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RESEARCH ARTICLE

Terrestrial Dispersal and Potential Environmental Transmission of the Amphibian Chytrid Fungus (*Batrachochytrium dendrobatidis*)

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Abstract

Dispersal and exposure to amphibian chytrid fungus (*Batrachochytrium dendrobatidis*, *Bd*) is not confined to the aquatic habitat, but little is known about pathways that facilitate exposure to wild terrestrial amphibians that do not typically enter bodies of water. We explored the possible spread of *Bd* from an aquatic reservoir to terrestrial substrates by the emergence of recently metamorphosed infected amphibians and potential deposition of *Bd*-positive residue on riparian vegetation in Cusuco National Park, Honduras (CNP). Amphibians and their respective leaf perches were both sampled for *Bd* presence and the pathogen was detected on 76.1% (35/46) of leaves where a *Bd*-positive frog had rested. Although the viability of *Bd* detected on these leaves cannot be discerned from our quantitative PCR results, the cool air temperature, closed canopy, and high humidity of this cloud forest environment in CNP is expected to encourage pathogen persistence. High prevalence of infection (88.5%) detected in the recently metamorphosed amphibians and frequent shedding of *Bd*-positive residue on foliage demonstrates a pathway of *Bd* dispersal between aquatic and terrestrial habitats. This pathway provides the opportunity for environmental transmission of *Bd* among and between amphibian species without direct physical contact or exposure to an aquatic habitat.

Introduction

Infection by the pathogenic amphibian chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) poses a major threat to global amphibian biodiversity [1,2]. Response to infection varies considerably between species; a minority of those tested generally carry *Bd* in the absence of

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morbidity and may serve as aclinical reservoir hosts, such as the American bullfrog, *Lithobates catesbeianus* [3], and the African clawed frog, *Xenopus laevis* [4], whereas others are highly susceptible to chytridiomycosis and have suffered dramatic decline following introduction in wild populations [5,6]. Variation in virulence has been observed, and exposure to certain isolates of the highly pathogenic *Bd*GPL clade causes mortality in amphibians more quickly than others [7,8]. *Bd* demonstrates low host species specificity and as of 2012, infection had already been reported in 516 species from 52 countries [9], and evidence suggests this pathogen is native in some parts of its range but emerging and spreading in others [10,11]. Identified 15 years ago [12], the geographic origin and subsequent pathways of global and local *Bd* dispersal remain largely speculative, although recent studies show *Bd* is now commonly spread via the international and domestic trade in live amphibians [13–16]. However, mechanisms of dispersal outside the amphibian host and in the absence of anthropogenic assistance are more obscure.

Direct and indirect modes of *Bd* dispersal and transmission within wild amphibian populations have been postulated, but few have been demonstrated. Direct contact between animals engaged in amplexus or territorial confrontation is thought to be a common mode of transmission [17]. Contact with contaminated water is another avenue, and *Bd*'s motile uniflagellated zoospores can disperse through a water body either by swimming short distances or by being carried in water currents [18]. Waterfowl might carry *Bd* between separate water bodies, either on their feathers or feet [19–21]. However, the high prevalence of *Bd* detected in terrestrial and arboreal amphibian species that infrequently contact each other and typically do not directly engage with other species or enter permanent water bodies [22–25], suggests the presence of additional avenues of *Bd* dispersal and environmental transmission. For example Burrowes et al. [26] detected a high prevalence of infection (44.1%) in *Eleutherodactylus coqui*, a direct-developing terrestrial anuran inhabiting leaf litter in the cloud forest in Puerto Rico and McCracken et al. [27] found 33% of canopy-dwelling amphibians infected in a lowland Ecuadorian rainforest. *Bd* has also been detected on 62% of terrestrial soil-dwelling caecilians sampled in Cameroon [28,29]. Collectively, the detection of *Bd* on amphibians that inhabit the forest canopy, terrestrial leaf littler, and soil suggests a common terrestrial existence where its dispersal and transmission are not constrained by the absence of permanent water.

The spread of *Bd* through Central and South America is associated with dramatic amphibian declines and extirpations [5,6,30,31] and interestingly, affected sites include remote wilderness areas and national parks where anthropogenic-assisted *Bd* spread is expected to be minimal [31–33]. Although a wave of *Bd* appears to have swept southeast through Central America during the 1980's [10,32], relatively little is known of its present distribution and ecological impact in Honduras. Infected amphibians have been reported from two locations, Pico Bonito National Park [34] and Cusuco National Park (CNP) [24], but the country boasts a mosaic of additional montane cloud forests likely to be similarly affected, but not yet surveyed. It has been estimated that nearly 50% of 111 amphibian species in Honduras have suffered declines in recent years from a combination of factors, including chytridiomycosis, and seven endemic anuran species were believed extinct [35], although one (*Craugastor milesi*) was recently rediscovered [36]. *Bd* has been detected in Honduran terrestrial anurans that undergo direct metamorphosis in leaf litter, including *Craugastor aurilegulus* and *C. rostralis* [24,34], and the source of pathogen exposure to these species remains enigmatic. Similarly, *Bd*-positive terrestrial frogs have been detected in Costa Rica (*Oophaga pumilio* and *Craugastor fitzingeri*), prompting the authors to suggest that *Bd* can survive on the moist forest floor where transmission might occur [32].

Since *Bd* occurs in the superficial skin of infected metamorphosed amphibians, there appears to be potential for infectious zoospores and sporangia within shedding skin to contaminate environmental substrates. Newly post-metamorphic anurans, in particular, often exhibit both elevated *Bd* prevalence and zoospore loads [24,37–39], so their emergence from water

might represent a considerable pathway of *Bd* dispersal into the terrestrial zone. To explore this potential avenue of terrestrial *Bd* spread we investigated whether terrestrial vegetation becomes contaminated with *Bd* following contact with recently metamorphosed amphibians under natural field conditions.

Materials and Methods

Ethics

Amphibian sampling in CNP adhered to established protocols [40] and were authorized by the Instituto Nacional de Conservacion y Desarollo Forestal Areas Protegidas y Vida Silvestre (ICF) of Honduras as part of the long-term Biodiversity Monitoring Programme performed by Operation Wallacea. Permission to export samples was granted by Honduran permit #'s 44735 and 19987.

Study Site

This investigation was performed from 9 July to 6 August 2013 in Cusuco National Park (CNP), a montane rainforest located in the Sierra de Omoa of northwestern Honduras. The altitude of CNP ranges from 550 m to 2200 m and fieldwork was performed between 1300 m and 1600 m at three different river sites (Rio Cusuco, N 15.495, W 88.213, elev. 1600 m; Rio Cortecito, N 15.523, W 88.288, elev. 1350 m; and Rio Danto, N 15.530, W 88.277, elev. 1545 m). Previous work identified widespread distribution and high prevalence of *Bd* in CNP at these locations [24] and its presence in the region for approximately two decades or greater [41]. Recently metamorphosed individuals of four tree frog species susceptible to *Bd* were targeted for sampling (*Duellmanohyla soralia*, *Plectrohyla dasypus*, *Plectrohyla exquisita*, and *Ptychohyla hypomykter*). Of these species, *P. dasypus*, previously demonstrated the highest prevalence of infection both at the species level (78%) and among recently metamorphosed individuals (94%) [24]. Most sampling was performed at night when animals were more active and likely to be encountered on riparian vegetation, although some opportunistic sampling occurred in the day. Most frogs were encountered within 5 m of the water's edge, but some were found up to 50 m from the river. Sampling was restricted to frogs resting on leaves, and not those perched on stalks or branches.

Leaf and Amphibian Sampling

Recently metamorphosed amphibians were removed from leaves by inverting them above a new plastic bag, into which the amphibian either jumped or was guided by a gentle tap on the underside of the leaf. Care was taken not to exert pressure between the frog and leaf, to prevent increased potential *Bd* shedding. Vegetation was sampled first, and then the corresponding amphibian was sampled. Nitrile gloves were worn and changed between every swab collected to reduce the risk of sample cross contamination. Leaves and frogs were each sampled with sterile fine-tipped rayon swabs (Medical Wire & Equipment Co., #MW113). For leaves, each swab was drawn across the leaf surface 20 times, where the amphibian was perched and in most instances, had left a small film of moisture visible on the leaf's surface, approximately 0.5 cm in diameter, marking the amphibians' location (Fig 1). For amphibians, the hands, feet, and pelvic patch were swabbed five times each following protocols established by Hyatt et al. [40]. Swab buds were snapped off into 2 mL microcentrifuge tubes filled with 1 mL 70% ethanol as a preservative. After sampling was completed, each amphibian was replaced to its original position in the vegetation.

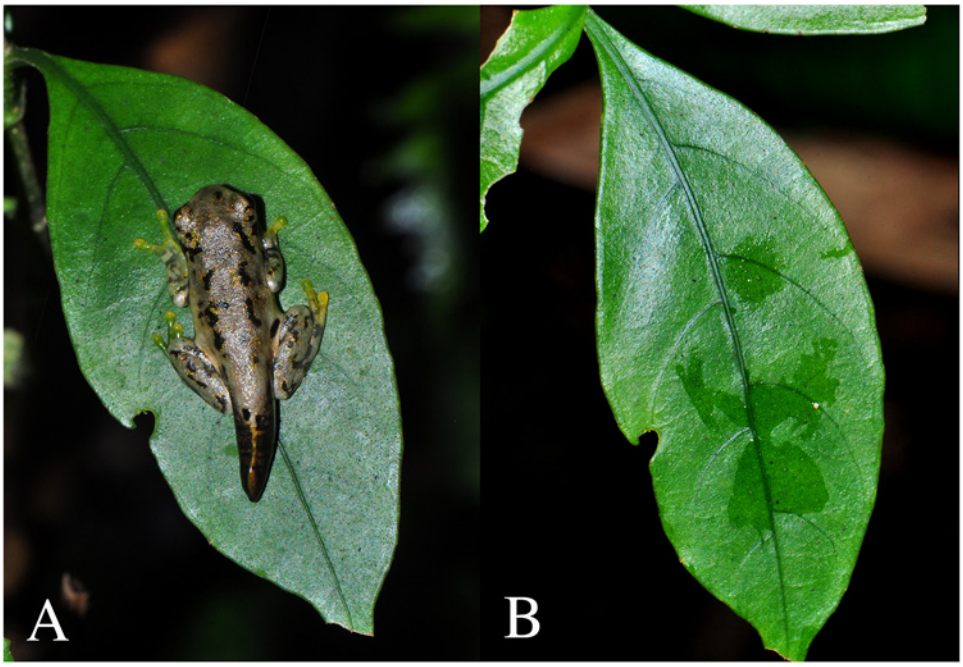

Fig 1. Recently metamorphosed *Plectrohyla dasypus* on terrestrial vegetation in Cusuco National Park, Honduras. (A) Amphibian as encountered on vegetation. (B) *Bd*-positive residue remaining on the leaf after amphibian removal.

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Temperature

Immediately upon encountering an amphibian perched on vegetation, the amphibian's dorsal body surface, the vegetation surface, and the air temperature were measured to characterize the environmental conditions *Bd* would be exposed to, if present. Temperatures were measured using a Raytek ST81 Non-contact Infrared Thermometer (RAYST81, emissivity set to 0.95), from a distance of 0.5 m or less. Accuracy of the thermometer is ± 1% of reading or ± 1°C, whichever is greater. This technique obtains amphibian body temperature readings within 0.5°C of cloacal temperatures [42]. Air temperature was measured with the attachable RTD temperature probe.

Water Sampling

Water samples from rivers at the three sites were collected and filtered for *Bd* detection. These samples were processed for *Bd* testing following established protocols [43], with the exception that a peristaltic pump was used to increase the efficiency of sampling efforts by maximizing the volume of water filtered. We used sterile silicone rubber peristaltic pump tubing and replaced a new length for the collection of each sample. Water was pumped through Sterivex filter capsules (0.22 micron pore size) until the flow rate greatly diminished. Then the volume filtered was measured and recorded. The content of each filter capsule was rinsed with 50 mL phosphate buffered saline solution and then pumped dry. A bead of clay sealant was used to plug the outlet spout of the capsule before being preserved by adding 0.9 mL of Qiagen ATL tissue lysis buffer with a sterile 1 mL syringe. Luer-Lok screw caps sealed the inlet spout of the capsules and a bead of quick-drying epoxy was applied behind each clay plug in the outlet spout to provide the seal with reinforcement during transit. Fresh pairs of Nitrile gloves were worn each time a water sample was collected. All water sampling was performed during

daytime hours. Water temperature was measured at the time of sampling using the attachable RTD temperature probe of the Raytek ST81 Non-contact Infrared Thermometer.

Real-Time PCR Analysis

Samples were processed via a sensitive quantitative PCR assay (qPCR) specific to *Bd* following the established protocol [44] and with the addition of BSA to the qPCR master mix as per Garland et al. (2010) [45]. Samples were extracted with 100 μl Prepman Ultra (Applied Biosystems, California, USA), with a final 20 μl of supernatant removed for downstream use. An aliquot of this supernatant was diluted 1:10 in DNase-free water for qPCR. The qPCR protocol used Sensi-Mix II Low Rox (Bioline, Massachusetts, USA) as the qPCR master mix [46]. For each sample, 5 μl of 1:10 dilution of swab DNA was added to each well for a final total qPCR volume of 20 μl. Samples and controls were run in triplicate with three positive, standard control samples (100, 10, and 1 zoospore/well, made from JAM81 pure culture; see Boyle et al. 2004 for standard control construction) and one non-template control (DNase free, molecular-grade water). When the qPCR assay failed to detect *Bd* in all three wells, the sample was deemed negative for *Bd*. Samples that produced a positive signal for *Bd* in either two or three wells on the first run were considered positive for *Bd*. When only one of three replicates detected *Bd*, the sample was rerun (in triplicate again) in a subsequent plate. For rerun samples that had at least a cumulative total of two of six replicates positive for *Bd* (from at least two separate plates), the sample was deemed positive for *Bd*. All zoospore loads described in this report have not been converted and reflect the actual zoospore loads present in 5 μl DNA (1:10 dilution), placed into 20 μl reaction volumes.

Data Analysis

We applied Chi-square test on a 2x2 contingency table to determine whether row and column marginal frequencies were equal. The values in the matrix included: number of *Bd*-negative frogs associated with *Bd*-negative leaves (5), number of *Bd*-negative frogs associated with *Bd*-positive leaves (1), number of *Bd*-positive frogs associated with *Bd*-negative leaves (11), number of *Bd*-positive frogs associated with *Bd*-positive leaves (35). Analysis was performed in R (R Development Core Team 2013 version 3.0.11 using package STATS (Chisq.test; version 3.0.3).

Results

Amphibian Swab *Bd* Results

Bd was detected on 46 of 52 (88.5%) amphibians and from all four species (Table 1). The average *Bd* zoospore equivalent load detected on *Bd*-positive amphibians was 103.94 and ranged from 0.06–1,574.62.

Leaf Swab *Bd* Results

Bd was detected on 36 of 52 (69.2%) leaves, 97.2% of which had a *Bd*-positive recently metamorphosed amphibian on them (35/36) (statistical significance of the association, df = 1, chi-squared = 6.23, p-value = 0.013) (Table 1). Only one *Bd*-positive leaf had an amphibian that tested *Bd*-negative. The average *Bd* zoospore equivalent load detected on *Bd*-positive leaves was 40.48 and ranged from 0.12–1,040.45.

River Water Filter *Bd* Results

The presence of *Bd* was detected in all three river water samples (Table 2). The average *Bd* zoospore equivalent load per liter of river water was 0.23 and ranged from 0.03–0.57. Daytime water temperature averaged 17.0°C and ranged from 16.3–17.5°C.

Table 1. Presence of *Batrachochytrium dendrobatidis* (*Bd*) detected on amphibians and vegetation sampled in Cusuco National Park, Honduras.

Date	Sample#	Species	Site	Frog qPCR	Leaf qPCR	Frog ZSE	Leaf ZSE
Jul 9 2013	HN13BD107	*Plectrohyla dasypus*	CO	+	-	0.45	n/a
Jul 9 2013	HN13BD109	*Ptychohyla hypomykter*	CO	+	-	0.54	n/a
Jul 9 2013	HN13BD110	*Plectrohyla dasypus*	CO	+	+	24.39	17.81
Jul 9 2013	HN13BD111	*Plectrohyla dasypus*	CO	+	-	2.38	n/a
Jul 9 2013	HN13BD112	*Plectrohyla dasypus*	CO	+	+	29.19	12.25
Jul 9 2013	HN13BD114	*Plectrohyla dasypus*	CO	+	+	2.38	16.11
Jul 9 2013	HN13BD115	*Plectrohyla dasypus*	CO	+	+	3.04	14.57
Jul 9 2013	HN13BD116	*Plectrohyla dasypus*	CO	+	+	2.10	0.81
Jul 9 2013	HN13BD117	*Plectrohyla dasypus*	CO	+	+	93.05	4.70
Jul 9 2013	HN13BD118	*Plectrohyla exquisita*	CO	+	+	53.94	1040.45
Jul 9 2013	HN13BD120	*Plectrohyla dasypus*	CO	+	+	0.39	1.52
Jul 10 2013	HN13BD121	*Duellmanohyla soralia*	CO	+	-	16.75	n/a
Jul 10 2013	HN13BD122	*Plectrohyla dasypus*	CO	+	-	0.35	n/a
Jul 10 2013	HN13BD123	*Duellmanohyla soralia*	CO	-	-	n/a	n/a
Jul 10 2013	HN13BD124	*Plectrohyla dasypus*	CO	-	-	n/a	n/a
Jul 10 2013	HN13BD125	*Duellmanohyla soralia*	CO	+	+	0.64	0.34
Jul 10 2013	HN13BD128	*Plectrohyla dasypus*	CO	+	-	3.00	n/a
Jul 10 2013	HN13BD129	*Duellmanohyla soralia*	CO	-	-	n/a	n/a
Jul 10 2013	HN13BD130	*Plectrohyla dasypus*	CO	-	+	n/a	0.12
Jul 10 2013	HN13BD131	*Plectrohyla dasypus*	CO	+	+	2.00	0.64
Jul 10 2013	HN13BD132	*Ptychohyla hypomykter*	CO	+	+	28.77	8.39
Jul 10 2013	HN13BD133	*Plectrohyla dasypus*	CO	+	+	13.79	1.97
Jul 10 2013	HN13BD134	*Plectrohyla dasypus*	CO	+	+	23.76	0.93
Jul 10 2013	HN13BD135	*Plectrohyla dasypus*	CO	+	+	33.18	1.68
Jul 10 2013	HN13BD136	*Plectrohyla dasypus*	CO	+	-	2.11	n/a
Jul 10 2013	HN13BD137	*Duellmanohyla soralia*	CO	+	+	22.42	23.06
Jul 11 2013	HN13BD144	*Plectrohyla dasypus*	CO	+	+	11.82	1.07
Jul 11 2013	HN13BD145	*Plectrohyla dasypus*	CO	+	+	1085.68	43.30
Jul 14 2013	HN13BD161	*Duellmanohyla soralia*	CO	+	-	0.06*	n/a
Jul 14 2013	HN13BD164	*Ptychohyla hypomykter*	CO	+	+	660.54	49.09
Jul 15 2013	HN13BD166	*Plectrohyla dasypus*	CO	-	-	n/a	n/a
Jul 15 2013	HN13BD170	*Plectrohyla dasypus*	CO	+	+	236.29	139.30
Jul 15 2013	HN13BD171	*Duellmanohyla soralia*	CO	+	+	1.20	0.47
Jul 15 2013	HN13BD172	*Plectrohyla dasypus*	CO	+	+	58.50	0.79
Jul 15 2013	HN13BD173	*Plectrohyla dasypus*	CO	+	+	57.69	4.38
Jul 15 2013	HN13BD174	*Plectrohyla dasypus*	CO	+	+	17.44	40.30
Jul 15 2013	HN13BD175	*Plectrohyla exquisita*	CO	+	+	38.66	1.00
Jul 15 2013	HN13BD177	*Plectrohyla dasypus*	CO	+	+	18.10	0.32
Jul 15 2013	HN13BD178	*Plectrohyla exquisita*	CO	+	+	281.79	10.93
Jul 15 2013	HN13BD179	*Ptychohyla hypomykter*	CO	+	+	0.64	0.25
Jul 15 2013	HN13BD180	*Ptychohyla hypomykter*	CO	+	-	1.37	n/a
Jul 15 2013	HN13BD181	*Duellmanohyla soralia*	CO	+	+	4.06	3.36
Jul 16 2013	HN13BD183	*Plectrohyla dasypus*	CO	+	+	1574.62	3.90
Jul 16 2013	HN13BD249	*Ptychohyla hypomykter*	CO	+	+	39.07	0.23
Jul 18 2013	HN13BD261	*Plectrohyla dasypus*	DA	+	+	147.26	7.84
Jul 14 2013	HN13BD323	*Plectrohyla dasypus*	DA	-	-	n/a	n/a
Aug 5 2013	HN13BD389	*Plectrohyla exquisita*	CU	+	-	27.10	n/a

(*Continued*)

Table 1. (*Continued*)

Date	Sample#	Species	Site	Frog qPCR	Leaf qPCR	Frog ZSE	Leaf ZSE
Aug 5 2013	HN13BD390	*Plectrohyla exquisita*	CU	+	+	34.02	0.44
Aug 5 2013	HN13BD391	*Plectrohyla exquisita*	CU	+	+	48.13	1.97
Aug 6 2013	HN13BD407	*Plectrohyla dasypus*	CU	+	+	25.23	0.29
Aug 6 2013	HN13BD408	*Plectrohyla exquisita*	CU	+	+	1.03	2.63
Aug 6 2013	HN13BD409	*Plectrohyla exquisita*	CU	+	-	52.55	n/a

Survey sites include Rio Cortecito (CO), Rio Danto (DA), and Rio Cusuco (CU). Average zoospore equivalent (ZSE) per qPCR reaction is reflected for all *Bd*-positive samples. Asterisk denotes the single sample that produced a positive reaction in 2/6 wells; all other samples produced *Bd*-positive reactions in 2/3, 3/3, or 3/6 wells.

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Amphibian and Vegetation Temperatures

Most animals were sampled during nocturnal surveys, from 20:00–2:00 hrs (n = 45), although some were occasionally encountered and sampled during the day, from 10:45–15:00 hrs (n = 7). Night temperatures of the frogs' dorsal surfaces, leaf surfaces, and air averaged 17.0°C, 17.1°C, and 16.9°C and ranged from 15.2–18.9°C, 15.8–19.1°C, and 15.3–17.8°C, respectively, whereas day temperatures averaged 20.8°C, 21.0°C, and 20.2°C and ranged from 18.4–26.6°C, 18.8–7.0°C, and 19.4–21.9°C, respectively.

Discussion

We frequently detected *Bd* on leaf surfaces after removal of recently metamorphosed *Bd*-positive frogs, indicating their emergence does contribute towards the spread of *Bd* from aquatic into terrestrial locations. Average zoospore loads detected on leaf surfaces were comparable to those from corresponding amphibian skin swabs, and sometimes greater. The presence of *Bd* on riparian vegetation allows exposure to occur in the absence of direct physical contact with *Bd*-positive animals or contaminated water. Accordingly, this pathway of *Bd* dispersal and terrestrial exposure provides one possible explanation for the source of infection previously detected in amphibians that do not demonstrate a strong association with water.

This pathway of *Bd* spread may occasionally facilitate transmission between aquatic and terrestrial species and from juvenile to adult frogs, if foliage maintains infectious *Bd* loads. On 11 July 2013, both a recently metamorphosed and adult *Plectrohyla dasypus* were observed perched together on the same plant at the same time, approximately 5 cm apart (Fig 2). The skin swab sample collected from this juvenile frog (HN13BD145) exhibited a considerable zoospore load (1,085.68), as did the leaf swab (43.30), demonstrating a high risk of exposure to the

Table 2. Presence of *Batrachochytrium dendrobatidis* (*Bd*) detected in water filter samples collected from amphibian survey sites in Cusuco National Park, Honduras.

Sample#	Site	Vol (ml)	T (°C)	ZSE/L
HN13W01	CO	11000	17.5	0.08
HN13W02	DA	2700	17.1	0.03
HN13W03	CU	4600	16.3	0.57

Survey sites include Rio Cortecito (CO), Rio Danto (DA), and Rio Cusuco (CU). Volume of water filtered, water temperature, and average *Bd* zoospore equivalent (ZSE) per liter of river water is reflected for all samples.

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Fig 2. Adult and recently metamorphosed *Plectrohyla dasypus* resting in close proximity in Cusuco National Park, Honduras. The skin swab collected from this juvenile (HN13BD145) tested positive for *Bd* infection and exhibited a considerable zoospore load (1,085.68 ZSE), as did the leaf swab (43.30 ZSE), demonstrating the risk of exposure to the nearby *Bd*-negative adult through contact with contaminated vegetation.

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nearby adult which tested *Bd*-negative at the time of sampling. Following metamorphosis, this species leaves the aquatic habitat and moves into arboreal vegetation, reducing the likelihood of subsequent *Bd* exposure from contaminated river water. The high prevalence of infection in *P. dasypus* juveniles detected in this and previous surveys [24] suggests that their seasonal emergence *en masse* may release a substantial quantity of *Bd* into the riparian zone shared with amphibians that approach the water's edge, but do not typically enter it.

Although we identified a potential mechanism of pathogen exposure to terrestrial amphibians, the role of contaminated vegetation in *Bd* transmission remains in question. Detection of *Bd* via qPCR indicates DNA presence, but does not reveal condition at the time of sampling. A lack of experimental work to evaluate the persistence and detectability of *Bd* DNA following cell death makes it difficult to discern whether we likely detected viable *Bd* or instead DNA fragments from expired cells that continued to react with *Bd* qPCR primers. This interpretation limitation is not exclusive to environmental *Bd* swab samples, but likewise applies to amphibian skin swabs; a positive qPCR result does not independently demonstrate the viability of *Bd* on that animal. Still, environmental conditions observed at all sampling localities in CNP were similar to those in the laboratory where *Bd* survived outside a host [19] and may aid persistence of *Bd* on leaf surfaces. Temperatures recorded in the field were all within or near the range for optimal in vitro growth of *Bd* (17–25°C) and well below its thermal maximum of 28°C [47], although optimal temperature regimes may vary between *Bd* isolates [48] and none from Honduras have yet been characterized. Desiccation poses the other well-defined abitoic limitation to *Bd* survival [19,49] but the presence of both high relative humidity and a dense forest canopy preventing direct sun exposure is correlated with higher *Bd* prevalence and infection loads [50]. These conditions are typical of CNP, a montane cloud forest, and expected to prolong

drying. Lastly, laboratory experiments have shown that when maintained under suitable temperature and moisture levels (and without bacteria), *Bd* can survive in the absence of a host for at least two months in water or moist sand [19]. Thus, additional laboratory work is needed to test the survival times of cultured *Bd* on leaf surfaces to identify the potential duration of this form of environmental persistence and evaluate the average *Bd* loads needed to cause successful transmission under naturalistic conditions.

Previous efforts to illustrate environmental *Bd* transmission have mainly focused on exposure to permanent water bodies inhabited by *Bd*-infected amphibians [18,19]. Laboratory trials demonstrated transmission of *Bd* between experimentally-infected and uninfected tadpoles of *Rana muscosa* and also from tadpoles to post-metamorphic animals, when occupying a shared water source [18]. Successful transmission required a 2–3 week duration of exposure, likely impeded by dilution of the pathogen in a naturalistic environment, similar to the low densities of *Bd* detected in the water samples collected at our survey sites in CNP (Table 2). To encourage transmission after short-term exposure, laboratory experiments have often employed highly concentrated inoculates of approximately 100 million *Bd* zoospores delivered in less than 100 mL of water [51–53] whereas the highest concentration detected in a natural body of water has been 3 million zoospores L^{-1} and less than 100 zoospores L^{-1} is common [54]. In this context, the concentrated *Bd* loads we detected on leaf surfaces in CNP relative to the adjacent *Bd*-positive river water suggests that contact with affected foliage might pose a greater threat of exposure and transmission to terrestrial amphibians than would a splash of water from these rivers.

We detected the presence of *Bd* on vegetation in the understory, but periods of heavy rain are expected to also flush *Bd* into the soil and leaf litter below. Surveys in CNP have identified the presence of live aquatic crustaceans (copepods and ostracods) inhabiting terrestrial water films on forest floor leaf litter [55,56], suggesting moisture persistence in this limnoterrestrial habitat. The persistence of these water films in humid rainforest environments would help protect *Bd* from desiccation in a seemingly terrestrial habitat, and also allow exposure to amphibians that occupy leaf litter and burrow into the ground. Accordingly, this mode of *Bd* dispersal and indirect exposure may explain the origins of infection documented in species of soil-dwelling salamanders [22,23,25] and caecilians [28,29].

Numerous biologic and abiotic factors are expected to influence the frequency of *Bd* dispersal from aquatic into terrestrial habitats and potential consequences. The prevalence and intensity of *Bd* detected in amphibian populations often demonstrates fluctuations due to seasonal changes in environmental conditions and these factors will affect the amount of zoospores available to be shed into the terrestrial environment [49,54,57]. Rowley et al. [58] investigated the presence of *Bd* in terrestrial retreat sites of two aquatic stream frog species (*Litoria lesueuri* and *L. nannotis*), and did not detect *Bd* in 122 environmental swab samples. As suggested by the authors, the observed *Bd* absence may have been influenced by the low prevalence and infection loads concurrently detected in the adult amphibians sampled at these locations. Our results show that in a locality where both *Bd* prevalence and infection loads are high, it is common for *Bd* to be shed into terrestrial locations, including amphibian retreat sites.

The presence of *Bd* in terrestrial habitats should be considered when identifying potential threats to amphibian species of concern. Although it has been suggested that *Bd* poses the greatest risk of infection to amphibians breeding in permanent streams [59], we caution against this generalization and encourage additional surveillance in terrestrial and arboreal amphibian habitats where animals continue to test positive for *Bd*, despite pathways of exposure being more obscure. The frequency of *Bd* exposure from terrestrial substrates is unknown but may be considerable where optimal environmental conditions are present, especially if it can survive as a saprobe as previously suggested [12]. An improved understanding of *Bd* dispersal and

persistence in the natural environment is essential to better explain and predict the continued spread of this pathogen in regions where the anthropogenic-assisted exposure to *Bd*-positive amphibians or substrates is unlikely.

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Author Contributions

Conceived and designed the experiments: JEK. Performed the experiments: JEK SDR. Analyzed the data: JEK MJ. Contributed reagents/materials/analysis tools: KLR. Wrote the paper: JEK SDR LB LS MJ KLR.

References

1. Stuart SN, Chanson JS, Cox NA, Young BE, Rodrigues ASL, Fischman DL, et al. (2004) Status and Trends of Amphibian Declines and Extinctions Worldwide. Science 306: 1783–1786. PMID: 15486254
2. Skerratt L, Berger L, Speare R, Cashins S, McDonald K, Phillot AD, et al. (2007) Spread of Chytridiomycosis Has Caused the Rapid Global Decline and Extinction of Frogs. EcoHealth 4: 125–134.
3. Daszak P, Strieby A, Cunningham AA, Longcore JE, Brown CC, Porter D (2004) Experimental evidence that the bullfrog (*Rana catesbeiana*) is a potential carrier of chytridiomycosis, an emerging fungal disease of amphibians. Herpetological Journal 14: 201–207.
4. Rollins-Smith LA, Ramsey JP, Reinert LK, Woodhams DC, Livo LJ,Carey C (2009) Immune defenses of *Xenopus laevis* against *Batrachochytrium dendrobatidis*. Frontiers in Bioscience 1: 68–91. PMID: 19482684
5. Lips KR, Brem F, Brenes R, Reeve JD, Alford RA, Voyles J, et al. (2006) Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. Proceedings of the National Academy of Sciences of the United States of America 103: 3165–3170. PMID: 16481617
6. Catenazzi A, Lehr E, Rodriguez LO, Vredenburg VT (2011) *Batrachochytrium dendrobatidis* and the collapse of anuran species richness and abundance in the upper Manu National Park, southeastern Peru. Conservation Biology 25: 382–391. doi: 10.1111/j.1523-1739.2010.01604.x PMID: 21054530
7. Fisher MC, Bosch J, Yin Z, Stead DA, Walker J, Selway L, et al. (2009) Proteomic and phenotypic profiling of the amphibian pathogen *Batrachochytrium dendrobatidis* shows that genotype is linked to virulence. Molecular Ecology 18: 415–429. doi: 10.1111/j.1365-294X.2008.04041.x PMID: 19161465
8. Farrer RA, Weinert LA, Bielby J, Garner TWJ, Balloux F, Clare F, et al. (2011) Multiple emergence of genetically diverse amphibian-infecting chytrids include a globalised hypervirulent lineage. Proceedings of the National Academy of Sciences USA 108: 18732–6. doi: 10.1073/pnas.1111915108 PMID: 22065772
9. Olson DH, Aanensen DM, Ronnenberg KL, Powell CI, Walker SF, Bielby J, et al. (2013) Mapping the Global Emergence of *Batrachochytrium dendrobatidis*, the Amphibian Chytrid Fungus. PLoS ONE 8 (2): e56802. doi: 10.1371/journal.pone.0056802 PMID: 23463502
10. Lips KR, Diffendorfer J, Mendelson JR III, Sears MW (2008) Riding the wave: reconciling the roles of disease and climate change in amphibian declines. PLoS Biology 6: 441–454.
11. Rosenblum EB, James TY, Zamudio KR, Poorten TJ, Ilut D, Rodriguez D, et al. (2013) Complex history of the amphibian-killing chytrid fungus revealed with genome resequencing data. Proceedings of the National Academy of Sciences USA 110: 9385–9390. doi: 10.1073/pnas.1300130110 PMID: 23650365
12. Longcore JE, Pessier AP, Nichols DK (1999) *Batrachochytrium dendrobatidis* gen. et sp. nov., a Chytrid Pathogenic to Amphibians. Mycologia 91: 219–227.
13. Schloegel LM, Picco A, Kilpatrick AM, Hyatt A, Daszak P (2009) Magnitude of the US trade in amphibians and presence of *Batrachochytrium dendrobatidis* and ranavirus infection in imported North American bullfrogs (*Rana catesbeiana*). Biological Conservation 142: 1420–1426.
14. Schloegel LM, Toledo LF, Longcore JE, Greenspan SE, Vieira CA, Lee M, et al. (2012) Novel, panzootic and hybrid genotypes of amphibian chytridiomycosis associated with the bullfrog trade. Molecular Ecology 21: 5162–5177. doi: 10.1111/j.1365-294X.2012.05710.x PMID: 22857789

15. Gilbert M, Bickford D, Clark L, Johnson A, Joyner PH, Keatts LO, et al. (2013) Amphibian pathogens in Southeast Asian frog trade. Ecohealth 9: 386–98.
16. Kolby JE, Smith KM, Berger L, Karesh WB, Preston A, Pessier AP, et al. (2014) First Evidence of Amphibian Chytrid Fungus (*Batrachochytrium dendrobatidis*) and Ranavirus in Hong Kong Amphibian Trade. PLoS ONE 9: e90750. doi: 10.1371/journal.pone.0090750 PMID: 24599268
17. Rowley J, Alford RA (2007a) Behaviour of Australian rainforest stream frogs may affect the transmission of chytridiomycosis. Diseases of Aquatic Organisms 77:1–9.
18. Rachowicz LJ, Vredenburg VT (2004) Transmission of *Batrachochytrium dendrobatidis* within and between amphibian life stages. Diseases of Aquatic Organisms 61:75–83. PMID: 15584413
19. Johnson M, Speare R (2005) Possible modes of dissemination of the amphibian chytrid *Batrachochytrium dendrobatidis* in the environment. Diseases of Aquatic Organisms 65: 181–186. PMID: 16119886
20. Garmyn A, Van Rooij P, Pasmans F, Hellebuyck T, Van Den Broeck W, Haesebrouck F, et al. (2012) Waterfowl: Potential Environmental Reservoirs of the Chytrid Fungus *Batrachochytrium dendrobatidis*. PLoS ONE 7: e35038. doi: 10.1371/journal.pone.0035038 PMID: 22514705
21. Wimsatt J, Feldman SH, Heffron M, Hammond M, Ruehling MPR, Grayson KL, et al. (2014) Detection of pathogenic *Batrachochytrium dendrobatidis* using water filtration, animal and bait testing. Zoo Biology 33: 577–585. doi: 10.1002/zoo.21154 PMID: 25231013
22. Cummer MR, Green DE, O'Neill EM (2005) Aquatic chytrid pathogen detected in a terrestrial plethodontid salamander. Herpetological Review 36: 248–249.
23. Weinstein SB (2009) An aquatic disease on a terrestrial salamander: individual and population level effects of the amphibian chytrid fungus, *Batrachochytrium dendrobatidis*, on *Batrachoseps attenuatus* (Plethodontidae). Copeia 4: 653–660.
24. Kolby JE, Padgett-Flohr GE, Field R (2010) Amphibian chytrid fungus *Batrachochytrium dendrobatidis* in Cusuco National Park, Honduras. Diseases of Aquatic Organisms 92: 245–251. doi: 10.3354/dao02055 PMID: 21268988
25. Caruso NM, Lips KR (2013) Truly enigmatic declines in terrestrial salamander populations in Great Smoky Mountains National Park. Divers. Distrib. 19: 34–48.
26. Burrowes PA, Longo AV, Rodríguez CA (2008) Fitness cost of *Batrachochytrium dendrobatidis* infection in *Eleutherodactylus coqui*, and comments on habitat-related risk of infection. Herpetotropicos 4: 51–57.
27. McCracken SF, Gaertner JP, Forstner MRJ, Hahn D (2009) Detection of *Batrachochytrium dendrobatidis* in amphibians from the forest floor to the upper canopy of an Ecuadorian Amazon lowland rainforest. Herpetological Review 40: 190–195.
28. Doherty-Bone TM, Gonwouo NL, Hirschfeld M, Ohst T, Weldon C, Perkins M, et al. (2013) *Batrachochytrium dendrobatidis* in amphibians of Cameroon, including first records for caecilians. Diseases of Aquatic Organisms 102: 187–194. doi: 10.3354/dao02557 PMID: 23446968
29. Gower D, Doherty-Bone TM, Loader S, Wilkinson M, Kouete M, Tapley B, et al. (2013) *Batrachochytrium dendrobatidis* infection and lethal chytridiomycosis in caecilian amphibians (Gymnophiona). EcoHealth 10: 173–183. doi: 10.1007/s10393-013-0831-9 PMID: 23677560
30. Berger L, Speare R, Daszak P, Green DE, Cunningham AA, Goggin CL, et al. (1998) Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. Proceedings of the National Academy of Sciences USA 95: 9031–9036. PMID: 9671799
31. Crawford AJ, Lips KR, and Bermingham E (2010) Epidemic disease decimates amphibian abundance, species diversity, and evolutionary history in the highlands of central Panama. Proceedings of the National Academy of Sciences USA 107: 13777–13782. doi: 10.1073/pnas.0914115107 PMID: 20643927
32. Puschendorf R, Bolanos F, Chaves G (2006a) The amphibian chytrid fungus along an altitudinal transect before the first reported declines in Costa Rica. Biological Conservation 132: 136–142.
33. Seimon TA, Seimon A, Daszak P, Halloy SRP, Schloegel LM, Aguilar CA, et al. (2007) Upward range extension of Andean anurans and chytridiomycosis to extreme elevations in response to tropical deglaciation. Global Change Biology 13: 288–299.
34. Puschendorf R, Castaneda F, McCranie JR (2006b) Chytridiomycosis in wild frogs from Pico Bonito National Park, Honduras. EcoHealth 3: 178–181.
35. Wilson LD, McCranie JR (2003) The conservation status of the herpetofauna of Honduras. Amphibian and Reptile Conservation 3: 6–33.
36. Kolby JE, McCranie JR (2009) Discovery of a surviving population of the Montane Streamside Frog *Craugastor milesi* (Schmidt). Herpetological Review 40: 282–283.

37. Briggs CJ, Knapp RA, Vredenburg VT (2010) Enzootic and epizootic dynamics of the chytrid fungal pathogen of amphibians. Proceedings of the National Academy of Sciences USA 107:9695–9700. doi: 10.1073/pnas.0912886107 PMID: 20457916

38. Russell DM, Goldberg CS, Waits LP, Rosenblum EB (2010) *Batrachochytrium dendrobatidis* infection dynamics in the Columbia spotted frog *Rana luteiventris* in north Idaho, USA. Diseases of Aquatic Organisms 92: 223–230. doi: 10.3354/dao02286 PMID: 21268985

39. Piovia-Scott J, Pope KL, Lawler SP, Cole EM, Foley JE (2011) Factors related to the distribution and prevalence of the fungal pathogen *Batrachochytrium dendrobatidis* in *Rana cascadae* and other amphibians in the Klamath Mountains. Biological Conservation 144: 2913–2921.

40. Hyatt AD, Boyle DG, Olsen V, Boyle DB, Berger L, Obendorf D, et al. (2007) Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis*. Diseases of Aquatic Organisms 73: 175–192. PMID: 17330737

41. Kolby JE, Padgett-Flohr GE (2009) Amphibian Chytrid Fungus (*Batrachochytrium dendrobatidis*) in Honduras: Historical Exposure in *Plectrohyla dasypus* and Subsequent Decline. Herpetological Review 40:307–308.1.

42. Rowley JJL, Alford RA (2007b) Non-contact infrared thermometers can accurately measure amphibian body temperatures. Herpetol. Rev. 38, 308–311 (2007b).

43. Kirshtein JD, Anderson CW, Wood JS, Longcore JE, Voytek MA (2007) Quantitative PCR detection of *Batrachochytrium dendrobatidis* DNA from sediments and water. Diseases of Aquatic Organisms 77: 11–15. PMID: 17933393

44. Boyle DG, Boyle DB, Olsen V, Morgan JA, Hyatt AD (2004) Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. Diseases of Aquatic Organisms 60: 141–148. PMID: 15460858

45. Garland S, Baker A, Phillott AD, Skerratt LF (2010) BSA reduces inhibition in a TaqMan assay for the detection of *Batrachochytrium dendrobatidis*. Diseases of Aquatic Organisms 92: 113–116. doi: 10.3354/dao02053 PMID: 21268973

46. Richards-Hrdlicka KL, Richardson JL, Mohabir L (2013) First survey for the amphibian chytrid fungus *Batrachochytrium dendrobatidis* in Connecticut (USA) finds widespread prevalence. Diseases of Aquatic Organisms 102: 169–180. doi: 10.3354/dao02552 PMID: 23446966

47. Piotrowski JS, Annis SL, Longcore JE (2004) Physiology of *Batrachochytrium dendrobatidis*, a chytrid pathogen of amphibians. Mycologia 96: 9–15. PMID: 21148822

48. Voyles J, Johnson LR, Briggs CJ, Cashins SD, Alford RA, Berger L, et al. (2012) Temperature alters reproductive life history patterns in *Batrachochytrium dendrobatidis*, a lethal pathogen associated with the global loss of amphibians. Ecology and Evolution 2: 2241–2249 doi: 10.1002/ece3.334 PMID: 23139882

49. Berger L, Speare R, Hines H, Marantelli G, Hyatt AD, McDonald KR, et al. (2004) Effect of season and temperature on mortality in amphibians due to chytridiomycosis. Australian Veterinary Journal 82:31–36. PMID: 15088954

50. Becker CG, Rodriguez D, Longo AV, Talaba AL, Zamudio KR (2012) Disease Risk in Temperate Amphibian Populations Is Higher at Closed-Canopy Sites. PLoS ONE 7: e48205. doi: 10.1371/journal.pone.0048205 PMID: 23118953

51. Carey C, Bruzgul JE, Livo LJ, Walling ML, Kuehl KA, Dixon BF, et al. (2006) Experimental exposures of boreal toads (*Bufo boreas*) to a pathogenic chytrid fungus (*Batrachochytrium dendrobatidis*). EcoHealth 3:5–21. doi: 10.1007/s10393-005-0006-4

52. Langhammer PF, Lips KR, Burrowes PA, Tunstall T, Palmer CM, Collins JP 2013. A fungal pathogen of amphibians, *Batrachochytrium dendrobatidis*, declines in pathogenicity with in vitro passages. PLOS One doi: 10.1371/journal.pone.0077630

53. DiRenzo GV, Langhammer PF, Zamudio KR, Lips KR (2014) Fungal Infection Intensity and Zoospore Output of *Atelopus zeteki*, a Potential Acute Chytrid Supershedder. PLoS ONE 9: e93356. doi: 10.1371/journal.pone.0093356 PMID: 24675899

54. Chestnut T, Anderson C, Popa R, Blaustein AR, Voytek M, Olson DH, et al. (2014) Heterogeneous Occupancy and Density Estimates of the Pathogenic Fungus *Batrachochytrium dendrobatidis* in Waters of North America. PLoS ONE 9: e106790. doi: 10.1371/journal.pone.0106790 PMID: 25222122

55. Fiers F, Jocque M (2013) Leaf litter copepods from a cloud forest mountain top in Honduras (Copepoda: Cyclopidae, Canthocamptidae). Zootaxa 3630: 270–290.

56. Jocque M, Fiers F, Romero M, Martens K. (2013) Crustacea in phytotelmata: a global overview. Journal of Crustacean Biology 33: 451–460.

57. Kriger KM, Hero JM (2006) Large scale seasonal variation in the prevalence and severity of chytridiomycosis. Journal of Zoology doi: 10.1111/j.1469-7998.2006.00220.x

58. Rowley JJL, Skerratt LF, Alford RA, Campbell R (2007) Retreat sites of rain forest stream frogs are not a reservoir for *Batrachochytrium dendrobatidis* in northern Queensland, Australia. Diseases of Aquatic Organisms 74:7–12. PMID: 17425258

59. Kriger KM, Hero JM (2007) The chytrid fungus *Batrachochytrium dendrobatidis* is non-randomly distributed across amphibian breeding habitats. Diversity and Distributions doi: 10.1111/j.1472-4642.2007:00394.x