Effects of invasive larval bullfrogs (*Rana catesbeiana*) on disease transmission, growth and survival in the larvae of native amphibians

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Abstract The mechanisms by which invasive species negatively affect native species include competition, predation, and the introduction of novel pathogens. Moreover, if an invasive species is a competent disease reservoir, it may facilitate the long-term maintenance and spread of pathogens in ecological assemblages and drive the extinction of less tolerant or less resistant species. Disease-driven loss of biodiversity is exemplified by the amphibian–chytrid fungus system. The disease chytridiomycosis is caused by the aquatic chytrid fungus *Batrachochytrium dendrobatidis* (Bd) in anurans and is associated with worldwide amphibian population declines and extinctions. For amphibian species that metamorphose and leave infected aquatic habitats, the

mechanisms by which Bd persists over winter in these habitats remains a critical open question. A leading hypothesis is that American bullfrogs (*Rana catesbeiana*), a worldwide invasive species, are tolerant to Bd and serve as a reservoir host for Bd during winter months and subsequently infect native species that return to breed in spring. Using outdoor mesocosms, we experimentally examined if two strains of Bd could overwinter in aquatic systems, in the presence or absence of bullfrog tadpoles, and if overwintered Bd could be transmitted to tadpoles of two spring-breeding species: Pacific treefrogs (*Pseudacris regilla*) and Cascades frogs (*Rana cascadae*). We found that only 4 of 448 total animals (one bullfrog and three spring breeders) tested positive for Bd after overwintering. Moreover, two of the three infected

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spring breeders emerged from tanks that contained overwintered Bd but in the absence of infected bullfrogs. This suggests that Bd can persist over winter without bullfrogs as a reservoir host. We found no effect of Bd strain on bullfrog survival after overwintering. For Pacific treefrogs, Bd exposure did not significantly affect mass at or time to metamorphosis while exposure to bullfrogs reduced survival. For Cascades frogs, we found an interactive effect of Bd strain and bullfrog presence on time to metamorphosis, but no main or interactive effects on their survival or mass at metamorphosis. In short, bullfrog tadpoles rarely retained and transmitted Bd infection in our experiment and we found limited evidence that Bd successfully overwinters in the absence of bullfrog tadpoles and infects spring-breeding amphibians.

Keywords Bullfrogs · Invasive species · Reservoir species · Disease ecology · *Batrachochytrium dendrobatidis*

Introduction

Biological invasions can alter the dynamics of ecological communities (Lodge 1993; Sakai et al. 2001; Simberloff 2006; Fukami et al. 2006; Lowry et al. 2013) and contribute to population declines and losses in biodiversity (Elton 1958; Gibbons et al. 2000; Blackburn et al. 2004; Simberloff 2013; Katsanevakis et al. 2014). The mechanisms by which invasive species negatively affect native species include competition, predation (Mooney and Cleland 2001) and the introduction of novel pathogens (Lymbery et al. 2014; Vilcinskas 2015; Dunn and Hatcher 2015; Searle et al. 2016). Competent reservoir species may facilitate the long-term maintenance and spread of novel pathogens in ecological assemblages (Keesing et al. 2006, 2010; Telfer and Bown 2012) and drive the extinction of less tolerant or less resistant species (McCallum 2012; Reeder et al. 2012; Telfer and Bown 2012; Gervasi et al. 2017).

Disease-driven loss of biodiversity is exemplified by the amphibian–chytrid fungus system. The chytrid fungus, *Batrachochytrium dendrobatidis* (Bd), is one of two fungi that cause the disease chytridiomycosis in amphibians. Bd is associated with worldwide amphibian population declines and extinctions (Berger et al.

1998; Stuart et al. 2004; Skerratt et al. 2007; Olson et al. 2013; Scheele et al. 2019). Bd grows on amphibian skin and mouthparts (because both contain keratin), produces aquatic zoospores (Berger et al. 2004; Piotrowski et al. 2004) and is found in a variety of terrestrial and aquatic habitats (Olson et al. 2013; Chestnut et al. 2014; Kolby et al. 2015; Sette et al. 2015). Bd may be saprophytic (Daszak et al. 1999; Longcore et al. 1999; Briggs et al. 2005) and is most pathogenic in moist, cool climates (Berger et al. 2004; Piotrowski et al. 2004; La Marca et al. 2005; Whitfield et al. 2016). Anuran susceptibility to Bd can vary with host life stage, species, population, age, Bd strain to which hosts are exposed, and environmental conditions (Blaustein et al. 2005, 2018; Garcia et al. 2006; Di Rosa et al. 2007; Searle et al. 2011; Gervasi et al. 2013a, 2017; Sapsford et al. 2013; Bradley et al. 2015; Dang et al. 2017; Mesquita et al. 2017). For instance, Rumschlag and Boone (2018) observed increased winter mortality of leopard frogs (*Rana pipiens*) exposed to Bd before overwintering, which could reduce population growth rates due to reduced recruitment. However, entire anuran populations may be infected with Bd but individuals may show few or no symptoms (e.g. Retallick et al. 2004; Di Rosa et al. 2007). Given the rapid demise caused by the spread of Bd among amphibian populations (Scheele et al. 2019), it is vital to understand what factors promote the spatial and temporal persistence of Bd.

The mechanisms by which Bd persists in aquatic environments and is transmitted across seasons remain critical open questions. Bd can persist in freshwater for weeks (Johnson and Speare 2005; Chestnut et al. 2014) and can remain in some larval amphibians and after metamorphosis between seasons (Briggs et al. 2010; Narayan et al. 2014). Vredenburg et al. (2010) suggested that patterns of Bd spread within a metapopulation in the Sierra Range of California is consistent with frog movement patterns as well as the possibility of unknown additional vectors.

A leading hypothesis is that American bullfrogs (*Rana catesbeiana*) may serve as an asymptomatic carrier or reservoir for Bd (Daszak et al. 2004; Hanselmann et al. 2004; Garner et al. 2006; Salla et al. 2015; Yap et al. 2018; Urbina et al. 2018). American bullfrogs have the ability to mount an immune response to Bd (Davis et al. 2010) and can take 2–3 years to complete their aquatic larval stage.

Bullfrogs may display tolerance (absence of mortality during a low-dose exposure to Bd) or resistance (ability to clear Bd infection). Furthermore, the presence of invasive bullfrogs in some regions has been correlated with the presence of Bd and, in some cases, a decline of native amphibian populations or mortality events (e.g. Borzée et al. 2017; Adams et al. 2017; Yap et al. 2018). If bullfrogs serve as an infection-tolerant reservoir species for Bd, it could have profound effects on disease dynamics. However, the actual mechanism by which Bd overwinters in aquatic environments has yet to be experimentally demonstrated. Identifying this mechanism is an important research priority for amphibian conservation efforts as the range of Bd is expected to increase, potentially placing more species at risk to Bd exposure (Rödger et al. 2009; Xie et al. 2016).

American bullfrogs are native to the central and eastern United States and were introduced west of their historic range for bullfrog farming in the late 1800's (Kats and Ferrer 2003). They have since been introduced to other regions of the world (Schloegel et al. 2012; Bucciarelli et al. 2014). In the wild, bullfrogs have tested positive for Bd (Hanselmann et al. 2004; Blaustein et al. 2005; Garner et al. 2006; Bai et al. 2010) although quantitative estimates of Bd infection load in bullfrogs are largely unknown (but see Garner et al. 2006; Gervasi et al. 2013b). Recent experimental evidence showed that bullfrog susceptibility varies with Bd strain and that infection loads detected in the skin decreased over time, suggesting that bullfrogs may be inefficient long-term carriers of Bd (Gervasi et al. 2013b; Urbina et al. 2018). Furthermore, Eskew et al. (2015) showed that recently metamorphosed American bullfrogs experimentally exposed to Bd had low infection prevalence, low infection loads and lacked clinical signs of chytridiomycosis. Therefore, the ability of American bullfrogs to serve as reservoir hosts that overwinter with the pathogen might vary with bullfrog population and Bd strain, among other factors.

We experimentally examined if Bd could overwinter in aquatic environments in the presence and absence of Bd-tolerant bullfrog tadpoles. Furthermore, we investigated if overwintered Bd could be transmitted to the larvae of other amphibian species the following spring. The native spring breeding species we examined lay their eggs in early spring and undergo metamorphosis in summer (Nussbaum et al.

1983). We hypothesized Bd would overwinter in keratinized tissues of bullfrog tadpoles, but not in the absence of amphibian hosts. We also expected infected bullfrog tadpoles to retain Bd infection over winter and transmit the fungus to the larvae of spring-breeding amphibians.

Methods

We investigated the transmission dynamics of Bd in simulated freshwater pond communities (mesocosms) at the Donald S. Wood Field Laboratory at the University of Pittsburgh's Pymatuning Laboratory of Ecology (Linesville, PA, USA). We employed a completely randomized experimental design with the factorial combination of three Bd treatments (no Bd, Bd strain JEL 627, Bd strain JEL 630) and two American bullfrog treatments (bullfrog absent, bullfrog present). The eastern strain (JEL 627) was isolated from an American bullfrog in its native range (Bethel, Maine, USA) in 2009. The western strain (JEL 630) was isolated from an American bullfrog from Finley National Wildlife Refuge (Oregon, USA) in 2009. Both strains were originally obtained from Dr. Joyce E. Longcore (University of Maine). The six Bd $\times$ bullfrog treatment combinations were replicated five times for a total of 30 experimental units. We added a replicate of the two treatments that contained Bd and bullfrogs to be destructively sampled prior to overwintering, increasing the total number to 32 experimental units (Fig. 1).

We conducted our experiment using a three-phase approach (Fig. 1). In *Phase 1*, we inoculated bullfrog tadpoles or water with Bd to introduce infection. In *Phase 2*, we moved the Bd-exposed or sham inoculated-exposed bullfrog tadpoles and their water from *Phase 1* to outdoor mesocosms to monitor the persistence of Bd in bullfrogs and the aquatic environment. In *Phase 3*, we added larvae of two other anuran species to each mesocosm the following spring to investigate the impact of overwintered Bd and bullfrog tadpoles on the potential transmission of Bd to spring-breeding frog species.

Husbandry

We collected eight freshly oviposited egg masses of American bullfrogs from local ponds on private land

Fig. 1 Experimental design. The number of individuals used and subsampled in each Phase are included. The number of Phase 2 and Phase 3 replicates differs due to the destructive

sampling of two mesocosms prior to overwintering that contained bullfrogs and the eastern or western Bd strain

(Crawford County, PA, USA) on 21 May 2013. Although Bd has been reported in other amphibian populations of northwest Pennsylvania (Groner and Relyea 2010), there are no records available for our bullfrog population. Thus, freshly oviposited egg masses were collected to avoid using previously exposed individuals. Each egg mass was placed into a 300-L pool filled with 200 L aged well water where it developed under ambient environmental conditions. We fed tadpoles Bunny 16 rabbit chow (Blue Seal; Muscatine, IA, USA) ad libitum and conducted full water changes as needed.

Phase 1: inoculation with Bd

Phase 1 experimental units were 14-L plastic tubs filled with 2 L of UV-irradiated, carbon-filtered aged well water. We assigned two Phase 1 tubs to each of the 32 experimental mesocosms for a total of 64 tubs. We added 8 bullfrog tadpoles (Gosner (1960) stage (GS) 25; 31.2 ± 1.9 mg (mean $\pm$ 1 SE) to each tub

assigned to a ‘bullfrog-present’ treatment (n = 272; Fig. 1). Tadpoles were randomly selected from a homogenized subset of individuals taken from all clutches to avoid genetic bias. We chose this experimental design (2 bins each containing 8 tadpoles) to reduce tadpole density and preserve water quality during the week-long Bd inoculation. All tubs received agar-based *Spirulina* food pellets at an ad libitum ration.

We exposed the 44 tubs assigned to Bd treatments to either the eastern or western Bd strain (Fig. 1). We cultured both strains on 1% tryptone agar Petri plates. Culture plates were incubated for 9–15 days at room temperature (20 °C). To obtain zoospores, we flooded each plate with 10 mL of filtered water. After 15 min, we collected released zoospores and zoosporangia from the agar using a rubber policeman scraper. We rinsed all plates with filtered water and poured the slurry into a 1-L glass beaker to create a homogenized sample. Zoospore concentration was determined using a hemocytometer. Inoculations were conducted on 13

August 2013 and repeated on 16 August 2013 (Fig. 2). We diluted the Bd slurry with filtered water to obtain a final concentration of 400,000 total zoospores in 2 L of water (concentration 200 zoospores/mL). Doses used were based on previous work from our lab (e.g. Dang et al. 2017) and were high enough to infect bullfrogs but not enough to cause mortality. We added 10 mL of Bd stock solution to each experimental unit assigned to a Bd treatment. Experimental units not assigned Bd were mock-dosed with 10 mL of UV-irradiated, carbon-filtered aged well water.

On 20 August 2013, following a 7-d Bd exposure (100% bullfrog survival), we haphazardly sampled 3 tadpoles from each tub to confirm Bd infection ($n = 36$ per Bd treatment, $n = 30$ for no-Bd control treatment). Tadpoles were individually euthanized using a MS-222 overdose (Tricaine-S; Western Chemical Inc., Ferndale, WA, USA) and preserved separately in 20-mL plastic scintillation vials (Fisher® Cat. No. 03-337-23C) using 95% EtOH. Preserved specimens were shipped to Oregon State University (Corvallis, OR, USA) for analysis of Bd load. Mouthparts were dissected and homogenized and tissue samples were tested for Bd using quantitative polymerase chain reaction (qPCR) (methods below). We then combined the contents of the two tubs (i.e., 10 remaining tadpoles and water) and added them to the respective outdoor mesocosm replicate.

Phase 2: persistence of Bd in freshwater communities

For *Phase 2*, we used 32, 1,300-L outdoor mesocosms as our experimental units. On 18 June 2013, we filled each mesocosm with 1000 L of aged well water and added 38 L of topsoil. To create semi-natural freshwater communities, we added leaf litter, zooplankton, and algae to each mesocosm on 26 June 2013. We added 300 mg of dried oak (*Quercus* spp.) for structure and an additional source of nutrients. We collected, filtered (64- μ m mesh), and homogenized water from three permanent waterbodies and then added 1.24 L to each mesocosm to introduce an algal community. We collected zooplankton from a nearby permanent waterbody, removed invertebrate predators, and then added 355 mL of the homogenized sample to colonize each mesocosm with a zooplankton community. We then covered each mesocosm with 60% shade cloth to prevent invertebrate oviposition and amphibian emigration.

To confirm the persistence of infection in bullfrog tadpoles before overwintering, we randomly sampled 3 individuals from each bullfrog treatment on 13 October 2013 (Figs. 1, 2). Using a 38 $\times$ 63 cm² dip net, we gently swept through each mesocosm and collected the first three tadpoles captured. We used four to seven sweeps to capture the 3 individuals. We then euthanized the bullfrog tadpoles individually using an MS-222 overdose and preserved them in 95% EtOH. To prevent cross-contamination, we soaked the dip net in quaternary ammonia (10%) for 30 s (Johnson et al. 2003) and rinsed the net thoroughly

Fig. 2 Experimental timeline

with aged well water before moving to the next mesocosm. To replicate the disturbance caused by our bullfrog sampling, we also made 3 sweeps in all of the mesocosms that lacked bullfrog tadpoles. Two experimental units (one assigned to a bullfrog-western Bd treatment and one to a bullfrog-eastern Bd treatment) were destructively sampled to assess bullfrog survival and Bd load prior to overwintering. All surviving individuals were collected, euthanized with MS-222 overdose, and preserved in 95% EtOH. In the two destructively sampled tanks, we observed 70% and 90% survival among bullfrog tadpoles exposed to the western and eastern Bd strains, respectively.

The outdoor mesocosms developed under ambient conditions (-24 to 33 °C) and we monitored water depth to prevent overflow. When necessary, we manually bailed each mesocosm using 19-L buckets and collected the treated water in adjacent empty mesocosms. We removed the same volume of water from each mesocosm. Before moving to the next mesocosm, we submerged each bucket in quaternary ammonia (10%) for 30 s (Johnson et al. 2003) and thoroughly rinsed each bucket with aged well water. Each researcher also rinsed their hands with ethanol (70%) and allowed their hands to dry prior to moving to the next experimental unit. Once all mesocosms had been bailed, we sterilized all removed water with quaternary ammonia. We inadvertently added quaternary ammonia to one mesocosm (a bullfrog-western Bd replicate) in November following the bailing of mesocosms, and thus, removed it from the experiment.

Phase 3: transmission of Bd to spring-breeding amphibians

To investigate the transmission of Bd to the larvae of spring-breeding amphibians, we added larvae of two spring-breeding species to each mesocosm in spring 2014 (Fig. 2). We chose Cascades frogs (RC; *Rana cascadae*) and Pacific treefrogs (PR; *Pseudacris regilla*), which are native to wetlands and ponds of the Pacific Northwest. PR are negatively affected by invasive bullfrogs (Bucciarelli et al. 2014) whereas bullfrogs have not yet invaded the range of RC at our collection sites. We collected five RC egg masses and ten PR egg masses on 15 April 2014 (Linn Co, OR, USA). Egg masses were shipped to the Pymatuning Laboratory of Ecology overnight on ice and were gradually warmed prior to being placed outdoors in

300-L pools filled with 200 L of aged well water. Tadpoles developed under ambient environmental conditions and were fed Bunny 16 rabbit chow ad libitum.

We added 20 individuals of each species to each outdoor mesocosm on 24 May 2014. For both species, we mixed tadpoles from all egg masses and size-selected 620 individuals; 580 tadpoles were used for the experiment, 20 individuals were held for 24 h to assess survival following handling (100%), and 20 individuals were euthanized for mass (RC 130 ± 6 mg, PR 59 ± 5 mg) and stage identification (GS 25).

We monitored anuran development daily and removed fully metamorphosed individuals ($>GS$ 45) from each mesocosm beginning 25 June 2014. To prevent cross-contamination, we rinsed collection equipment (aquarium nets) and hands with EtOH (70%) before advancing to the next mesocosm. We euthanized collected amphibians with an MS-222 overdose and preserved individuals in scintillation vials filled with 95% EtOH.

We terminated the experiment on 28 July 2014. We collected any metamorphosed individuals and remaining tadpoles from each mesocosm, euthanized them with an MS-222 overdose, and preserved each in scintillation vials filled with 95% EtOH. To confirm our removal of all amphibians from each mesocosm, we siphoned the water from each experimental mesocosm into an adjacent empty tank. All equipment used to collect and euthanize amphibians and to siphon and remove water from experimental units was sanitized between mesocosms using quaternary ammonia. Once all tanks had been double-checked for amphibians, we sanitized all mesocosms and water with quaternary ammonia.

Water sample analysis

To confirm the presence or absence of Bd in our freshwater communities, we sampled the water of our experimental units three times during the experiment. We first sampled water on 14 August 2013 to confirm our *Phase 2* mesocosms had not been exposed prior to the addition of *Phase 1* water and bullfrogs. We then sampled the *Phase 2* mesocosms on 12 October 2013 to assay Bd prevalence prior to overwintering. Lastly, we sampled the mesocosms on 28 July 2014 prior to the experimental takedown.

We collected 200 mL of water from the four cardinal directions and middle of each mesocosm by submersing an inverted 200-mL container. We homogenized the five 200-mL samples and withheld 60 mL. Once 60 mL had been collected from each treatment replicate, we pooled all samples together to create a single sample for each experimental treatment (300–360 mL). We passed the homogenized sample through a prepared syringe fitted with a Sterivex 0.22- μ m capsule filter (Fisher Scientific; Cat. No. SVGPL10RC). Using a separate syringe, we then passed 50 mL of phosphate buffer saline (Sigma-Aldrich; SKU P5368) through the filter to remove excess dissolved carbon and pumped air through the chamber to dry the filter. We capped the filter with Hemataseal capillary sealant (Fisher Scientific; Cat. No. 23-550-112), and added 0.9 mL of cell lysis solution (Fisher Scientific; Cat. No. FP2301320) before closing the filter with a sterile luer-lock cap. Closed filters were individually placed in sterile Whirl-Pak and frozen (-20°C). We shipped frozen filters overnight on ice to the USGS Reston Microbiology Lab (Reston, VA, USA) for independent qPCR analysis.

Quantifying amphibian Bd load

We used qPCR to determine infection status and load for preserved animals. We assessed Bd infection load prior to overwintering by analyzing the 3 destructively sampled bullfrogs collected from outdoor mesocosms ($n = 61$). Specifically, we sampled 3 tadpoles from 15 mesocosms ($n = 45$) plus 16 individuals from the surplus mesocosms ($n = 61$). Preserved metamorphs were swabbed for Bd to assess infection load after overwintering. We analyzed samples from all bullfrogs ($n = 27$ total) and a subsample of half of the spring-breeding species ($n = 421$ total). For each preserved metamorph, we swabbed the abdomen five times, each leg five times, and each foot five times for a total of 25 passes. Quantifying Bd infection status and load followed the methods of Boyle et al. (2004), except that extractions were done using 60 μL of Prepman Ultra (Applied Biosystems). Real time qPCR was conducted using an ABI PRISM 7500 Fast machine. Samples were run in triplicate against Bd standard titrations from 10^{-1} to 10^2 (USGS Reston Microbial Laboratory, Reston, VA, USA). Samples were considered positive if Bd amplified in two of the

three wells. Samples were considered negative if Bd amplified in one or none of the three wells.

Statistical analysis

To test for treatment effects on amphibian survival, we conducted generalized linear mixed-effects models (GLMM) for each species. Amphibian survival was recorded at the end of the experiment and included all experimental replicates. For bullfrogs (only including bullfrog-present treatments), we assessed the main effect of ‘Bd treatment’ on the survival of individuals represented by a binomial survival response (0 = dead, 1 = alive). For PR and RC survival, we examined the main and interactive effects of ‘Bd treatment’ and ‘bullfrog presence’ on the binomial survival response (0 = dead, 1 = alive). We included ‘mesocosm’ as a random effect term in each model using the *glmer* function in the *lme4* package of R (R 3.5.1, RStudio 1.2.1335; R Core Team 2018). We assessed model significance using the *Anova* function in the *car* package at $\alpha = 0.05$.

To determine the effects of each treatment on the life history traits of each species, we also conducted multivariate analysis of variance (MANOVA) for each species. Tank means for the life history traits snout-to-vent length (SVL), mass at metamorphosis, and time to metamorphosis were based on fully-metamorphosed individuals (< 2 mm tail; GS 46). Life history measurements were recorded after termination of the study. The SVL and mass of individuals were measured with digital calipers (nearest 0.1 mm) and on an electronic balance (0.1 mg; Sartorius CPA 124S), respectively. Time to metamorphosis (d) was calculated by subtracting the initial addition date (bullfrogs = August 20, 2013; PR and RC = May 24, 2014) from the collection date of metamorphosed individuals. Mesocosms without metamorphosed individuals were excluded. We examined the main effect of ‘Bd treatment’ on bullfrog life-history traits, whereas the main and interactive effects of ‘Bd treatment’ and ‘bullfrog presence’ were included for both models examining PR and RC life history traits. Significant MANOVA results were subsequently examined with ANOVA for each life history trait, and pair-wise comparisons were conducted using Tukey’s HSD tests. We also calculated effect sizes (η^2) to examine the relative degree in which the total variance found in ANOVA models was associated

with each of the main effects and their interactions. All statistical analyses were conducted using R (R 3.5.1; RStudio 1.2.1335; R Core Team 2018) with the *car*, *stats*, and *sjstats* packages.

Results

Water sample analysis

Prepared filters revealed no detectable levels of Bd (< 1 copy μL^{-1}) in the water samples collected from mesocosms on 14 August 2013, 12 October 2013, and 28 July 2014.

Confirmation of Bd infection following Phase 1 inoculation

Bd analysis on bullfrog tadpoles subsampled on 20 August 2013, prior to the addition to the *Phase 2* outdoor mesocosms, revealed that infection prevalence differed between strains at the completion of *Phase 1*. Of 36 bullfrog tadpoles exposed to the eastern strain, 25 (69%) had detectable Bd infection with an average of 1.45 ± 0.33 Bd-genome equivalents. Of 36 bullfrog tadpoles exposed to the western strain, 15 (41%) had detectable Bd infection with an average of 6.23 ± 3.9 Bd-genome equivalents.

Bullfrog subsample to examine Bd infection pre-overwintering

The destructive sampling of 3 individuals from mesocosms containing bullfrog tadpoles on 12 October 2013 ($n = 61$) revealed one tadpole from both the eastern and western Bd treatments with detectable Bd infections. The Bd loads were 1.20 and 0.67 Bd-genome equivalents, respectively.

Bd infection at experimental takedown

Swabs of the 448 animals that metamorphosed (all three species) revealed that only 4 had detectable Bd infections (Table 1). Out of the 9 mesocosms that contained Bd and bullfrogs, we observed a single bullfrog and RC to be infected with the eastern Bd strain. In the 10 mesocosms that were exposed to Bd only, one RC and one PR were infected with the eastern and western Bd strain, respectively.

Amphibian response variables

For American bullfrogs, Bd treatment did not influence survival (Wald $\chi^2 = 0.04$, $p = 0.979$), which averaged $28.6 (\pm 7.8)\%$ across all treatments. Bd exposure did not influence the SVL, mass, or time to metamorphosis of bullfrogs (Wilks' $\lambda = 0.588$, $p = 0.856$).

For PR, survival in mesocosms was negatively affected by bullfrogs (Wald $\chi^2 = 6.14$, $p = 0.013$), but not by Bd exposure or the Bd-by-bullfrog interaction (Wald $\chi^2 \leq 4.8$, $p \geq 0.091$). Survival of PR in the absence of overwintered bullfrog tadpoles was $66.7 (\pm 7.7)\%$ but reduced to $42.1 (\pm 9.0)\%$ in the presence of bullfrogs. The MANOVA on life-history traits revealed a significant effect of Bd (Wilks' $\lambda = 0.498$, $p = 0.024$) but not bullfrog presence or their interaction ($p \geq 0.107$). Subsequent ANOVAs revealed that SVL length and mass were not influenced by the treatments or the interaction ($p \geq 0.147$). However, time to metamorphosis was marginally influenced by bullfrogs (Fig. 3; $F_{1,22} = 4.0$, $p = 0.058$, $\eta^2 = 0.121$) and Bd exposure ($F_{2,22} = 2.9$, $p = 0.074$, $\eta^2 = 0.177$), but not their interaction ($F_{2,22} = 0.7$, $p = 0.520$, $\eta^2 = 0.041$). Treefrog tadpoles exposed to bullfrogs metamorphosed 4.8 d later than those not exposed. Exposure to the western strain decreased time to metamorphosis by approximately 7 d compared to non-exposed individuals ($p = 0.067$). Time to metamorphosis did not differ between individuals exposed to eastern and western strains ($p = 0.255$), or between the eastern and the no-Bd treatment ($p = 0.760$).

For RC, survival was reduced by bullfrog presence (Wald $\chi^2 = 10.9$, $p = 0.001$), but not by Bd exposure or the bullfrog-by-Bd interaction (Wald $\chi^2 \leq 3.78$, $p > 0.151$). Survival of RC was $95.3 (\pm 1.65)$ and $64.3 (\pm 9.62)\%$ in the absence and presence of overwintered bullfrog tadpoles, respectively. The MANOVA on life-history traits showed a significant effect of Bd exposure (Wilks' $\lambda = 0.373$, $p = 0.003$), no main effect of bullfrog presence (Wilks' $\lambda = 0.846$, $p = 0.354$), and a marginal interaction (Wilks' $\lambda = 0.555$, $p = 0.068$). ANOVAs revealed mass at metamorphosis and SVL were not influenced by the treatments or their interaction ($p \geq 0.135$). However, time to metamorphosis of RC was affected by Bd exposure (Fig. 4; $F_{2,21} = 8.673$, $p = 0.002$, $\eta^2 = 0.351$) and Bd-by-bullfrog interaction

Table 1 Bullfrog treatment (presence/absence), Bd treatment [eastern strain (JEL 627), western strain (JEL 630), or unexposed] and the number of animals, species, and genome equivalents from metamorphosed amphibians with detectable Bd infection

Bullfrog treatment	Bd treatment	Bd positive species	Bd genome equivalents
Bullfrog	No Bd	–	–
	Eastern strain	1 <i>R. catesbeiana</i>	0.43
		1 <i>R. cascadae</i>	0.06
	Western strain	–	–
No bullfrog	No Bd	–	–
	Eastern strain	1 <i>R. cascadae</i>	0.02
	Western strain	1 <i>P. regilla</i>	0.59

Fig. 3 Pacific treefrog time to metamorphosis following exposure to overwintered bullfrogs and Bd treatments (no-Bd control, Eastern chytrid strain and Western chytrid strain). Letters represent significant differences between treatments

($F_{2,21} = 4.647$, $p = 0.021$, $\eta^2 = 0.188$), but not by the main effect of bullfrogs ($F_{1,21} = 1.737$, $p = 0.202$, $\eta^2 = 0.035$). In the absence of bullfrogs, exposure to the western strain of Bd significantly reduced time to metamorphosis of individuals compared to tadpoles exposed to the no-Bd treatment ($p = 0.043$) but did not differ from those exposed to the eastern strain ($p = 0.146$). Time to metamorphosis did not differ between RC in the no-Bd treatment and exposed to the eastern Bd strain ($p = 0.756$). Individuals exposed to the western Bd strain metamorphosed 9.7 d earlier than those in the no Bd treatment in the absence of bullfrogs. With bullfrogs, exposure to the eastern Bd strain significantly increased RC time to metamorphosis compared to the no-Bd treatment ($p = 0.010$)

observed using Tukey's HSD test. Filled circles represent the mean treatment response and error bars are representative of ± 1 SE

and the western strain ($p = 0.004$). The time to metamorphosis of RC did not differ in the later treatments ($p = 0.630$). When exposed to bullfrogs, the time to metamorphosis of individuals in the eastern Bd strain treatment was increased by 12.5 and 15.3 days on average compared with individuals in the no-Bd and western Bd strain treatments, respectively.

Discussion

There is evidence that Bd can persist freely in water (Chestnut et al. 2014), on non-amphibian hosts (e.g., Kilburn et al. 2011; McMahon et al. 2013) and on

Fig. 4 Cascades frog time to metamorphosis following exposure to overwintered bullfrogs and Bd treatments. Letters represent significant differences between treatments observed using Tukey's HSD test. Filled circles represent the mean treatment response and error bars are representative of ± 1 SE

amphibian hosts (e.g., Narayan et al. 2014). Thus, unexposed spring-breeding amphibians returning to water to breed could be exposed to Bd that has persisted on hosts or in the environment for many months following initial infection. Hosts carrying Bd could be larvae or adult bullfrogs. We tested the hypotheses that Bd persists on overwintering invasive bullfrog larvae and is subsequently transmitted to spring-breeding amphibians. Thus, we examined if American bullfrogs, which can take 2–3 years to complete their larval stage, serve as a reservoir for Bd. Out of a total of 448 animals, only one bullfrog tested positive for Bd after winter and only three individuals of the other species (two in the no-bullfrog treatment) tested positive for Bd.

The persistence of Bd over winter was low in bullfrogs and transmission to other hosts was low. Infection intensity was low in the four animals where Bd was detected when compared with laboratory experiments. In two of the animals (one bullfrog and one PR), infection intensity was comparable to infection in other mesocosm experiments (e.g. Buck et al. 2015). Although, it was not possible to tell if Bd was present on any bullfrogs when the spring breeders were introduced, our results suggest the possibility that bullfrog larvae can carry Bd over winter.

However, most of the initially infected bullfrogs lost their infection prior to overwintering. Only 2 of 46 Bd-exposed bullfrogs tested positive for Bd in October, 2 months after addition to the mesocosms. Furthermore, only one bullfrog retained infection to the spring following overwintering. These results corroborate earlier experimental work that also found low infection loads and a decrease in infection load in bullfrogs over time (Gervasi et al. 2013b; Urbina et al. 2018). Bullfrog tadpoles in our study were inefficient reservoirs for Bd and that successful overwintering of Bd outside of amphibian hosts was infrequent.

The impact of bullfrogs on native amphibian species is complex and influenced by a combination of direct and indirect effects (Bucciarelli et al. 2014; Anderson and Lawler 2016). Bullfrog larvae can compete with and prey upon native species and may stress larvae of other amphibian species through pathogen transmission, chemical interference, or food resource alteration (Kiesecker and Blaustein 1997; Chivers et al. 2001; Kiesecker et al. 2001). Although the presence of bullfrogs did not affect RC and PR metamorph SVL and mass, bullfrogs did negatively affect the survival of both spring breeders. Moreover, RC and PR time to metamorphosis was influenced by bullfrog presence. In nature, the time to metamorphosis and size at metamorphosis may be keys to survival. Thus, faster growing individuals may be competitively superior to others that grow slower and reach metamorphosis later due to exposure to bullfrogs (Wilbur and Collins 1973; Wells 2007). Furthermore, the interaction between Bd exposure and bullfrog exposure in our study significantly affected time to metamorphosis in RC, illustrating the complex dynamics in systems with an invasive species that harbors pathogens that can be potentially transmitted to other species.

Our results have implications for the generalization that bullfrogs maintain high infection with Bd without signs of morbidity or mortality. American bullfrogs exhibit variation in responses to Bd depending on pathogen strain but also due to individual-level heterogeneity in resistance to infection, with some individuals able to clear Bd infections in the skin, even after repeated high inoculations (Gervasi et al. 2013b, 2017; see also Eskew et al. 2018). In different ecological systems there may be variation in which host species or populations represent the most relevant reservoirs for Bd (Reeder et al. 2012; Bradley et al.

2015). Subsampling individuals could potentially influence overall Bd infection if we preferentially removed infected sensitive individuals from our experiment; however, our haphazard selection of individuals should have prevented such bias. While bullfrogs may be important carriers of Bd in some systems, alternative hosts may be just as important for the maintenance and global spread of this pathogen (Kilburn et al. 2011; Reeder et al. 2012; Gervasi et al. 2013b; Liu et al. 2013; McMahon et al. 2013; Burrowes and De la Riva 2017; O'Hanlon et al. 2018). The infection of PR and RC in mesocosms void of bullfrog hosts illustrates the important role algae, zooplankton, or soil may play in the preservation of Bd in aquatic systems (Johnson and Speare 2003; Chestnut et al. 2014). Moreover, Bd has been found in water (e.g. (Kirshtein et al. 2007; Chestnut et al. 2014; Kamoroff and Goldberg 2017). Chestnut et al. (2014) showed that Bd occurs in the water year-round in North America. In that study Bd exhibited temporal and spatial heterogeneity in density, but did not exhibit seasonality in occupancy.

In conclusion, we provide evidence that bullfrog tadpoles have a limited capacity to retain Bd infection over winter and transmit the fungus to other species. However, bullfrog presence was detrimental to spring-breeding species and reduced their survival. In one species, the presence of bullfrogs increased its time to metamorphosis. Complex dynamics of predation, competition, disease and stress may have affected spring breeders in our study. In our study, under semi-natural experimental conditions, we have shown that the chytrid fungus can overwinter in bullfrogs and be transmitted to other amphibian species but these processes were limited.

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