Chytrid fungus *Batrachochytrium dendrobatidis* has nonamphibian hosts and releases chemicals that cause pathology in the absence of infection

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***Batrachochytrium dendrobatidis*, a pathogenic chytrid fungus implicated in worldwide amphibian declines, is considered an amphibian specialist. Identification of nonamphibian hosts could help explain the virulence, heterogeneous distribution, variable rates of spread, and persistence of *B. dendrobatidis* in freshwater ecosystems even after amphibian extirpations. Here, we test whether mosquitofish (*Gambusia holbrooki)* and crayfish (*Procambarus* spp. and *Orconectes virilis*), which are syntopic with many amphibian species, are possible hosts for *B. dendrobatidis*. Field surveys in Louisiana and Colorado revealed that zoosporangia occur within crayfish gastrointestinal tracts, that *B. dendrobatidis* prevalence in crayfish was up to 29%, and that crayfish presence in Colorado wetlands was a positive predictor of *B. dendrobatidis* infections in cooccurring amphibians. In experiments, crayfish, but not mosquitofish, became infected with *B. dendrobatidis*, maintained the infection for at least 12 wk, and transmitted *B. dendrobatidis* to amphibians. Exposure to water that previously held *B. dendrobatidis* also caused significant crayfish mortality and gill recession. These results indicate that there are nonamphibian hosts for *B. dendrobatidis* and suggest that *B. dendrobatidis* releases a chemical that can cause host pathology, even in the absence of infection. Managing these biological reservoirs for *B. dendrobatidis* and identifying this chemical might provide new hope for imperiled amphibians.**

alternative hosts | field correlation | vectors | Bd toxin

Although some pathogens are highly host-specific, those infecting multiple host species can profoundly affect disease dynamics by increasing pathogen persistence, virulence, and movement between host populations (1). Furthermore, when there are multiple hosts for a pathogen, some can serve as reservoir hosts. Reservoir hosts can sustain the parasite when particular hosts of interest are absent or temporarily resistant to infection and are often necessary for pathogens to drive other host populations or species extinct (2, 3).

The chytrid fungus *Batrachochytrium dendrobatidis* is an example of a parasite that putatively causes host extinctions. Indeed, it has been implicated in the declines of hundreds of amphibian species worldwide (4-10). *B. dendrobatidis* is able to persist without amphibian hosts (11, 12), which could prevent successful amphibian reintroductions (3). One possible mechanism for persistence is the presence of nonamphibian hosts of *B. dendrobatidis*. *B. dendrobatidis* is generally thought of as an amphibian specialist that consumes host keratin for sustenance (13), despite it commonly being maintained in the laboratory on nonkeratinized media, such as tryptone. Numerous vertebrate and invertebrate taxa possess keratin or keratin-like compounds in their gastrointestinal (GI) tracts (14). Hence, it is not surprising that previous researchers have hypothesized that there might be nonamphibian hosts or vectors of *B. dendrobatidis* (15, 16). However, this idea appeared to be temporarily abandoned after Rowley et al. (17) retracted their initial report of the detection of *B. dendrobatidis* on nonamphibian hosts (18). Recently, it was reported that *B. dendrobatidis* can be carried on algae (12), terrestrial reptiles (19), waterfowl (20), and nematodes (21), but there is currently no evidence that these carriers actually supported pathogen growth or transmission, which would be necessary to explain the long-term persistence of *B. dendrobatidis* in the absence of amphibians. Other studies have grown *B. dendrobatidis* on boiled snake skin (11, 22), sterilized bird feathers (23), and toe scales from waterfowl (20), but none of these studies demonstrated *B. dendrobatidis* growth on live hosts with functioning immune systems. Consequently, we lack studies that demonstrate *B. dendrobatidis* growth on living, nonamphibian hosts, transmission of *B. dendrobatidis* from these hosts to amphibians, and links between nonamphibian hosts and *B. dendrobatidis* prevalence in the field.

Here, we test whether mosquitofish (*Gambusia holbrooki)* and crayfish (*Procambarus* spp. and *Orconectes virilis*) are hosts for *B. dendrobatidis* by field-collecting each species, examining them for embedded zoosporangia, screening them for *B. dendrobatidis* using quantitative (q)PCR, and testing for associations between crayfish and *B. dendrobatidis* occurrence in nature. We selected these species because they cooccur with many amphibian species and have been widely introduced beyond their native ranges (24). We then attempted to experimentally infect mosquitofish and crayfish (*Procambarus alleni*) with *B. dendrobatidis* and determined whether these potential hosts could transmit *B. dendrobatidis* to amphibians.

Results and Discussion

We found *B. dendrobatidis*$^{+}$ *Procambarus* spp. (*P. alleni* and *P. clarkii*) in three of the five southeastern Louisiana sites sampled in September 2011 and in one of two sites sampled in April 2012. Conservative estimates of average prevalence based on qPCR of swabs from the carapace and GI tract and light microscopy of the GI tracts were 17.3% and 10% for these two surveys, respectively (see Table S1 for information by site and mean *B. dendrobatidis* intensity data and *SI Methods*). The crayfish from all of the *B. dendrobatidis*$^{+}$ Louisiana sites had distinct zoosporangia with discharge tubules in their GI tracts (Fig. 1*A*). During light microscopy, the zoosporangia could not be rinsed away and did not move independent of the GI tract when agitated with a probe (Fig. 1*A*), and histological sections verified that these zoosporangia were embedded in the GI tract of the crayfish (Fig. 1*B*). The zoosporangia grew colonially just below the GI epithelial surface (Fig. 1*B*), similar to their growth in frog skin. Furthermore,

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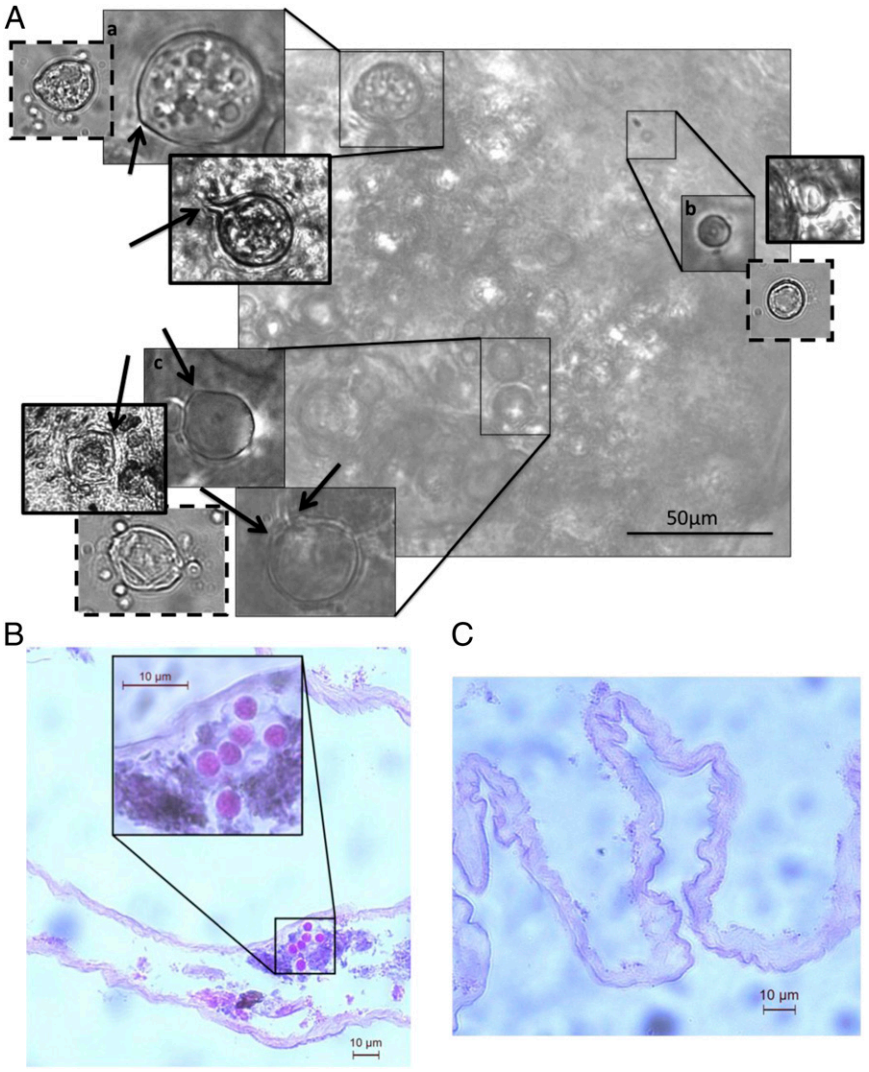

Fig. 1. Light microscopy of laboratory-infected and wild-caught *P. alleni* (collected in September) (*A*), a histological section of a wild-caught *Procambarus* spp. intestinal tract embedded with developing *B. dendrobatidis* (collected in April) (*B*) and an uninfected intestinal tract (*C*). (*A*) The laboratory-infected *P. alleni* have thin solid borders, the wild-caught *Procambarus* spp. have thick solid borders, and the same life stages grown in culture have dashed borders. Light microscopy images show zoosporangia filled with developing zoospores (*a*), encysted zoospore beginning to form rhizoids (*b*), and empty zoosporangia (*c*); arrows point to potential zoospore discharge tubules on the zoosporangia. (*B* and *C*) Histology images are stained with hematoxylin and eosin, and *B* shows what appears to be developing zoosporangia that grew colonially just below the GI epithelial surface.

crayfish that were considered *B. dendrobatidis*$^+$ based on light microscopy were also *B. dendrobatidis*$^+$ based on qPCR. Despite *B. dendrobatidis* prevalence in crayfish being >17% in September, frogs (n = 11) collected at the same time from these sites were *B. dendrobatidis*$^-$ (see Table S1 for information by site). The fact that we did not detect *B. dendrobatidis* on the frogs is consistent with a multisite seasonal survey in southeastern Louisiana that showed that *B. dendrobatidis* prevalence on amphibian skin approaches zero in September, despite being high (~45%) in the spring (Fig. S1 and *SI Methods*). These results suggest that crayfish could function as hosts for *B. dendrobatidis* during the time of the year when *B. dendrobatidis* prevalence in amphibians is low, supporting the hypothesis that crayfish are reservoir hosts for this pathogen.

Field-collected crayfish (*O. virilis*) from two of three sites surveyed in Colorado in May 2012 also had embedded zoosporangia with discharge tubules visible in their GI tracts (Fig. S2). A conservative estimate of prevalence based on light microscopy was 20%, although sample inhibition prevented verification of *B. dendrobatidis* presence by qPCR (*SI Text*). To test whether *Orconectes* spp. (*O. virilis* and *Orconectes immunis*) presence was a positive predictor of *B. dendrobatidis* infections in amphibians, we sampled 97 wetlands in Colorado, swabbing 9,174 amphibians for *B. dendrobatidis* (representing five species; *SI Methods*). Amphibians were *B. dendrobatidis*$^+$ at 40 wetlands, including six sites with positive results from more than one amphibian species (see Table S2 for frequency of positive results among species). The occurrence of *Orconectes* spp. was a significant positive predictor of *B. dendrobatidis* detection in one or more amphibian species ($\chi^2 = 10.87$; df = 1; $P = 0.001$; Fig. 2*A*). There was no evidence of overdispersion, and no other variables, including larval amphibian density, occurrence of bullfrogs (a known reservoir host for *B. dendrobatidis*), wetland area, or amphibian species richness, significantly improved model fit. When included as univariate predictors, each of these variables had ΔAIC_c (Akaike information criterion) values of >7.2 relative to the crayfish-only model, reinforcing the hypothesis of a positive association between crayfish and *B. dendrobatidis* infections.

To test whether *B. dendrobatidis* could use crayfish carapace and GI tract as a resource, *B. dendrobatidis* growth was quantified on agar alone, agar plus autoclaved crayfish carapace, and agar plus autoclaved crayfish GI tracts. *B. dendrobatidis* grew and reproduced for a minimum of 7 d (the duration of the experiment) on agar mixed with crayfish carapace or GI tracts but died within 3 d on the plates containing only agar (Fig. S3), verifying that *B. dendrobatidis* can be sustained on crayfish tissues in the absence of an immune response.

To test whether *P. alleni* and *G. holbrooki* could be infected with *B. dendrobatidis*, noninfected *P. alleni* and *G. holbrooki* were exposed to either *B. dendrobatidis*$^+$ or *B. dendrobatidis*$^-$ water for

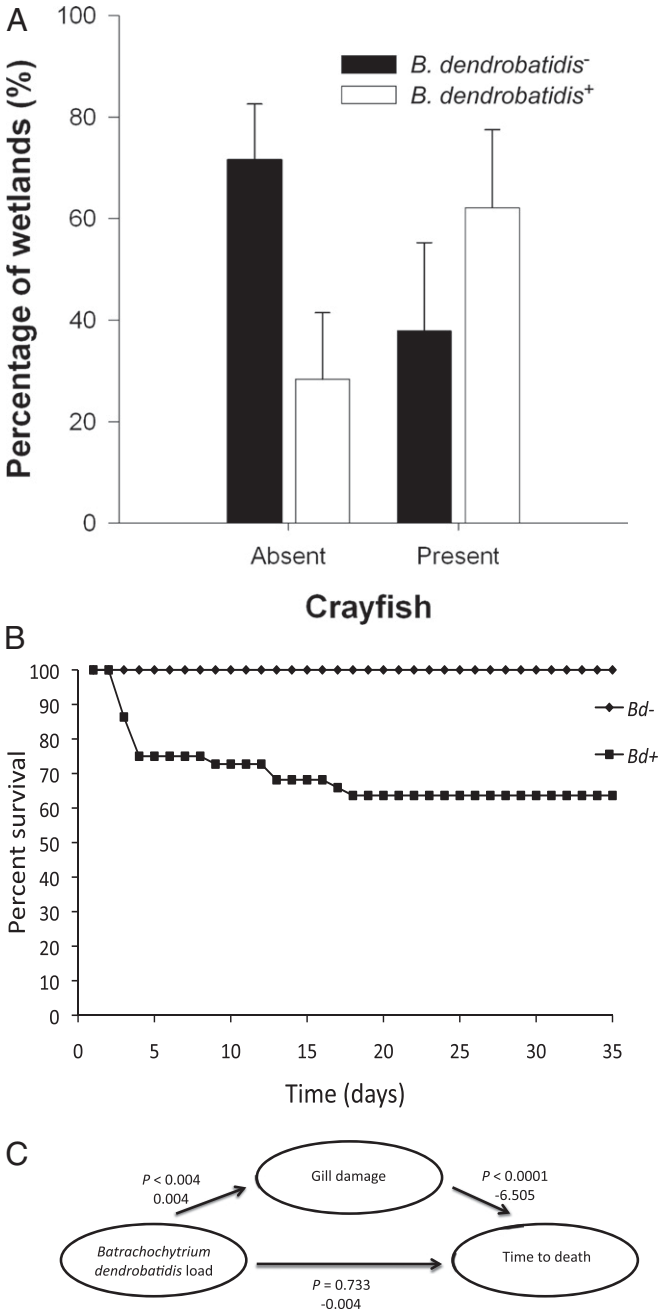

Fig. 2. Relationship between crayfish and amphibian *B. dendrobatidis* infections in the field and the effects of *B. dendrobatidis* on crayfish survival in the laboratory. (*A*) Percentage of wetlands with positive and negative *B. dendrobatidis* detections in the amphibian community as a function of crayfish (*O. virilis*) presence or absence (positive upper 95% confidence interval for a proportion). Ninety-seven wetlands were sampled with 40 yielding a positive detection of *B. dendrobatidis* in one or more amphibian species. All wetlands categorized as being negative for *B. dendrobatidis* had a minimum of 20 amphibians tested. (*B*) Percentage of survival through time of the crayfish *P. alleni* when exposed to *B. dendrobatidis*$^+$ (*Bd+*) ($n = 44$) or *B. dendrobatidis*$^-$ (*Bd–*) inoculates ($n = 21$). (*C*) Path model suggesting that effect of *B. dendrobatidis* load on time to death was mediated by gill recession. Probability values and unstandardized path coefficients are provided next to each path.

2 wk, after which they were transferred to new containers with *B. dendrobatidis*$^-$ artificial spring water (ASW) (25) and were given weekly water and container changes to ensure that we were not detecting the initial *B. dendrobatidis* inoculate. After 7 wk, animals were euthanized and swabbed externally and internally (GI tract), and *B. dendrobatidis* load (genome equivalents) on the swabs was determined using qPCR.

No *G. holbrooki* ($n = 13$ per treatment) were *B. dendrobatidis*$^+$ nor did any die during the experiment. In contrast, 91% of *B. dendrobatidis*–exposed *P. alleni* ($n = 44$) had detectable *B. dendrobatidis* based on qPCR, whereas no control *P. alleni* were *B. dendrobatidis*$^+$ ($n = 21$). By week 7, *B. dendrobatidis*–exposed *P. alleni* experienced 36% mortality compared with 0% mortality in the controls ($\chi^2_1 = 15.53$; $P < 0.0001$; Fig. 2*B*). Moreover, 100% of the dead and 84% of the live *B. dendrobatidis*–exposed *P. alleni* were *B. dendrobatidis*$^+$ and the *P. alleni* that died had higher *B. dendrobatidis* loads at their time of death than the loads of *P. alleni* that lived with the infection ($\chi^2_1 = 28.03$; $P < 0.001$; mean $\log_{10}$ *B. dendrobatidis* intensity ± SE: dead external, 3.31 ± 0.33; dead internal, 2.90 ± 0.47; live external, 2.63 ± 0.33; live internal, 0.58 ± 0.39). Light microscopy of the GI tract of *P. alleni* revealed zoosporangia filled with zoospores, empty zoosporangia with discharge tubules, and encysting zoospores (Fig. 1*A*), demonstrating infection of the crayfish GI tract. No zoosporangia were found in control *P. alleni* (Fig. 1*C* for uninfected section of GI tract).

To examine whether *P. alleni* could transmit *B. dendrobatidis* to amphibians, we exposed uninfected tadpoles to either infected or uninfected *P. alleni* (three tadpoles per replicate crayfish). The frogs were collected as egg masses from a *B. dendrobatidis*–free pond and maintained in the laboratory under *B. dendrobatidis*–free conditions until the experiment began. *B. dendrobatidis* was successfully transmitted from infected *P. alleni* to tadpoles in 7 of 10 replicates (crayfish mean $\log_{10}$ external intensity ± SE: 2.07 ± 0.66; mean $\log_{10}$ intensity/tadpole mouthpart ± SE: 0.79 ± 0.07), whereas all 12 tadpoles in the four replicates with uninfected *P. alleni* were negative for *B. dendrobatidis*.

Examination of the gills of *P. alleni* from our initial infection experiment revealed that the *B. dendrobatidis*–exposed crayfish, especially those that died early in the experiment, had significantly more gill recession (mean distance between epithelium and gill tip ± SE: 1.15 ± 0.4 μm; $n = 18$; Fig. S4*A*) than did those that were not exposed to *B. dendrobatidis* (mean ± SE: 0.12 ± 0.12 μm; $n = 7$; Fig. S4*B*; see Fig. 2*C* for statistics). To test whether death alone or fouling of the crayfish between death and preservation could explain the gill recession, a group of *P. alleni* were euthanized by pithing and allowed to sit for 24 h ($n = 5$). These crayfish had no more gill recession than the crayfish that were not exposed to *B. dendrobatidis* (mean ± SE: 0.06 ± 0.007 μm; Fig. S4*C*), suggesting that death alone or fouling of the crayfish between death and preservation could not explain the gill recession. This suggests that gill recession contributed to *B. dendrobatidis*–induced mortality rather than mortality causing the gill recession. Indeed, a path model supports the hypothesis that *B. dendrobatidis* exposure indirectly lead to reduced survival time by causing gill recession that was a negative predictor of time of death (Fig. 2*C*). Gill damage has been associated with other crayfish parasitic infections (26), where it reduced gill functioning and oxygen intake resulting in acute mortality (27).

Although *B. dendrobatidis* exposure seemed to cause gill recession, *B. dendrobatidis* was not observed to infect the gills directly. Consequently, as Berger et al. (28) hypothesized in their seminal study discovering *B. dendrobatidis*, we postulated that *B. dendrobatidis* might be producing a factor that can cause pathology in the absence of actual infection; for instance, *B. dendrobatidis* produces proteolytic enzymes known to degrade host tissues (22, 29-31). To test this hypothesis, we exposed *P. alleni* to (*i*) an unfiltered *B. dendrobatidis*$^+$ inoculum, (*ii*) a *B. dendrobatidis*$^+$ inoculum where all of the zoospores and zoosporangia were removed with a 0.7 μm filter, or (*iii*) a control *B. dendrobatidis*$^-$ inoculum filtered through a 0.7 μm filter ($n = 5$ per treatment). We found that the filtered and unfiltered *B. dendrobatidis*$^+$ inocula

induced similar, elevated levels of gill recession compared with the *B. dendrobatidis*$^{-}$ inoculum ($F_{2,11} = 17.28$; $P = 0.0004$; Fig. 3*A* and Fig. S4*D*). Moreover, all of the crayfish exposed to the filtered and unfiltered *B. dendrobatidis*$^{+}$ inocula died within 3 d, whereas all of the crayfish exposed to the filtered *B. dendrobatidis*$^{-}$ control inoculum survived until the end of the 4-d experiment ($\chi^2_1 = 16.01$; $P = 0.0003$; Fig. 3*B*). The higher mortality seen in this experiment (100%) compared with the infection experiment (36%) was probably because we exposed the crayfish to a filtered *B. dendrobatidis* inoculum that previously had more *B. dendrobatidis* zoospores than used in the infection experiment (1.2×10^5 and 1.2×10^3 zoospores per milliliter, respectively). We conducted a follow up dose–response experiment, exposing two separate populations of *P. alleni* to serially diluted, filtered *B. dendrobatidis*$^{+}$ inocula (concentration of zoospores removed: 10^6, 1.5×10^5, 10^5, 10^4, 10^3, 10^2 zoospores per milliliter; $n = 4$, $n = 10$, $n = 10$, $n = 11$, $n = 10$, and $n = 9$, respectively) and a filtered *B. dendrobatidis*$^{-}$ control inoculum ($n = 10$). We found that the populations responded similarly and that the concentration of *B. dendrobatidis* filtered from the inoculum was associated positively with molting frequency ($\chi^2_1 = 4.26$; $P = 0.03$; Fig. S5), gill recession ($F_{1,51} = 33.28$; $P < 0.001$; Fig. 3*C*), and mortality ($\chi^2_1 = 26.49$; $P < 0.0001$; Fig. 3*D*; see *SI Text* for caveat on population-level differences). Molting might be an important stage for parasitic infections (32) because after molting, crayfish have a soft exoskeleton and might be immunosuppressed (33). These results indicate that *B. dendrobatidis* can induce pathology in the absence of direct host contact or infection. This could help explain rapid mortality of tadpoles (within 48 h) exposed to *B. dendrobatidis* (34) and amphibian pathology associated with *B. dendrobatidis* exposure without infections (e.g., refs. 35 and 36). Whether these pathology-inducing chemicals released by *B. dendrobatidis* are known proteases (22, 29–31) and are the cause of *B. dendrobatidis*–induced electrolyte imbalance and cardiac arrest in amphibians (37) remains to be tested.

Building upon the results of our initial infection experiment, we conducted a 12-wk study to evaluate whether *P. alleni* could maintain *B. dendrobatidis* infections long-term and thereby potentially function as reservoir hosts. At 7 wk, 89% (25/28) of *B. dendrobatidis*–exposed *P. alleni* were *B. dendrobatidis*$^{+}$. At 12 wk, 64% (18/28) of *B. dendrobatidis*–exposed *P. alleni* had survived and 22% (4/18) of those survivors still had detectable *B. dendrobatidis* (mean $\log_{10}$ internal intensity ± SE: 1.79 ± 0.20; Fig. S6), indicating that some *P. alleni* cleared the infection, whereas others maintained the infection for at least 3 mo. Although there was a significant decrease in external *B. dendrobatidis* load between weeks 7 and 12 ($\chi^2_1 = 18.53$; $P < 0.0001$; Fig. S6), there was a significant increase in internal *B. dendrobatidis* loads over this same time period ($\chi^2_1 = 6.37$; $P = 0.01$; Fig. S6). Between weeks 7 and 12, control *P. alleni* gained weight, whereas *P. alleni* exposed to *B. dendrobatidis* lost weight (mean mass change between 7 and 12 wk ± SE; control: 11.56 ± 12.1%; *B. dendrobatidis*–exposed: −10.63% ± 14.4%; $F_{1,12} = 7.97$; $P = 0.01$), indicating a cost of infection even for the surviving individuals. Whereas the gill recession described above is likely the cause of the acute crayfish mortality, *B. dendrobatidis* infections of the GI tract might have contributed to the reduced growth rates of surviving *P. alleni*.

Overall, our results indicate that crayfish become infected with *B. dendrobatidis* in nature, can maintain these infections for months in the laboratory, and can transmit infections to amphibians. Furthermore, crayfish presence was a positive predictor of *B. dendrobatidis* occurrence in cooccurring amphibians among field sites in Colorado, even after considering competing factors such as host density or amphibian reservoir hosts (e.g., bullfrogs). Previous studies investigating potential nonamphibian hosts for *B. dendrobatidis* have not (*i*) tested live nonamphibian species with functioning immune systems, (*ii*) demonstrated *B. dendrobatidis* growth on nonamphibian species, or (*iii*) transmitted *B. dendrobatidis* from any nonamphibian host to amphibians. Our study is unique in demonstrating all three.

Mathematical models indicate that alternative hosts can allow for increased pathogen virulence and can cause host extinctions because the pathogen can persist in the remaining host species (2, 3). This might explain why *B. dendrobatidis* is so virulent, causes host extirpations, and can persist in local environments after amphibians have been extirpated. The abundance and distribution of alternative hosts might also help explain geographic variation in the distribution and rates of spread of *B. dendrobatidis*. For example, both crayfish and *B. dendrobatidis*–related amphibian declines are more common in stream than pond systems (38, 39). Additionally, crayfish infection with *B. dendrobatidis* might explain how crawfish frogs

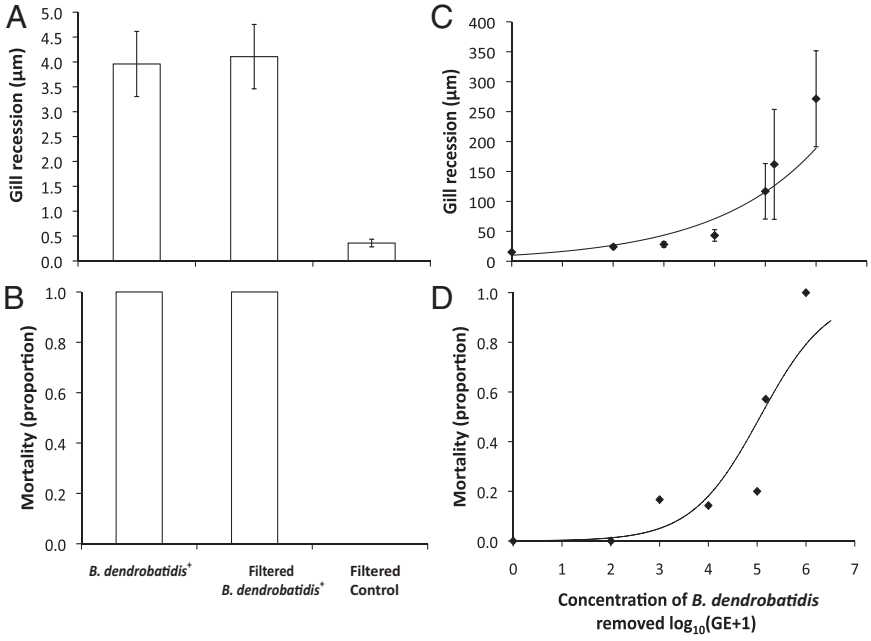

Fig. 3. Effects of an unfiltered *B. dendrobatidis*$^{+}$ inoculum, a *B. dendrobatidis*$^{+}$ inoculum where all of the zoospores and zoosporangia were removed, and a *B. dendrobatidis*$^{-}$ inoculum on *P. alleni* gill recession (distance between epithelium and gill tip) (*A*) and mortality (*B*). Dose–response relationship between *B. dendrobatidis* inocula with all of the zoospores and zoosporangia removed and *P. alleni* gill recession (*C*) and mortality (*D*). Values are mean ±1 SE. Shown is the best fit line in *C* and a logistic regression fit [$y = \exp(-7.1962833 + (1.4220071 \times x)/(1 + \exp(-7.1962833 + (1.4220071 \times x))$] in *D*.

(*Lithobates areolatus*) obtained *B. dendrobatidis* infections while overwintering in crayfish burrows (40).

Although more work is needed to generalize these results and the role of crayfish in *B. dendrobatidis* epizootics in amphibians, alternative hosts might help elucidate the emergence of the global *B. dendrobatidis* pandemic. Crayfish are regularly moved among water bodies as fish bait (41), and crayfish are regularly transported nationally and internationally in the live food, aquaculture, and aquarium and pond trade, where crayfish escapes and releases are not uncommon (24). These different methods of live crayfish relocations could rapidly move *B. dendrobatidis* great distances and contribute to the global *B. dendrobatidis* pandemic. Most efforts to conserve and restore amphibian populations challenged by *B. dendrobatidis* have been unsuccessful, but managing alternative hosts offers a new and potentially more effective approach to managing *B. dendrobatidis*. Likewise, identifying the specific pathology-inducing chemical released by *B. dendrobatidis* might facilitate the development of new strategies to reduce the risk posed by this devastating pathogen.

Methods

General. All crayfish (mean initial mass ± SE: 3.35 ± 0.75 g) in the laboratory studies were exposed to *B. dendrobatidis* isolate SRS 812 (isolated from *Lithobates catesbeianus*; see *SI Methods* for *B. dendrobatidis* culture and inoculation methodology) and were maintained individually in 1-L polyethylene containers filled with 500 mL of ASW (25) at 23 °C and on a 14:10 h light:dark cycle. All of the crayfish and tadpoles were fed organic spinach ad libitum and were checked daily for mortality. Zoospore densities in the *B. dendrobatidis*$^+$ inoculums were estimated with a hemocytometer and were diluted with deionized water to the targeted concentrations in each experiment (Table S3).

Crayfish and mosquitofish were euthanized by freezing and MS222 overdose, respectively, were swabbed externally (30 swipes from the snout to tail) and internally (15 swipes of the entire length of the inside of the GI tract), and were preserved individually. In between each swab, gloves were cleaned with 10% bleach and rinsed with 1% Novaqua (neutralizes the bleach) and then water. To ensure that the GI tract was not contaminated with *B. dendrobatidis* from the exoskeleton or scales, they were removed with sterilized forceps by one experimenter and were swabbed by a second experimenter. *B. dendrobatidis* abundance on swabs was determined using qPCR following the methods of Hyatt et al. (42) (see *SI Methods* for qPCR methodology).

Crayfish Screening. *P. alleni* and *P. clarkii* (9–20 crayfish per site from five sites in September 2011 and 10 crayfish per site from two sites in April 2012) were collected from southeastern Louisiana (Table S1). *O. virilis* (4–18 crayfish per site from three sites in May 2012) were collected from Colorado (Table S1). Crayfish were swabbed externally and internally (GI tract) as described above, and light microscopy, histology (see *SI Methods*), and qPCR were used to determine prevalence and abundance of *B. dendrobatidis*.

Colorado Field Surveys. Between 2007 and 2010, 97 wetlands distributed across an 11 county region of Colorado were sampled to evaluate the importance of biotic and abiotic factors in predicting *B. dendrobatidis* occurrence on amphibians (Table S2). Standard methods (visual encounter surveys, dip-net sampling, and seine hauls; *SI Methods*) were used to characterize amphibian and invertebrate communities (43) over the course of two visits to each site. Larval, metamorphic, or adult amphibians were tested for *B. dendrobatidis* using nonlethal swabs followed by a qPCR assay (*SI Methods*). The goal was to detect *B. dendrobatidis* when present rather than to estimate prevalence; thus, species swabs were batch-pooled for each wetland and targeted a minimum of 20 swabbed individuals per site.

***B.dendrobatidis* Culture and Inoculation.** *B. dendrobatidis* inoculum was prepared by growing 1 mL of *B. dendrobatidis* stock (strain SRS 812 isolated from *L. catesbeianus*) on a 1% tryptone agar plate for 8 d at 23 °C. Each plate was flooded with 3 mL of ultrapure water to suspend the zoospores and the water from each plate was homogenized to generate the *B. dendrobatidis*$^+$ inoculum. The *B. dendrobatidis*$^-$ inoculum was simultaneously prepared using the same method but no *B. dendrobatidis* was added to the agar plates (see Table S3 for zoospore concentrations).

Infection Experiment. We collected *G. holbrooki* and *P. alleni* from a pond in Tampa, FL (28°06.759′N, 082°23.014′W) that is free of *B. dendrobatidis*. Each animal received 10 mL of either the *B. dendrobatidis*$^-$ inoculum (control; *G. holbrooki*: $n = 13$; *P. alleni*: $n = 12$) or the *B. dendrobatidis*$^+$ inoculum (*G. holbrooki:* $n = 13$; *P. alleni*: $n = 22$). After 2 wk of exposure to *B. dendrobatidis* with no water changes, all animals were moved to new containers with fresh *B. dendrobatidis*$^-$ ASW for 5 more weeks and water and container changes occurred weekly. The animals were weighed at the end of the experiment.

The gills from each crayfish were removed and photographed (100× magnification). The greatest distance between the epithelium and the external surface of the gill was measured on three randomly selected gill filaments per crayfish using ImageJ software. A follow-up study was conducted to determine whether gill recession was an artifact of animal death and/or fouling of *B. dendrobatidis*–exposed animals (given that no control animals died during the experiment). Five *P. alleni* (collected from the same Tampa, FL population) were euthanized (pithed) and held for 24 h in the same conditions as the control animals in the experiment. The gills were removed and the distance between the epithelium and the external surface of the gill was then measured as described above.

***B.dendrobatidis* Culture Experiment.** *B. dendrobatidis* was cultured on agar plates containing either ($n = 3$ per treatment) autoclaved crayfish GI tract, autoclaved crayfish carapace, 1% tryptone (positive control), or agar alone (negative control) to test whether *B. dendrobatidis* is able to use these substrates for growth and reproduction (*SI Methods*). Each plate was inoculated with *B. dendrobatidis* (*SI Methods*), maintained at 23 °C for 7d, and monitored daily for zoospore activity. On day 7, each plate was flooded with 7 mL of ultrapure water to suspend all zoospores. A 150-μL aliquot was taken from each plate and the number of living zoospores was counted using a hemocytometer.

Transmission Experiment. Three *L. sphenocephalus* egg masses were collected from a *B. dendrobatidis*–free pond (28°06.759′N, 82°23.014′W) and raised in the laboratory under *B. dendrobatidis*–free conditions until the tadpoles reached Gosner (44) stage 28 (*SI Methods*). Three tadpoles were haphazardly selected and placed in each of fourteen 1-L polyethylene cups filled with 750 mL of ASW. One *P. alleni* was added to each of these cups directly above the tadpoles (*B. dendrobatidis*$^+$: $n = 10$; *B. dendrobatidis*$^-$: $n = 4$; these crayfish were also part of the infection maintenance experiment and were verified 7 wk after exposure to be *B. dendrobatidis*$^+$ or *B. dendrobatidis*$^-$ by external swabs) and crayfish and tadpoles were separated by a window screen to prevent predation. The crayfish were placed above the tadpoles to ensure that the crayfish feces dropped into the foraging arena of the tadpoles. After 5 d, the species were separated and the tadpoles were maintained in separate 1-L polyethylene cups filled with 750 mL of *B. dendrobatidis*$^-$ ASW for 10 d. Tadpoles were euthanized in MS222, and their mouthparts were removed and stored in 70% (vol/vol) ethanol for qPCR analysis. The *P. alleni* were maintained in *B. dendrobatidis*$^-$ ASW until 12 wk after the initial *B. dendrobatidis* exposure, at which time they were euthanized, weighed, and swabbed (these crayfish were used in the infection maintenance experiment as well).

Filtered *B. dendrobatidis* Experiment. *P. alleni* housed in 500 mL of ASW were exposed to 15 mL of each of the following: (*i*) a *B. dendrobatidis*$^+$ inoculum (1.2×10^5 zoospores/mL); (*ii*) a *B. dendrobatidis*$^+$ inoculum (1.2×10^5 zoospores/mL) where all of the zoospores and zoosporangia were removed with a 0.7 μm filter (G6 Glass Fiber Filter; Fisher Scientific); or (*iii*) a control *B. dendrobatidis*$^-$ inoculum filtered through a 0.7-μm filter ($n = 5$). All inocula were prepared as described in *SI Methods*, and the crayfish were exposed to the filtered inocula 10 min after filtering was complete. Fifteen 20-μL aliquots of the filtered *B. dendrobatidis*$^+$ inoculum were examined for *B. dendrobatidis* using a compound microscope and a hemocytometer, and none had any detectable zoospores or zoosporangia. The exposures lasted 4 d, after which, the crayfish were euthanized and gill recession was assessed as described above.

Filtered *B. dendrobatidis* Dose–Response Experiment. This experiment was run in two temporal blocks where crayfish were exposed to serially diluted filtered *B. dendrobatidis*$^+$ inocula or a filtered *B. dendrobatidis*$^-$ control inoculum. For block 1, *P. alleni* were purchased from The Marine Warehouse (Tampa, FL), and the exposure lasted for 22 d (concentration of zoospores removed: 1.5×10^5, 10^5, 10^4, 10^3, 10^2 zoospores per milliliter; $n = 6$, $n = 6$, $n = 7$, $n = 6$, and $n = 6$, respectively; *B. dendrobatidis*$^-$ control: $n = 6$). For block 2, *P. alleni* were collected from the same population used in the *Filtered* B. dendrobatidis *experiment*, and exposures lasted for 9 d (concentration of zoospores removed: 10^6, 10^5, 10^4, 10^3, 10^2 zoospores per milliliter; $n = 4$, $n = 4$, $n = 4$, $n = 4$, and $n = 3$, respectively; *B. dendrobatidis*$^-$ control: $n = 4$). For both blocks, the crayfish were exposed to the inocula 25 min after filtering was complete, and fifteen 20-μL aliquots of the filtered *B. dendrobatidis*$^+$

inoculum were examined for *B. dendrobatidis* as described above, and none had any detectable zoospores or zoosporangia. Gill recession was assessed as described above.

Infection Maintenance Experiment. *P. alleni* were housed and exposed to either *B. dendrobatidis*$^-$ or *B. dendrobatidis*$^+$ inoculum (*B. dendrobatidis*$^-$: $n = 9$; *B. dendrobatidis*$^+$: $n = 28$) using the same methodology as described for the infection experiment, except that 7 wk after initial exposure, animals were swabbed externally (to verify infection), and 12 wk after initial exposure, animals were euthanized and swabbed internally and externally (as described above). After the week 7 swabbing, a subset of these *P. alleni* (*B. dendrobatidis*$^+$: $n = 10$; *B. dendrobatidis*$^-$: $n = 4$) were selected for use in the transmission trials.

Statistical Analysis. For the Colorado survey, we used generalized linear models with a binomial response and a logit-link function to test whether crayfish presence (*Orconectes* spp.), bullfrog (*L. catesbeianus*) presence, larval amphibian density (summed among species; $\log_{10}$-transformed), wetland area ($\log_{10}$-transformed), and amphibian species richness were significant predictors of *B. dendrobatidis* occurrence in amphibians at each wetland. In the laboratory experiments, we tested for the effect of *B. dendrobatidis* exposure on crayfish survival using censored survival regression, using a Weibull distribution. Analysis of variance was used to determine whether *B. dendrobatidis* exposure affected weight change relative to the controls. We analyzed weight change between weeks 7 and 12. We conducted a path analysis to evaluate the level of support for the hypothesis that *B. dendrobatidis* exposure was indirectly related to crayfish death via gill recession [using the Lavaan package in R (45)]. We tested whether population and $\log_{10}$ concentration of filtered *B. dendrobatidis* inoculum affected crayfish mortality and molting compared with the filtered controls with Cox proportional hazards regression (function: coxph). We also tested whether $\log_{10}$ concentration of filtered *B. dendrobatidis* affected $\log_{10}$ gill recession with a linear regression model (function: lm). Statistical analyses were conducted in R statistical software (45).

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Supporting Information

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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).

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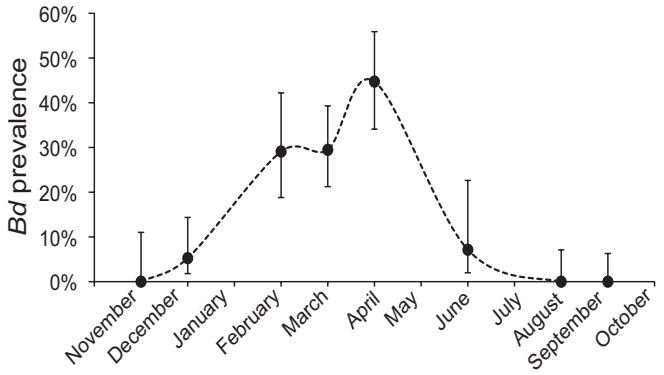

Fig. S1. Seasonality of *B. dendrobatidis* (*Bd*) prevalence in amphibians collected from Southeastern Louisiana (n = 31, 57, 55, 95, 76, 28, 50, 57 per month, respectively). Means ± 95% CI.

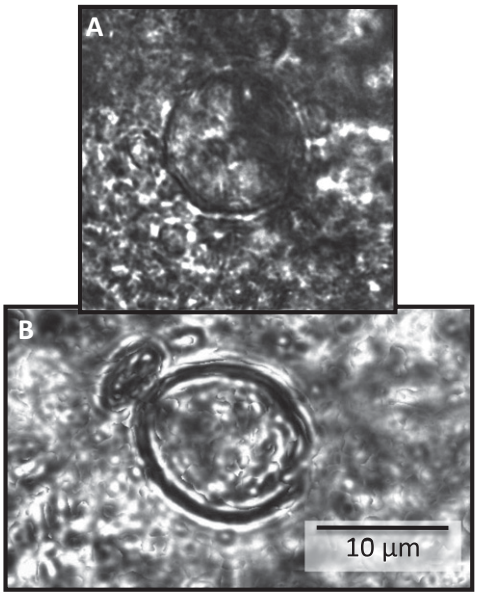

Fig. S2. *B. dendrobatidis* on the intestinal wall of wild caught *O. virilis*. (*A*) Developing zoosporangia. (*B*) Empty zoosporangia. Images were taken by T.A.M. and M.B.J.

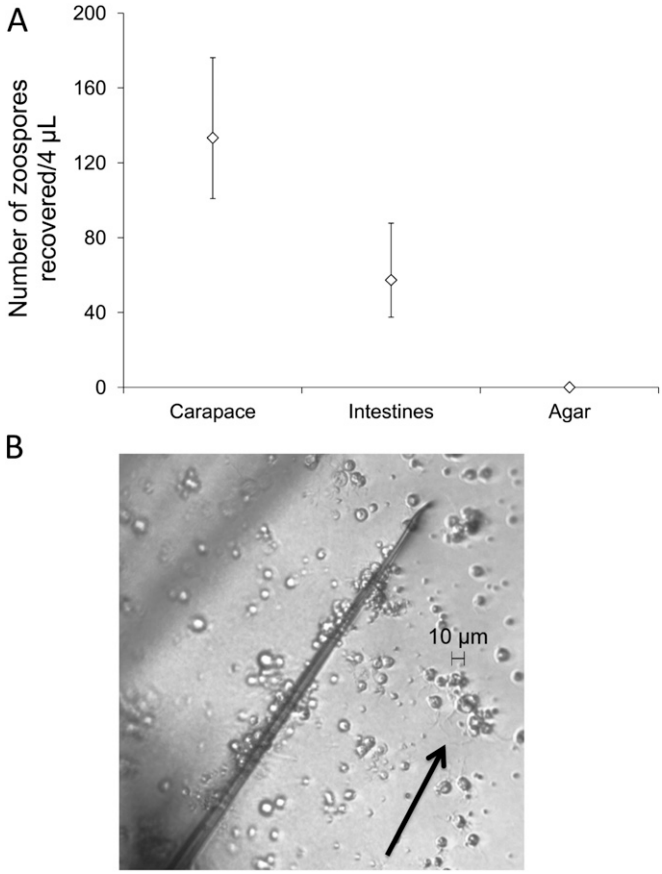

Fig. S3. Results of our experiment testing whether *B. dendrobatidis* could grow on agar plates with pieces of autoclaved crayfish tissue. (*A*) Mean number of motile *B. dendrobatidis* zoospores (±95% CI) viewed in a hemocytometer after 7 d of growth on agar and carapace, agar and intestines, and agar 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.

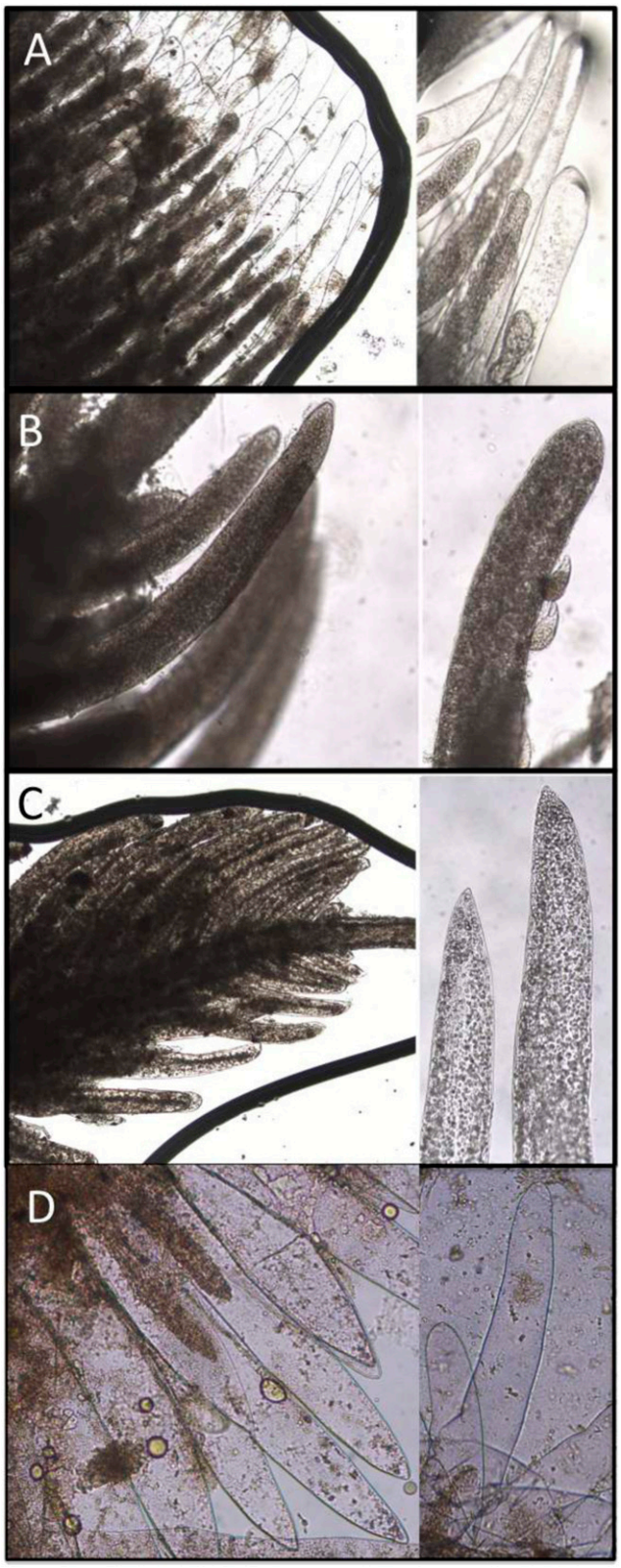

Fig. S4. *Procambarus alleni* exposed to *B. dendrobatidis* have significantly more gill damage than those that were not exposed to *B. dendrobatidis*. (*A*) Crayfish gills exposed to *B. dendrobatidis* (died early in the experiment). (*B*) Gills of control, *B. dendrobatidis*–free crayfish that survived 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.

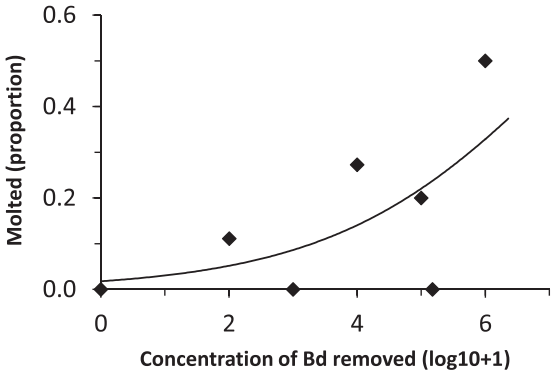

Fig. S5. Molting occurrence in *P. alleni* exposed to a *B. dendrobatidis*$^{+}$ (Bd) inoculum where all of the zoospores and zoosporangia were removed with a 0.7-μm filter (concentration of zoospores removed: 0, 10^6, 1.5×10^5, 10^5, 10^4, 10^3, 10^2 zoospores per milliliter). Provided is the logistic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

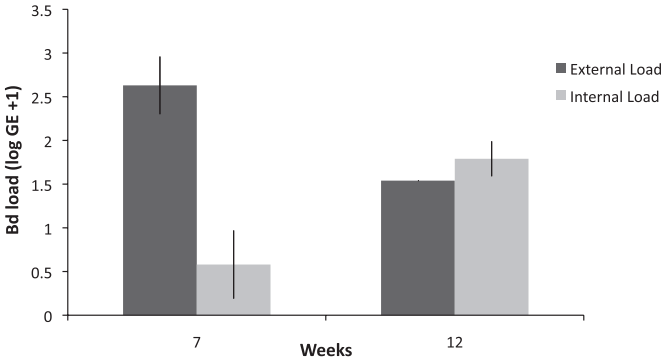

Fig. S6. Internal (inside the GI tract) and external *B. dendrobatidis* (Bd) load (shown is mean log zoospore equivalents ± SE) in *P. alleni* 7 and 12 wk after exposure.

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