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, 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. L4). 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. L4), and histological sections verified that these zoosporangia were embedded in the GI tract of the crayfish (Fig. L8). The zoosporangia grew colonially just below the GI epithelial surface (Fig. L9), similar to their growth in frog skin. Furthermore,
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. 2A). 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 $\Delta$AIC$_c$ (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
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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 (*χ^2^ = 15.53; *P* < 0.0001; Fig. 2B). 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 (*χ^2^ = 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. 1A), demonstrating infection of the crayfish GI tract. No zoosporangia were found in control *P. alleni* (Fig. 1C 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. S4A) than those that were not exposed to *B. dendrobatidis* (mean ± SE: 0.12 ± 0.12 μm; *n* = 7; Fig. S4B; see Fig. 2C 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. S4C), suggesting that death alone 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. 2C). 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. S4D). 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 ($\chi^2 = 16.01; P = 0.0003$; Fig. 3B). 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 ($1.2 \times 10^3$ and $1.2 \times 10^7$ 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: $10^6$, $1.5 \times 10^6$, $10^7$, $10^8$, $10^9$, $10^7$ zoospores per milliliter; $n = 4, n = 10, n = 10, n = 11, n = 10, n = 9$, 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 motility frequency ($\chi^2 = 4.26; P = 0.03$; Fig. S5), gill recession ($F_{1,51} = 33.28; P < 0.001$; Fig. 3C), and mortality ($\chi^2 = 26.49; P < 0.0001$; Fig. 3D); 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 immuno-suppressed (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 $\pm$ SE: $1.79 \pm 0.20$; 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 ($\chi^2 = 18.53; P < 0.0001$; Fig. S6), there was a significant increase in internal B. dendrobatidis loads over this same time period ($\chi^2 = 6.37; P = 0.01$; 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 $\pm$ SE: control: $11.56 \pm 12.11\%$; B. dendrobatidis–exposed: $-10.63\% \pm 14.4\%$; $F_{1,12} = 7.97; P = 0.01$), 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 a 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 crayfish frogs
(Lithobates aranelatus) 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 pathogenicity-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 aranelatus; SI Methods) in broth (an 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 mosquito fish were euthanized by freezing and MS222 overdose, respectively, were swabbed externally and internally (15 swipes from the snout to tail) and interally (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 (neutralized 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, 82°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) autocalved crayfish G6 tracts, autocalved 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 (10 μL aliquots of the inoculum; Table S1) and maintained 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 selected from each plate and the number of living zoospores was counted using a hemocytometer.

Transmission Experiment. Three L. sphenocoelatus 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 selected from each plate and the number of living zoospores was counted using a hemocytometer.

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 × 104 zoospores/mL); (ii) a B. dendrobatidis+ inoculum (1.2 × 106 zoospores/mL) that was then filtered (collected from the same Tampa, FL population) 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 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 × 102, 104, 106, 108 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: 106, 108, 1010, 1012, 1014 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 log-link function to test whether crayfish presence (Orconectes spp.), bullfrog (L. catesbeianus) presence, larval amphipod density (summed across species; log10-transformed), wetland area (log10-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 log10 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 log10 concentration of filtered B. dendrobatidis affected log10 gill recession with a linear regression model (function: lm). Statistical analyses were conducted in R statistical software (46).

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