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Effect of Simultaneous Amphibian Exposure to Pesticides and an Emerging Fungal Pathogen, *Batrachochytrium dendrobatidis*

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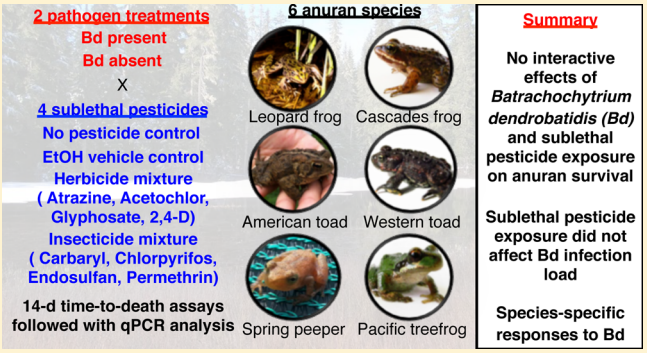
Supporting Information

ABSTRACT: Amphibian declines have been linked to numerous factors, including pesticide use and the fungal pathogen *Batrachochytrium dendrobatidis* (Bd). Moreover, research has suggested a link between amphibian sensitivity to Bd and pesticide exposure. We simultaneously exposed postmetamorphic American toads (*Anaxyrus americanus*), western toads (*A. boreas*), spring peepers (*Pseudacris crucifer*), Pacific treefrogs (*P. regilla*), leopard frogs (*Lithobates pipiens*), and Cascades frogs (*Rana cascadae*) to a factorial combination of two pathogen treatments (Bd^+, Bd^-) and four pesticide treatments (control, ethanol vehicle, herbicide mixture, and insecticide mixture) for 14 d to quantify survival and infection load. We found no interactive effects of pesticides and Bd on anuran survival and no effects of pesticides on infection load. Mortality following Bd exposure increased in spring peepers and American toads and was dependent upon snout−vent length in western toads, American toads, and Pacific treefrogs. Previous studies reported effects of early sublethal pesticide exposure on amphibian Bd sensitivity and infection load at later life stages, but we found simultaneous exposure to sublethal pesticide concentrations and Bd had no such effect on postmetamorphic juvenile anurans. Future research investigating complex interactions between pesticides and Bd should employ a variety of pesticide formulations and Bd strains and follow the effects of exposure throughout ontogeny.



INTRODUCTION

Understanding the factors that influence a species' health, abundance, and distributions is an important concern among environmental disciplines. Anthropogenic stressors, such as habitat destruction and fragmentation, introduction of invasive species, and contaminant exposure, can negatively influence individuals, populations, and communities. Moreover, natural and anthropogenic factors can interact in complex ways.[1−3] This is illustrated by the dynamics of amphibian population declines occurring worldwide. The percentage of amphibian species that are threatened or endangered exceeds that of mammals and birds,[4,5] and amphibian population declines have several contributing factors, including climate and atmospheric changes, contaminant exposure, invasive species, and disease.[6−8] As human activities continue to increase, we need a better understanding of how species will respond to these impacts and how these factors may interact synergistically with natural factors.

The emerging fungal pathogen *Batrachochytrium dendrobatidis* (Bd), which causes the disease chytridiomycosis, is especially important among those pathogens that have been linked to global amphibian population declines.[9,10] The pathogen is transmitted in aquatic environments following the release of zoospores from zoosporangia, which encyst in keratinized tissues of amphibians (e.g., tadpole mouthparts, postmetamorphic amphibian skin).[10−12] Bd infection causes a number of pathogenic symptoms, including lethargy, roughening and sloughing of the skin, and death.[10−12] Previous research has proposed two competing hypotheses for the spread and emergence of Bd: (1) the inadvertent introduction of highly virulent and transmissible strains into naïve populations and (2) immunological or behavioral changes of endemic Bd and host species under varying ecological conditions that have increased

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Table 1. Collection of Anuran Species in Pennsylvania and Oregon[a]

common name	scientific name	family	collection location	collection date	no. of egg masses/clutches	initial mass (mg)
			Eastern			
spring peeper	*P. crucifer*	Hylidae	41°34′09.53″ N 80°27′22.84″ W	4/9/13 to 4/10/13	20	20.8 (2.5)
American toad	*A. americanus*	Bufonidae	41°34′09.53″ N 80°27′22.84″ W	4/15/13 to 4/21/13	8	52.2 (2.1)
leopard frog	*L. pipiens*	Ranidae	41°41′30.33″ N 80°30′03.38″ W	4/12/13 to 4/19/13	12	61.5 (5.5)
			Western			
Pacific treefrog	*P. regilla*	Hylidae	44°31′19.34″ N 122°01′53.42″ W	5/13/13	12	37.7 (3.5)
western toad	*A. boreas*	Bufonidae	44°26′01.39″N 121°54′01.65″ W	5/13/13	2	63.1 (3.2)
Cascades frog	*R. cascadae*	Ranidae	44°31′19.34″ N 122°01′53.42″ W	5/13/13	20	70.1 (4.6)

[a]Initial mass data represents mean values (±1 SE) of 20 tadpoles.

the pathogenicity and virulence of Bd.[13−16] Furthermore, the evolutionary differences among host species, pathogens, or some combination of the two factors can shape pathogen infectivity and virulence.[17−19] For example, the susceptibility of amphibians to pathogens differs among species,[20−23] populations,[24] developmental stages,[25−28] and size classes.[21,23,29] Moreover, the effects of pathogens are dependent upon the identity of the pathogen strain.[22,30,31] Given its rapid spread and high virulence, Bd has been identified as one of the "100 worst invasive alien species".[32]

Pathogen virulence is also influenced by numerous human activities. While much attention has been given to the effects of species introductions, habitat loss and fragmentation, and climate change on disease dynamics, less work has explored the influence of pesticide use.[18,23,33,34] The use of pesticides to increase crop yields through the control of disease vectors and pest species has increased since the 1940s and continues to grow with the introduction of genetically modified organisms.[35−38] Furthermore, improper use and disposal of pesticides, runoff, and atmospheric drift and deposition have led to the ubiquitous contamination of habitats worldwide.[7,39,40] Exposure to pesticides (e.g., atrazine, glyphosate, carbaryl, chlorothalonil) can have direct lethal effects on pathogens, reducing their abundance,[19,41] and can also influence host survival through the modification of the immune response and skin bacteria and peptides that inhibit pathogen colonization.[42,43] The ubiquity of pesticide use underscores the importance for understanding its influence on host−pathogen dynamics.

Since exposure to pathogens and pesticides can occur throughout ontogeny, previous researchers have examined how pesticide exposure in early life stages influences amphibian sensitivity to Bd at later life stages. For example, McMahon et al.[19] reported reduced infection loads of *Osteopilus septentrionalis* tadpoles exposed simultaneously to Bd and atrazine (herbicide) or chlorothalonil (fungicide). However, the sublethal exposure to pesticides as tadpoles and subsequent Bd exposure as postmetamorphic amphibians did not reduce Bd load or affect survival of six anuran species.[23] These contrasting results indicate two important factors: first, amphibian responses to Bd and pesticides are dependent upon exposure regime (i.e., sequential versus simultaneous exposure); second, amphibian sensitivity to pesticides and Bd varies with developmental stage. For instance, earlier developmental stages can be more tolerant to Bd than later stages for some amphibian species.[26] Moreover, it is probable that postmetamorphic amphibians are exposed simultaneously to both pesticides and pathogens under natural conditions, especially near agricultural systems. Thus, it is important to know how simultaneous exposure to Bd and pesticides affects organisms at sensitive life stages.

We investigated the effects of simultaneous exposure to Bd and pesticides on six species of recently metamorphosed amphibians. We asked the following questions: (1) Does simultaneous exposure to Bd and sublethal pesticide mixtures increase amphibian mortality compared to their individual effects, and (2) does exposure to various sublethal pesticide mixtures alter Bd infection load in postmetamorphic amphibians?

MATERIALS AND METHODS

We investigated the effects of simultaneous exposure to Bd and sublethal pesticide concentrations on six North American anuran species from three families (Table 1) during the summer of 2013. All species were raised to metamorphosis (Gosner[44] stage 46) at the Donald S. Wood Field Laboratory at the University of Pittsburgh's Pymatuning Laboratory of Ecology and were subsequently exposed to Bd and pesticides as metamorphs at Oregon State University (OSU).

Anuran Collection and Husbandry. Anuran species were collected as freshly oviposited egg masses or clutches from populations in both Pennsylvania and Oregon (Table 1). Egg masses of Oregon species were shipped overnight to the PA laboratory. All species were raised outdoors under ambient conditions in 300-L pools filled with 200 L of aged well water. Tadpoles were fed rabbit chow (Bunny 16, Blue Seal, Muscatine, IA) ad libitum before being transferred to common-garden pools.

Tadpoles were raised to metamorphosis under common-garden conditions using 60 100-L wading pools filled with approximately 85 L of aged well water. Five grams of rabbit chow and 100 g of dried oak leaves were added to each pool as an initial and slow-release nutrient source, respectively. To add a zooplankton assemblage similar to natural systems, water was collected from four nearby ponds, screened for invertebrate predators, homogenized, and added to each pool in 177-mL aliquots. Additionally, 354 mL of water collected from the same four ponds was passed through a 64-μm mesh to remove invertebrates, homogenized, and added to each tank to introduce an algal community. All pools were covered with 65% shade cloth to prevent oviposition by invertebrates and amphibians and emigration of amphibians from pools. Zooplankton and algal

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Table 2. Pesticides and Concentration Means (± 1 SE) Used in the Present Study

pesticide	CAS no.	mode of action	concentration (μg/L)		
			nominal	actual	max observed in environment
		Herbicides			
atrazine	1912-24-9	inhibits photosystem II	5	3.9 (0.10)	250[a]
acetochlor	34256-82-1	inhibits cellular growth	5	4.9 (0.05)	25.1[b]
glyphosate	1071-83-6	inhibits amino acid synthesis	5	3.5 (0.15)	5200[c]
2,4-D	94-75-7	auxin mimic promotes uncontrollable growth	5	4.9 (0.05)	692[d]
		Insecticides			
carbaryl	63-25-2	inhibits acetylcholine esterase	5	5.0 (0.15)	2500[e]
chlorpyrifos	2921-88-2	inhibits acetylcholine esterase	5	4.8 (0.10)	2.8[f]
endosulfan	115-29-7	nervous system antagonist	5	3.6 (0.05)	9[g]
permethrin	2645-53-1	sodium channel inhibitor	5	0.9 (0.10)	17.5[h]

[a]Murphy, M. B.; Hecker, M.; Coady, K. K.; Tompsett, A. R.; Jones, P. D.; Du Preez, L. H.; Everson, G. J.; Solomon, K. R.; Carr, J. A.; Smith, E. E.; Kendal, R. J.; Van Der Kraak, G.; Giesy, J. P. *Aquat. Toxicol.* **2006**, *76* (3−4), 230−245. [b]Battaglin, W. A.; Furlong, E. T.; Burkhardt, M. R.; Peter, C. J. *Sci. Total Environ.* **2000**, *248* (2−3), 123−133. [c]Edwards, W. M.; Triplett, G. B.; Kramer, R. M. *J. Environ. Qual.* **1980**, *9* (4), 661−665. [d]Hazardous Substances Data Bank (https://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB). [e]Norris, L. A.; Lorz, H. W.; Gregory, S. V. USDA, General Technical Report PNW-149, 1983 [f]Giesy, J. P.; Solomon, K. R.; Coats, J. R.; Dixon, K. R.; Giddings, J. M.; Kenaga, E. E. *Rev. Environ. Contam. Toxicol.* **1999**, *160*, 1−129. [g]Muschal, M. Australian Department of Land and Water Conservation, Sydney, 1997. [h]Delgado-Moreno, L.; Lin, K.; Veiga-Nascimento, R.; Gan, J. *Agric. Food Chem.* **2011**, *59* (17), 9448−9456.

communities developed for 2 weeks before the addition of anurans.

Tadpoles of American toads (*Anaxyrus americanus*), western toads (*A. boreas*), spring peepers (*Pseudacris crucifer*), Pacific treefrogs (*P. regilla*), and leopard frogs (*Lithobates pipiens*) were added to the wading pools on May 23, and Cascades frogs (*Rana cascadae*) were added on May 27 (see Table 1 for collection information and initial tadpole masses). Because the species grow to different metamorphic sizes, we raised them at one of two densities, which affected the number of mesocosms used for each species with a goal of generating a similar number of metamorphs for all six species. American toads, western toads, spring peepers, and Pacific treefrogs all metamorphose at a relatively small size, so they were raised at a density of 25 individuals per pool; we set up 7 pools each for American toads and spring peepers, and 8 pools each for western toads and Pacific treefrogs. In contrast, leopard frogs and Cascades frogs metamorphose at a much large size, so tadpoles of these species were added at a density of 10 individuals per pool, and we reared 15 pools of tadpoles for each these species.

Daily visual checks were conducted to collect metamorphosing anurans. Collected metamorphs were held communally in 15-L plastic containers filled with moist sphagnum moss and were fed pin-head crickets ad libitum. Sphagnum moss was changed twice per week. Following the collection of all metamorphs for a given species, animals were shipped overnight to OSU with moist sphagnum. Due to variation in time to metamorphosis, American toads, western toads, and Pacific treefrogs were shipped on July 1, spring peepers and Cascades frogs were shipped on July 8, and leopard frogs were shipped on July 29.

Experimental Exposure. Once the anurans arrived at OSU, we employed a completely randomized design combining two pathogen treatments [Bd present (Bd^+), Bd absent (Bd^-)] with four pesticide treatments (no-pesticide control, ethanol vehicle control, herbicide mixture, and insecticide mixture) to create eight Bd−pesticide treatments for each species. Due to the low water solubility of some pesticides, we used an ethanol vehicle to dissolve our pesticides. In doing so, we added an ethanol vehicle treatment to test for effects of ethanol on response variables. Due to differences in the number of metamorphs collected among the species, the sample size for the 8 treatments ranged from 8 to 25 replicates [Table S1, Supporting Information (SI)].

At OSU, each species was held for a 4-d acclimation period, which was followed by a 14-d time-to-death (TTD) assay. Anurans were housed communally in the lab (14:10 light/dark photoperiod) in 37.8-L glass aquaria at 13.5 °C and fed crickets ad libitum. Three days after arriving at OSU, we measured the mass and snout−vent length (SVL) of all individuals (Table S2, SI). The following day (day 0), each individual was randomly assigned to one of the eight Bd−pesticide treatments, and housed individually in clear, sterile polystyrene Falcon culture dishes (150 × 25 mm; Corning Inc., Corning, NY). Each dish contained 25 mL of treated water (5 mL of pesticide-treated water, 10 mL of Bd-treated water, and 10 mL of no-pesticide, no-disease control water). Pesticide mixtures consisted of either four herbicides (atrazine, acetochlor, glyphosate, 2,4-D) or four insecticides (carbaryl, chlorpyrifos, endosulfan, permethrin). These eight pesticides (Table 2) are commonly applied in the agricultural, private, and home sectors,[35,37] and many are detected in field surveys of aquatic ecosystems.[39,40] Furthermore, the use of pesticide mixtures is likely representative of exposure under natural conditions[39,40] and allowed us to efficiently screen a number of pesticides while limiting the size of our experiment. To create our pesticide-treated water, we first prepared stock solutions for all eight pesticides by dissolving each solid chemical active ingredient (Sigma-Aldrich, Pestanal analytical standards; St. Louis, MO) in 5 mL of ethanol. We then added a specific volume of each stock solution to 265 mL of filtered water to obtain a concentration of 25 μg/L. To create our ethanol vehicle and no-pesticide control treatments, we added 26.5 μL ethanol or filtered water, respectively, to 265 mL of filtered water. After the addition of the four pesticides to make each mixture, we thoroughly homogenized the mixture before adding 5 mL of treated water into each experimental unit assigned a pesticide treatment.

Following methods used previously to create Bd-treated water,[21,23,45] we cultured Bd on 1% tryptone agar Petri plates (strain JEL 274 originally isolated from *A. boreas* in Colorado, 1999). Bd plates were incubated for 9−15 d at room temperature (20 °C). Prior to inoculation, a subset of plates (6−41 plates depending on the number of individuals to be challenged) was

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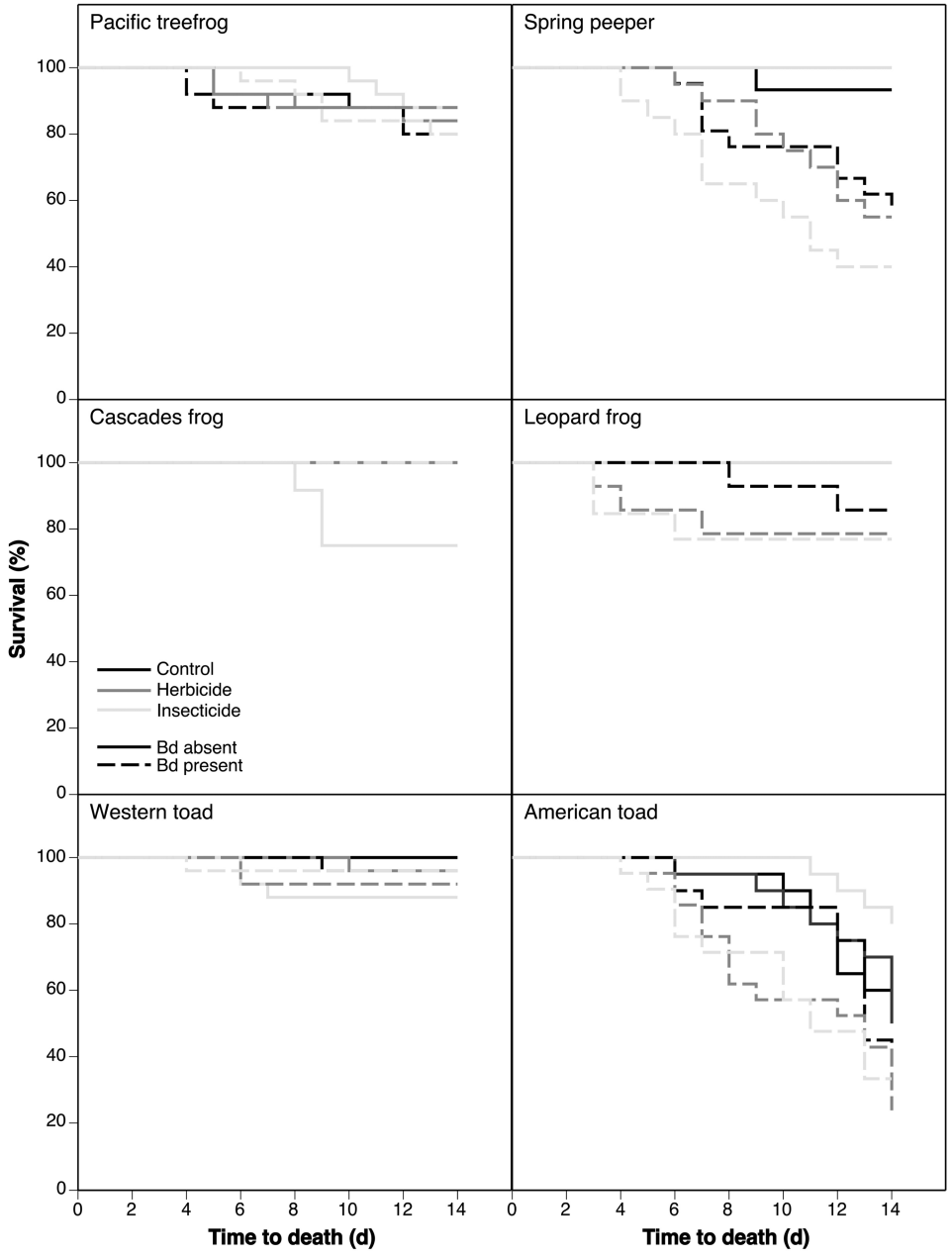

Figure 1. Survival of six anuran species exposed simultaneously to sublethal pesticide concentrations and *B. dendrobatidis* (Bd) over a 14-d period. Solid lines represent Bd-absent treatments, whereas dashed lines represent Bd-present treatments. Data for the ethanol vehicle is not shown, as it was not statistically different from the control treatment. Although some may overlap, figures contain lines for each treatment combination.

flooded with 10 mL of filtered water. After 15 min, we harvested zoospores and zoosporangia from the agar using a rubber policeman scraper and poured the Bd slurry from each flooded plate into a 1-L glass beaker to created a homogenized sample. Zoospore concentration was determined using a hemocytometer and diluted with filtered water to make an inoculum with a final concentration of 10 000 zoospores mL^{-1}. We added 10 mL of Bd-treated water to each Bd^+ experimental unit for a total of 100 000 zoospores mL^{-1}, bringing the liquid volume of the experimental dish to 15 mL (10 mL of Bd-treated water + 5 mL of pesticide-treated water). Bd-absent treatments received 10 mL of untreated (control) water. An additional 10 mL of filtered water was added to each dish, bringing the total volume to 25 mL, to completely cover the bottom of the experimental unit. This ensured that anurans remained in contact with the treated water throughout the experiment.

Throughout the TTD assay, we fed anurans and conducted water exchanges. On days 2, 5, 9, and 12, individuals were fed 0.1 g of cricket per 1 g of frog mass (approximately two or three crickets per frog). A full water change was conducted on day 7, which included reapplication of pesticides. To observe differences in Bd load following a single infection event in a contaminated environment, we did not renew Bd concentrations during the water exchange. Survival was monitored daily, and deceased individuals were preserved in 95% ethanol. The TTD assay was concluded on day 14, and all surviving individuals were euthanized with MS-222 overdose in accordance with IACUC protocols and were preserved in 95% ethanol.

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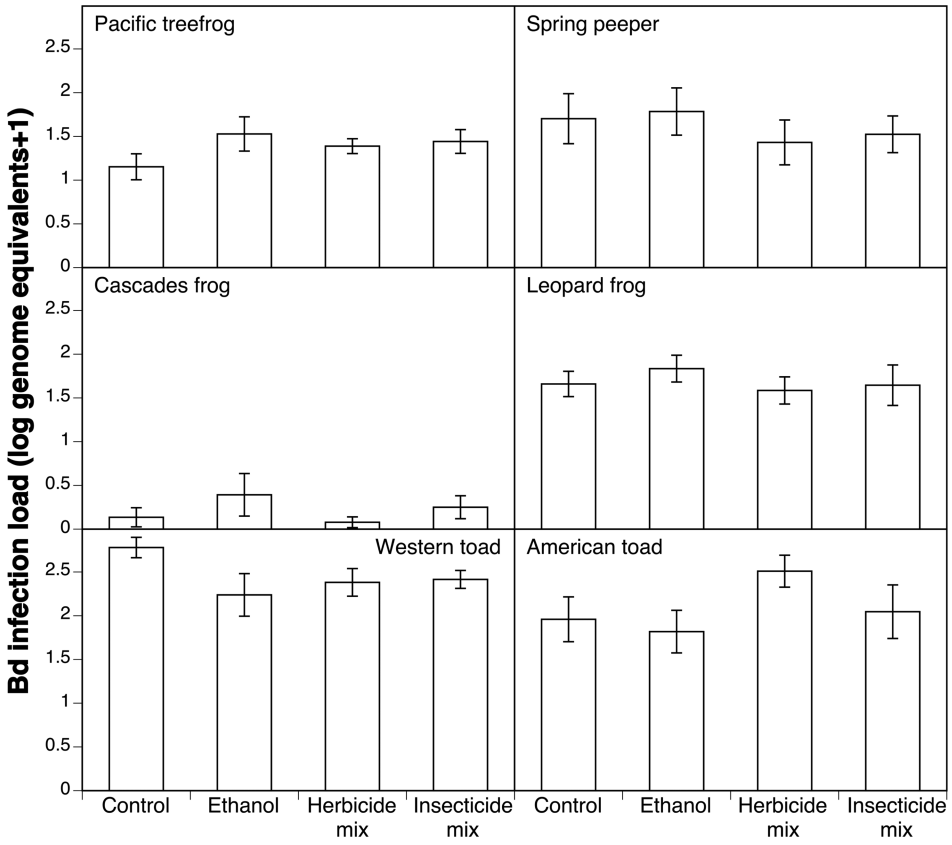

Figure 2. Effects of sublethal pesticide exposure on mean Bd infection load (±1 SE) for six anuran species. All unexposed anurans tested negative for Bd.

Quantifying Bd Load. For each anuran species, we used a quantitative polymerase chain reaction (qPCR) to determine the mean Bd load for a subset of Bd-exposed and control individuals (see Buck et al.[23] for a detailed description of the methods). Briefly, we selected up to 12 Bd-exposed individuals per pesticide treatment per species. We used 11 or 12 Cascades frogs for each treatment due to the lower number of replicates. We then selected 3 unexposed metamorphs from the Bd-absent treatment for a total sample of 358 individuals. To minimize pathogen load variation, we selected individuals that were euthanized following the conclusion of the assay when possible; 21 individuals used for qPCR analysis died between days 7 and 13. Each individual was swabbed 10 times on the right ventral surface (abdomen to toes) using a fine-tip sterile swab (Medical Wire and Equipment, Wiltshire, England, UK). We followed qPCR methods established by Boyle et al.,[46] with the exception that swabs were immersed in 60 μL of Prepman Ultra (Applied Biosystems, Carlsbad, CA) instead of 40 μL during DNA extraction. qPCR was performed using the Applied Biosystems StepOnePlus real-time PCR system. Samples were run in triplicate and these three runs were averaged to determine the Bd load for each animal. If a replicate tested negative, a second triplicate was run; a sample was considered Bd positive if four of six replicates tested positive.

Pesticide Testing. To confirm pesticide concentrations used in each mixture, four 500-mL samples were taken from each prepared pesticide mixture on July 6, 2013. Each sample was prepared by placing 2 mL of methylene chloride (CAS no. 75-09-2; Fisher Scientific, Waltham, MA) into a precleaned amber glass jar to stabilize and reduce the breakdown of pesticide-treated water. One sample (500 mL) of the no-pesticide control and two samples (2 × 500 mL) of each pesticide mixture were held at 4 °C before being sent overnight on ice to the University of Connecticut's Center for Environmental Science and Engineering (Storrs, CT) on July 10. Independent testing of pesticide samples (see Supporting Information) on July 18 revealed that actual concentrations of seven of the eight pesticides were within 70−98% of the nominal concentrations. The exception was permethrin, with an actual concentration that was 18% of the nominal concentrations. On the basis of previous studies, permethrin breaks down in water very rapidly, often resulting in water tests that produce concentrations that are substantially lower than nominal concentrations.[47] There were no detectable levels of any pesticide in the control treatment (Table 2).

Statistical Analysis. To examine the effect of simultaneous exposure to Bd and pesticides on anurans, we compared the rate of survival of each anuran species measured with TTD assays. Cox's[48] proportional hazards model (including Bd treatment and pesticide treatment as main effects, the interaction term, and snout−vent length as a covariate) was used to analyze survival for each anuran species (SPSS Statistics, IBM Corp., Armonk, NY). Snout−vent length (SVL) was included in each model to assess the influence of anuran size on treatment-induced mortality.

We used analyses of variance (ANOVA) followed by Tukey's honest significant difference tests to determine if Bd load differed among pesticide treatments (IBM SPSS Statistics, version 21). We log-transformed (data +1) Bd load for all species except Cascades frogs. Bd load for Cascades frogs did not meet the assumption of homogeneous errors; therefore, we rank-transformed the data.

RESULTS

Anuran Survival. We observed high survival for spring peepers, Pacific treefrogs, western toads, Cascades frogs, and

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leopard frogs in the control (88−100%) and ethanol vehicle (90−100%) treatments but reduced survival of American toads with the same treatments (60%).

When testing the effects of simultaneous exposure to sublethal pesticide mixtures and Bd, we found no Bd-by-pesticide interaction for any species (all $p > 0.200$). Given that the interaction term was not significant, we removed it from the model and then examined the main effects of pesticide mixtures, Bd exposures, and the covariate snout−vent length. The low concentrations of the different pesticide mixtures had no effect on the survival of the six species (all $p \geq 0.223$; Figure 1). Bd exposure did not affect the survival of Pacific treefrogs, western toads, Cascades frogs, and leopard frogs (all $p \geq 0.084$), but it did reduce the survival of spring peepers and American toads (all $p \leq 0.001$; Figure 1). Spring peeper survival decreased from 98% in non-Bd treatments to 52% in Bd treatments. American toad survival decreased from 63% in non-Bd treatments to 33% in Bd treatments. As snout−vent length of Bd-exposed anurans increased, survival decreased for American toads ($p < 0.001$) and Pacific treefrogs ($p = 0.079$), whereas survival increased for western toads ($p < 0.001$). Snout−vent length did not affect the survival of spring peepers, leopard frogs, or Cascades frogs exposed to Bd.

Anuran Bd Load. The ANOVA analysis revealed that pesticide exposures and snout−vent length had no effect on Bd load of any anuran species (all $p \geq 0.126$; Figure 2). All unexposed anurans tested negative for Bd.

DISCUSSION

To understand how Bd and sublethal pesticide mixtures interact to influence anuran mortality, we simultaneously exposed six species of postmetamorphic anurans to Bd and a sublethal pesticide mixture. We found no interactive effects of sublethal pesticide mixtures and Bd on anuran mortality. Exposure to Bd decreased the survival of spring peepers and American toads, but it did not affect the survival of the other anuran species, indicating species-level differences in susceptibility to the pathogen. Lastly, we did not find any effect of sublethal pesticide exposure on Bd load.

B. dendrobatidis has been linked to amphibian declines worldwide.[2,6,49−51] We observed reduced survival of spring peepers and American toads when exposed to Bd. Spring peeper mortality following Bd exposure is consistent with a previous experiment[23] but in contrast to the results of Gahl et al.[22] The discordant results may be due to different Bd strains in each study. The current study and that of Buck et al.[23] used JEL 274 isolated from western toads (*A. boreas*) in Colorado, whereas Gahl et al.[22] used JEL 404 isolated from American bullfrogs (*L. catesbeianus*) in Maine and JEL 434 isolated from *Phyllomedusa lemur* in Panama. Ecological differences (e.g., location, previous exposure history) among tested species may have also contributed to the differences between studies. Though Bd is endemic in some amphibian populations,[52−54] increased virulence of Bd may occur due to the introduction of novel strains and changing environmental conditions.[14,15,55]

Sensitivity to Bd may also be due in part to evolutionary differences among amphibian species and populations.[20−24,52] Although the studies of Gahl et al.[22] and Wise et al.[56] and the current study used different Bd strains, all studies observed decreased American toad survival when exposed to Bd. These results suggest that evolutionary processes, rather than Bd strain, may drive American toad tolerance to Bd. Our results further support the importance of Bd strain[22,30,31] and host identity.[20,21,23,45] Future research identifying highly virulent Bd strains[55] and at-risk amphibian species using phylogenetic analyses[57] will improve conservation efforts for declining amphibian populations.

Pathogen virulence is not only influenced by the identity of strain and host species but can be mediated by the size of host species. As SVL increased, we observed an increase in Bd-induced mortality of American toads and Pacific treefrogs but a decrease in the mortality of western toads. There was no effect of SVL on mortality in leopard frogs, Cascades frogs, and spring peepers. Previous researchers have reported differences in Bd sensitivity among amphibian size classes.[21,23,29] Larger individuals may have the capacity to carry higher Bd infection loads due to their greater skin surface area, which may lead to infection loads that overcome innate and acquired immunity.[58] Alternatively, larger individuals may have the resources available to respond and clear infections more efficiently.[21,49] Understanding how size modifies Bd sensitivity is a key first step in predicting how anuran will respond to Bd under additional environmental and anthropogenic stressors.

Exposure to sublethal pesticide concentrations can also cause behavioral, morphological, and physiological changes in amphibians.[59−61] Sublethal pesticide exposure may increase the pathogenicity of Bd by decreasing immune function of amphibians by affecting the thymus,[62] decreasing the production of immune cells,[63−65] and reducing antimicrobial skin defenses.[66,67] In addition, sublethal pesticide concentrations may decrease mortality following Bd exposure by directly killing Bd zoosporangia and zoospores.[19,41] In the current study, none of the anuran species showed increased sensitivity to Bd when simultaneously exposed to pesticides. Moreover, sublethal pesticide exposure did not reduce Bd infection load in any anuran species. In contrast to our results, Hanlon and Parris[28] found that gray treefrog tadpoles (*Hyla versicolor*) first exposed to Bd had increased survival in the presence of the herbicide Roundup (glyphosate, 2.3 mg/L) relative to unexposed tadpoles or those exposed to Sevin (carbaryl, 4.0 mg/L). Furthermore, McMahon et al.[19] reported reduced Bd load on Cuban treefrog tadpoles (*Osteopilus septentrionalis*) following sublethal exposure to the fungicide chlorothalonil (0.0176−17.6 μg/L) and herbicide atrazine (1.06−106 μg/L). Although both glyphosate and atrazine were components of the herbicide mixture used in our study, we did not find any interactive effect with Bd exposure on anuran survival or Bd infection load. While our atrazine concentration (3.5 μg/L) was in a similar range to that of McMahon et al.,[19] our glyphosate concentration (3.9 μg/L) was 3 about orders of magnitude lower than that of Hanlon and Parris,[28] which may explain the difference in outcomes.

Discordance between our results and past studies on the interaction between Bd-induced mortality and pesticides may be in part due to differences in anuran developmental stage (i.e., larvae vs metamorphs) tested and the timing of pesticide exposure. Ontogeny can affect sensitivity to pesticides[68] and Bd.[25−27,51]

Timing of exposure after metamorphosis may also impact whether pesticides affect the pathogenicity of Bd. Buck et al.[23] also used post-metamorphic anurans in their study, but the timing of pesticide exposure differed from our study. Specifically, Buck et al.[23] first exposed tadpoles or newly metamorphosed anurans to sublethal pesticide mixtures and then subsequently exposed individuals to Bd. Though our pesticide mixtures did not influence postmetamorphic amphibian survival, our results add to the body of research discussed above that suggests effects of

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pesticides and Bd on amphibian survival change with pesticide identity and mode-of-action (e.g., organophosphates versus pyrethroids), host identity (e.g., species-specific sensitivity), and developmental stage (i.e., tadpole versus metamorph). Knowing these sensitive developmental windows among amphibians will be vital when investigating the effects of pesticides on amphibian responses to Bd in future experiments.

Our study aimed to screen for interactions between Bd and the most commonly applied pesticides in the U.S. home and garden and agricultural sectors.[35] The use of pesticide mixtures at environmentally relevant concentrations is a commonly employed tactic within ecotoxicology to screen a large number of pesticides while also limiting experimental size and number of replicates needed when outcomes are unknown.[69] Following a significant effect of a given pesticide mixture, researchers could then test single pesticides to confirm which active ingredient is driving the effects on amphibians and Bd. Though we found no interactive effects of Bd and sublethal concentrations of herbicides and insecticides on amphibian survival, there are a number of factors future studies could investigate that may shed light on this complex interaction. Researchers could modify the current experimental design through the addition of fungicides. Found in numerous freshwater systems,[40,70] fungicides have been shown to decrease Bd zoosporangia and zoospore abundance under laboratory conditions[19,71] but are associated with increased Bd load under natural conditions.[70] Researchers may also choose to increase the range of concentrations employed to determine how each pesticide influences sensitivity to Bd above environmentally relevant concentrations. For example, McMahon et al.[19] reported dose-dependent responses of Bd when exposed to chlorothalonil and atrazine in culture and on tadpoles (*O. septentrionalis*). Using pesticides that differ in mode-of-action or a concentration−response design in future studies would strengthen our understanding of Bd and pesticides as chemical use continues to increase.

Understanding how human activities affect pathogen infectivity and virulence is vital to conserving amphibians, which are important members of aquatic and terrestrial food webs.[72] Though we report no effect of sublethal pesticide concentrations on the sensitivity to Bd in six anuran species, our work supports previous research that indicates the importance of Bd strain, host identity, and timing of exposure. Taken together, these results suggest that the interplay between the pathogen Bd and anthropogenic chemicals is highly context-dependent. Future research that investigates various concentrations and class-specific (i.e., mode-of-action) effects of pesticides throughout amphibian development may lead to a better understanding of the complexity of pesticide and pathogen interactions.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b06055.

Water quality assurances, detailed pesticide sampling protocols, the number of replicates per treatment per species, and amphibian body size measurements (Tables S1 and S2) (PDF)

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