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Individual and combined effects of multiple pathogens on Pacific treefrogs

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Abstract In nature, individual hosts often encounter multiple pathogens simultaneously, which can lead to additive, antagonistic, or synergistic effects on hosts. Synergistic effects on infection prevalence or severity could greatly affect host populations. However, ecologists and managers often overlook the influence of pathogen combinations on hosts. This is especially true in amphibian conservation, even though multiple pathogens coexist within amphibian populations, and several pathogens have been implicated in amphibian population declines and extinctions. Using an amphibian host, *Pseudacris regilla* (Pacific treefrog), we experimentally investigated interactive effects among three pathogens: the trematode *Ribeiroia* sp. (hereafter, *Ribeiroia*), the fungus *Batrachochytrium dendrobatidis* (hereafter, BD), and the water mold *Achlya flagellata*. We detected no effects of *A. flagellata*, but did find effects of *Ribeiroia* and BD that varied depending on context. Low doses of *Ribeiroia* caused relatively few malformations, while higher *Ribeiroia* doses caused numerous deformities dominated by missing and reduced limbs and limb elements. Exposure to low doses of BD accelerated larval host development, despite there being no detectable BD infections, while exposure to higher BD doses caused infection but did not alter developmental rate. Hosts exposed to both *Ribeiroia* and BD exhibited the highest mortality, although overall evidence of interactive effects of multiple pathogens was limited. We suggest further research on the influence of multi-pathogen assemblages on amphibians, particularly under a variety of ecological conditions and with a wider diversity of hosts and pathogens.

Keywords Coinfection · Concomitant infections · Polyparasitism · Emerging infectious disease · Amphibian decline

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Introduction

Organisms often encounter multiple pathogens simultaneously, with pathogens combining to produce additive, antagonistic, or synergistic effects within individual hosts (Petney and Andrews 1998; Bentwich et al. 1999; Jolles et al. 2008). Synergistic effects of multiple pathogens on

individual hosts can increase infection prevalence and severity and have the potential to reduce host population size, as recently described in *Bison bison athabascae* (wood bison) affected by bovine tuberculosis and brucellosis (Joly and Messier 2005) and *Panthero leo* (African lions) impacted by canine distemper and babesiosis (Munson et al. 2008). However, the possibility that interactive effects of multiple pathogens are contributing to the worldwide decline of amphibian populations has received little attention, even though several different pathogens have been implicated as causative factors in the crisis (Worthylake and Hovingh 1989; Cunningham et al. 1996; Daszak et al. 2003; Green et al. 2002; Greer et al. 2005). Duffus (2009) advocated a more comprehensive approach to investigation of amphibian mortality events that evaluates a number of pathogens as possible causative agents in place of the current narrow focus on a single pathogen, the fungus *Batrachochytrium dendrobatidis*. We further suggest that researchers should also test whether amphibian disease events arise from interactive effects of two or more pathogens, especially because infection and disease in amphibians are highly influenced by environmental stressors (e.g., Kiesecker and Blaustein 1995; Rohr et al. 2008a; Kerby and Storfer 2009). Surprisingly, researchers rarely investigate additional pathogens as environmental stressors that might trigger amphibian disease (but see Cunningham et al. 1996; Johnson et al. 1999). However, the recent discovery that symbiotic skin bacteria provide important defenses for amphibians against *B. dendrobatidis* (Becker and Harris 2010; Lam et al. 2010) emphasizes that there is much to learn about the interactive effects of the diverse components of microbial communities, including pathogen combinations, on amphibians (Belden and Harris 2007).

We performed two experiments, using a set of three pathogens consisting of trematodes (*Ribeiroia* sp.; hereafter, *Ribeiroia*), the chytridiomycete fungus *B. dendrobatidis* (hereafter, BD), and the oomycete *Achlya flagellata* to test for interactive effects of multiple pathogens on Pacific treefrogs (*Pseudacris regilla*). All three pathogens are implicated in amphibian mortality or pathology (Tiffney and Wolf 1939; Johnson et al. 2002a; Berger et al. 1998) and the cosmopolitan *A. flagellata* (Post 1987; Khulbe 1994; Noga 1996) and globally distributed BD (Fisher et al. 2009; Pearl et al. 2009) are likely to co-occur extensively with *Ribeiroia* throughout its wide range in North America (Johnson et al. 2002a, 2003).

Ribeiroia uses snails of the family Planorbidae as first intermediate hosts, fish and amphibians as second intermediate hosts, and birds and mammals as definitive hosts (reviewed in Johnson et al. 2004). Infected snails shed free-swimming *Ribeiroia* cercariae, which infect frog and salamander larvae and encyst as metacercariae, resulting in a variety of limb deformities, elevated mortality, delayed metamorphosis, and delayed regeneration following limb injury (Johnson et al. 1999, 2001, 2003, 2006; Kiesecker 2002; Blaustein and Johnson 2003; Schotthoefer et al. 2003). *Ribeiroia*-associated limb deformities are widespread in the United States (Johnson et al. 2002a, 2003; Kiesecker 2002), and qualitative evidence supports the hypothesis that the prevalence of *Ribeiroia*-induced limb deformities in amphibians in the western USA has recently increased (Johnson et al. 2003; Johnson and Lunde 2005).

The fungus *B. dendrobatidis* has been linked to catastrophic declines of amphibian populations (Daszak et al. 2003; Rohr et al. 2008b; Fisher et al. 2009; Kilpatrick et al. 2010). BD infects the keratinized mouthparts of frog tadpoles and the keratinized skin of postmetamorphic amphibians (Berger et al. 1998; Davidson et al. 2003) and can kill amphibians in multiple life stages (Berger et al. 1998; Rachowicz and Vredenburg 2004; Blaustein et al. 2005; Garcia et al. 2006; Garner et al. 2009). Moreover, BD can cause a variety of sublethal effects in frog larvae (e.g., Berger et al. 1998; Parris and Baud 2004; Parris and Cornelius 2004; Blaustein et al. 2005).

Achlya flagellata is a water mold in family Saprolegniaceae that is closely related to *Saprolegnia*, which can kill amphibians at multiple life stages (Kiesecker and Blaustein 1995; Romansic et al. 2006, 2007, 2009). *A. flagellata* is capable of saprobism and parasitism (Johnson et al. 2002b), can contribute to mortality in amphibians and fishes (e.g., Srivastava and Srivastava 1975, 1976; Khulbe 1992; Khulbe et al. 1995; Bisht et al. 1996; see Johnson et al. 2002b for additional references), and has been linked to large die-offs of fish in natural and hatchery environments (Tiffney and Wolf 1939; Khulbe 1992; Bisht et al. 1996). Tiffney and Wolf (1939) found diseased *Notopthalamus viridescens* (red-spotted newts) with *A. flagellata/Saprolegnia parasitica* co-infections and other *Achlya* spp. have been found on dead amphibian embryos (Green and Converse 2005; Touchon et al. 2006; Gomez-Mestre et al. 2006).

The species in our study were chosen because of their high potential to be involved in interactive host-pathogen effects. *P. regilla* larvae are susceptible to infection by *Ribeiroia* (Johnson et al. 2002a) and BD (Blaustein et al. 2005; T.S.T., unpublished data), incur mortality and develop deformities following *Ribeiroia* exposure (Johnson et al. 1999, 2002a, 2003), and are susceptible to mortality from water mold (Romansic et al. 2009). All three of our pathogens infect epidermal tissue and *A. flagellata* opportunistically infects wounds (Tiffney and Wolf 1939). *A. flagellata* might colonize wounds caused by the entry of *Ribeiroia* cercariae, while *Ribeiroia* and BD may have synergistic effects if infection with one impedes the host from combating or repairing damage caused by the second. Alternatively, if zoospores of *A. flagellata* and BD, which infects frog larvae only in the oral region (Berger et al.

1998), compete for limited colonization sites, competitive exclusion may lead to less-than-additive effects on hosts. Indeed, Nieto et al. (2007) found a negative association between BD infection and diversity of oral parasites in *Rana aurora* (northern red-legged frog) larvae.

In our first experiment, we tested for individual and interactive effects of *Ribeiroia* and BD on survival, deformities, developmental rate, growth, and BD infection status in *Pseudacris regilla*. We next ran a second, similar experiment to test whether combining either *Ribeiroia* or BD with the pathogenic oomycete *Achlya flagellata* leads to interactive effects on these host parameters.

Materials and methods

Experimental 1: exposure to *Ribeiroia* and BD

We used a 2 × 2 randomized factorial design with two treatments (exposure and control; denoted with superscripts $^{+}$ and $^{-}$, respectively) for each pathogen (*Ribeiroia* and BD). We used one larva per experimental unit and 16 replicates of each treatment combination (64 larvae total). *P. regilla* larvae were haphazardly selected from laboratory stocks (developmental stages 26–28, Gosner 1960; mean total length ± 1SE = 21.3 ± 0.3 mm) and added to individual, randomly positioned 1-l plastic beakers filled with 200 ml of water (see Collection and maintenance of *Pseudacris regilla,* Electronic Supplementary Material, ESM). We pipetted 12 *Ribeiroia* cercariae (see Pathogen sources, ESM) into each *Ribeiroia*$^{+}$ beaker along with 5 ml of water used to house source snails. *Ribeiroia*$^{-}$ beakers received 5 ml of water from snails not shedding cercariae (no cercariae). Three standard-sized Petri dishes (diameter 9 cm) containing BD cultures (see Pathogen sources, ESM) were each flooded with 3.0 ml of ultrapure water and resulting zoospore solutions were combined and added to each BD^{+} beaker (0.1 ml/beaker), producing an estimated initial BD zoospore concentration of 2.6×10^7 zoospores/l (see Pathogen counting methods, ESM, for details of all BD and *Achlya* propagule estimates). We treated BD^{-} beakers identically, except that they received solution from sterile dishes lacking BD. We applied BD treatments within 30 min of *Ribeiroia* treatments. After 10 h, beakers were checked for cercariae visually and using a dissecting microscope. We found no cercariae in beakers, consistent with all cercariae entering larvae. Larvae were transferred to individual beakers filled with 6 l of water 24 h after treatment applications and fed 12 h later (see Collection and maintenance of *Pseudacris regilla*, ESM). Thereafter, larvae received food daily such that it was always present. Animals were checked daily, and metamorphs (forelimb emergence, stage 42, Gosner 1960) were transferred to individual 9-l glass tanks filled with 600 ml of water and tilted such that half the tank bottom was dry. Each metamorph, upon tail resorption, was measured for snout–vent length and transferred to an individual, large plastic Petri dish (diameter 15 cm, height 3 cm, with moist paper towels covering half the bottom). Each metamorph was maintained until 28 days after resorption of its tail, at which point its final snout–vent length was recorded and it was euthanized using MS-222 and preserved in 70% ethanol. Dead individuals (larvae and metamorphs) were also preserved in 70% ethanol. We inspected all individuals for abnormalities using a dissecting microscope.

Experiment 2: exposure to *Ribeiroia*, BD, and *Achlya*

Methods were the same as in experiment 1 except for the following. We used a 2 × 2 × 2 randomized factorial design with two treatments (exposed and control) each for *Ribeiroia*, BD, and *Achlya* and 20–21 replicates of each treatment combination (161 larvae total). Haphazardly chosen larvae (developmental stages 26–28, Gosner 1960), ~18–27 days post-hatching, 14–22 mm in total length, and 30–100 mg in mass) were added to individual 120-ml plastic beakers filled with 100 ml of water. The *Ribeiroia*$^{+}$ dose was 15 cercariae/larva. Then, 10 h after *Ribeiroia* treatments, larvae were transferred to individual 1-l plastic beakers each containing 600 ml of water. Six hours later, *Achlya* dishes (see Pathogen sources, ESM) were stirred and their water was combined and added to *Achlya*$^{+}$ beakers (1 ml/beaker), producing an initial concentration of *Achlya* zoospores and zoospore cysts of 5.8×10^6/l. *Achlya*$^{-}$ beakers received 1 ml of water treated identically except that it housed sterile, sham-inoculated hemp seeds. Six hours later, 10 BD dishes were used to apply 0.25 ml of BD inoculum per beaker, producing an estimated initial BD zoospore concentration of 1.1×10^6 zoospores/l. Larvae were fed after BD treatments were complete. The experiment ended 54 days after treatment application was complete, when 87% of individuals were dead or metamorphosed. Metamorphs (live or dead) were removed, swabbed (see Swabbing methods, ESM), and preserved in 70% ethanol. Dead larvae and all larvae remaining at the end of the experiment were removed, swabbed, and preserved using the same methods. All preserved specimens were massed to the nearest 0.1 mg and inspected with a dissecting microscope for deformities and hyphae. Hyphae were removed and examined under a compound microscope (×400 magnification) to check for structures consistent with *Achlya* (Johnson et al. 2002b).

Quantitative PCR measurement of BD infection

We sampled tissue for all individuals in experiment 1 ($n = 64$ individuals) and, for experiment 2, 10–11

randomly selected individuals in each BD^- treatment combination and each BD^+ individual ($n = 121$ individuals). Whole larval mouthparts were taken when present (some individuals lost their larval mouthparts due to metamorphosis). For each individual that reached stage 37, we took either the two outermost toes (experiment 1) or the entire foot (experiment 2) of one randomly chosen hind foot. Hindfoot samples of two BD^+ metamorphs in experiment 2 were rendered unusable due to experimental error. DNA was extracted from tissue and swabs using PrepMan Ultra (Applied Biosystems) and one-eighth of the resulting template was assayed for BD at 1:10 dilution using quantitative real-time PCR (Boyle et al. 2004). We checked the validity of our assays by using positive and negative control *P. regilla* and testing for PCR inhibition (see Additional controls in PCR assays for BD, ESM). We considered samples to be weakly positive if they yielded <1 ge (zoospore genome equivalents) of BD DNA in the reaction well, which translates to <80 ge in the total sample due to the sample division and dilution described above. Weakly positive samples were re-assayed in triplicate and results were averaged across all runs. We scored a sample as infected if its average load was ≥0.5 ge in the reaction well (corresponding to ≥40 ge in the total sample). Samples that were positive, but below this cutoff, were scored as uninfected. Thus, 0.5 ge in the reaction well (40 ge in the total sample) served as a threshold for assigning infection. Previous authors have used a standard threshold of 80 ge in the total sample to avoid misidentifying low level contamination as infection (Knapp and Morgan 2006; Rachowicz et al. 2006). We lowered this threshold to 40 ge in the total sample to decrease the chance of misidentifying light infections as non-infections.

Statistical analyses

We analyzed larval survival, postmetamorphic survival (experiment 1 only), proportion of individuals infected with BD, and the proportion of individuals that were deformed (proportion of individuals with ≥1 deformity) using logistic regression. For experiment 2, to closely examine an apparent trend of a *Ribeiroia* × BD synergism influencing survival, we further analyzed larval survival with post-hoc, pairwise logistic regression comparisons among all combinations of *Ribeiroia* and BD treatments, using a Bonferroni adjustment to maintain $\alpha = 0.05$ while making multiple pairwise comparisons (Quinn and Keough 2002). Each logistic regression in the multiple comparisons procedure used only the experimental units in the two *Ribeiroia*/BD treatment combinations being compared. For proportion of individuals infected with BD, we used life stage (larva or metamorph) as a categorical covariate. We also analyzed another dependent variable involving deformities, mean number of deformities per individual, using Poisson log-linear regression. Each individual deformity was counted, with some exceptions (Tables 1 and 2). All regression models used maximum likelihood-ratio tests to evaluate the significance of treatments and interactions. We employed Firth-adjusted maximum likelihood estimates due to separation (Firth 1993; Heinze 2006). For experiment 2, we analyzed deformities for stage 42 individuals only, to facilitate comparison of deformity patterns with previous studies (e.g., Johnson et al. 1999, 2001). However, most deformed individuals (60%) in experiment 1 died before reaching stage 42, so for experiment 1, our analyses of deformities included all individuals reaching stage 37 (separation of all toes on hind limbs). For ease of interpretation, we present all proportion results as percentages.

Rate of development was investigated by adapting Cox Proportional Hazards (Cox PH) models used in survival analysis (Parmar and Machin 1996). Our Cox PH models analyzed the hazard (risk) of metamorphosis not occuring (failure to metamorphose by the end of the experiment), censoring individuals that died or did not metamorphose. Thus, hazard of metamorphosis not occuring served as a single measurement of developmental rate that incorporated the time to metamorphosis of individuals that successfully metamorphosed and the proportion of individuals that failed to metamorphose. We used ten separate Cox PH models (see Cox Proportional Hazards models of developmental rate, ESM). Length at tail resorption (developmental stage 46, Gosner 1960), final length (only individuals that survived to 28 d after tail resorption), mass at metamorphosis, and postmetamorphic growth (growth after tail resorption, defined as $\frac{\text{final length} - \text{length at tail resorption}}{\text{length at tail resorption}} \times 100$) met parametric assumptions of normality and homoscedasticity without transformation and were analyzed using ANOVA with and without time to tail resorption included as a continuous covariate. Inclusion of this covariate controlled for length of the larval period. All regression, ANOVA, and Cox PH models included all possible two and three-way interaction terms involving pathogen treatments. Interactions involving covariates were excluded, except that the life stage × BD interaction was included in the analyses of BD infection because frog larvae may lose BD infections during metamorphosis due to resorption of their keratinized mouthparts (Gosner 1960; Warburg et al. 1994).

Results

Experiment 1

Neither larval nor postmetamorphic survival was significantly influenced by *Ribeiroia*, BD, or their interaction

Table 1 Deformities in *Pseudacris regilla* reaching stage 37 in experiment 1, with numbers (and relative frequencies, in percentages) of each deformity type

	R^+/BD$^-$	R^+/BD$^+$	All R^+
Deformity type			
Brachydactyly (abnormally short toe)	1 (12.50)	0 (0.00)	1 (8.33)
Ectrodactyly (completely missing toe)	1 (12.50)	0 (0.00)	1 (8.33)
Polydactyly (extra toe)	1 (12.50)	0 (0.00)	1 (8.33)
Syndactyly (fused toe)	0 (0.00)	0 (0.00)	0 (0.00)
Brachymelia (abnormally short limb)	0 (0.00)	0 (0.00)	0 (0.00)
Polymelia (extra limb or part of limb)	1 (12.50)	1 (25.00)	2 (16.67)
Cutaneous fusion (skin webbing)	1 (12.50)	1 (25.00)	2 (16.67)
Taumelia (bony triangle)	0 (0.00)	1 (25.00)	1 (8.33)
Micromelia (abnormally small limb)	1 (12.50)	0 (0.00)	1 (8.33)
Permanent extension	1 (12.50)	0 (0.00)	1 (8.33)
Permanent flexion	0 (0.00)	1 (25.00)	1 (8.33)
Other hind limb deformities	1 (12.50)[b]	0 (0.00)	1 (8.33)
No. deformed	3	2	5
No. of individuals	14	13	27
Percentage of individuals deformed[a]	21.43 ± 10.97	15.39 ± 10.01	18.52 ± 7.48
Total no. of deformities	8	4	12
No. of deformities per individual[a]	0.57 ± 0.34	0.31 ± 0.21	0.44 ± 0.20
No. of deformities per deformed individual[a]	2.67 ± 0.88	2.00 ± 0.00	2.40 ± 0.51

Ribeiroia is abbreviated as *R*. No R^- individuals were deformed ($n = 13$ and 11 for the R^-/BD$^-$ and R^-/BD$^+$ treatment combinations, respectively). All deformities were hind limb deformities. Deformity categories followed Johnson et al. (2001) with the following exceptions: (1) "limb hyperextension" was not used as a category, (2) "permanent extension" was used for joints that did not fully flex, and (3) "permanent flexion" was used for joints that did not fully extend. Femoral projections and other finger-like projections on limbs were not counted as extra limb elements. To avoid over-counting deformities that may arise from only one deformation event, multiple missing or multiple extra toes on the same foot were counted as only one deformity. Similarly, because taumelia is strongly linked with the occurrence of multiple deformity types distal to the bony triangle, non-taumelia deformities distal to a bony triangle were not counted. No extra limbs or limb elements occurred distal to a bony triangle in either experiment

[a] ±SE

[b] No range of motion in joint

(Fig. 1; all $p \geq 0.34$). However, among individuals reaching stage 37, *Ribeiroia* increased the mean number of deformities per individual by 0.44 (Table 1; $n = 51$, $\chi^2_1 = 8.29$, $p = 0.004$) and the proportion of individuals that were deformed was marginally higher in the *Ribeiroia*$^+$ treatment (18.52%) compared to the *Ribeiroia*$^-$ treatment (0%) ($n = 51$, $\chi^2_1 = 3.76$, $p = 0.052$). Neither the mean number of deformities per individual nor the proportion of individuals that were deformed was significantly influenced by BD or interactive effects of *Ribeiroia* and BD (all $p \geq 0.75$). All deformities were hind limb malformations. Polymelia and cutaneous fusion were the most common types of malformation, each comprising 16.67% of all deformities (Table 1). We found no effects of either pathogen or their interaction on rate of development, body length at tail resorption, final body length, or post-metamorphic growth (ESM Figs. S1, S2; all $p \geq 0.07$).

The proportion of individuals infected with BD increased from zero in the BD$^-$ treatment to 31.25% (SE = 8.19) in the BD$^+$ treatment (logistic regression, $n = 64$, $\chi^2_1 = 7.55$, $p = 0.006$). Among BD-exposed individuals, proportion infected was 38.1% higher in larval than metamorph specimens (percent infected ± 1SE = 53.85 ± 13.83 and 15.79 ± 8.37% for larvae and metamorphs, respectively); however, this difference was not statistically significant ($p \geq 0.33$ for both life stage and the life stage × BD interaction) and we found no evidence that proportion infected was influenced by *Ribeiroia* or a BD × *Ribeiroia* interaction (both $p \geq 0.80$). Among the 10 BD-infected individuals, BD load ranged from 207 to 31,826 ge. Mean load ± 1SE of BD-infected individuals was 610 ge ± 338 among *Ribeiroia*$^-$ larvae ($n = 2$), 16,169 ge ± 15,657 in *Ribeiroia*$^-$ metamorphs ($n = 2$), and 669 ge ± 349 among *Ribeiroia*$^+$ larvae ($n = 5$). The

Table 2 Deformities in *Pseudacris regilla* metamorphs (stage 42) in experiment 2

	All R^-	$A^-/R^+/BD^-$	$A^-/R^+/BD^+$	$A^+/R^+/BD^-$	$A^+/R^+/BD^+$	All R^+
Deformity type						
Cephalic and axial						
Edema	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	1 (3.70)	1 (1.02)
Other deformities	0 (0.00)	1 (3.45)[c]	1 (5.00)[f]	2 (9.09)[h]	1 (3.70)[k]	5 (5.10)
Forelimb						
Ectromely (completely missing limb)	1 (33.33)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Permanent flexion	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	1 (3.70)	1 (1.02)
Other forelimb deformities	0 (0.00)	0 (0.00)	0 (0.00)	2 (9.09)[i]	0 (0.00)	2 (2.04)
Hind limb						
Brachydactyly (abnormally short toe)	0 (0.00)	0 (0.00)	0 (0.0)	1 (4.55)	0 (0.00)	1 (1.02)
Ectrodactyly (completely missing toe)	0 (0.00)	2 (6.90)	1 (5.00)	1 (4.55)	1 (3.70)	5 (5.10)
Polydactyly (extra toe)	0 (0.00)	0 (0.00)	0 (0.00)	1 (4.55)	1 (3.70)	2 (2.04)
Syndactyly (fused toe)	0 (0.00)	2 (6.90)	0 (0.00)	0 (0.00)	0 (0.00)	2 (2.04)
Brachymelia (abnormally short limb)	0 (0.00)	1 (3.45)	2 (10.00)	0 (0.00)	0 (0.00)	3 (3.06)
Hemimelia (partially missing limb)	0 (0.00)	2 (6.90)	3 (15.00)	6 (27.27)	4 (14.82)	15 (15.31)
Ectromelia (completely missing limb)	0 (0.00)	4 (13.79)	2 (10.00)	2 (9.09)	7 (25.93)	15 (15.31)
Polymelia (extra limb or part of limb)	0 (0.00)	0 (0.00)	1 (5.00)	1 (4.55)	2 (7.41)	4 (4.08)
Mirror-image duplication	0 (0.00)	1 (3.45)[d]	0 (0.00)	0 (0.00)	0 (0.00)	1 (1.02)
Femoral projection[a]	0 (0.00)	0 (0.00)	0 (0.00)	2 (9.09)	0 (0.00)	2 (2.04)
Cutaneous fusion (skin webbing)	0 (0.00)	0 (0.00)	1 (5.00)	1 (4.55)	0 (0.00)	2 (2.04)
Taumelia (bony triangle)	0 (0.00)	3 (10.35)	0 (0.00)	0 (0.00)	0 (0.00)	3 (3.06)
Micromelia (abnormally small limb)	1 (33.33)	1 (3.45)	1 (5.00)	1 (4.55)	0 (0.00)	3 (3.06)
Permanent extension	0 (0.00)	3 (10.35)	1 (5.00)	0 (0.00)	2 (7.41)	6 (6.12)
Permanent flexion	1 (33.33)	5 (17.24)	3 (15.00)	0 (0.00)	3 (11.11)	11 (11.23)
Other hind limb deformities	0 (0.00)	4 (13.79)[e]	4 (20.00)[g]	2 (9.09)[j]	4 (14.82)[l]	14 (14.29)
No. deformed	1	8	6	9	10	33
No. of metamorphs	75	13	14	14	13	54
Percentage of metamorphs deformed[b]	1.33 ± 1.32	61.54 ± 13.49	42.86 ± 13.23	64.29 ± 12.81	76.92 ± 11.69	61.11 ± 6.63
Total no. of deformities	3	29	20	22	27	98
No. of deformities per metamorph[b]	0.04 ± 0.04	2.23 ± 0.74	1.43 ± 0.59	1.57 ± 0.44	2.08 ± 0.42	1.82 ± 0.28
No. of deformities per deformed metamorph[b]	3.00 ± 0.00	3.63 ± 0.89	3.33 ± 0.92	2.45 ± 0.48	2.70 ± 0.34	2.97 ± 0.31

Scoring and format follows Table 1. *Achlya* is abbreviated as "*A*". $A^-/R^-/BD^-$, $A^-/R^-/BD^+$, $A^+/R^-/BD^-$, and $A^+/R^-/BD^+$ treatment combinations had samples sizes of 17, 20, 18, and 19 metamorphs, respectively. The single deformed R^- metamorph was in $A^+/R^-/BD^-$

[a] Finger-like appendage (projection) on the dorsal skin of a hind limb

[b] ±SE

[c] Finger-like projection on ventrum near hind limb

[d] Mirror-image duplication of toes in the digit pattern 5-4-3-3-4-5, digits 1 and 2 present

[e] Finger-like projection on lower limb (2 individuals), foot rotated with respect to lower limb (2 individuals)

[f] Bulge in abdomen near hind limb

[g] Abnormally shaped foot, bump on lower limb, finger-like projection on lower limb segment, finger-like projection on truncated limb

[h] Bump on sacrum, finger-like projection on cloacal tail piece

[i] Toe abnormally bent at base and abnormally shaped

[j] Abnormal orientation of joint

[k] Ridge on ventrum near forelimb

[l] Bump on lower limb (2 cases), bump on knee, no range of motion in joint

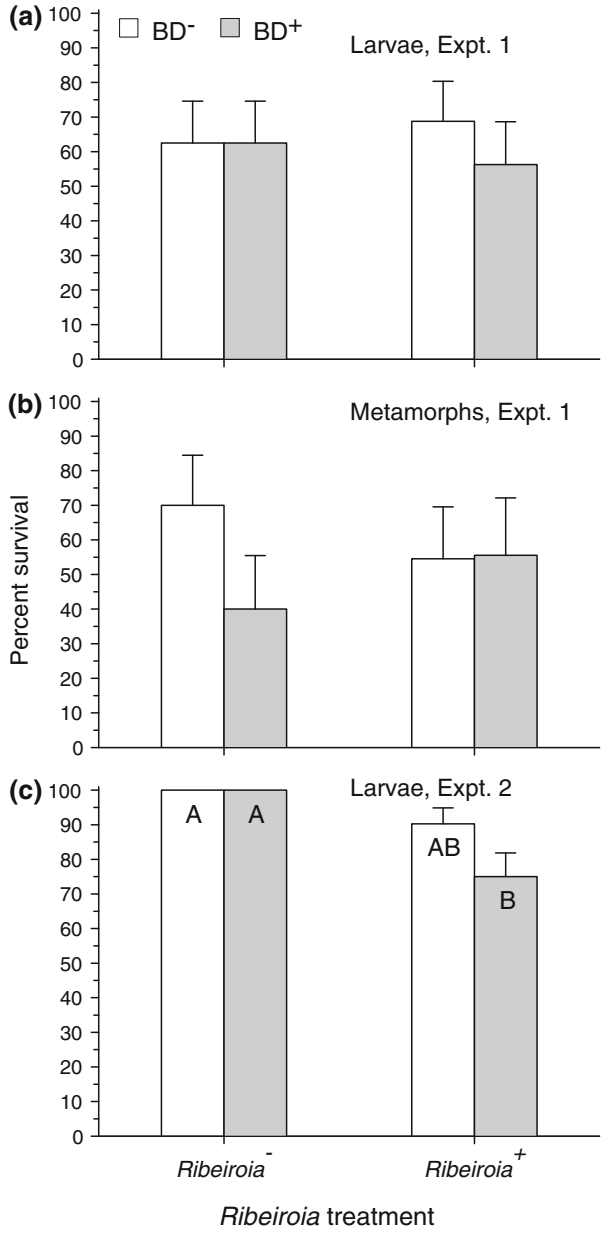

Fig. 1 Experiment 1: survival of *Pseudacris regilla* **a** larvae and **b** metamorphs (mean ± 1SE) in control (−) and exposure (+) treatments of *Ribeiroia* trematodes and *Batrachochytrium dendrobatidis* (BD) fungus. Survival of larvae represents the percentage of all larvae that survived to metamorphosis, while survival of metamorphs represents the percentage of individuals that survived 28 days post-metamorphosis (to the end of the experiment) out of all the individuals that survived to metamorphosis. **c** Experiment 2: survival of *Pseudacris regilla* larvae (mean ± 1SE) in control and exposure treatments of *Ribeiroia* and BD. *Achlya* had no observable effects, so *Achlya* control and *Achlya* exposure treatments are combined for readability. Each of the four treatment combinations presented includes both *Achlya* control and *Achlya* exposure treatments. Treatment combinations that share a *letter* did not differ in survival according to the multiple comparisons procedure ($\alpha = 0.05$)

single BD-infected *Ribeiroia*$^+$ metamorph had a load of 1,883 ge.

Experiment 2

Larval survival

Ribeiroia lowered survival of *P. regilla* larvae by 17% (Fig. 1; overall logistic regression using all treatment combinations, $n = 161$, $\chi^2_1 = 10.45$, $p = 0.001$). The odds of dying were 2.91 (95% CI: 1.48–7.46) times greater for larvae exposed to *Ribeiroia* compared to those not exposed to *Ribeiroia*. The overall logistic regression found no evidence that larval survival was influenced by BD, *Achlya*, or interactive effects of pathogens (BD: $p = 0.495$; *Ribeiroia* × BD: $p = 0.495$; all other $p = 0.774$). However, survival was lowest in the *Ribeiroia*$^+$/BD$^+$ treatment combination (75%) and the multiple comparisons procedure assessing survival among *Ribeiroia*/BD combinations was partially consistent with an interactive effect of *Ribeiroia* and BD. Specifically, *Ribeiroia* had a significant effect on survival only when BD was present (Fig. 1; $p = 0.052$ in the test for a *Ribeiroia* effect when BD was absent, $p < 0.001$ in the test for a *Ribeiroia* effect when BD was present). This suggests that *Ribeiroia* and BD acted synergistically to reduce survival either through BD allowing *Ribeiroia* to have a negative effect or by BD intensifying the negative effect of *Ribeiroia*. However, survival of *Ribeiroia*-exposed larvae was not significantly different when they were also exposed to BD compared to when BD was absent ($p = 0.075$, far from statistical significance after Bonferroni adjustment), which is inconsistent with a *Ribeiroia*-BD synergism. Pairwise comparisons of survival among *Ribeiroia*/BD combinations had a critical p value (threshold p value for rejection of null hypotheses) of 0.0083 after Bonferroni adjustment for six pairwise comparisons.

Deformities

Ribeiroia raised the proportion of *P. regilla* metamorphs that were deformed by 60%, which amounted to a 46-fold increase (Table 2; logistic regression, $n = 129$, $\chi^2_1 = 58.59$, $p < 0.0001$). The odds of having one or more deformities were 6.73 (95% CI: 3.67–16.10) times greater for *Ribeiroia*$^+$ individuals compared to *Ribeiroia*$^-$ individuals. Similarly, *Ribeiroia* increased the mean number of deformities per individual by 1.78 (Poisson log-linear regression, $n = 129$, $\chi^2_1 = 105.87$, $p < 0.0001$). There was little evidence that the proportion of metamorphs that were deformed or the mean number of deformities per metamorph were influenced by BD, *Achlya*, or interactive

effects among pathogens (all $p \geq 0.36$). Only one *Ribeiroia*$^-$ individual was deformed; it had an abnormally small hind limb with a permanently flexed joint and was missing a forelimb (Table 2).

Deformities in the Ribeiroia$^+$ *treatment*

Among the *Ribeiroia*-exposed metamorphs exhibiting malformations, hind limb deformities dominated; most (32 of 33, 96.96%) deformed metamorphs had at least one limb deformity, most (92 of 98, 93.88%) deformities were limb deformities, and most (89 of 92, 96.74%) limb deformities involved a hind limb rather than a forelimb (Table 2). Hind limb ectromelia (completely missing limb) and hind limb hemimelia (partially missing limb) were the most common malformations, each comprising 15.31% of all deformities.

Rate of development

Ribeiroia slowed larval development, as demonstrated by increased hazard of metamorphosis not occurring in *Ribeiroia*$^+$ compared to *Ribeiroia*$^-$ individuals (Cox PH model 2, $n = 161$, $\chi^2_1 = 15.71$, $p < 0.0001$). We suggest this effect arose from a combination of increased time to metamorphosis in individuals that successfully metamorphosed and an increased proportion of larvae failing to metamorphosis by the end of the experiment (Fig. 2). Hazard of metamorphosis not occurring was 2.03 times higher for *Ribeiroia*$^+$ relative to *Ribeiroia*$^-$ individuals. In addition, BD exposure accelerated development by decreasing time to metamorphosis in individuals that successfully metamorphosed and increasing the proportion of larvae that successfully metamorphosed (Cox PH model 2, $\chi^2_1 = 8.08$, $p = 0.005$). Hazard of metamorphosis not occurring was 1.67 times higher for BD$^-$ relative to BD$^+$ individuals. Evidence that *Achlya* or interactive effects among pathogens influenced rate of development was weak at best (all $p \geq 0.07$), so the *Achlya* factor was dropped from the analysis and subsequent pairwise comparisons of developmental rate were made for the four remaining treatment combinations (Fig. 2). Pairwise comparisons of developmental rate among *Ribeiroia*/BD combinations (Fig. 2) had a critical p value of 0.0083 after Bonferroni adjustment for six pairwise comparisons.

Ribeiroia$^+$ individuals that did not die or display deformities nevertheless developed more slowly than *Ribeiroia*$^-$ individuals (Cox PH model 9, $n = 107$, $\chi^2_1 = 12.26$, $p = 0.001$, data not shown), indicating that *Ribeiroia* delayed development even in *P. regilla* that did not show obvious pathology. Hazard of metamorphosis not occurring was 2.34 times higher for *Ribeiroia*$^+$ individuals that did not die or show deformities relative to *Ribeiroia*$^-$ individuals. We did not find effects of deformity status, BD, or interactions on rate of development in this restricted dataset (*Ribeiroia*$^+$ individuals that did not die or exhibit deformities and *Ribeiroia*$^-$ individuals, Cox PH model 9, all $p \geq 0.13$). Among *Ribeiroia*$^+$ *P. regilla*, deformity status, *Achlya*, BD, and interactions all were all non-significant predictors of developmental rate (Cox PH model 10, $n = 72$, all $p \geq 0.12$, data not shown).

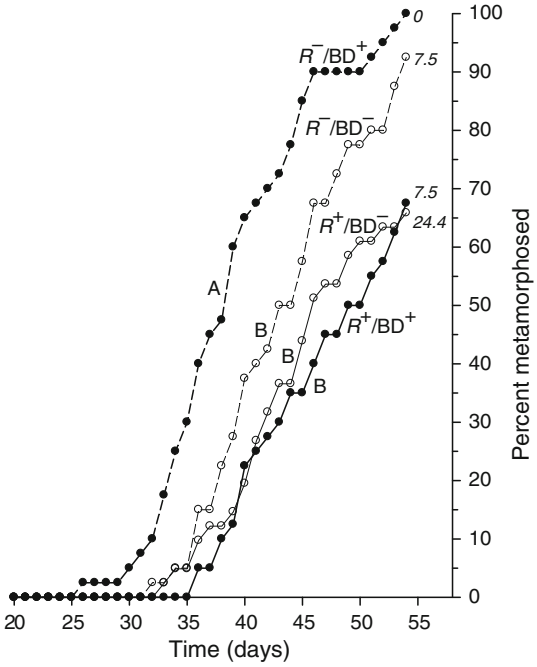

Fig. 2 Percentage of *Pseudacris regilla* larvae reaching metamorphosis over time in experiment 2. Percentages are out of the total number of individuals at the start of the experiment. Italicised numbers denote the percentage of larvae that did not metamorphose despite surviving. *Dashed* and *solid lines* represent, control (−) and exposure (+) treatments of *Ribeiroia*, respectively, while *open* and *closed circles* represent control (−) and exposure (+) treatments of *Batrachochytrium dendrobatidis* (BD). *Line labels* use the abbreviation *R* for *Ribeiroia*. *Achlya*$^-$ and *Achlya*$^+$ treatments are combined as in Fig 1. Rates of development were analyzed using Cox PH models that incorporated whether or not metamorphosis occurred and, if so, the time to metamorphosis. Treatment combinations that share a letter did not differ in hazard of metamorphosis not occurring according to the multiple comparisons procedure (Cox PH models 3–8, $\alpha = 0.05$)

Mass at metamorphosis

There was little evidence that mass at metamorphosis was influenced by any treatments or interactions, regardless of whether the time to metamorphosis covariate was included (ESM Fig. S3, $n = 129$, all $p \geq 0.07$).

Infection

Hyphae were observed on only one live individual. This individual was in the *Ribeiroia*$^+$/*Achlya*$^+$/BD$^+$ combination

and had white hyphal growth on the foot of its left forelimb. No clearly identifiable oomycete reproductive structures were visible by microscopy, but coenocytic hyphae consistent with *Achlya* infection were abundant. No BD^+ individuals showed infection in the PCR assays, indicating that they were not infected when preserved and thus either failed to become infected or lost their infections prior to preservation. Also, no BD^- or negative control individuals showed infection, but all positive controls did (see Additional controls in PCR assays for BD, ESM).

Discussion

In this study, we found no strong evidence that *Ribeiroia*, BD, or *Achlya* had interactive effects on *P. regilla* hosts. The greatest evidence for interactive effects was in experiment 2, in which the multiple comparisons analysis of larval survival was partially consistent with a *Ribeiroia*–BD synergism. Survival among all *Ribeiroia*/BD combinations was lowest in larvae exposed to both *Ribeiroia* and BD. Furthermore, according to the multiple comparisons analysis of survival, *Ribeiroia* caused significant mortality only when BD was present. However, the multiple comparisons did not fully support a *Ribeiroia*–BD synergism, because it did not detect a difference in survival of *Ribeiroia*-exposed larvae when BD was present compared to when it was absent. Most importantly, the direct test for a *Ribeiroia* × BD interaction, from the overall logistic regression analysis, was far from significance. Therefore, we do not have sufficient statistical evidence to conclude that *Ribeiroia* and BD had an interactive effect. Furthermore, the non-significance of the pairwise comparison that tested for an effect of *Ribeiroia* when BD was absent should be interpreted cautiously. This test had less power than the overall regression, due to a lower sample size and the Bonferroni adjustment, so it does not provide evidence against the overall regression's finding of a significant effect of *Ribeiroia* that was not significantly altered by the presence or absence of BD. However, we highlight the observed pattern of low survival when both *Ribeiroia* and BD were present as warranting further investigation.

Although we did not find clear evidence for interactive effects of pathogens, we did find main pathogen effects of *Ribeiroia* on survival and deformities and main effects of both *Ribeiroia* and BD on developmental rate, the sole endpoint that was significantly influenced by the combined effects of multiple pathogens. Developmental rate was not significantly affected by any of the pathogens in experiment 1, but in experiment 2, the combined effects of *Ribeiroia* and BD on developmental rate were additive, although opposite in direction. However, as we explain below, the additive effects on developmental rate found in this laboratory study could translate into synergistic or less-than-additive effects in nature.

Among our set of pathogens, *Ribeiroia* had the greatest number of effects on *P. regilla* hosts. One-time exposure to small numbers of *Ribeiroia* cercariae (12) in experiment 1 caused malformations and one-time exposure to slightly higher numbers of *Ribeioroia* cercariae (15) in experiment 2 caused mortality and delayed development in addition to deformities, supporting previous findings of dose-dependent effects of *Ribeiroia* on survival and morphology in amphibians (Johnson et al. 1999, 2001; Johnson and Buller 2010). Many of these deformities, especially missing limbs, extra limbs, and joint abnormalities, severely impede locomotion and make survival in nature highly unlikely (Johnson and Lunde 2005). Direct mortality and indirect mortality via malformations could contribute to population declines in *Ribeiroia*-sensitive species, but more research is needed to determine the influence of this emergent trematode on amphibian populations (Johnson and Lunde 2005).

Ribeiroia might contribute to amphibian population declines not only by causing mortality and deformities but also by slowing development. Our finding that *Ribeiroia* delayed development of *P. regilla* larvae in experiment 2 is consistent with negative effects on rate of development previously found in frog and salamander larvae (Johnson et al. 2006, 2008; Johnson and Hartson 2009). Although the effects of *Ribeiroia* on developmental rate are more subtle than their effects on survival and deformities, they could lead to substantial effects at the population level because they may ultimately impair survival, growth, and reproduction, as evidenced by the positive correlations found between rapid metamorphosis and adult recruitment and fitness in amphibians (Smith 1987; Semlitsch et al. 1988). If density-dependent survival limits the number of larvae that successfully metamorphose or eventually reach adulthood (Wilbur 1977; Petranka and Sih 1986), delayed development might have a greater negative effect on population size and persistence than direct mortality or mortality resulting from severe malformations (Johnson and Lunde 2005).

Delayed development following *Ribeiroia* exposure might occur because amphibians shift resources away from development toward repair of wounds caused by entry of *Ribeiroia*. Lower doses of *Ribeiroia* in experiment 1 did not alter developmental rate, perhaps because lower numbers of cercariae cause less tissue damage that demands fewer resources to repair. We found no evidence that the rate of development among *Ribeiroia*-exposed *P. regilla* in experiment 2 was related to the presence or absence of limb deformities, BD treatment, *Achlya* treatment, initial developmental stage, or their interactions. Thus, limb deformities resulting from exposure to *Ribeiroia* were not

associated with delayed development, suggesting that the mechanisms by which *Ribeiroia* alters morphology and developmental rate are somewhat independent.

Our results further suggest that *Ribeiroia* slows amphibian development even when it does not cause deformities. In addition to slowing development overall, *Ribeiroia* in experiment 2 delayed development of *P. regilla* that did not die or show obvious limb deformities, suggesting that *Ribeiroia* might have insidious effects on amphibian populations by slowing development in lesser-affected individuals. Such an effect may lead to severe consequences for populations that breed in temporary aquatic habitats because their larvae will die if they fail to metamorphose before the habitat dries (Blaustein et al. 2001). Moreover, delayed development could lengthen the amount of time amphibian larvae are exposed to *Ribeiroia* cercariae, likely increasing *Ribeiroia* infection load and the severity of *Ribeiroia*-induced effects, including delayed development, thus creating a positive feedback. Importantly, limb deformities might be particularly influenced by delayed development. Frog larvae are susceptible to *Ribeiroia*-induced limb deformities only if *Ribeiroia* infects during early hind limb development (Schotthoefer et al. 2003; Bowerman and Johnson 2003). Because the effects of *Ribeiroia* on limb development in frogs are dose-dependent (Johnson et al. 1999, 2001), extending the amount of time larvae are in these sensitive stages of development likely would increase the prevalence, number, and severity of limb deformities in habitats where *Ribeiroia* is present. Moreover, slow-developing larvae would have more time for exposure to other pathogens, potentially creating synergistic yet indirect interactions between *Ribeiroia* and other pathogens (Johnson and Buller 2010).

However, not all pathogen effects on developmental rate were negative. Unlike exposure to *Ribeiroia*, exposure to low doses of BD in experiment 2 accelerated development. Although we did not detect a significant difference in developmental rate between BD^+ and BD^- individuals when *Ribeiroa* was present, our omnibus Cox PH results found that the effects of BD and *Ribeiroia* were opposite in direction, suggesting that BD exposure partially counteracted the negative effects of *Ribeiroia.* Yet, none of the BD^+ *P. regilla* in experiment 2 tested positive for BD infection, even though all positive control individuals and some BD^+ individuals in experiment 1 showed infection, indicating that our sampling and PCR methods were capable of detecting BD. Most experiment 2 samples were from individuals that were at a relatively advanced larval stage (stage 42, Gosner 1960) and had lost their larval mouthparts. During metamorphosis of frog larvae, keratin is lost via resorption of the keratinized mouthparts (Gosner 1960; Warburg et al. 1994), which may have eliminated BD infection and thereby prevented its detection in BD-exposed animals. Nevertheless, our results suggest that exposure to the BD dose used in experiment 2 increased the rate of host development. In nature, such a response could be advantageous to the host. Hastened metamorphosis in the presence of BD could speed the departure of hosts from habitats with high densities of BD zoospores or BD-infected individuals and thus could reduce the risk of BD infection or, for hosts already infected, the risk of infection becoming more severe. Such compensatory host responses to the presence of BD could decrease the impact of BD on amphibian populations.

Based on our findings of increased developmental rate following exposure to BD, we suggest that amphibian larvae can speed up development in response to increased disease risk. This seems likely because researchers have already discovered similar kinds of phenotypic plasticity in amphibians (Wilbur and Collins 1973). Adult and larval amphibians can respond behaviorally to increased disease risk (e.g., Kiesecker et al. 1999; Pfennig 2000; Rohr et al. 2009), and embryonic amphibians can decrease time to hatching in response to pathogens (behavioral hatching, Warkentin et al. 2001; Touchon et al. 2006; Gomez-Mestre et al. 2006). Amphibian larvae can also speed up development in response to falling water levels, low food resources, or the presence of predators or competitors (Sokol 1984; Werner 1986; Newman 1988; Crump 1989a, b; Rohr et al. 2004). However, the net effect of BD on rate of development might depend on infection status. In experiments in which BD infection is confirmed, the net effect of BD on rate of development in amphibian larvae has been negative (e.g., Parris and Baud 2004; Parris and Beaudoin 2004; Parris and Cornelius 2004) or neutral (experiment 1), not positive as in experiment 2. Perhaps exposure to a threshold level of BD induces faster host development, but once the host becomes infected its development slows due to the physiological effects of infection.

Our study moves beyond the single-pathogen approach that has dominated investigations of disease ecology in amphibians (e.g., Kiesecker and Blaustein 1995; Rachowicz et al. 2006; Johnson et al. 2007) by testing how a single host is affected by pathogen combinations. Although we found that when *Ribeiroia* and BD had combined effects those effects were additive, different levels of exposure might result in interactive effects of these pathogens. Furthermore, abiotic environmental stressors, which were not manipulated in our study, can strongly influence susceptibility to disease in amphibians (e.g., Kiesecker 2002; Rohr et al. 2008c) and might control whether interactions between pathogens occur. Nevertheless, we did find effects on development that could lead to interactive effects in nature. Hastened development from BD exposure will shorten the amount of time larvae spend in aquatic habitats with *Ribeiroia* cercariae, which could

reduce *Ribeiroia* loads and lessen effects of *Ribeiroia*. On the other hand, *Ribeiroia*-induced delays in development might trap frog larvae in aquatic habitats where BD infection risk is high. Thus, the net effects of *Ribeiroia* and BD on amphibians in nature could be less-than-additive or synergistic, perhaps depending on intensity of exposure to each pathogen.

Besides potentially controlling the presence of interactive pathogen effects, dose-dependency also appeared to play a role in the complex set of main effects we detected in our laboratory study, as *Ribeiroia* and BD affected survival, infection, deformities, and developmental rate differently in the two experiments, which used different pathogen doses. In particular, the effects of BD on larval development might depend on dose and whether or not larvae become infected. Thus, we need further experiments that examine a range of BD and *Ribeiroia* exposure regimes to evaluate how these two pathogens combine to affect amphibians, especially considering that our study provides some evidence consistent with a synergistic effect of these two pathogens on larval survival. Such a synergism between these widespread pathogens could have severe negative consequences for amphibian populations. Furthermore, we emphasize that our study focused on a single host species and three pathogen species. Researchers must investigate a diversity of pathogens and hosts to adequately evaluate how multiple pathogens might act in combination to influence the population-level biology of amphibians, especially when additional stressors such as predators and contaminants are present.

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