogs were collected.

Pond	February	March	April	May	June	July	August	Average
A-12	12 (± 6)	2,100 (± 1,400)	1,600 (± 850)	170 (± 36)	40 (± 20)	120 (± 36)	210 (± 65)	870
B-16	1,300 (± 980)	9,100 (± 10,000)	13,000 (± 12,000)	360 (± 340)	100 (± 63)	Nd	160 (± 47)	3,800
C-1	270 (± 120)	780 (± 1,000)	670 (± 790)	5,500 ± 9,600	Nd	Nd	Nd	1,200
D-5	150 (± 67)	270 (± 170)	120 (± 57)	85 (± 47)	Nd	Nd	Nd	180
E-11	Nd	6,600 (± 7,400)	1,300 (± 850)	3,300 (± 3,100)	38 (± 17)	180 (± 26)	200 (± 40)	1,600
F-WEL	2,200 (± 2,100)	30,000 (± 43,000)	1,500 (± 2,600)	2,400 (± 2,700)	280 (± 310)	220 (± 23)	140 (± 55)	5,200
Average	760	9,400	4,000	1400	85	174	180	2,400

the study, with monthly average intensities peaking in March at 9,400 *Bd* GE per individual and decreasing in the summer to a minimum of 85 *Bd* GE per individual in June (Table 3). Our previous studies documented seasonal changes in infection rate but not in intensity, which was not previously analyzed, with high infection rates in spring and no *Bd* detections during summer when one cured individual was identified (Gaertner et al. 2009). Although these previous results seem to contradict our current investigation with high rates of *Bd* detection throughout the year, differences among infection rates are most likely attributable to different sensitivities of the detection methods. The *q*PCR detection method developed by (Boyle et al. 2004) has widely been accepted and used as adequate detection and quantification method for *Bd* (e.g., Kirshtein et al. 2007; Kriger et al. 2006a, b; Walker et al. 2007), with a sensitivity about 2–5 times higher than the nested PCR approach applied in our previous study (Gaertner et al. 2009). The low numbers detected during summer by *q*PCR in the current study (i.e., usually less than 10 GE) might therefore not have been detectable by nested PCR in our previous study (Gaertner et al. 2009).

Our PCA analysis of relationships between *Bd* and environmental characteristics demonstrated that ponds differed along two primary environmental gradients (Fig. 1). The first Principal Component explained 31% of the variation in *Bd* occurrence and described a gradient from relatively deep ponds with consistently lower nutrient levels to ponds that were shallow and thereby more prone to nutrient loading including organic matter (OM), total phosphorus (TP), and non-volatile suspended solids (NVSS). The second Principal Component explained 19% of the variation and contrasted aquatic habitats dominated by high respiration (high ammonium, nitrate, and pH) to those of high primary production (high dissolved organic carbon and chlorophyll *a*). Low-use ponds (ponds A-12 and B-16) were, on average, deeper (2.3–2.5 m) and had lower average concentrations of OM (10–27 mg l^{-1}), TP (80–110 µg l^{-1}), ammonium (36–133 µg l^{-1}), and nitrate (260–270 µg l^{-1}), whereas cattle ponds (C-1 and D-5) and those used by feral hogs (E-11 and F-WEL) were generally shallower (0.46–1.1 m) with high concentrations of OM (15–50 mg l^{-1}), TP (210–370 µg l^{-1}), ammonium (175–1,600 µg l^{-1}), and nitrate (385–1,100 µg l^{-1}).

The number of *Bd* GE per individual did not differ among low-use ponds, cattle ponds, or feral hog ponds ($F_{2, 3} = 3.8$, $P = 0.14$), but differences were apparent among months ($F_{7, 25} = 3.8$, $P < 0.01$). Whereas an association between numbers of *Bd* GE per individual and precipitation was not detected ($F_{1,36} = 0.6$, $P = 0.46$), we found that across pond type, numbers of *Bd* GE per individual were inversely related to day ($F_{1,36} = 7.4$, $P = 0.01$) and air temperature ($F_{1,36} = 6.7$, $P = 0.01$) (Fig. 2). Seasonal variation has been documented for *Bd* occurrence on amphibian hosts with peak prevalence of disease levels at temperatures less than 19.4°C (Kriger and Hero 2007b) and 21.6°C (Gaertner et al. 2009). These temperatures are in agreement with those in our study; for all sites, we found that the monthly peak in overall intensity of infection occurred in March (15.4°C) and April (17.1°C) (Table 1).

Air temperatures are often auto-correlated with water temperature. However, whereas all ponds warmed at the same general rate despite differences in size (not depicted), the strongly negative correlation for *Bd* GE for temperatures greater than 25°C was not observed for all ponds. Two of the ponds had relatively more stable year-round environmental conditions, likely a result of the larger volume of water for those sites (low use ponds A-12 and B-16). The seasonality of infection by *Bd* was fairly predictable in these ponds with the average intensity of infection rising to a peak in March and April and then declining through the summer months. These ponds showed negative correlations between *Bd* GE and water temperature (Figure 2). The remaining ponds were characterized by much more dynamic environmental characteristics. This included two ponds (cattle ponds C-1 and D-5) in which the greatest total *Bd* GE detections occurred at temperatures above 25°C. Admittedly, those *Bd* GE values were not notably high for either pond when scaled against all ponds, reaching only ~5,500 GE and ~275 GE, respectively. It is possible

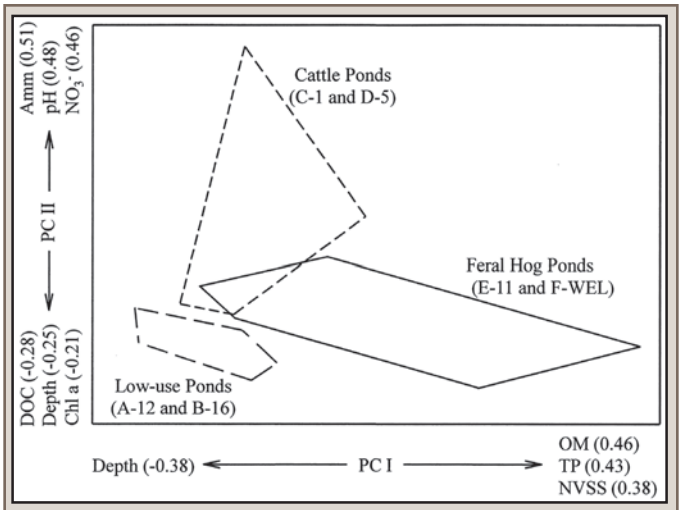

FIG. 1. Plot of sample scores from six sites, enveloped by pond type, on principal component axis I and II of environmental data (H+ [*pH*], ammonium [*Amm*], nitrates [NO_3^-], soluble reactive phosphorus [*SRP*], non-volatile suspended solids [*NVSS*], total phosphorus [*TP*], water temperature [*WaterTemp*], organic matter [*OM*], chlorophyll *a* [*Chla*], soil moisture [$SoilH_2O$], soil organic matter [*SOM*], dissolved organic carbon [DOC], and depth [*Depth*]) collected monthly from ephemeral to semi-permanent ponds located in Bastrop County, Texas.

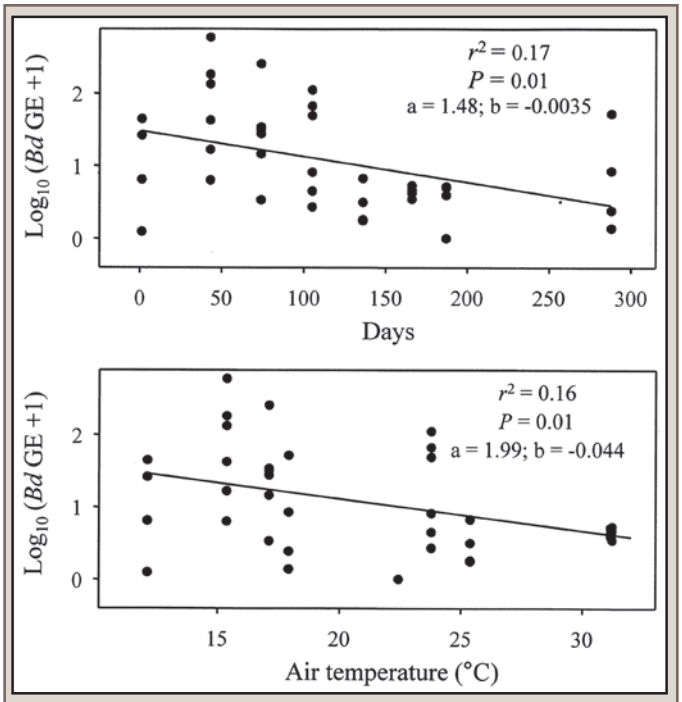

FIG. 2. Relationships between *Batrachochytrium dendrobatidis* genome equivalents (*Bd* GE) per individual *Acris crepitans blanchardi* and Julian date (starting February 1 and ending November 15) (top panel) and between *Bd* GE per individual *A. c. blanchardi* and air temperature averaged from 30 d preceding each sampling date (bottom panel) collected from ephemeral to semi-permanent ponds located in Bastrop County, Texas.

that the overall lower *Bd* GE rates for these two sites reflected their more ephemeral nature when compared with the other ponds examined here.

It also may be possible that the frogs sampled in the later, warmer periods at those two sites were migrants from larger, cooler ponds rather than residents and this could also explain the increased *Bd* GE values despite the seemingly unsuitable temperatures. We consider these more dynamic patterns of *Bd* prevalence to be consequent of their smaller size and the concomitant effect of evaporation and precipitation events (ponds C-1and F-WEL). The environmental variables measured in these ponds fluctuated widely between monthly sampling events as did the intensity of infection by *Bd*. Aside from temperature, we did not detect a strong trend among alternative environmental parameters for these ponds in association with the level of infection over time. None of the water quality assessment measures appeared to influence the prevalence or occurrence of *Bd* at those sites. Overall, the seasonal pattern in abundance was more pronounced in deeper ponds than in shallow or ephemeral ponds and was correlated with consistently lower nutrient levels in deeper ponds.

Bd has been found to be more prevalent in amphibians occurring in flowing rather than in standing waters, and more individuals of amphibians were infected with *Bd* and at higher levels in permanent water bodies than in ephemeral water bodies where detection of *Bd* was extremely rare (Kriger and Hero 2007a). Since the aquatic zoospore of *Bd* cannot survive desiccation (Johnson et al. 2003), desiccation may prevent *Bd* from causing significant infections at sites without standing water despite there being enough moisture to support amphibian populations. Impoundment construction and modifications of ephemeral ponds into permanent livestock water sources have increased the number of permanent water bodies over the last century with some negative potential impacts to rare amphibians (Gaston et al. 2010). Since central Texas has a large number of endangered and endemic amphibian species (Brown et al. 2012; Chippindale et al. 2000), an increase of permanent water sources might consequently result in an increase of the abundance and transmission of *Bd*, and might thus have detrimental effects on the amphibian assemblages in the area.

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Surveys for Frog Diversity and *Batrachochytrium dendrobatidis* in Jamaica

Jamaica is home to the world's second most endangered frog assemblage, with 16 of 21 (76%) endemic species recognized as threatened (IUCN 2011). There are 17 endemic *Eleutherodactylus* (Hedges 1989), five endemic *Osteopilus*, one of which is unnamed and thus has not been assessed by the IUCN (Moen and Wiens 2008; S. B. Hedges, unpubl. data) and four invasive anurans on the island (Mahon and Aiken 1977). We conducted the first large-scale assessment of the island's amphibians since the 1980s while sampling for the amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*). Prior to our work, *Bd* was known from all of the other Greater Antillian islands (Burrowes et al. 2004; Diaz et al. 2007; Joglar et al. 2007), several of the Lesser Antillian islands (Alemu et al. 2008; Garcia et al. 2009), and mainland North, Central and South America (Carnaval et al. 2006; Longcore et al. 1999; Fisher et al. 2009), but was not confirmed in Jamaica.

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Given the documented extinctions and population declines in congeners to Jamaica's frogs from chytridiomycosis in Puerto Rico (Burrowes et al. 2004; Longo et al. 2010) and Central America (Lips et al. 2004; Puschendorf et al. 2006), we were concerned about the conservation implications of epidemic outbreaks of *Bd* to Jamaica's amphibians. As of September 2010, 6 of Jamaica's 21 described endemic species had not been recorded in over two decades (Hedges and Diaz 2011). The timeframe of their last sighting was similar to that of some chytridiomycosis-related extinctions and extirpations elsewhere in the Caribbean (Burrowes et al. 2004). We conducted this project to investigate the occurrence of *Bd* per species and location, assess the status of Jamaican frog species, and to provide information to focus future conservation efforts directed at extant endemic species.

We sampled for amphibians across Jamaica, spending at least one person-day in the field in the known ranges of every endemic species on the island (Fig. 1; Table 1). We conducted our field work between October 2010 and June 2011. We defined a