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DISEASES OF AQUATIC ORGANISMS
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Complex interactive effects of water mold, herbicide, and the fungus *Batrachochytrium dendrobatidis* on Pacific treefrog *Hyliola regilla* hosts

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ABSTRACT: Infectious diseases pose a serious threat to global biodiversity. However, their ecological impacts are not independent of environmental conditions. For example, the pathogenic fungus *Batrachochytrium dendrobatidis* (*Bd*), which has contributed to population declines and extinctions in many amphibian species, interacts with several environmental factors to influence its hosts, but potential interactions with other pathogens and environmental contaminants are understudied. We examined the combined effects of *Bd*, a water mold (*Achlya* sp.), and the herbicide Roundup® Regular (hereafter, Roundup®) on larval Pacific treefrog *Hyliola regilla* hosts. We employed a 2 wk, fully factorial laboratory experiment with 3 ecologically realistic levels (0, 1, and 2 mg l^{-1} of active ingredient) of field-formulated Roundup®, 2 *Achlya* treatments (present and absent), and 2 *Bd* treatments (present and absent). Our results were consistent with sublethal interactive effects involving all 3 experimental factors. When Roundup® was absent, the proportion of *Bd*-exposed larvae infected with *Bd* was elevated in the presence of *Achlya*, consistent with *Achlya* acting as a synergistic cofactor that facilitated the establishment of *Bd* infection. However, this *Achlya* effect became nonsignificant at 1 mg l^{-1} of the active ingredient of Roundup® and disappeared at the highest Roundup® concentration. In addition, Roundup® decreased *Bd* loads among *Bd*-exposed larvae. Our study suggests complex interactive effects of a water mold and a contaminant on *Bd* infection in amphibian hosts. *Achlya* and Roundup® were both correlated with altered patterns of *Bd* infection, but in different ways, and Roundup® appeared to remove the influence of *Achlya* on *Bd*.

KEY WORDS: Amphibian decline · Multipathogen · Sapronosis · Oomycete · Environmental stressor

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INTRODUCTION

Infectious diseases comprise one of the greatest threats to biodiversity conservation, through their direct effects on host populations as well as community and ecosystem-mediated effects on non-host species (Smith et al. 2009a, Cobb et al. 2012). However, pathogens do not act upon hosts independent of other environmental factors. The etiology of infectious diseases in general is multifactorial; disease is produced by the interactive effects of pathogens and environmental conditions on hosts (Dobson &

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Foufopoulos 2001, Altizer et al. 2013). In some host–pathogen systems, the impact of one pathogen on host individuals or populations is exacerbated, or in some cases reduced, by a second pathogen, a parasite, or an abiotic stressor (Acevedo-Whitehouse & Duffus 2009, Telfer et al. 2010, Maas et al. 2012, Fenton 2013). However, the interactive effects of multiple pathogens on hosts and how these effects vary over environmental gradients are poorly understood. We tested for interactive effects in a host–pathogen–stressor system that included amphibian hosts, the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*), a potentially pathogenic water mold in the genus *Achlya*, and the herbicide Roundup® Regular.

Bd, a pathogenic fungus associated with population declines of many amphibian species (Van Rooij et al. 2015), is considered by some to be the world's single most destructive pathogen in terms of biodiversity loss (Smith et al. 2009b, Hyatt et al. 2010, Kilpatrick et al. 2010). *Bd* is the main pathogen that causes the disease known as amphibian chytridiomycosis (Longcore et al. 1999), although a recently discovered congener, *B. salimandrivorans*, also causes the disease (Martel et al. 2013). Spread of virulent strains of *Bd* into naïve host populations appears to have aided the emergence of this disease as a global conservation threat (Farrer et al. 2011). The effects of *Bd* on amphibians are influenced by several biotic and abiotic factors, including host species (Gervasi et al. 2013), population (Bradley et al. 2015) and life-history stage (Searle et al. 2013), host diversity (Searle et al. 2011), predators of *Bd* zoospores (Buck et al. 2011), amphibian skin bacteria (Harris et al. 2009), temperature (Woodhams et al. 2003, Raffel et al. 2013, Catenazzi et al. 2014), ultraviolet-B radiation (Ortiz-Santaliestra et al. 2011), moisture (Bustamante et al. 2010), and environmental contaminants (McMahon et al. 2013). However, few studies have investigated possible interactive effects of *Bd* and other pathogens or parasites on amphibians (but see Romansic et al. 2011, Paetow et al. 2013), despite observations of amphibians co-infected with *Bd* and other pathogens (e.g. Nieto et al. 2007, Reshetnikov et al. 2014, Rothermel et al. 2016, Warne et al. 2016).

We tested the combined effects of *Bd* and a water mold species isolated from a natural amphibian breeding site in the central Oregon Cascade Range, USA, and identified to the genus *Achlya* using morphological characteristics (Johnson et al. 2002). Several species of *Achlya* are facultative parasites that infect fish (Johnson et al. 2002) and some species in this genus infect amphibians (Tiffney & Wolf 1939, Czeczuga et al. 1998, Petrisko et al. 2008). Water molds use live and dead amphibians of all life-history stages as substrates for growth and zoospore production. Heavy infections can produce growths of whitish, thread-like hyphae on embryos and the skin of larvae and post-metamorphic individuals (Blaustein et al. 1994, Berger et al. 2001, Kim et al. 2008, Ruthig 2009). Besides killing amphibians (Kiesecker & Blaustein 1995, Romansic et al. 2006, 2007), water molds can also produce sublethal effects in amphibian hosts that may make them more susceptible to other stressors such as *Bd*. For example, embryos exposed to water mold can hatch sooner, resulting in hatchlings that are smaller and less advanced in development (Gomez-Mestre et al. 2006). Furthermore, Uller et al. (2009) demonstrated that moor frog *Rana arvalis* exposed to water mold at the embryo life-history stage do not hatch earlier but still exhibit decreased mass at metamorphosis, suggesting that water mold exposure early in development produces long-lasting sublethal effects. In addition, exposure to water molds could damage the epidermis of larval and post-metamorphic amphibians and aid colonization and growth of *Bd*, even if water mold infection does not become established.

Pathogenic water molds are most accurately described as sapronotic disease agents (sensu Kuris et al. 2014) because of their ability to grow and reproduce saprobically on dead organic matter (Johnson et al. 2002), which allows them to persist in the absence of live hosts (Gleason et al. 2014) and may also allow them to reach high zoospore densities in amphibian breeding habitats. Indeed, the shallow littoral zones of lentic amphibian breeding habitats often accumulate large numbers of dead amphibian embryos laden with water mold (e.g. Blaustein et al. 1994). In addition, the larvae of many amphibian species remain attached to their embryo jelly mass after hatching, where they may receive extremely large doses of water mold zoospores because of their proximity to dead embryos, especially when large aggregates of communally laid embryo masses are present and embryo mortality is high (e.g. Kiesecker & Blaustein 1997).

Few studies have investigated how water molds and environmental contamination combine to affect amphibians (but see Romansic et al. 2006, Karraker & Ruthig 2009), but a variety of contaminants, including heavy metals, insecticides, fungicides, and herbicides have been tested as cofactors for amphibian chytridiomycosis (e.g. Parris & Baud 2004, Buck et al. 2012, McMahon et al. 2013). Most of these studies have found that the contaminant did not magnify the effects of *Bd* (but see Rohr et al. 2013, Wise et al.

2014, Buck et al. 2015). Indeed, contaminants that have direct negative effects on *Bd*, such as the antimicrobial triclosan, the herbicide atrazine, and the fungicide chlorothalonil, appear to decrease *Bd* infection load or diminish the negative effects of *Bd* on amphibian survival when initial *Bd* exposure occurs in the presence of the contaminant (Brown et al. 2013, McMahon et al. 2013). Similarly, none of the few experimental studies on the combined effects of contaminants and water molds on amphibians have detected synergistic effects (e.g. Romansic et al. 2006, Puglis & Boone 2007, Karraker & Ruthig 2009). Indeed, abiotic stressors may in general reduce impacts on hosts from pathogens and parasites such as *Bd* and water molds that have small, free-living propagules because of the high sensitivity of these life-history stages to environmental stressors (Lafferty & Kuris 1999, Lafferty & Holt 2003). However, there are notable counter-examples. In amphibians, temperature fluctuations can increase *Bd* infection loads (Raffel et al. 2013), and ultraviolet-B radiation magnifies water mold-induced embryo mortality (Kiesecker & Blaustein 1995). Thus, reliance of a pathogen on small, free-living stages such as zoospores does not necessarily preclude it from interacting synergistically with an abiotic stressor.

We used the herbicide Roundup® Regular as the abiotic stressor in our study because it is representative of the glyphosate-based class of herbicides, which have received considerable attention for their direct negative effects on amphibian survival (reviewed in Relyea 2011). Glyphosate-based herbicides are one the world's most heavily used classes of herbicides (Grube et al. 2011). The active ingredient glyphosate has low toxicity to amphibians (Mann & Bidwell 1999, Howe et al. 2004), but glyphosate-based herbicides are usually applied in field formulations that contain surfactants such as polyethoxylated tallow amine, which is in Roundup® Regular. Various field formulations of Roundup® have caused mortality and sublethal effects on growth in amphibians when tested at ecologically realistic concentrations (e.g. Mann & Bidwell 1999, Cauble & Wagner 2005, Relyea 2005a). Furthermore, the lethal toxicity of Roundup® formulations is magnified by natural biotic stressors, including predators and competitors (Relyea 2005b, Jones et al. 2011). Therefore, we hypothesized that Roundup® herbicides might cause sublethal damage to amphibians that increases their susceptibility to becoming infected with *Bd* or *Achlya*, inhibits their ability to keep infection load low, or decreases their ability to tolerate infection without dying. However, Roundup® Regular had direct negative effects on production of *Bd* zoosporangia and zoospores in a previous study (Hanlon & Parris 2012), and we hypothesized that the herbicide might affect *Achlya* similarly. Thus, the net effects of Roundup® herbicides in combination with *Bd* and *Achlya* on amphibian hosts are difficult to predict.

Larvae of Pacific treefrog *Hyliola* (*Pseudacris*) *regilla* were used as hosts because they potentially play a key role in *Bd* transmission dynamics. Larval and post-metamorphic *H. regilla* can be infected by *Bd*, but can often tolerate high *Bd* infection loads without dying (Reeder et al. 2012). Although *Bd* has caused mortality and sublethal effects in *H. regilla* in experiments (e.g. Kleinhenz et al. 2012, Gervasi et al. 2013, Searle et al. 2013, Buck et al. 2015), no *Bd*-associated mass mortality events have been observed in this species. Indeed, populations of *H. regilla*, including *Bd*-infected individuals free of obvious disease, persist during *Bd*-associated extirpations of southern mountain yellow-legged frog *Rana muscosa*, suggesting that *H. regilla* is a reservoir host that maintains *Bd* and transmits it to other host species that are more sensitive to the pathogen (Reeder et al. 2012). Thus, identification of factors that influence *Bd* infection status and load in *H. regilla*, a relatively common amphibian species with a wide geographic range, could aid control of chytridiomycosis in *Bd*-sensitive species that co-occur with *H. regilla*. For example, managers might be able to remove or lessen factors found to increase *Bd* zoospore production in *H. regilla* and thereby reduce the risk of infection in co-occurring amphibian species.

MATERIALS AND METHODS

Collection and maintenance of *Hyliola regilla*

Thirty-two masses of *Hyliola regilla* embryos (developmental stages 16–20 according to Gosner 1960) were collected on 1 June 2007 at Little Three Creek Lake, a natural amphibian breeding site in the Deschutes National Forest in the Central Oregon Cascade Range (44.102° N, 121.642° W), 26.4 km west northwest of Bend, Oregon. Prior to experimentation, *H. regilla* were kept in 38 l glass aquaria filled with approximately 35 l of aerated water (8 embryo clutches per aquarium) and transferred to new tanks with new water every 7–8 d. Four days after completion of hatching, the larvae in each aquarium were evenly divided between 2 new tanks and maintained thereafter at a density of 1.6–5.7 larvae l^{-1} of water. Before and during experimentation, larvae were fed a mixture (3:1 by volume) of rabbit chow and Tetramin® (Tetra) fish flakes and kept under a natural photoperiod at 14.5–17.0°C. Water, unless otherwise noted, was dechlorinated tapwater conditioned with NovAqua® and Amquel® (Novalek; 0.12 ml of each conditioner per l of water) to remove any residual chlorine, protect against pH changes, and prevent buildup of ammonia.

Pathogen sources

Water mold was isolated from a water sample taken on 20 May 2007 at Lost Lake in the Willamette National Forest (44.434° N, 121.901° W), 32.1 km northwest of Sisters, Oregon. Isolation used sterile hemp seeds and yeast-glucose agar media following Fuller & Jaworski (1987). We identified the resulting water mold isolate as a member of the genus *Achlya* using available keys and standard methods (Johnson 1956, Johnson et al. 2002). *Achlya* dosages were prepared by adding a yeast-glucose agar plug containing actively growing *Achlya* hyphae to each of 35 sterile, standard-sized (diameter = 9 cm) Petri dishes filled with 46 ml of sterile ultrapure water and 30 sterile hemp seeds. *Achlya* dosages were incubated at 21.5–23.0°C for 11 d, which produced clumps of seeds connected by *Achlya* hyphae. *Bd* isolate JEL 274 (originally isolated from *Anaxyrus boreas* from Colorado) was grown on sterile, standard-sized (diameter = 9 cm) Petri dishes containing 1% tryptone agar media. *Bd* cultures were incubated for 10 d at 21.0–23.5°C and subsequently maintained at 4–5° C for 12 d before experimentation to prevent overgrowth.

Experimental design

We used a 2 × 2 × 3 randomized factorial design with 2 treatments (present and absent) for *Bd* and *Achlya* and 3 treatments of Roundup® Regular (Monsanto; hereafter Roundup®) with nominal Roundup® concentrations of 0, 1, and 2 mg active ingredient l^{-1} (hereafter; a.i. l^{-1}), which are equal to 0, 0.75, and 1.5 acid equivalents a.i. l^{-1}). These ecologically realistic levels are within the range of glyphosate active ingredient concentrations measured in aquatic habitats, although they are close to the upper limit of this range (Thompson et al. 2004, Relyea 2006). The experiment had 5 replicates of each treatment combination, for a total of 60 experimental units. Each experimental unit consisted of a 9 l aquarium filled (tank) with 2 l of water and stocked with 6 *H. regilla* larvae. Groups of 6 larvae were chosen haphazardly from laboratory stocks (33–49 d post-hatching; developmental stages 25–29 [Gosner 1960]; mean weight ± SE = 117 ± 12 mg; mean total length ± SE = 15.3 ± 0.9 mm) and then randomly assigned to experimental units.

To ensure that food was always available, we added 25 mg of food per live larva at the start of the experiment and 5 and 9 d later. Larvae were counted and examined visually for hyphae consistent with *Achlya* infection daily. The rest of the contents of the aquaria were also inspected daily for hyphae consistent with the growth of *Achlya* or other water molds. Dead larvae were removed and preserved in 70% ethanol. After 14 d, the experiment was ended and surviving larvae were euthanized using MS-222 and preserved in 70% ethanol. All preserved specimens were re-examined visually for hyphae, and each preserved specimen not used for quantification of *Bd* infection was re-examined again for hyphae under a dissecting microscope at 10× and 40× magnification for at least 5 min. We used visual and microscopic examination to check for *Achlya* infection because molecular methods for quantifying *Achlya* infection have not yet been developed. Visual and microscopic examination are often sufficient to detect water mold infection (e.g. Blaustein et al. 1994, Berger et al. 2001, Gomez-Mestre et al. 2006). Indeed, Romansic et al. (2006) found that visual inspection of live larvae combined with examination of dead individuals under a dissecting microscope successfully identified hyphae consistent with infection by the water mold *Saprolegnia diclina* in northern red-legged frog *Rana aurora*. *Bd* infection status (infected or not infected) and load were measured using quantitative real-time PCR (Boyle et al. 2004, Hyatt et al. 2007) on preserved specimens for a subset of individuals in the *Bd*-absent treatment and all individuals in the *Bd*-present treatment, except for 4 that died and were completely eaten by conspecifics before they could be removed from experimental units and 2 for which preserved specimens were lost because of experimental error (see 'Quantification of *Bd* infection'). Following Catenazzi et al. (2014), we scored a sample as infected with *Bd* if any amount of *Bd* was detected.

Application of treatments

Each of 60 9 l glass aquaria were randomly assigned to a treatment combination and filled with 2 l of water containing the appropriate concentration of Roundup® prior to addition of larvae. Roundup®

solutions were prepared using a 100 mg a.i. l^{-1} stock solution of commercially obtained Roundup® diluted with ultrapure water. Due to the large volume of water required for the experiment, Roundup® concentrations of 1 and 2 mg a.i. l^{-1} were each prepared by adding the appropriate amount of stock solution (248 and 500 ml, respectively) to each of two 38 gallon glass aquaria containing 24.5 l of water and mixing using a glass stirring rod. Water from the 2 aquaria was then homogenized by transferring water back and forth between the 2 aquaria using clean glass beakers. Control water containing no Roundup® was prepared for the 0 mg a.i. l^{-1} Roundup® treatment in the same way, except that no Roundup® stock solution was added.

Each aquarium in the *Achlya*-present treatment received 30 *Achlya*-laden hemp seeds lifted out of their incubation dish using stainless steel clean forceps. Aquaria in the *Achlya*-absent treatment each received 30 hemp seeds treated identically to those in the *Achlya*-present treatment, except that they were prepared using a sterile agar plug. *Bd* inoculum was obtained by flooding each of 35 Petri dishes containing *Bd* isolate JEL 274 with 2.0 ml of ultrapure water, stirring the dishes, and combining the resulting zoospore solutions. Each aquarium in the *Bd*-present treatment received 1.0 ml of *Bd* inoculum. We treated *Bd*-absent aquaria the same way, except that they each received a sham inoculum from sterile Petri dishes containing 1% tryptone agar media.

Larvae were transferred to new aquaria with new water lacking Roundup® and pathogen treatments after 8 d. For simplicity, treatments were not renewed; thus, the experiment employed pulse-type exposures to Roundup® and the pathogens. However, we expect that the *Achlya* included a press-type component because *Achlya* inocula likely continued to release zoospores over the entire first 8 d.

Zoospore densities

Because of logistical constraints, zoospore density was not estimated directly from the *Achlya* and *Bd* inocula. Instead, unused cultures from the same pathogen stocks used in experimentation were selected randomly and used to estimate zoospore densities in the *Achlya*-present and *Bd*-present treatments. One *Achlya* dosage was lifted out of its Petri dish, placed in a new Petri dish, and rinsed with 10.0 ml of ultrapure water. For *Bd*, 1 Petri dish containing a *Bd* culture was flooded with 3.0 ml of ultrapure water and stirred. We counted zoospores in these representative zoospore solutions using a hemacytometer. Initial concentrations of *Achlya* zoospores in the *Achlya*-present treatment and *Bd* zoospores in the *Bd*-present treatment were calculated using extrapolation based on the volume of water in the aquaria. The initial concentration of *Achlya* zoospores in the *Achlya*-present treatment was approximately 1.4×10^7 zoospores l^{-1} and the initial concentration of *Bd* zoospores in the *Bd*-present treatment was approximately 1.2×10^6 zoospores l^{-1}.

Quantification of *Bd* infection

DNA was extracted from excised mouthpart tissue 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). To check for *Bd* contamination in the *Bd*-absent treatment (the control treatment for the *Bd*-present treatment), we analyzed a subset of individuals in the *Bd*-absent treatment for *Bd* infection. One randomly chosen preserved specimen in each of 2 randomly chosen experimental units in each *Bd*-absent treatment combination (12 individuals in total) was analyzed in addition to 1–6 randomly chosen individuals from 4 other *Bd*-absent experimental units chosen at random (15 additional individuals). In addition, blank extraction controls were introduced during DNA extraction and processed alongside specimens to check for *Bd* DNA cross-contamination. Blank extraction controls consisted of microcentrifuge tubes identical to and treated identically to those used for processing specimens, except that they contained no specimen material.

Statistical analyses

The percentage of larvae infected with *Bd* and average *Bd* load were determined for each experimental unit, arcsine square-root transformed (percentage of larvae infected) or natural $\log(x+1)$ transformed (average load) to meet parametric assumptions, and analyzed using linear regression. Thus, inferences about *Bd* load pertain to the median of tank-wide averages. We treated *Achlya* and *Bd* as nominal factors and Roundup® concentration as a continuous factor. Interactive effects of *Achlya* and Roundup® were investigated using simple effect tests (Keppel & Wickens 2004) to evaluate the *Achlya* effect at each Roundup® concentration. To maintain α at 0.05 while performing 3 separate tests for *Achlya* effects, simple effect tests used a Bonferroni-adjusted critical p-value of 0.017 for rejection of null hypotheses. We analyzed survival using Cox proportional hazards modeling, with a maximum likelihood ratio test to compare the explanatory power of the resulting model to that of null model containing no treatment effects. A nonsignificant maximum likelihood test indicates a lack of significant treatment effects (Ramsey & Schafer 1997). All analyses started with a full model including all interaction factors and a quadratic Roundup® term. However, the quadratic Roundup® term was always nonsignificant (all $p \geq 0.330$) and was therefore dropped from all analyses. Nonsignificant interactions with $p > 0.1$ were also dropped. Thus nonsignificant interaction factors were liberally included in analyses. However, use of the more conservative approach of dropping all interaction factors with $p > 0.05$ did not change any qualitative interpretations of results. All statistical analyses were performed using R (version 2.15.3; R Core Team 2013).

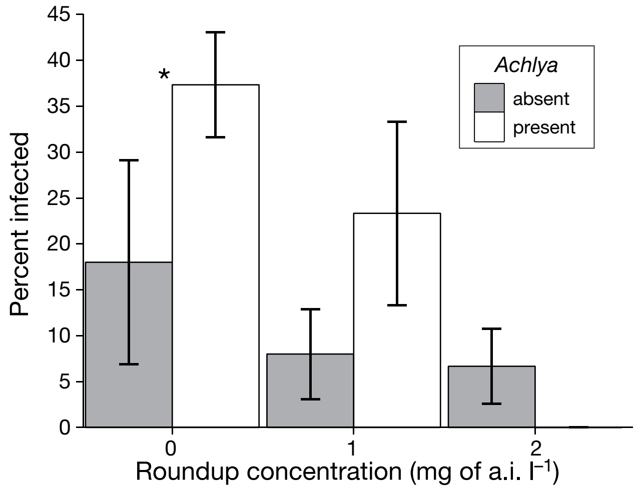

Fig. 1. Percentage of *Bd*-exposed *Hyliola regilla* larvae infected with *Bd*. No larvae were infected in the *Achlya*-present treatment at the highest Roundup® concentration. Error bars are ±1 SE. Total number of larvae represented in each column, from left to right, is 29, 29, 28, 29, 29, and 30. a.i.: active ingredient. * denotes the significant difference between the *Achlya*-present and *Achlya*-absent treatments

RESULTS

Bd infection prevalence

No *Bd* infection was detected in *Hyliola regilla* larvae in the *Bd*-absent treatment. Within the *Bd*-present treatment, the mean percentage of larvae infected with *Bd* ranged from 0 to 37.3% across the different combinations of Roundup® and *Achlya* (Fig. 1). Regression results indicated a significant Roundup × *Achlya* interaction factor consistent with less-than-additive (antagonistic) interactive effects of Roundup® and *Achlya* on *Bd* prevalence ($t_{26} = -2.250$, $p = 0.033$). When Roundup® was absent, the proportion of *Bd*-exposed larvae infected with *Bd* was higher in the *Achlya*-present treatment compared with the *Achlya*-absent treatment ($t_{26} = 2.642$, $p = 0.014$). This difference was significant after application of the Bonferroni method to maintain $\alpha = 0.05$ (see 'Statistical analyses'). Similarly, more *Bd*-exposed larvae were infected with *Bd* in the *Achlya*-present treatment compared with the *Achlya*-absent treatment when the Roundup® concentration was low (1 mg a.i. l^{-1}), but this difference was smaller in magnitude than the difference between the *Achlya*-present and *Achlya*-absent treatments in the no-Roundup® control and nonsignificant ($t_{26} = 1.422$, $p = 0.167$). In addition, the pattern of elevated *Bd* prevalence in the *Achlya* treatment was eliminated when the Roundup® concentration was further increased; in the high Roundup® treatment (2 mg a.i. l^{-1}), more *Bd*-exposed larvae were infected with *Bd* when *Achlya* was absent compared with when it was present, although this difference was nonsignificant ($t_{26} = -0.843$, $p = 0.407$).

Overall, *Bd* infection prevalence dropped as Roundup® concentration increased. Only 2 individuals in the high Roundup® treatment, both of which were in the high Roundup®–*Achlya*-absent treatment combination, were infected with *Bd*. Nevertheless, the effect of Roundup® alone was not significant ($t_{26} = -0.758$, $p = 0.455$). *Bd* was not detected in any individuals in the *Bd*-absent treatment or any blank extraction controls, consistent with a lack of *Bd* contamination in the *Bd*-absent treatment and a lack of cross-contamination in the PCR analysis.

Bd infection load

Only 2 individuals in the high Roundup® treatment were infected with *Bd*, but one of these individuals had the highest *Bd* load in the experiment (3.00×10^4 genome equivalents [ge]), which produced an outlying observation in the multiple regression analysis of average *Bd* load (Fig. 2). Nevertheless, we detected a robust dose-dependent negative effect of Roundup® on *Bd* infection loads. Exclusion of the outlying observation, which had a positive influence on the Roundup® regression coefficient, did not in-

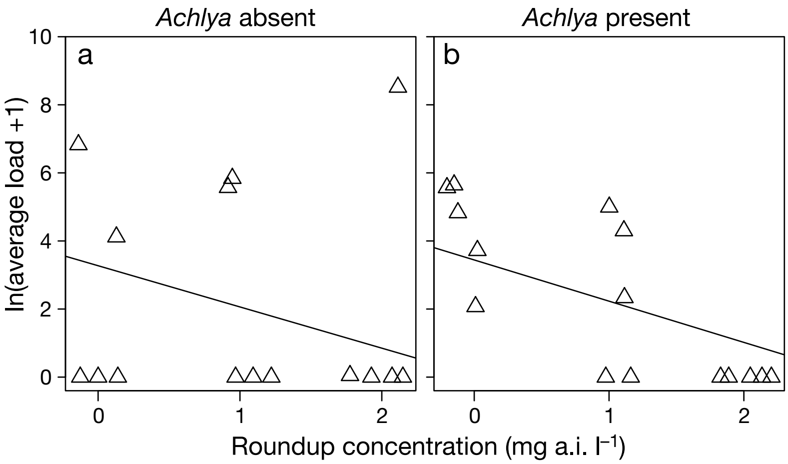

Fig. 2. *Bd* infection load of *Bd*-exposed *Hyliola regilla* larvae in the (a) *Achlya*-absent and (b) *Achlya*-present treatments. Each triangle represents the average *Bd* load for a single experimental unit. Average loads have been natural log(x+1) transformed and are thus shown on the natural log(x+1) scale. Data points are jittered horizontally for clarity. Lines indicate least-squared multiple regressions. In accordance with the model selected by multiple regression analysis, lines reflect only the main (significant) effect of Roundup® and the main (nonsignificant) effect of *Achlya*

fluence the qualitative interpretation or results, so the outlier was retained. The standard error of raw zoospore loads within individual experimental units in the *Bd*-present treatment ranged from 0 to 843 ge, and the average standard error across these 30 experimental units was 73 ge. Increasing Roundup® concentration caused a multiplicative decrease in *Bd* loads of *Bd*-exposed larvae (t_{27} = −2.059, p = 0.049). Each increase in Roundup® concentration of 1 mg a.i. l^{-1} was associated with an 87.24% decrease in the median *Bd* load. In contrast to Roundup®, *Achlya* did not affect median *Bd* load (Fig. 2; t_{27} = 0.176, p = 0. 861). The Roundup® × *Achlya* interaction was also nonsignificant, indicating that the effect of Roundup® did not depend on the presence or absence of *Achlya* (t_{26} = −1.712, p = 0.137).

Achlya infection

Visual inspection of larvae during the experiment and during excision of mouthparts revealed no hyphae consistent with *Achlya* infection. Similarly, microscopy revealed such hyphae on only 1 individual. This individual, which died 2 d after the start of the experiment, was in the *Achlya*-present–*Bd*-absent treatment combination in the low (1 mg a.i. l^{-1}) Roundup® treatment and had coenocytic hyphae on its mouthparts and snout and the side of its body. Continued growth of hyphae on the *Achlya*-laden hemp seeds in the *Achlya*-present treatment was visually observed during the 8 d exposure period at the start of the experiment. No other hyphae were observed on any larvae or other tank contents. All individuals that died during the experiment were partially or completely eaten by conspecifics before removal and preservation, which might have limited the detectability of hyphae.

Survival

Survival of *H. regilla* larvae ranged from 80 to 96.7% across the different treatment combinations. Cox Proportional Hazards modeling resulted in a full model containing all main and interaction factors. However, comparison of this model with the null model indicated that none of the experimental factors significantly influenced survival (maximum likelihood ratio test, χ^2_7 = −10.6, p = 0.157).

DISCUSSION

Effects of *Achlya* on *Bd* infection

Our results are consistent with synergistic effects of *Achlya* and *Bd* that were removed by Roundup®. In the absence of Roundup®, *Bd* infection among *Bd*-exposed larvae was more common when larvae were also exposed to *Achlya* compared with when *Achyla* was absent, suggesting that *Achlya* increased the susceptibility of larvae to becoming infected with *Bd*. However, this *Achlya* effect diminished and became nonsignificant when Roundup® was present and disappeared completely at the highest Roundup® concentration. Although *Achlya* did not influence *Bd* infection loads, its effect on the prevalence of *Bd* infection indicates that it could play an important facilitative role in chytridiomycosis dynamics. Our study, the first to describe experimental effects consistent with synergistic effects of *Bd* and another microbe, suggests that *Achlya* spp. and other water molds could contribute to the widespread and often severe effects of *Bd* on individual amphibian hosts and host populations.

Our experiment was not designed to determine the mechanism behind interactive effects of *Achlya* and *Bd*. However, we postulate that germ tubes from colonizing *Achlya* zoospores or hyphae from *Achlya* infections may have physically damaged the epidermis of larvae, including the mouthparts, opening pathways that facilitated colonization of the mouthparts by *Bd* zoospores. Chemicals produced by *Achlya* that promote invasion of the host by germ tubes or digestion of host tissues might have contributed to such damage. Alternatively, *Achlya* zoospores landing on larvae might have disrupted microflora on the larval epidermis that protect against *Bd* infection.

Hyphae consistent with *Achlya* infection were detected on only one *Achlya*-exposed larva. However, some *Achlya* infections might have gone undetected because of consumption of hyphae during feeding of larvae on dead and dying conspecifics. All individuals that died during the experiment were at least partially eaten by conspecifics before the carcass was found and removed, consistent with observations in several frog species that larvae sometimes feed on conspecific larvae by scavenging their carcasses or through active cannibalism (reviewed in Heinen & Abdella 2005). Furthermore, Gomez-Mestre et al. (2006) observed wood frog *Rana sylvatica* larvae eating water mold hyphae off infected American toad *Bufo americanus* eggs. In addition, light *Achlya* infections might have gone undetected by visual inspections during the experiment and been cleared before it ended. Additional studies that elucidate the effects of *Achlya* and other water molds on the epidermis and immune system of amphibian hosts are needed to determine the mechanism behind the observed correlation between exposure to water mold and increased prevalence of *Bd* infection.

Effects of Roundup® on *Bd* infection

Unlike *Achlya*, Roundup® exerted a negative influence on *Bd*. Roundup® removed the positive effect of *Achlya* on the proportion of *Hyliola regilla* larvae infected with *Bd*, perhaps by killing *Achlya* zoospores, reducing their production or infectivity, or causing a shift from parasitism to saprobism. In addition, Roundup® alone decreased *Bd* loads of *Bd*-exposed larvae in a dose-dependent manner. Similarly, *Bd* infection in the absence of water mold became less common as Roundup® concentration increased, although this relationship was not statistically significant. Nevertheless, the pattern of reduced prevalence of *Bd* infection contributed to the observed decrease in *Bd* loads as Roundup® concentration increased. In addition, Roundup® may have reduced the infection severity of *Bd*-infected individuals, but too few larvae were infected with *Bd* to allow effective testing of this hypothesis.

The negative influence of Roundup® on *Bd* loads probably arose from the direct negative effects of Roundup® on *Bd* (Hanlon & Parris 2012), which may have included decreased survival, motility, or infectivity in free-swimming zoospores, as well as mortality, slowed development, or decreased zoospore production in zoosporangia growing on larvae. However, even the highest Roundup® concentration did not completely eliminate *Bd* infection. Also, because individuals that escape or clear *Bd* infection during exposure to Roundup® could become infected with the fungus later, further study is needed to determine whether the negative effects of Roundup® on *Bd* lead to long-term changes in disease dynamics.

Host survival

The effects of Roundup® and *Achlya* on *Bd* infection did not change host survival. Cox proportional hazards modeling of survival found no evidence for effects of any of the experimental factors. Thus, *H. regilla* larvae were less susceptible to Roundup® in our study compared with a previous study in which no *H. regilla* larvae survived exposure to Roundup® at concentrations of 1.0 mg a.i. l^{-1} or greater (King & Wagner 2010). However, these 2 studies used different methods. For example, larvae in our study were larger and more advanced in development than those in King & Wagner (2010). In addition, *H. regilla* larvae in our study were susceptible to becoming infected with *Bd* but were resistant to the lethal effects of this fungus, consistent with some but not all previous studies on this species (e.g. Blaustein et al. 2005, Garcia et al. 2006, Romansic et al. 2011, Reeder et al. 2012). Larval and newly metamorphosed *H. regilla* have exhibited *Bd*-induced mortality in other studies (Kleinhenz et al. 2012, Gervasi et al. 2013, Searle et al. 2013, Buck et al. 2015), including Rumschlag et al. (2014), in which *Bd* was lethal under some but not all temperature conditions. This suggests that interactive effects of *Achlya* and Roundup® on *Bd* infection could, in some situations, influence survival in this species. Regardless, in species that are less tolerant of *Bd* infection than *H. regilla*, *Achlya*-induced facilitation of *Bd* would likely have strong effects on survival.

CONCLUSIONS

The negative influence of Roundup® on *Bd* load, coupled with the lack of a synergistic effect of Roundup® on the proportion of larvae infected with *Bd*, suggests that the direct negative effects of Roundup® on *Bd* were more important than the direct negative effects of Roundup® on the amphibian host in determining patterns of chytridiomycosis in larval hosts. The relative importance of these opposing effects may have been influenced by the relatively low toxicity of Roundup® to host larvae in our study. However, Hanlon & Parris (2014) found that exposure of eastern gray treefrog *Hyla versicolor* larvae to 2.0 or 3.5 mg a.i. l^{-1} of a similar Roundup® formulation, despite being toxic enough to cause mortality, led to decreased *Bd*-induced mortality, a result similar to our finding of reduced *Bd* load. Our results also fit those of other previously published experiments performed in laboratory, mesocosm, and field venues in which glyphosate-based herbicides, including various Roundup® formulations, inhibited amphibian chytridiomycosis or had no detectable effect on the disease (Edge et al. 2011, 2013, Gahl et al. 2011, Paetow et al. 2012, Hanlon & Parris 2014). Roundup®-induced reductions in amphibian survival, although not evident in the *H. regilla* larvae used in this study, could also limit the ability of Roundup® to promote *Bd*, because reductions in host density will reduce the rate of *Bd* transmission in amphibian host populations if *Bd* transmission is density dependent. Thus, the available evidence points away from Roundup® formulations being cofactors that intensify the effects of *Bd* on amphibians. However, the full range of realistic exposure scenarios has not been adequately investigated yet. For example, although 2 studies exposed amphibians to Roundup® after previous exposure to *Bd*, these studies allowed only 24 (Edge et al. 2013) or 48 h (Hanlon & Parris 2014) between exposures. Because *Bd* may be protected from Roundup® surfactants if it is within amphibian tissue, experiments are needed that allow infections to become well-established before amphibians are challenged with the herbicide. Even if Roundup® does not facilitate *Bd* in such a scenario, the long-term effects of *Bd* infection could make amphibians more susceptible to the toxic effects of Roundup®.

Our finding of an association between *Achlya* exposure and *Bd* infection prevalence, combined with the near ubiquity of water molds in amphibian habitats, including aquatic water bodies and moist soils (Johnson et al. 2002), underscores the potential importance of water molds in chytridiomycosis and amphibian population declines. Therefore, we propose further investigation of water molds as potential environmental cofactors in the *Bd*–amphibian host–pathogen system. Based on the moderating effect of Roundup® observed in our study, we predict that water mold–*Bd* interactions are highly dependent on environmental context. Indeed, Romansic et al. (2011) found that *Achlya flagellata* did not facilitate *Bd* infection in *H. regilla* larvae. But Romansic et al. (2011) had key differences in pathogen dosage, larval density, water mold strain, and temperature. In some cases, water molds could outcompete *Bd* on amphibian hosts and thereby reduce chytridiomycosis impacts. However, positive effects of water molds on *Bd* could intensify and prolong *Bd*-associated mass mortality events and population declines in *Bd*-susceptible species, especially because pathogenic water molds can proliferate without live hosts. Moreover, increased *Bd* prevalence in *Bd*-tolerant species such as *H. regill*a could lead to increased transmission of the fungus to amphibian species that are less tolerant of *Bd* infection, further exacerbating amphibian losses. Because of the numerous ecological pathways by which interactive effects of pathogens might impact amphibian populations, we call for further investigation of multifactor exposures involving not only water molds and *Bd*, but also other disease-causing organisms, including bacteria, viruses, and trematodes.

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