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First survey for the amphibian chytrid fungus *Batrachochytrium dendrobatidis* in Connecticut (USA) finds widespread prevalence

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ABSTRACT: The amphibian chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) is an emerging infectious fungal pathogen of amphibians and is linked to global population declines. Until now, there has only been 1 survey for the fungus in the northeastern USA, which focused primarily on northern New England. We tested for *Bd* in a large number of samples (916 individuals from 116 sites) collected throughout the state of Connecticut, representing 18 native amphibian species. In addition, 239 preserved wood frog *Lithobates sylvaticus* tadpoles from throughout the state were screened for the fungus. *Bd* presence was assessed in both the fresh field swabs and the preserved samples using a sensitive quantitative PCR assay. Our contemporary survey found widespread *Bd* prevalence throughout Connecticut, occurring in 14 species and in 28% of all sampled animals. No preserved *L. sylvaticus* specimens tested positive for the fungus. Two common species, bullfrogs *R. catesbeiana* and green frogs *R. clamitans* had particularly high infection rates (0.21–0.39 and 0.33–0.42, respectively), and given their wide distribution throughout the state, we suggest they may serve as sentinels for *Bd* occurrence in this region. Further analyses found that several other factors increase the likelihood of infection, including life stage, host sex, and host family. Within sites, ponds with ranids, especially green frogs, increased the likelihood of *Bd* prevalence. By studying *Bd* in populations not facing mass declines, the results from this study are an important contribution to our understanding of how some amphibian species and populations remain infected yet exhibit no signs of chytridiomycosis even when *Bd* is widely distributed.

KEY WORDS: Frog · Toad · Salamander · Chytridiomycosis · Emerging infectious disease · New England · qPCR · *Lithobates catesbeiana* · *Lithobates clamitans*

INTRODUCTION

The chytridiomycete fungus *Batrachochytrium dendrobatidis* (*Bd*) is implicated in the global decline of amphibian populations (Berger et al. 1998, Stuart et al. 2004, Wake & Vredenburg 2008). Identified in the late 1990s (Berger et al. 1998, Longcore et al. 1999), *Bd* has received much research attention, as many scientists are working to identify its range and what host species are susceptible to infection (e.g. Woodhams et al. 2008, Bielby et al. 2009, Gaertner et al. 2009, Murphy et al. 2009). Many of the regions and species that are the focus of extensive research efforts are those either currently undergoing declines or are species of conservation concern (e.g. Berger et al. 1998, Bosch et al. 2001, Lips et al. 2006, Voordouw et al. 2010). However, to fully understand its ecology, it is helpful to identify where *Bd* does and where it does not occur. This includes screening populations that

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are not undergoing disease-related dieoffs and not solely focusing on active decline sites.

New England is among the regions that has not been well surveyed for *Bd*, perhaps because there have been no apparent dieoffs consistent with chytridiomycosis, such as those noted in Panama (Lips et al. 2006). Until now, our only understanding of the range and prevalence of *Bd* in this region of the USA comes from a single survey by Longcore et al. (2007). They sampled 86 sites between 2000 and 2002 from 5 states (New York, Vermont, New Hampshire, Maine, and Massachusetts); of these, 74 sites were located in Maine. Rhode Island and Connecticut (CT) were not surveyed. They analyzed 751 animals representing both pre- and post-metamorphic stages and 3 species of caudates and 9 anuran species. Specimens were analyzed for *Bd* via histology or microscopy of fresh tissues; no molecular tests were performed. *Bd* was not found in any caudate. Anurans with the greatest infection prevalence were the northern leopard frog *Lithobates pipiens* (0.26 prevalence), green frog *L. clamitans* (0.26), pickerel frog *L. palustris* (0.23) and the bullfrog *L. catesbeiana* (0.22).

To date, no data are available for *Bd* in CT. Our goal was to screen for *Bd* in as many species as possible, including species of all abundances, and from as many sites as possible. CT hosts 23 (1 is a recognized hybrid) amphibian species, of which 4 are listed as species of conservation concern in the state (Klemens 2000). Both recognized blue-spotted salamander species (*Ambystoma laterale* and its hybrid complex) and the spring salamander *Gyrinophilus porphyriticus* are listed primarily because CT is at their range limit (Klemens 2000). The Jefferson salamander *A. jeffersonianum* is listed as it is especially susceptible to land fragmentation and habitat loss (Klemens 2000). The northern leopard frog is thought to have been more prevalent in the state at one time, but is sensitive to acidic environmental conditions (i.e. acid rain) and is now mostly found in areas dominated by limestone (Klemens 2000). Some of the other species, such as the green frog, bullfrog, and pickerel frog, dominate the landscape and can be found in nearly all types of wetlands, from early summer to mid-fall (Klemens 2000).

MATERIALS AND METHODS

Study area

Our study area in CT was covered by the Laurentide ice sheet during the last glacial maximum, creating the post-glacial terrain encountered today (see Fig. 1). In general, this area is dominated by mixed deciduous-coniferous forests intermixed with lowland areas and developed landscapes. The study region includes 2 upland areas on the east and west sides of the state separated by the wide Central Valley of CT containing the Connecticut River and much human development. Sites were randomly selected for survey, but ultimately the surveyed sites were those where we had the landowner's permission or the site was on state-owned land. Sampled sites spanned a wide range of ecosystems and environmental settings, including forested to open canopy, urban to rural settings, overgrown to minimal emergent vegetation, streams and ponds of all sizes and hydroperiods (permanent and temporary), lakes, and terrestrial areas surrounding waterbodies.

Sample collection

Contemporary survey

Because CT and several of the focal species had never been surveyed for *Bd*, our approach to data collection was purely opportunistic, based entirely on access to the site and the presence of accessible animals to swab. The goal of this survey was to gather as much baseline data from as wide a geographic and species range as possible, rather than to test various drivers of prevalence (e.g. host species, site type, seasonal differences). Much of the sampling occurred as part of a partnership with the CT Department of Energy and Environmental Protection, and surveys were integrated with their standard statewide monitoring programs. There was neither a specific pattern for how the state was surveyed nor was any site resampled.

From May to November 2010, field researchers used gear decontaminated between site visits (10% bleach solution; Cashins et al. 2008). Amphibians were captured, either by hand, with a new pair of latex or nitrile gloves, or by dipnet. All available species and individuals at a site were targeted, regardless of age or sex, and swabbed according to standardized practices (see Hyatt et al. 2007): the captured animal was restrained and a wooden toothpick rubbed over its body, focusing on the limbs, feet, and drink patch (groin) for metamorphosed anurans, only the mouthparts for anuran larvae, or the entire body for caudates. These respective locations on the body have been found to increase the likelihood of detecting *Bd* zoospores in these taxa and age classes

(Hyatt et al. 2007). The animals were returned to their site of capture. While cotton-tipped, wire swabs (e.g. Medical Wire & Equipment MW 100–100) are a common collection tool, toothpicks have been used successfully in previous studies to collect *Bd* from amphibians (Retallick et al. 2006, Woodhams et al. 2006, Retallick & Miera 2007, Hyman & Collins 2012). Despite the popularity of wire swabs, there is no evidence demonstrating that wooden toothpicks are any better or worse at collecting *Bd*. Toothpicks were placed into a 2 ml screw cap tube containing 70% ethanol. Each sampling effort at a site concluded once all accessible animals were swabbed; there were no time limits imposed per sampling effort. In total, 916 individuals were sampled from 116 sites, and swabs were transported back to the lab and kept at room temperature until DNA extraction. All animals were captured and handled according to an approved protocol (no. 2009-11306) from the Institutional Animal Care and Use Committee of Yale University or the Connecticut General Statute 26-3.

Preserved specimen survey

Wood frog tadpoles (n = 239) previously collected (and unused) for another study were screened for *Bd*. These CT specimens, preserved in 70% ethanol, were collected in May 2007 from Berlin (n = 35), East Haven (n = 28), Meriden (n = 61), North Branford (n = 72), and Wallingford (n = 43). Tissues taken from these tadpoles were half a mouthpart (cut down the sagittal plane) and, if available, 1 of each fore- and hindlimb. Tissues were excised using sterile equipment, including a new razor blade for each individual. All tissues were extracted and analyzed for *Bd* via quantitative PCR (qPCR; see below).

DNA extraction and qPCR

For the preserved amphibian tissues, DNA was extracted using the Gentra PureGene protocol (Qiagen). DNA from swabs was extracted according to Retallick et al. (2006) with a minor modification: we used 100 µl of PrepMan Ultra (Applied Biosystems) rather than 40 µl. *Bd* DNA was detected via qPCR according to Boyle et al. (2004) and Garland et al. (2010). Briefly, 20 µl reaction volumes consisted of 5 µl of DNA template (diluted 1:10 for field swabs or full strength for preserved tadpoles), 10 µl qPCR master mix (initially, TaqMan Universal No AmpErase, Applied Biosystems, then a final switch to SensiMix II Probe Low ROX, Bioline), 1 µl of each primer (forward and reverse; 900 nm each), 2 µl of probe (250 nm), and 1 µl BSA (500 ng μl^{-1}). Diluting DNA from swab samples and the addition of BSA helps to reduce inhibition in qPCR (Garland et al. 2010). The qPCR protocol was: 10 min activation at 95°C followed by 45 cycles of 15 s at 95°C and 60 s at 60°C. qPCR assays included a negative control (molecular grade DNase-free water) and 3 dilutions (100, 10, or 1 zoospore per 20 µl reaction) of a positive control standard (*Bd* isolate JAM081). The protocol for preparing standard positive controls followed Boyle et al. (2004): zoospores were harvested by flooding the agar plate with DNase free water and pipetting the supernatant into 15 ml tubes. Harvested zoospores were counted 4 times using a hemocytometer. All samples, including the controls, were run in triplicate. Presence of *Bd* in each sample was determined by successful amplification of the 146 base pair fragment within *Bd*'s ribosomal DNA (rDNA) (Boyle et al. 2004). Any sample in which at least 2 of the 3 wells (replicates) had successful amplification was deemed positive for *Bd*. Any sample that reported only 1 positive well was rerun. Samples that had no amplification in all 3 wells were deemed negative for *Bd*. The reported numbers of zoospores per swab have not been adjusted; the reported numbers of zoospores come from a 5 µl aliquot from a 1:10 dilution of the original DNA extract, all in a 20 µl reaction volume.

Statistical analyses

The qPCR assay detects *Bd* and through the use of standard positive control dilutions, it quantifies how much *Bd* is in every well, providing the mean number of zoospores from all replicates. In addition to the mean zoospore load per sample, data collected include: (1) host life stage, (2) host order, (3) host family, (4) host species, (5) host sex, (6) the presence/absence of each taxonomic order at a site, (7) the presence/absence of each taxonomic family at a site, and (8) the presence/absence of each species at a site. Variables 1 to 5 represent individual-level variables and 6 to 8 represent site-level variables. We did not test for temporal patterns of *Bd* prevalence because every site was sampled once, leading to autocorrelation between site and date. All statistical tests and models were calculated using Minitab 16. Graphics were created in ESRI ArcMap v10 and Microsoft Excel 2007.

Analysis of infection probability

Due to high degrees of colinearity, unbalanced sampling, over-representation of particular observations (i.e. species, sex), and insufficient degrees of freedom, there were no significant interactions among variables; therefore, variables were tested in individual univariate analyses of variance (ANOVAs) on the same response variable, *Bd*-positive or -negative, using a Tukey-Kramer post hoc test. Following a significant univariate effect, binary logistic regression models were run to assess how the levels of each variable influenced the likelihood of *Bd* infection, using the binary outcome, *Bd*-positive or -negative, as the response variable; interaction terms were not included. Binary logistic regressions set 1 level as the reference, meaning that comparisons within a predictor variable (e.g. host family) are only possible between the reference and another variable level, not between 2 non-reference variables. For example, when evaluating how the likelihood of infection changes within anuran families, comparisons can only be made between the reference value, bufonids, and either hylids or ranids, not between hylids and ranids. Non-significant variable levels were removed until the model achieved significance. In addition, we used Bonferroni tests to correct for multiple comparisons and to obtain an adjusted alpha level.

Probability of carrying higher zoospore loads

A series of general linear models were fit to the above individual-level predictor variables (1 to 5) to assess how each variable influenced the likelihood of an individual carrying more zoospores; no interac-

Table 1. Number of *Batrachochytrium dendrobatidis* (*Bd*)-positive and negative swabs collected from Connecticut (CT), USA, amphibians in 2010. Data from 18 species are organized by species and age class. Values in **bold** font exclude data from the larval age class. Missing values indicate no samples taken. N: northern; Neg: negative for *Bd*; Pos: positive for *Bd*; Juv: juvenile; Meta: metamorph

Species	Adult		Juv/Meta		Larva		Grand total		
	Neg	Pos	Neg	Pos	Neg	Pos	Total	Proportion pos	95% CI
Frogs and toads	266	116	206	121	61	1	771	0.31	0.28–0.34
							709	**0.33**	**0.30–0.37**
American toad	2		8	1			11	0.09	0.002–0.41
Bullfrog	28	26	38	5	8		105	0.30	0.21–0.39
							97	**0.32**	**0.21–0.39**
Fowler's toad			9				9	0	0–0.28
Green frog	188	78	93	93	10		462	0.37	0.33–0.42
							452	**0.38**	**0.33–0.42**
Grey tree frog	1		9		2	1	13	0.08	0.002–0.36
							10	**0**	**0–0.26**
N. leopard frog[a]	11	6	4	3			24	0.38	0.19–0.59
Pickerel frog	13	6	35	17			71	0.32	0.22–0.45
Rana spp.	3		5		40		48	0	0–0.06
							8	**0**	**0–0.31**
Spring peeper	11		2				13	0	0–0.21
Wood frog	9		3	2	1		15	0.13	0.02–0.40
							14	**0.14**	**0.01–0.43**
Salamanders	68	18	49	5	5	0	145	0.16	0.10–0.23
							140	**0.16**	**0.11–0.24**
Blue-spotted[a]	1		3				4	0	0–0.53
Dusky	7	4	2	1			14	0.36	0.13–0.65
Four-toed		1	1				2	0.50	0.02–0.99
Marbled	1		3				4	0	0–0.53
N. two-lined	14		1	1			16	0.06	0.002–0.30
Red-backed	36	7	6				49	0.14	0.06–0.27
Red-spotted newt	9	5	27	2	5		48	0.15	0.06–0.28
							43	**0.16**	**0.07–0.31**
Spotted			6	1			7	0.14	0.004–0.58
Spring[a]		1					1	1.0	0.05–1
Grand total	334	134	255	126	66	1	916	0.28	0.26–0.32
							849	**0.31**	**0.28–0.34**

[a]Species of conservation concern in CT

tion terms were included in the models, nor did any model test more than 1 predictor variable at a time. The response variable, number of zoospores per swab (range 0.006 to 29 420 zoospores), was log-transformed to meet model assumptions. As before, the larval stage was removed, and we corrected for multiple comparisons using Bonferroni tests. Lastly, differences in zoospore load between infected species were assessed using a 1-sided Student's *t*-test to specifically test whether a particular species carried more zoospores than another species.

RESULTS

We did not detect *Bd* in any of the larval wood frogs collected in 2007. From the 2010 contemporary sampling effort, 261 of all 916 individuals (a prevalence of 0.28, with a 95% confidence interval of 0.26 to 0.32,), were found positive for *Bd* (Table 1). Of the 916 swabs, 67 were from pre-metamorphic individuals and of those, only 1 grey tree frog *Hyla versicolor* tadpole was found *Bd*-positive. If the larval stage is not considered, the overall prevalence was 0.31 (0.28 to 0.34; Table 1). The majority, 50.4%, of swabs were collected from green frogs (Table 1). *Bd* prevalence in 4 of the most often captured metamorphosed species, red-spotted newts *Notophthalmus viridescens*, red-backed salamanders *Plethodon cinereus*, bullfrogs, and green frogs, ranged from 0.14 to 0.38 (Table 1). Of the 260 swabs from infected post-metamorphs, 210 were measured quantitatively. The range in zoospore loads per swab was 0.006 to 29 420 (Table 2). Four species were negative for *Bd* across all age classes: Fowler's toads *Bufo fowleri*, spring peepers *Pseudacris crucifer*, blue-spotted salamanders, and marbled salamanders *Ambystoma opacum* (Table 1). Three sampled species (blue-spotted salamanders, spring salamanders, and northern leopard frogs) are listed as species of conservation concern in CT (Table 1). Sample sizes were low for each of these listed species, but both spring salamanders and northern leopard frogs had at least 1 infected post-metamorphic individual. No captured animal showed any signs of infection or decrement in physical condition during the course of the survey.

In total we surveyed 116 sites in 2010 for *Bd* (Fig. 1, Table 3). Per site, the range in *Bd* prevalence was 0.0 to 1.0, and of any site with at least 1 infected individual, the lowest detected prevalence was 0.04 (0.005 to 0.13; Table 3). In infinitely large populations, sampling 30 individuals can detect, with 95% confidence, at least 1 infected individual if *Bd* is in ≤0.10 of the population (Cannon & Roe 1982). Therefore, prevalence of infection in the 75 *Bd*-positive sites was likely ≥0.10 because we detected at least 1 *Bd*-positive animal in each of those sites (the number of sampled animals from each of those sites ranged from 1 to 53; Table 3). Of the 41 sites that had no *Bd*-positive animals, the number of swabs collected ranged from 1 to 20 (Table 3). Collecting 1 or 20 swabs per site decreases detection probability to 9 and 85%, respectively (Cannon & Roe 1982); thus, given the sample sizes for the *Bd*-negative sites, we can assert with far less certainty that *Bd* was prevalent in ≤0.10 of each site's population.

Infection prevalence and zoospore load

Infection prevalence among the life stages varied significantly ($F_{2,913}$ = 14.34, p = 0.000). All but 67 individuals sampled were post-metamorphic, and the infection prevalence of those larvae was very low (Table 1). Larvae were at least 26.48 times less likely to be infected than either other post-metamorphic life stage (Table 4). For all subsequent models, records with larvae were excluded, leaving 849 post-metamorphic individual records and 116 site records. Similarly, because there was a significant difference of infection prevalence between anurans and caudates ($F_{1,847}$ = 16.17, p = 0.000;

Table 2. Measured *Bd* zoospore load per swab of only post-metamorphic age classes. Pos: no. of individuals tested positive for *Bd*; N.: northern

Species	Pos	Min	Max	Mean	Median	SD
Frogs and toads						
American toad	1				0.31	
Bullfrog	27	0.068	535.00	42.88	1.41	135.86
Green frog	152	0.026	29419.74	358.58	4.26	2536.75
N. leopard frog[a]	8	0.085	10588.50	2210.70	108.34	3726.23
Pickerel frog	13	0.107	7087.83	651.24	86.77	1943.40
Salamanders						
Dusky	4	0.037	131.70	33.07	0.27	65.75
N. two-lined	1				33.48	
Red-backed	1				0.97	
Red-spotted newt	3	0.006	6.23	2.75	2.01	3.18
Total across all species	210	0.006	29419.74	390.42	4.38	2342.56

[a]Species of conservation concern in Connecticut

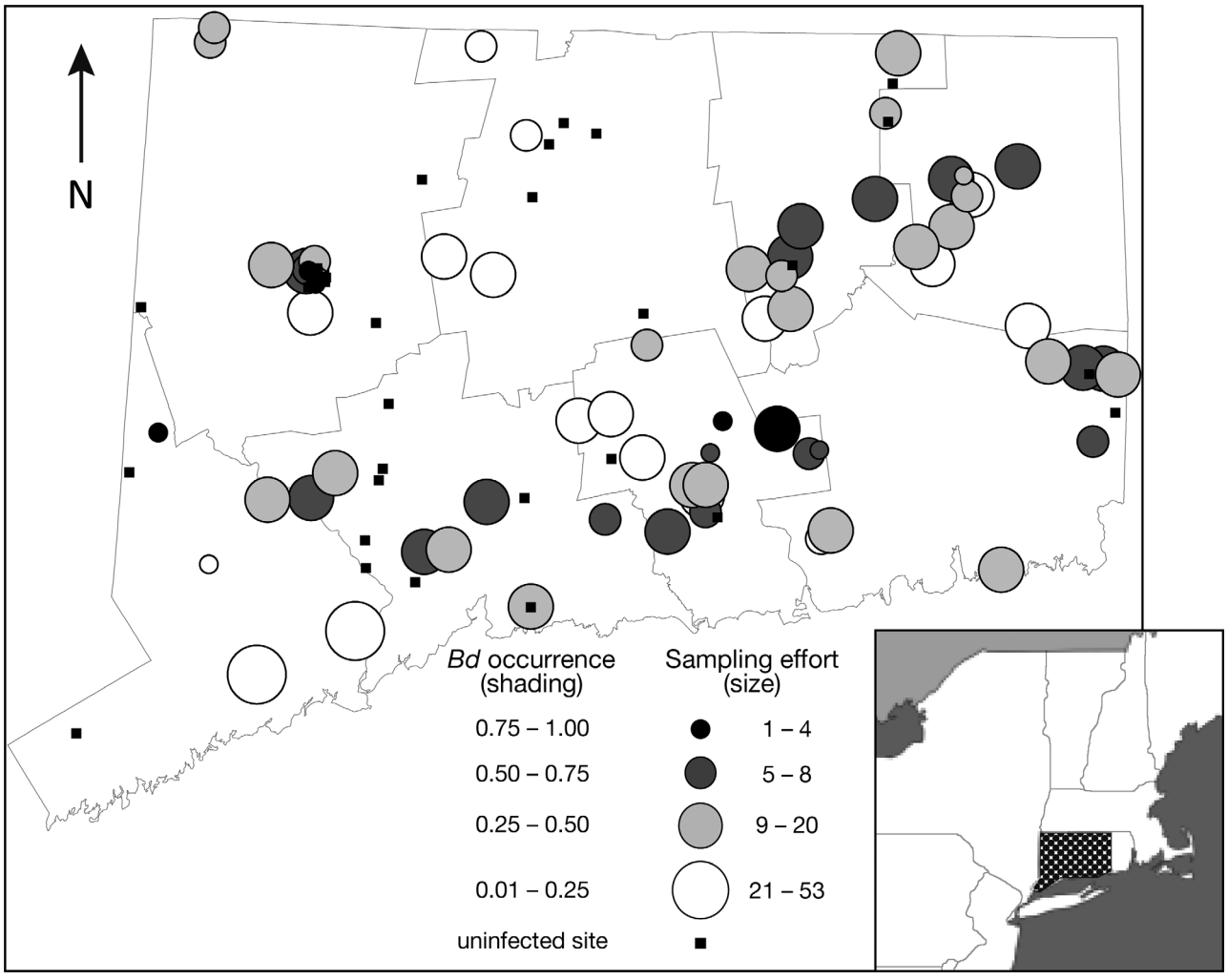

Fig. 1. Sampling locations of 99 field sites throughout Connecticut, USA (stippled area in inset). Sites without GPS coordinates were excluded (*Batrachochytrium dendrobatidis* [*Bd*]-positive sites n = 9; *Bd*-negative sites n = 8). Circle shading indicates percent *Bd*-positive individuals per site and circle size indicates sampling effort (no. of individuals)

Table 4), all subsequent models were separated based on taxonomic order. Within caudates, there were no significant differences among family ($F_{2,137}$ = 0.62, p = 0.541), species ($F_{8,131}$ = 1.75, p = 0.094), life stage ($F_{1,138}$ = 3.32, p = 0.071), or sex ($F_{2,137}$ = .039, p = 0.677). Within anurans, there were significant differences among family ($F_{2,706}$ = 10.26, p = 0.000), species ($F_{9,699}$ = 3.38, p = 0.000), and sex ($F_{2,706}$ = 7.56, p = 0.001). The differences among anuran species, however, were solely between the most sampled (and infected) species (green frogs, bullfrogs, northern leopard frogs, and pickerel frogs) and the species that were rarely sampled and had no detectable infections (Fowler's toad, grey tree frogs, spring peepers, and unidentified ranids). When these poorly sampled and *Bd*-negative species were removed from the model, the pattern disappeared ($F_{5,663}$ = 1.62, p = 0.154); post hoc Student's *t*-tests also supported there being no significant differences among the *Bd*-positive species. Prevalence among anuran life stages was not significantly different ($F_{1,707}$ = 3.49, p = 0.062). Since 19 species from both orders were sampled, we investigated whether any species had a greater incidence of infection. *Bd* prevalence did vary among species ($F_{18,830}$ = 3.23, p = 0.000), namely among green frogs, red-backed salamanders, and red-spotted newts ($F_{2,541}$ = 8.97, p = 0.000). Following ANOVAs with significant effects, binary logistic regressions were run to determine how each level within the predictor variable compared in infection odds. The following increased the likelihood of

infection: ranids versus bufonids or hylids, and the unknown/unrecorded sex or female anurans versus male anurans (Table 4). Among all species, of the 3 that varied in their infection odds, red-backed salamanders and red-spotted newts were less likely to be infected than green frogs (Table 4). Bonferroni corrections did not alter model significance.

General linear models tested whether various predictors resulted in increased zoospore loads per individual (Table 5). As before, the larval stage was removed from all models. We found no differences in zoospore loads among orders or families ($p > 0.05$); hence, models were run with all species together. Three models were significant: host life stage ($F_{1,208}$ = 71.05, p = 0.000), host sex ($F_{2,207}$ = 21.40, p = 0.000), and host species ($F_{8,201}$ = 2.80, p = 0.006; Table 6). One-sided Student's *t*-tests confirmed that northern leopard frogs (t = 2.25, p = 0.027) and pickerel frogs (t = 3.21, p = 0.002) carried more zoospores than bullfrogs. Bonferroni corrections did not alter model significance.

Table 3. Prevalence of *Bd* per site, grouped into prevalence bins, e.g. there were 11 sites where between 0.201 and 0.300 of swabbed individuals tested positive for *Bd*. The number of individuals swabbed in any of these 11 sites ranged from 4 to 31

Bd prevalence	No. sites	Sampling effort range
0	41	1–20
0.001–0.10	5	10–53
0.101–0.20	9	5–13
0.201–0.30	11	4–31
0.301–0.40	10	5–10
0.401–0.50	9	2–20
0.501–0.60	9	5–10
0.601–0.70	8	3–12
0.701–0.80	5	5–10
0.801–0.90	1	10
0.901–1.0	8	1–2
Total	116	1–53

Within-site infection prevalence

Bd prevalence was dependent on which order(s) were present at a site ($F_{2,113}$ = 3.40, p = 0.037); when

Table 4. Binary logistic regressions on the individual level with the response variable *Bd*-positive or -negative. Larvae were removed from all but the first test. Only variables with significance ($p < 0.05$) or close to significance were included. Comparisons within a predictor variable can only be made to the reference value. n: no. of records; juv: juvenile; F: female; U: unknown; Coeff: coefficient

Model	Coeff	SE Coeff	*Z*	p	Odds ratio	95% CI
Three life stages, n = 916						
Constant (null model)	−4.19	1.01	−4.16	0.000		
Life stage, adult	3.28	1.01	3.24	0.001	26.48	3.64 – 192.72
Life stage, juv	3.48	1.01	3.44	0.001	32.61	4.47 – 237.68
Log-likelihood = −527.262, G = 40.195, df = 2, p = 0.000						
Host order, n = 849						
Constant (null model)	−1.63	0.23	−7.13	0.000		
Host order, Anura	0.94	0.24	3.88	0.000	2.55	1.59 – 4.10
Log-likelihood = −514.288, G = 17.495, df = 1, p = 0.000						
Anuran families, n = 709						
Constant (null model)	−2.94	1.03	−2.87	0.000		
Hylids	−10001.40	208514.00	−0.05	0.962	0	0
Ranids	2.34	1.03	2.28	0.023	10.43	1.39 – 78.38
Log-likelihood = −436.937, G = 29.624, df = 2, p = 0.000						
Anuran sex, n = 709						
Constant (null model)	−1.44	0.22	−6.50	0.000		
Sex, F	0.90	0.28	3.25	0.001	2.47	1.43 – 4.25
Sex, U	0.90	0.24	3.67	0.000	2.45	1.52 – 3.96
Log-likelihood = −443.728, G = 16.043, df = 2, p = 0.000						
Green frogs, red-backed salamanders, and red-spotted newts, n = 544						
Constant (null model)	−0.50	0.10	−5.12	0.000		
Red-backed salamander	−1.29	0.42	−3.09	0.002	0.27	0.12 – 0.62
Red-spotted newt	−0.14	0.42	−2.69	0.007	0.32	0.14 – 0.73
Log-likelihood = −338.981, G = 19.537, df = 2, p = 0.000						

both orders were found at a site, the site was more likely to be infected when compared to sites with just caudates (Table 6). However, after correcting for multiple comparisons using Bonferroni, the model was marginally significant (p = 0.031, adjusted alpha = 0.017; Table 6). When testing whether the presence or absence of each of the families impacted *Bd* prevalence, only ranids increased its prevalence ($F_{1,114}$ = 14.02, p = 0.000; Table 5). Specifically, when green frogs were at a site, *Bd* was found in more hosts ($F_{1,114}$ = 9.45, p = 0.003) than when green frogs were absent from sites (Table 5).

Table 5. General linear models on the individual level with the response variable: log-transformed number of *Bd* zoospores. Only infected individuals were included, and larvae were removed from all tests. Only variables with significance (p < 0.05) or close to significance were included. n: no. of records; N.: northern; F: female; U: unknown; Coeff: coefficient

Model	Coeff	SE Coeff	*t*	p
Two life stages, n = 210				
Constant (null model)	0.67	0.08	8.85	0.000
Life stage, adult	−0.64	0.08	−8.43	0.000
Host sex, n = 210				
Constant (null model)	0.22	0.12	1.82	0.070
Sex, F	−0.07	0.15	−0.47	0.638
Sex, U	0.85	0.13	6.45	0.000
Host species, n = 210				
Constant (null model)	0.55	0.27	2.09	0.038
American toad	−1.06	1.11	−0.96	0.340
Bullfrog	−0.21	0.34	−0.64	0.525
Dusky salamander	−0.68	0.60	−1.13	0.262
N. leopard frog	1.30	0.46	2.81	0.006
N. two-lined salamander	0.97	1.11	0.87	0.383
Pickerel frog	1.04	0.40	2.60	0.010
Red-backed salamander	−0.57	1.11	−0.51	0.609
Red-spotted newt	−0.93	0.67	−1.37	0.171

DISCUSSION

In our contemporary survey, 18 species were sampled at 116 sites, and *Bd* was found in a large proportion of all swabs (0.28). An infection prevalence of 0.28, or 0.31 if only the post-metamorphs are considered, is very similar to the only other survey for *Bd* in the region. Longcore et al. (2007) found an infection prevalence of as high as 0.257 among the 74 screened northern leopard frogs. Given this result, we were not surprised to find an overall infection proportion of 0.28 across all species and age classes. We did not use an internal positive control in any qPCR, which possibly leads to underestimations of *Bd* prevalence. However, as per Boyle et al. (2004) and Garland et al. (2010), DNA from our field swabs was diluted 1:10 and BSA added to every qPCR well to help reduce inhibition. Despite the possibility of underestimating *Bd* prevalence, our survey across CT represents one of the most intensive regional surveys for *Bd* and is the first for CT.

Table 6. Binary logistic regressions on the site level with the response variable: *Bd*-positive or -negative. Larvae were removed. Only variables with significance (p < 0.05) or close to significance were included. Comparisons within a predictor variable can only be made to the reference value. n: no. of records; Coeff: coefficient

Model	Coeff	SE Coeff	*Z*	p	Odds ratio	95% CI
Presence/absence of each or both orders, n = 116						
Constant (null model)	1.73	0.63	2.77	0.006		
Anurans, present	−1.18	0.67	−1.78	0.076	0.31	0.08 – 1.13
Caudates, present	−2.02	0.83	−2.45	0.014	0.13	0.03 – 0.67
Log-likelihood = −71.865	*G* = 6.966	df = 2	p = 0.031			
Presence/absence of ranids, n = 116						
Constant (null model)	−0.85	0.49	−1.74	0.082		
Ranids, present	1.79	0.54	3.32	0.001	5.96	2.08 – 17.12
Log-likelihood = −69.254	*G* = 12.189	df = 1	p = 0.000			
Presence/absence of green frogs, n = 116						
Constant (null model)	−0.24	0.35	−0.68	0.494		
Green frogs, present	1.24	0.43	2.91	0.004	3.45	1.50 – 7.96
Log-likelihood = −71.019	*G* = 8.660	df = 1	p = 0.003			

The most common ranid species at our sampling sites (bullfrogs, green frogs, northern leopard frogs, and pickerel frogs) had comparatively high levels of infection. It was not surprising that bullfrogs were in this group, as they have been shown in previous research to be a carrier, even an international carrier in the pet and food trade, of *Bd* (Daszak et al. 2004, Schloegel et al. 2009). Our data perhaps indicate that along with bullfrogs, these other ranids are also effective carriers of *Bd*. Since these anuran species are equally likely to be infected, the most widely distributed species in this region (green frogs, bullfrogs, and pickerel frogs) could serve as sentinel species for monitoring programs concerned with *Bd* presence or absence across the state. These ranid species are relatively abundant and easy to find throughout the summer months, reasonably easy to swab given their larger body size, and they harbor *Bd*. If monitoring programs wish to maximize their resources and simply screen for the presence of *Bd*, the following factors could also be included in sampling schemes: focus on juveniles and adults (i.e. avoid larval stages), anurans, the female of any anuran species, and sites dominated by ranids (especially green frogs). To further investigate trends in zoospore loads, juveniles or northern leopard frogs or pickerel frogs should be targeted, as these carry greater zoospore loads than any other life stage or species. Reduced body size and an underdeveloped immune system have been hypothesized as reasons why juveniles carry greater zoospore loads than adults (Carey et al. 2006, Rachowicz et al. 2006, Pearl et al. 2009, Briggs et al. 2010).

Many zoospore loads were quite low (>0 to 1 per swab), but the extracts were re-run and zoospore values were replicated, demonstrating that in this study, low zoospore loads are real and common. It is important to note, however, that metrics of zoospore load are associated with substantial variation related to sampling protocol. Different researchers swab differently — some swab more thoroughly or with greater pressure than those with a lighter hand or less skill (Retallick et al. 2006) — but there is probably a species, life stage, or even an individual host effect. Recent research found that zoospore load variation could not be explained solely by species, nor habitat, climate, or elevation; zoospore load may be entirely an individual host characteristic (Gründler et al. 2012). For example, it would not be surprising that a large, infected bullfrog would yield more zoospores than a small, infected red-backed salamander. In this case, body size could explain zoospore load differences, and it would not imply a greater or lesser 'infection intensity.' Alternatively, suppose the salamander was in an environment well suited to *Bd* growth (17 to 25°C, Piotrowski et al. 2004, Andre et al. 2008) versus a bullfrog that recently shed its skin and was sunning itself in temperatures at or beyond the thermal limit of *Bd* (>28°C, Piotrowski et al. 2004, Andre et al. 2008, Meyer et al. 2012). In this case, the resulting zoospore load comparison may be more similar; this would still not suggest any equality (or difference) in 'infection intensity.' Following a standard swabbing protocol (which was done here) in the field is valuable and necessary, but there are many factors outside the researcher's control that may influence measured zoospore loads. Therefore, we caution any interpretation of our tests evaluating how predictor variables likely increase zoospore load. Our model results, however, provide suggestive trends (e.g. adults carry fewer zoospores than juveniles), that require further research.

The low zoospore loads and the lack of *Bd*-related dieoffs support the hypothesis that *Bd* is endemic in this region. In a related study system, Briggs et al. (2010) measured the infection dynamics for 5 yr at 3 sites with persistent *Bd* (endemic sites). The infection prevalence among the 3 sites ranged from 0.60 to 0.76 of all adult frogs, and the average zoospore load per post-metamorph was 4913 ± 820 zoospores. In another region similarly persisting with *Bd* and asymptomatic individuals, the average *Bd* prevalence was high (0.83 of post-metamorphic individuals), and the average *Bd* load ranged between 4 and 3861 zoospores (mean = 514; Puschendorf et al. 2011). These studies report both a greater infection prevalence and higher average zoospore load than that found in CT. Of other surveys for *Bd* done elsewhere in the world (95% CI range reported here, except where noted), 0.20–0.36, 0.04–0.14, 0.96–0.99, 0–0.92 (not CI), and 0.02–0.03 of all sampled individuals were found to be *Bd*-positive in Gabon, Peninsular Malaysia, Panama (El Cope), southeastern Brazil, and among 15 Asian countries, respectively (Lips et al. 2006, Bell et al. 2011, Savage et al. 2011, Swei et al. 2011, Gründler et al. 2012). While our reported prevalence does not mirror the upper limit measured during a dieoff in Panama (Lips et al. 2006) or levels measured at other persistent regions (California, Briggs et al. 2010; Australia, Puschendorf et al. 2011), a prevalence of 0.28 is moderate and within previously detected ranges. The presence of asymptomatic individuals with moderate prevalence and zoospore loads hints at some time having passed since the initial date of *Bd* emergence or arrival to the region, as zoospore load, prevalence, and mortality

peak during the initial outbreak in naïve populations and subsequently taper off (Lips et al. 2006, Vredenburg et al. 2010, Puschendorf et al. 2011). For CT, at least 4 decades may have passed since these values likely peaked (Richards-Hrdlicka 2012).

While large-scale dieoffs are not currently documented in New England, it is unclear whether population declines in the past can be attributed to *Bd* infection. Three sampled species, northern leopard frogs, spring salamanders, and blue-spotted salamanders, are of conservation concern in CT, largely because CT is near the range limit for these species. The single spring salamander sampled was infected, as were 9 of the 24 sampled northern leopard frogs; the 4 blue-spotted salamanders tested negative for *Bd*. Although our sample sizes for these 3 species are small and we did not observe any clinical signs of infection, our preliminary data should be considered in a broader conservation context since this is the first time 2 of these species in this region were found to be *Bd*-positive. Our work was not aimed at addressing whether *Bd* played a role in leading these species to a state of 'conservation concern.' However, given that *Bd* was not identified until the late 1990s (Berger et al. 1998, Longcore et al. 1999), it is possible that some of these species are also rare because of their susceptibility to chytridiomycosis. In the case of northern leopard frogs, past studies have highlighted the role of habitat loss and pond water acidification as possible causes of population decline in this region (Dunson et al. 1992, Simon et al. 2002, Brodkin et al. 2003, Gibbs et al. 2005). However, in light of our results, the potential for a combined effect of habitat stress and infection with *Bd* should not be overlooked.

Our sampling scheme was opportunistic, with an emphasis on gathering as much spatial and species coverage as possible. We did not resample any site or control for wetland type or dominant host species. Three inferences can be made from our collected samples: (1) green frogs are very abundant across the sampled region, (2) the sites sampled are those often preferred by green frogs and other ranids (i.e. few terrestrial environments were surveyed), and (3) although our sampling protocol was haphazard, we were able to detect *Bd* at 72 sites where <30 swabs (the minimum number to detect *Bd* in 0.10 of the population, see above) were collected. Perhaps we were lucky with these swabs, perhaps the true prevalence of *Bd* at those sites was very high, or perhaps our detection success is explained by the hosts from which those swabs came, i.e. primarily ranids. Our data do not allow for tests of whether there is a temporal pattern to *Bd* prevalence or whether species richness or abundance or certain wetland types (e.g. open versus closed canopy, semi-permanent, ephemeral) influence the prevalence of *Bd*. However, our baseline data suggest that these are interesting hypotheses worthy of additional research. Future research into these putative trends will also inform whether there are hot spots of *Bd* infection and whether a single sampling event is sufficient to define the infection status of a given site.

None of the tadpoles collected in 2007 were positive for *Bd*, suggesting that *Bd* was not in the host at the time of collection. The earliest published record of *Bd* in this region of the world is 1961 from Saint-Pierre-de-Wakefield, Quebec (Ouellet et al. 2005), Canada, and the earliest record of *Bd* in CT is 1968 (Richards-Hrdlicka 2012). Given that *Bd* has been in this area for decades and it has been successfully extracted and detected from other ethanol-preserved amphibian tissues (Soto-Azat et al. 2009, Voordouw et al. 2010), we are confident that our methods were sufficient to detect *Bd* if it was present in these samples. These tadpoles were quite small (Gosner stages 25 to 28; Gosner 1960), and half a mouthpart may not have captured a nascent infection at this early larval stage. To detect *Bd* in individuals, we recommend targeting larger animals and gathering a sample from a greater percentage of susceptible tissue, such as by employing epidermal swabs.

Our work adds to other documented cases where *Bd* prevalence can be widespread and infects many species, yet can be non-lethal to its hosts (Retallick et al. 2004, Briggs et al. 2010, Puschendorf et al. 2011). Not succumbing to lethal chytridiomycosis infections may be explained by the host's biology (including host defenses, life stage, sex), environmental conditions, or the pathogen itself (including pathogenesis), or some combination of these factors (Voyles et al. 2011). Recently, investigations have started to focus on the molecular basis of individual *Bd* isolates (Fisher et al. 2009, Rosenblum et al. 2010), and this line of research will be able to identify whether there are different strains of *Bd*, whether they vary regionally, and whether some are more virulent than others. It may be that strains existing in CT, or more broadly throughout New England, are less lethal than strains found in dieoff locations. Since the northeastern US is a suspected origin for the original emergence of *Bd* (James et al. 2009), this region should be monitored closely and future research planned to investigate whether *Bd* in the northeast is genetically distinct from strains in dieoff zones, perhaps warranting efforts to limit pathogen dispersal. Until then, we

support recent efforts to prevent spreading *Bd* to new areas or even introducing novel strains to regions that are already infected with an undetermined strain (Phillott et al. 2010). Having baseline data about the geographic extent and species susceptibility to *Bd* infection in a region can be critically important in assessing population health, identifying concentrated infection zones, and providing useful guidelines to wildlife managers asked to prevent species declines. Understanding the dynamics of *Bd* through space and time could ultimately enable conservation managers to protect susceptible, endangered species in particular areas, or at the very least enforce decontamination of aquatic equipment between and within *Bd* hot spots.

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