Supporting Information

McMahon et al. 10.1073/pnas.1200592110

SI Methods

Several lines of evidence support a lack of *Batrachochytrium dendrobatidis* in the crayfish and tadpoles before their use in the experiment: (*i*) we collected amphibians as egg masses and reared them in the laboratory in a *B. dendrobatidis*–free environment; (*ii*) we have never collected *B. dendrobatidis* positive amphibians or crayfish from the Tampa, FL area; and (*iii*) all 12 of the control tadpoles in the transmission study were free of *B. dendrobatidis* according to qPCR.

Amphibian Seasonal Prevalence. Amphibians (n = 449) from five sites in southeastern Louisiana were captured, swabbed for *B. dendrobatidis* presence, and released at eight time points during the 11 mo before crayfish sampling (number amphibians per month: November: 31; December: 57; February: 55; March: 95; April: 76; June: 28; August: 50; and September: 57; Fig. S1).

Quantitative PCR for *Procambarus* and *Gambusia*. We followed the procedure described by Hyatt et al. (1) to quantify *B. dendrobatidis* using qPCR (with a StepOne Real-Time PCR System; Applied Biosystems). DNA was extracted from the swabs or tadpole mouthparts with 40 μL of PrepMan Ultra (Applied Biosystems). The tissue samples were beat with 30 g of 0.5-mm zirconia/silica beads (BioSpec Products) using a bead beater (Disruptornie; Scientific Industries) for 45 s and then centrifuged at 15,871 × g for 30 s (repeated two additional times). All samples were diluted 1:100 to reduce PCR inhibition. We added TaqMan Exogenous Internal Positive Control Reagents (Applied Biosystems) to every reaction well to assess inhibition of the PCR (1). There was no inhibition in any of these reactions.

Quantitative PCR for *Orconectes*. We followed the procedure described by Hyatt et al. (1) to quantify *B. dendrobatidis* in Colorado crayfish using qPCR as described above (see the section Quantitative PCR *Procambarus* and *Gambusia*). However, all crayfish samples were inhibited at both 1:100 and 1:1,000 dilutions, and only one of 41 samples was detected as weakly positive. We then processed all of the extracted DNA samples with GeneReleaser (BioVentures), a product developed to reduce inhibition of PCR reactions (also used in ref. 2). We processed the extracted DNA samples twice following the general GeneReleaser protocol and then again following the GeneReleaser 96-well plate protocol, which includes a microwaving step. All samples remained inhibited. We then spiked the extracted crayfish DNA samples with known quantities of *B. dendrobatidis* DNA (640, 64, 6.4 and 0.64 zoospores, respectively; n = 10). On the same plate, samples of the *B. dendrobatidis* DNA used to spike the crayfish samples were run alone as a positive control. The *B. dendrobatidis* DNA was amplified appropriately when run in the absence of the extracted DNA from *Orconectes virilis*. However, the samples with known quantities of already extracted *B. dendrobatidis* DNA added to the already extracted *O. virilis* DNA did not amplify and again were inhibited. These results indicate that there is something in the *O. virilis* samples that inhibits the qPCR reaction even when purified with GeneReleaser and diluted. Given this information, we believe that the qPCR assay, in its current form, is not an accurate way to estimate prevalence in these samples, leading us to rely on light microscopy results for the *O. virilis* samples.

Colorado Wetland Surveys. Wetland surveys were conducted in Colorado to determine the distribution of amphibian species, the chytrid fungal pathogen (*B. dendrobatidis*), and macroinvertebrates, including crayfish (*Orconectes* spp.). In total, 97 wetlands were surveyed between 2007 and 2010 in a variety of landscapes including grasslands, forested sites, suburban areas, and agricultural areas across 11 counties (Boulder, Larimer, Routt, Rio Grande, Rio Blanco, Moffat, Jefferson, Gilpin, Garfield, Douglas, and Broomfield). Wetland sites ranged in elevation from high plains regions (1,519 m) to montane regions (up to 3,140 m). A field crew comprised of three to four people sampled all wetlands between the months of May and August using the same sampling protocol (established by 3). At each wetland, a visual encounter survey (VES) was conducted to establish the presence any and all stages of amphibians. During the VES, the perimeter was walked of each wetland and recorded the number and species of all amphibians seen or heard within 3 m of shoreline, as well as the presence of crayfish near the water's edge. Dip-net sweeps were conducted by pulling a 1.4-mm mesh size dip net rapidly through the water in a 1.5-m line perpendicular to the shore. Dip-net sweeps were conducted every 15 m around the circumference of the pond. The contents of each sweep were placed into a white plastic tray and recorded the number and identity of all larval and adult amphibians captured as well as macroinvertebrates. Amphibians encountered in the surveys included: western chorus frog (*Pseudacris triseriata*), northern leopard frog (*Lithobates pipiens*), Woodhouse's toad (*Anaxyrus woodhousii*), tiger salamander (*Ambystoma tigrinum*), and the nonnative North American Bullfrog (*Lithobates catesbeianus*). Macroinvertebrates encountered included two species of crayfish, *O. virilis* and *O. immunis*. In many wetlands with accessible, shallow edge-habitat, three seine net hauls (with a net measuring 0.8 × 2 m) were completed, by stretching the net between two people and dragging it a distance of 3–8 m. The number and identity of all amphibians and macroinvertebrates captured in each seine net haul were recorded. At each site, amphibians were sampled to test for the presence of *B. dendrobatidis*, using a nondestructive swabbing technique following the protocol described in Johnson et al. (3). Across all sites and amphibian species, 9,174 amphibian individuals were swab sampled (Table S2); the aim was to swab 20 individuals per species per site to maximize our detection of *B. dendrobatidis*. Site-level presence/absence of *B. dendrobatidis* was of interest rather than prevalence per site, and so samples were pooled per amphibian species per site. The Qiagen Blood and Tissue Kit was used for DNA extraction, and a PCR assay was used to detect the presence of *B. dendrobatidis* at each site following the method described by Annis et al. (4). After completion of sampling at each pond, all waders, nets, and other equipment were decontaminated with a 10% bleach solution and the gear was sun-dried to reduce the risk of spreading material and pathogens between wetlands.

Microscopy and Histology of *Procambarus* Intestines. The GI tract from each crayfish was removed and opened lengthwise, and all fecal matter was cleared away. The tissue was washed with deionized (DI) water and the sample was fixed in 70% (vol/vol) ethanol. Using a compound microscope, zoosporangia were located within the GI tract. To verify that the zoosporangia were embedded in the tissue, they were rinsed with DI water and agitated with a probe. The GI tract was then fixed in 10% formalin, embedded in paraffin wax, and sections (5 μm thick) were plated and stained with hematoxylin and eosin.

SI Results

The Cox-proportional hazards model did not detect any differences between the two populations in time of death. However, this is likely a product of censoring crayfish that did not die by the end of the shorter of the two experiments. Two separate generalized linear models were conducted with $\log_{10}$ concentration (continuous predictor) and population as crossed factors and mortality (binomial error distribution) or days alive (normal error distribution) as response variables. Crayfish that survived until the end of the experiment were assumed to die the day after the last day of the experiment, which is a conservative estimate of their true time of death. For mortality, concentration (χ^2_1 = 21.75; $P < 0.0001$) and the interaction between concentration and population were significant (χ^2_1 = 7.44; P = 0.006), but the main effect of population was not (χ^2_1 = 0.933; P = 0.334). The interaction seemed to be driven by greater mortality at lower concentrations for the Marine Warehouse population relative to the Tampa population, but most of this occurred later in the experiment and, thus, could simply be attributable to the differences in the durations of the studies (9 vs. 22 d). For day of death, there were significant effects of concentration ($F_{1,56}$ = 9.65; P = 0.003) and population ($F_{1,56}$ = 139.99; $P < 0.0001$), but the interaction was not significant ($F_{1,56}$ = 0.030; P = 0.863). The effect of population was driven by individuals from the Tampa population that died during the experiment dying sooner when exposed to the *B. dendrobatidis* inoculate (mean ± SE: 2.25 ± 0.25 d) than individuals from the Marine Warehouse population (mean ± SE: 11.43 ± 2.05 d).

1. Hyatt AD, et al. (2007) Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis*. *Dis Aquat Organ* 73(3):175–192.
2. Kirshtein JD, Anderson CW, Wood JS, Longcore JE, Voytek MA (2007) Quantitative PCR detection of Batrachochytrium dendrobatidis DNA from sediments and water. *Dis Aquat Organ* 77(1):11–15.
3. Johnson PT, et al. (2011) Regional decline of an iconic amphibian associated with elevation, land-use change, and invasive species. *Conserv Biol* 25(3):556–566.
4. Annis SL, Dastoor FP, Ziel H, Daszak P, Longcore JE (2004) A DNA-based assay identifies Batrachochytrium dendrobatidis in amphibians. *J Wildl Dis* 40(3):420–428.

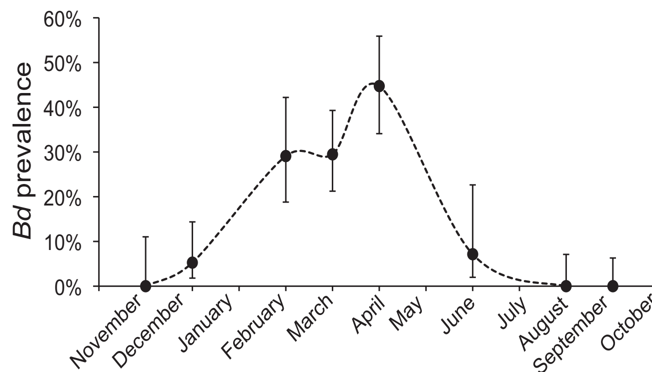

Fig. S1. Seasonality of *B. dendrobatidis* (*Bd*) prevalence in amphibians collected from Southeastern Louisiana (*n* = 31, 57, 55, 95, 76, 28, 50, 57 per month, respectively). Means ± 95% CI.

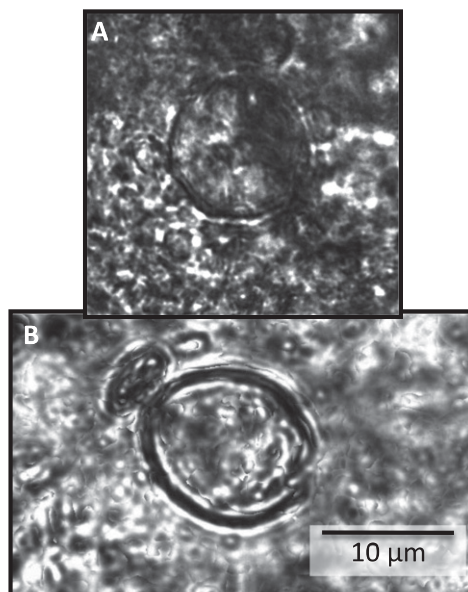

Fig. S2. *B. dendrobatidis* on the intestinal wall of wild caught *O. virilis*. (*A*) Developing zoosporangia. (*B*) Empty zoosporangia. Images were taken by T.A.M. and M.B.J.

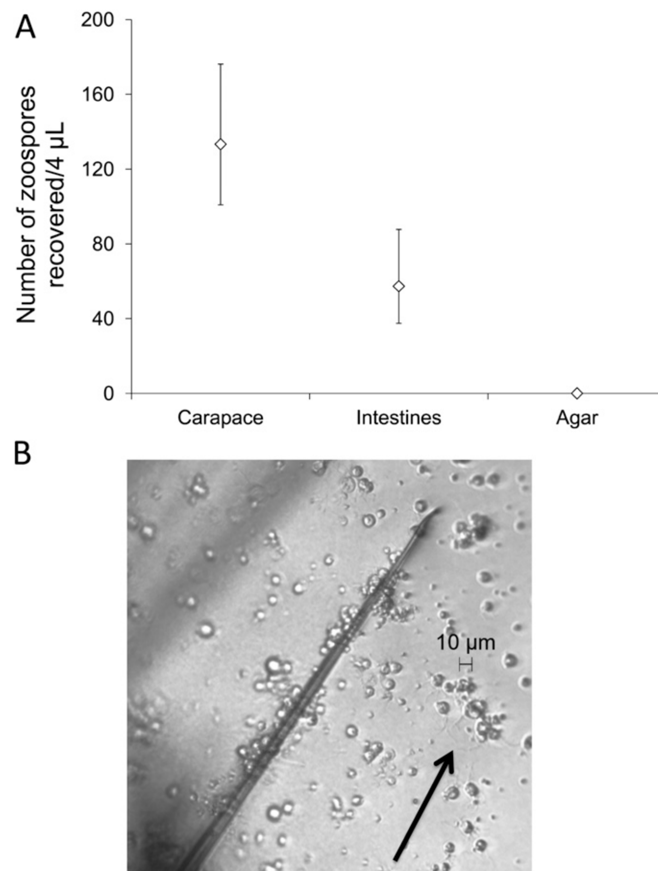

Fig. S3. Results of our experiment testing whether *B. dendrobatidis* could grow on agar plates with pieces of autoclaved crayfish tissue. (*A*) Mean number of motile *B. dendrobatidis* zoospores (±95% CI) viewed in a hemocytometer after 7 d of growth on agar and carapace, agar and intestines, and agar alone. Because we did not standardize the amount of crayfish tissue within or between treatments, this result only demonstrates that *B. dendrobatidis* can use crayfish tissue as a food source. (*B*) Image of developing *B. dendrobatidis* zoosporangia clustered around a piece of crayfish carapace that was embedded in agar. The large circles are *B. dendrobatidis* zoosporangia, the small circles are motile zoospores, and the arrow points to a group of rhizoids. The image was taken 7 d postinoculation at 100× magnification by MDV.

PNAS PNAS PNAS PNAS

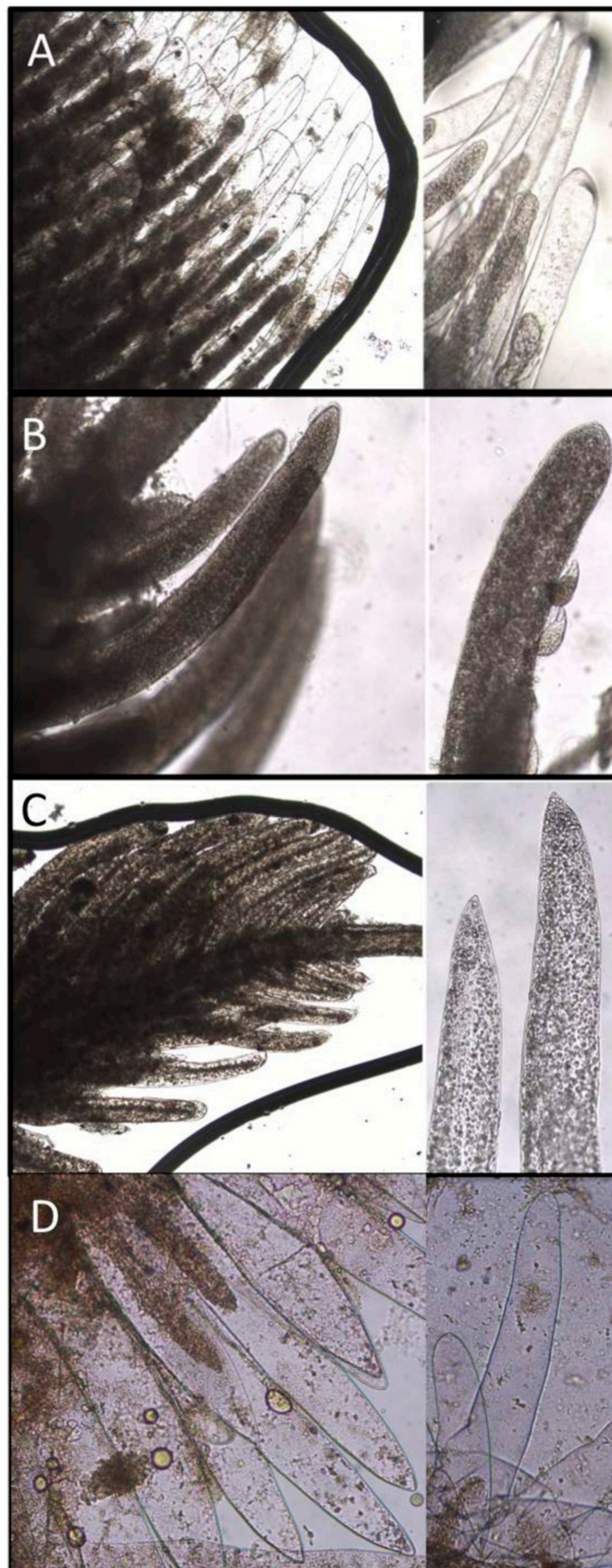

Fig. S4. *Procambarus alleni* exposed to *B. dendrobatidis* have significantly more gill damage than those that were not exposed to *B. dendrobatidis*. (*A*) Crayfish gills exposed to *B. dendrobatidis* (died early in the experiment). (*B*) Gills of control, *B. dendrobatidis*–free crayfish that survived until the end of the experiment. (*C*) Gills of control, *B. dendrobatidis*–free crayfish that were euthanized by pithing and left to foul for 24 h to demonstrate that gill damage was not caused by animal death or fouling. (*D*) Gills from a crayfish exposed to a *B. dendrobatidis*$^+$ inoculum where all of the zoospores and zoosporangia were removed with a 0.7-μm filter.

Fig. S5. Molting occurrence in *P. alleni* exposed to a *B. dendrobatidis*+ (Bd) inoculum where all of the zoospores and zoosporangia were removed with a 0.7-μm filter (concentration of zoospores removed: 0, 10^6, 1.5×10^5, 10^5, 10^4, 10^3, 10^2 zoospores per milliliter). Provided is the logistic regression fit [$y = \exp(-4.0064963 + (0.548814368 \times x))/(1 + \exp(-4.0064963 + (0.548814368 \times x)))$].

Fig. S6. Internal (inside the GI tract) and external *B. dendrobatidis* (Bd) load (shown is mean log zoospore equivalents ± SE) in *P. alleni* 7 and 12 wk after exposure.

Table S1. Infection intensity and prevalence of *B. dendrobatidis* by site in amphibians and crayfish

Collection state	Month and year collected	GPS location for site	No. of crayfish	Collection method*	Crayfish infection intensity ($\log_{10}$ GE ± SE)	*B. dendrobatidis* prevalence in crayfish (%)	No. of amphibians (amphibian genus)	Amphibian infection intensity ($\log_{10}$ GE ± SE)
Louisiana	September 2011	28°09.384′N, 82°19.378′W	17	SNW	1.52 ± 0.36	29.4	1 (*Acris*)	0 ± 0
Louisiana	September 2011	30°06.450′N, 90°26.463′W	20	SNW	1.43 ± 0.34	10	9 (*Rana*)	0 ± 0
Louisiana	September 2011	29°53.313′N, 89°57.358′W	11	SNW	0 ± 0	0	0	N/A
Louisiana	September 2011	30°06.450′N, 90°29.453′W	8	SNW	1.19 ± 0	12.5	1 (*Gastrophryne*)	0 ± 0
Louisiana	September 2011	29°53.438′N, 89°57.892′W	15	SNW	0 ± 0	0	0	N/A
Louisiana	April 2012	30°108′N, 90°435′W	10	SNW	0 ± 0	0	0	N/A
Louisiana	April 2012	29°89′N, 89°95′W	10	SNW	12.01 ± 0	10	0	N/A
Colorado	May 2012	40°012′N, 105°245′W	4	BCTs	N/A	50	0	N/A
Colorado	May 2012	39°978′N, 105°230′W	18	BCTs	N/A	0	0	N/A
Colorado	May 2012	40.009′N, 105.245′W	18	BCTs	N/A	11.1	0	N/A

Procambarus spp. was collected from southeastern Louisiana, and *O. virilis* was collected from Colorado. N/A, not available.
*Collection method: BCTs, baited crayfish traps; SNW, sweep netting the entire boarder of wetland.

Table S2. Colorado amphibians swabbed for *B. dendrobatidis* as a function of species and life stage

Species	Stage	No. swabbed	No. of sites	No. of sites *B. dendrobatidis*$^+$
A. tigrinum	Adults	214	13	6
	Larvae/metamorphs	2,760	48	3
A. woodhousii	Adults	21	13	3
	Larvae/metamorphs	663	17	1
P. triseriata	Adults	18	16	7
	Larvae/metamorphs	3,294	48	3
L. catesbeianus	Adults	23	22	5
	Larvae/metamorphs	1,104	40	10
L. pipiens	Adults	57	36	10
	Larvae/metamorphs	1,020	23	0

Listed are the number of individuals swabbed and the number of wetlands at which that species-by-stage combination was detected with *B. dendrobatidis*. Samples were batch-pooled by species and site. A total of 49 positive detections were recorded from 40 wetlands (of 97 total wetlands with at least 20 individual amphibians swabbed).

Table S3. Concentration of zoospores used in each experiment

Experiment	Zoospore concentration (zoospores per milliliter)
Infection experiment	5.3×10^3
Maintenance experiment	5.3×10^3
Transmission experiment	1.2×10^3
Filtered *B. dendrobatidis* experiment	1.2×10^5
Filtered *B. dendrobatidis* dose–response experiment	1×10^6