Herpetological Conservation and Biology 12:68–77.
Submitted: 7 September 2016; Accepted: 6 February 2017; Published: 30 April 2017.

Amphibians, Pesticides, and the Amphibian Chytrid Fungus in Restored Wetlands in Agricultural Landscapes

Rebecca A. Reeves[1], Clay L. Pierce[2], Mark W. Vandever[3], Erin Muths[3], and Kelly L. Smalling[4,5]

[1]Department of Natural Resource Ecology and Management, Iowa State University, Ames, Iowa 50011, USA
[2]U.S. Geological Survey, Iowa Cooperative Fish and Wildlife Research Unit, Iowa State University, Ames, Iowa 50011, USA
[3]U.S. Geological Survey, Fort Collins Science Center, Fort Collins, Colorado 80526, USA
[4]U.S. Geological Survey, New Jersey Water Science Center, Lawrenceville, New Jersey 08648, USA
[5]Corresponding author, email: ksmall@usgs.gov

***Abstract.*—Information on interactions between pesticide exposure and disease prevalence in amphibian populations is limited, especially from field data. Exposure to certain herbicides and insecticides has the potential to decrease the immune response in frogs, which can potentially lead to increased abundance of *Batrachochytrium dendrobatidis* (*Bd*) zoospores on individuals and in the wetlands. In contrast, exposure to certain fungicides can decrease *Bd* abundance on frog skin. We examined the relationships between the abundance of *Bd* on the skin of individual Boreal Chorus Frogs (*Pseudacris maculata*) and the concentrations of pesticides in the water and in frog tissue at six agriculturally dominated wetlands in Iowa, USA. We collected frogs from each wetland, swabbed them for *Bd*, and analyzed their tissues for a suite of fungicides, herbicides, and insecticides. We collected surface water from the wetlands and we analyzed it for the same suite of pesticides. We observed no relationship between *Bd* zoospores on the skin of individual frogs and the concentrations of total pesticides, total herbicides/insecticides and total fungicides in frog tissue. Similarly, we observed no relationship between *Bd* zoospore abundance in water and the concentration of total pesticides or total herbicides in water. However, we observed a negative relationship between *Bd* zoospore abundance in water and neonicotinoid concentrations in surface water. Negative results are seldom reported but can be important contributors to a more complete understanding of the complex and potentially synergistic relationships between disease and pesticides. Data from field studies on these relationships are particularly scarce. As our laboratory understanding of these relationships expands, the need for field based, or applied, studies grow.**

Key Words.—Batrachochytrium dendrobatidis; Boreal Chorus Frog; fungicides; herbicides; insecticides; *Pseudacris maculata*; tissue concentration; zoospore

Introduction

Amphibians are declining worldwide from a variety of factors including habitat loss and degradation, climate change, and emergent diseases (Collins and Storfer 2003; Grant et al. 2016). Between 1850 and 1950 the amount of farmland in the United States increased from < 120 to > 445 million ha (300 million to > 1.1 billion ac; United States Department of Agriculture. 2013. Census of Agriculture Historic Archive. Available from http://agcensus.mannlib.cornell.edu/AgCensus/homepage.do; [Accessed 5 December 2013]). In response to increases in human population growth, global agricultural production is projected to increase by 60–110% over the next three decades (Ray et al. 2013). Land-use changes from increased agricultural production may not destroy habitat entirely, but they usually alter habitat physically or chemically such that survival of resident organisms (e.g., amphibians) may be compromised.

Twenty-two species of amphibians are found in Iowa, USA, eight of which live in the Prairie Pothole region of central Iowa (Christiansen and Bailey 1991) and most can be found within the agricultural landscape (Knutson et al. 1999). Some amphibian declines and abnormalities have been attributed to contaminants (Hopkins et al. 2006; Brühl et al. 2013), often with a focus on water quality at breeding sites. Amphibian breeding sites in Iowa can be natural or man-modified for the purpose of collecting and removing nitrogen from agricultural field run-off (Otis et al. 2010. Assessment of Environmental Services of CREP Wetlands in Iowa and the Midwestern Corn Belt. Final report prepared for the United States Department of Agriculture Farm Services Agency. Available at http://lib.dr.iastate.edu/cgi/viewcontent.cgi?article=1002&context=cfwru_reports. [Accessed 6 June 2016). In this landscape, breeding sites are often situated in the middle of agricultural fields (Mark Vandever, Rebecca Reeves, pers. obs.).

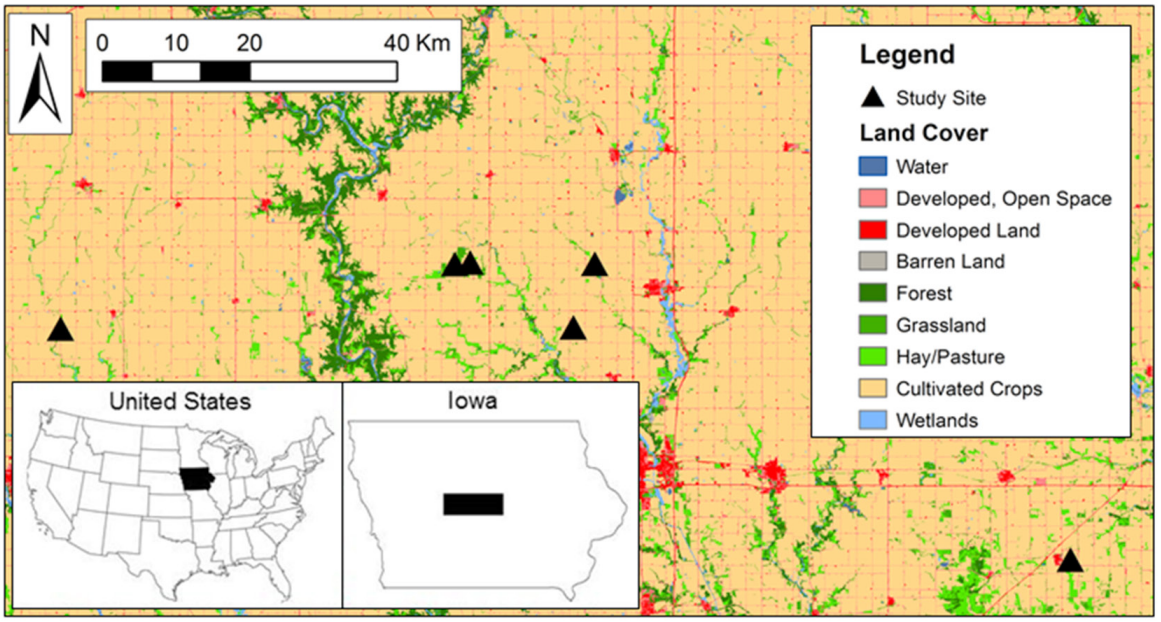

Figure 1. Map of the six wetlands sampled in Iowa, USA (insets) during 2012 and 2013 including land cover information (Homer et al. 2015).

Agricultural chemicals have variable impacts on amphibians and aquatic communities including indirect effects through synergies with pathogens. Exposure to pesticides can have positive or negative effects on disease transmission, infectivity, and host susceptibility (Clements and Rohr 2009; Blaustein et al. 2011). For example, certain fungicides can reduce the number of *Batrachochytrium dendrobatidis* (*Bd*) zoospores, the cause of chytridiomycosis, on frog skin (Berger et al. 2010; Hanlon et al. 2012; Brannelly et al. 2012), while some herbicides and insecticides, notably atrazine and DDT (dichlorodiphenyltrichloroethane), can cause immunosuppression in amphibians and may increase susceptibility to chytridiomycosis (Albert et al. 2007; Brodkin et al. 2007; Mann et al. 2009). Although laboratory mesocosm studies have examined the effects of one or a suite of chemicals (Christin et al. 2004; Buck et al. 2012), there have been limited field assessments of the relationship between pesticides and disease in amphibian populations.

We explored the relationship between pesticides and the presence of *Bd* in agricultural wetlands in Iowa where 75% of the landscape is human-modified (U.S. Department of Agriculture. 2007. Economic Research Service. Major uses of land in the United States. Available from http://www.ers.usda.gov/data-products/major-land-uses.aspx#.U39Xx_ldXg8 [Accessed 5 December 2016]), but where there is also a diversity of amphibians (Lannoo 1998). Results from previous studies indicate that pesticides can inhibit immune response in amphibians, making them more susceptible to disease (Hayes et al. 2006; Albert et al. 2007; Brodkin et al. 2007; Davidson et al., 2007), but that certain fungicides are capable of reducing the number of *Bd* zoospores on frogs (Brannelly et al. 2012). Therefore, we hypothesized that pesticides will alter pathogen prevalence in agricultural wetlands. To test this hypothesis, we compared the prevalence of *Bd* on frogs and in the water to total pesticide concentrations, total herbicide and insecticide concentrations, and total fungicide concentrations in water and frog tissue.

Materials and Methods

Study site.—We collected frogs and surface water from six wetlands in the Des Moines Lobe landform in Central Iowa (Fig. 1) in 2012 and 2013 (Reeves et al. 2016; Smalling et al. 2015). Site selection occurred opportunistically based on landowner permission, wetland surface area, and whether or not chorus frogs were present. Thus, inference is limited to the sampled sites. To minimize the transmission of pathogens between wetlands, we disinfected all equipment using household bleach at concentrations > 1% or by allowing equipment to dry completely between wetlands (Johnson et al. 2003). We scrubbed all equipment to remove soil or organic matter prior to either bleaching or drying.

Sample collection and analysis.—In May 2013, we collected five adult male Boreal Chorus Frogs (*Pseudacris maculata*) from each wetland and swabbed each one to test for the presence of *Bd* (Hyatt et al. 2007). Frogs were then euthanized, and immediately frozen for pesticide analysis (Smalling et al. 2015). We shipped

swabs to the Amphibian Disease Diagnostic Laboratory at Washington State University (Pullman, Washington, USA). Swabs were assessed for the *Bd* using a modified version of the Boyle et al. (2004) protocol. For DNA extraction, we used DNeasy 96 blood and tissue kits. We cut off swab tips and digested them twice as long as recommended in the Boyle et al. (2014) protocol. We extracted the DNA and we ran each sample in triplicate using the qPCR primers and protocols from Boyle et al. (2014). The qPCR reaction was run for 40 cycles. We obtained a *Bd* standard from Alex Hyatt at the Australian Animal Health Laboratories (Andrew Storfer, pers. com.) and we ran it in duplicate on each plate with the following titrations: 10^4, 10^3, 10^2, 10^1, and $10^{0.1}$. Each plate contained a positive and negative control. To ensure proper accuracy of the standard curve, an $r^2 \geq 0.97$ was needed. We re-ran plates if r^2 was < 0.97. We re-ran unknown samples with a coefficient of variation > 0.15 among the three replicates. Samples with a relative *Bd* equivalent > 10 were deemed positive. We considered those between 0.1 and 10 Suspect and those that were 0 negative.

We extracted and analyzed whole frog tissue samples (n = 30) for 98 pesticides and pesticide degradates at the USGS California (Sacramento, California) by gas chromatography mass spectrometry (GCMS; Smalling et al. 2013, 2015). During April and June of 2012 and June of 2013, we collected and filtered water samples (three samples per wetland) for *Bd* analysis at the USGS Reston Microbiology Laboratory (Reston, Virginia). Depending on the wetland, we collected between 0.1 and 0.9 L of water for analysis. We extracted DNA from the capsule water filter for *Bd* analysis using a Puregene kit for tissue (Gentra Systems, Valencia, California), and amplified and analyzed by quantitative polymerase chain reaction (qPCR) technique (Kirshtein et al. 2007). Quantitative PCR assays were conducted using the Qiagen QuantiTect SYBR Green PCR kit following standard protocols with 1.2 µM each of primers ITS1-3Chytr and 5.8Schytr (Kirstein et al. 2007). These primers target a 146 bp region of the ITS-1 region of the *Bd* ribosomal operon. To quantify the *Bd* zoospore equivalents, we calculated a conversion factor from a dilution series of known zoospore numbers (i.e., 169), which is within the range of 60 to 220 for rRNA fungal gene locus copy numbers. *Bd* genomic equivalents are therefore 169 times greater than the numbers we report (Chestnut et al. 2014). A site was considered *Bd*-positive if at least one of three replicate filters returned a positive qPCR result. The negative controls that we analyzed from each *Bd* batch for water were considered free of *Bd*. The relative standard deviations between the three water samples collected at each wetland ranged from 2 to 50%. We also collected an additional water sample at each site during April and June of 2012 and June of 2013, filtered and analyzed them for current-use pesticides and pesticide degradates by GCMS (Reilly et al. 2012), including neonicotinoid insecticides (Hladik and Calhoun 2012) at the USGS Organic Chemistry Research Laboratory (Sacramento, California) and glyphosate by liquid chromatography mass spectrometry (Meyer et al. 2009) at the USGS Organic Geochemistry Research Laboratory (Lawrence, Kansas).

Statistical analysis.—We determined that the data were not normally distributed using a Shapiro-Wilk test. For this reason, we used non-parametric, two-sided Spearman rank correlations to assess the relationship between tissue concentrations of total pesticides (combined herbicides, insecticides, and fungicides), total fungicides, and total herbicides/insecticides and the mean abundance of *Bd* zoospores on the skin of that individual. We also assessed the relationships between total dissolved pesticide concentrations and total dissolved insecticide concentrations (sum of the two neonicotinoids detected) in water samples with median *Bd* zoospore abundances (median of the 3 replicates/site collected) in water samples using two-sided Spearman rank correlations and simple linear regressions with log-transformed data. We considered the results to be statistically significant if the P-value was ≤ 0.05. We censored pesticide and *Bd* non-detection values in water and tissue at their reported method detection limits (Tables 1 and 3). We also used both Suspect and positive *Bd* swab data in our analysis. We used R (R Core Team 2013) for all statistical analyses.

Results

We detected *Bd* zoospores on the skin of 36% of the frogs tested for pesticides, and average zoospore counts per individual ranged from below detection limits to 18.2 zoospores (Table 1). Tissue concentrations for total pesticides (sum of 13 compounds), total herbicides/insecticides (sum of 8 compounds) and total fungicides (sum of 5 compounds) ranged from 0.1 to 961 µg/kg, 0.1 to 109 µg/kg, and 7.8 to 948 µg/kg, respectively (Tables 1 and 2). We detected *Bd* in water samples from all sites during the spring/summer with median zoospore counts ranging from 2.2 to 478 zoospores/L (Table 3). Of the 34 compounds detected in water samples (Smalling et al. 2015), we used only the compounds that were detected in greater than 15% of the samples collected for our statistical analysis. Water concentrations for total pesticides (sum of 6 compounds), total herbicides (sum of 4 compounds) and total insecticides (sum of 2 neonicotinoid compounds) ranged from 162 to 19,440 ng/L, 160 to 19,440 ng/L, and 2 to 16.0 ng/L,

Table 1. Total pesticide concentrations in tissue (µg/kg wet weight) and mean *Bd* zoospore abundance in tissue collected from six wetlands in Iowa during June 2013. Abbreviations are nd = not detected; BJB = Bjork Boda; BOP = Bob Pyle; GRE = Greene; MAR = Marshall; MCH = Boone; SCH = Story.

Biota ID	Mean *Bd* Zoospore Abundance	Total Fungicides	Total Insecticides/ Herbicides	Total Pesticides
BJB A	0.007	273	41.2	314
BJB B	7.40	173	4.8	178
BJB C	nd	948	13.0	961
BJB D	18.2	187	61.1	248
BJB E	0.13	8.0	14.3	21.9
BOP A	nd	52.0	0.1	52.3
BOP B	nd	98.9	9.3	108
BOP C	0.13	31.6	0.2	31.8
BOP D	0.33	nd	4.9	4.9
BOP E	nd	86.2	5.9	92.1
MCH A	nd	40.9	19.3	60.2
MCH B	nd	135	11.9	147
MCH C	nd	nd	4.7	4.7
MCH D	nd	nd	7.1	7.1
MCH E	4.76	121	3.6	125
GRE A	nd	68.0	13.2	81.2
GRE B	nd	20.7	23.0	43.7
GRE C	nd	nd	34.6	34.6
GRE D	nd	94.4	10.1	105
GRE E	nd	538	3.3	542
MAR A	10.9	157	109	266
MAR B	nd	nd	nd	nd
MAR C	nd	203	26.5	229
MAR D	nd	75.4	7.4	82.8
MAR E	nd	195	8.3	203
SCH A	nd	11.2	15.2	26.5
SCH B	1.78	7.8	0.1	7.9
SCH C	0.023	176	11.2	187
SCH D	nd	126	4.7	130
SCH E	0.242	nd	0.1	0.1

respectively (Table 3). We observed no fungicides in water at a detection frequency greater than 15% (Smalling et al. 2015).

We observed no relationship ($P > 0.05$) between the abundance of *Bd* zoospores on frog skin and the concentration of total pesticides, total insecticides/ herbicides or total fungicides in tissue of individual frogs. Similarly, we observed no relationships ($P > 0.05$) between the median abundance of *Bd* zoospores detected in water and the concentrations of either total herbicides or total pesticides in water samples.

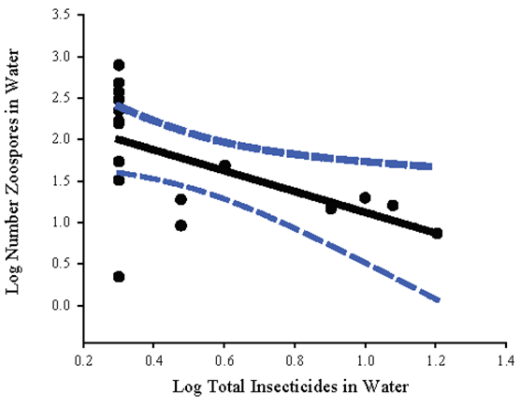

Figure 2. The relationship ($P = 0.02$, $R^2 = 0.304$) between the log transformed median abundance of *Batrachochytrium dendrobatidis* (*Bd*) zoospores in water and the log transformed total insecticide concentration (ng/L) in water (n = 17). The blue dotted lines represent the 95% confidence interval for the regression line.

However, we did observe a negative relationship between total insecticides (i.e., neonicotinoids) and the median abundance of *Bd* zoospores detected in water ($P = 0.02$; Fig. 2).

Discussion

Information on the susceptibility of Boreal Chorus Frogs to *Bd* infections or death by chytridiomycosis is limited. The Pacific Chorus Frog (*Pseudacris regilla*) appears unaffected by chytridiomycosis even though in the laboratory it can carry *Bd* loads up to an order of magnitude higher than loads found lethal to sympatric species (Reeder et al. 2012). However, in a laboratory study, host susceptibility to *Bd* was tested in six amphibian species, and Western Chorus Frogs were susceptible to infections although less than Southern Toads (*Anaxyrus terrestris*) and Wood Frogs (*Lithobates sylvaticus*; Searle et al. 2011). We found that *Bd* abundance varied and occurred in 36% of the individuals swabbed, but there were no relationships between the abundance of *Bd* zoospores and the pesticides detected in frog tissue. We detected *Bd* in every water sample during the spring and summer of both years, which is higher than predicted based on previous work. Nationally, *Bd* occupancy was estimated at 61% in amphibian habitats with no seasonal influence (Chestnut et al. 2014). Our sampling design was limited both temporally and spatially, which could explain the higher detection frequency. We observed no relationship between *Bd* zoospore abundance in water and the total concentration of pesticides and total concentrations of herbicides in water. However, we observed a significant negative relationship between *Bd* zoospore abundance in water and neonicotinoid concentrations in surface water. Thus, our data do not support our hypotheses.

Table 2. Individual pesticide concentrations in tissue (µg/kg wet weight) collected from six wetlands in Iowa in June 2013. Method detection limits are noted under the pesticide name in parentheses. Values in parentheses are less than the method detection limits and are reported as estimates. Abbreviations are nd = not detected; BJB = Bjork Boda; BOP = Bob Pyle; GRE = Greene; MAR = Marshall; MCH = Boone; SCH = Story; CAP=captan; FLX = fluoxastrobin; IMZ = imazalil, MET = metalaxyl; PYR = pyraclostrobin; ALC = alachlor; MEA = metolachlor; TRI = trifluralin; BIF = bifenthrin; CAR = carbofuran; FIP = fipronil; DDTs = total DDTs.

Biota ID	CAP (3.7)	FLX (2.3)	IMZ (2.6)	MET (1.1)	PYR (1.7)	ALC (1.6)	MEA (1.1)	TRI (0.7)	BIF (1.1)	CAR (2.4)	FIP (1.7)	DDTs (1.5)
BJB A	136	nd	nd	nd	137	15.1	(0.4)	0.7	10.0	9.2	nd	5.9
BJB B	173	nd	nd	nd	nd	nd	nd	(0.5)	nd	4.1	(0.2)	nd
BJB C	470	nd	nd	382	96.0	nd	nd	1.1	nd	nd	(0.4)	11.5
BJB D	164	23.1	nd	nd	nd	18.0	nd	(0.5)	nd	nd	(0.7)	41.9
BJB E	nd	nd	nd	nd	7.6	nd	nd	(0.2)	nd	nd	nd	14.1
BOP A	nd	nd	39.3	nd	12.9	nd	nd	nd	(0.1)	nd	nd	nd
BOP B	nd	nd	74.8	nd	24.2	nd	(0.2)	nd	nd	nd	nd	9.1
BOP C	nd	nd	31.6	nd	nd	nd	nd	nd	(0.2)	nd	nd	nd
BOP D	nd	nd	nd	nd	nd	nd	nd	nd	(0.2)	nd	nd	4.7
BOP E	nd	nd	nd	51.8	34.4	nd	5.3	nd	(0.6)	nd	nd	nd
MCHA	nd	nd	nd	nd	40.9	7.4	(0.6)	nd	1.3	9.9	nd	nd
MCHB	nd	nd	nd	nd	135	nd	(0.4)	nd	nd	8.0	nd	3.5
MCHC	nd	nd	nd	nd	nd	nd	nd	nd	(0.4)	nd	(0.2)	4.1
MCHD	nd	nd	nd	nd	nd	nd	nd	nd	nd	3.8	nd	3.3
MCHE	nd	nd	58.2	nd	63.2	nd	nd	nd	(0.3)	nd	nd	3.3
GRE A	nd	nd	68.0	nd	nd	nd	nd	nd	(0.8)	8.3	nd	4.1
GRE B	nd	nd	nd	20.7	nd	nd	nd	nd	(0.5)	9.1	nd	13.4
GRE C	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	34.6
GRE D	94.4	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	10.1
GRE E	nd	234	94.4	nd	210	nd	nd	nd	nd	nd	nd	3.3
MARA	nd	nd	nd	nd	157	nd	41.6	nd	nd	nd	nd	66.9
MARB	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
MARC	nd	nd	nd	nd	203	nd	nd	nd	nd	nd	nd	26.5
MARD	nd	nd	nd	nd	75.4	nd	nd	nd	nd	nd	nd	7.4
MARE	105	89.4	nd	nd	nd	nd	(0.5)	nd	nd	nd	nd	7.8
SCH A	nd	nd	nd	11.2	nd	3.0	nd	(0.4)	nd	nd	(0.3)	11.5
SCH B	nd	nd	nd	7.8	nd	nd	nd	(0.1)	nd	nd	nd	nd
SCH C	nd	22.4	nd	25.6	128	nd	nd	0.7	nd	nd	(0.3)	10.2
SCH D	nd	36.3	89.4	nd	nd	nd	nd	(0.5)	nd	nd	(0.2)	nd
SCH E	nd	nd	nd	nd	nd	nd	nd	(0.1	nd	nd	nd	nd

Although the reported effects of fungicides on *Bd* are equivocal, certain azole fungicides (e.g., fluconazole and itraconazole) reduce *Bd* zoospore counts on frog skin, but other fungicides (e.g., vinclozolin) have the potential to cause immunosuppression and endocrine disruption (Van Wyk et al. 2003; Berger et al. 2009; Brannelly et al. 2012; Holden et al. 2014). Itraconazole and fluconazole are not registered for agricultural use in the United States and vinclozolin has not been used agriculturally since 2008 (Baker and Stone 2015). Pyraclostrobin and captan have lethal effects on frogs at environmentally relevant concentrations (Belden et al. 2010; Brühl et al. 2013) and we detected both these compounds in 20 and 47% of the tissues analyzed, respectively (Smalling et al. 2015). The other fungicides we observed in tissue included imazalil, fluoxastrobin and metalaxyl (Smalling et al. 2015). Currently, little is known about the sub-lethal effects of these and other fungicides and how they interact with other stressors, such as disease or even just the presence of a pathogen. Fungicides tend to be the most frequently detected pesticides in frog tissue (Smalling et al. 2013, 2015; Battaglin et al. 2016), so we hypothesized that fungicides might ameliorate the effect of chytridiomycosis by reducing *Bd* abundance on individual animals. Our results did not support this relationship and we observed no relationships

Table 3. Dissolved concentrations (ng/L) of pesticides detected in greater than 15% of the samples and median *Bd* zoospores counts (zoospores/L) in water collected during 2012 and 2013. Method detection limits are noted under the pesticide name in parentheses. Values less than the method detection limit are in parentheses and reported as estimates. Abbreviations are nd = not detected; BJB = Bjork Boda; BOP = Bob Pyle; GRE = Greene; MAR = Marshall; MCH = Boone; SCH = Story; MED ZOO = median zoospores; AMPA = aminomethylphosphonic acid CLO = clothianidin; GLY = glyphosate; MEA = metolachlor; THX = thiomethoxam; TOT HERB = total herbicides; TOT INSECT = total insecticides and TOT PEST = total pesticides

Site	Date	MED ZOO (0.06)	AMPA (20)	CLO (1.0)	GLY (20)	MEA (1.5)	THX (1.0)	TOT HERB	TOT INSECT	TOT PEST
BJB	4/17/2012	376	40	nd	nd	140	nd	450	nd	450
	6/5/2012	156	50	nd	390	nd	nd	19,440	nd	19,440
	6/24/2013	7.4	290	6.0	120	200	10.0	860	16.0	876
BOP	4/18/2012	303	nd	nd	70	140	nd	410	nd	410
	6/24/2013	2.2	90	nd	210	150	nd	650	nd	650
MCH	4/18/2012	166	30	nd	50	120	nd	340	nd	340
	6/6/2012	478	40	nd	150	nd	nd	520	nd	520
	6/24/2013	48.7	130	3.0	260	120	nd	830	3.0	833
GRE	4/17/2012	18.8	nd	2.0	180	90	nd	200	2.0	202
	6/6/2012	223	30	nd	40	nd	nd	390	nd	390
	6/24/2013	14.6	40	6.0	nd	130	2.1	400	8.1	408
MAR	4/18/2012	352	50	nd	nd	60	nd	220	nd	220
	6/7/2012	54.0	nd	nd	70	nd	nd	310	nd	310
	6/24/2013	19.8	310	8.0	80	270	2.3	1,060	10.3	1,070
SCH	4/17/2012	9.2	nd	2.0	20	30	nd	160	2.0	162
	6/6/2012	32.3	60	nd	90	nd	nd	290	nd	290
	6/24/2013	16.0	30	10.0	50	200	2.2	470	12.2	482

between zoospore abundance on frog skin and the total concentration of fungicide in tissues. However, the lack of relationships between fungicide concentration and *Bd* abundance may be because no azole fungicides (considered to inhibit *Bd* growth) were observed in water or in frog tissue samples.

Field data and information about relationships among tissue pesticide concentrations and *Bd* load on an individual, or the prevalence of disease in a habitat are rare, and even limited datasets can provide insights. Such direct assessments are important because dermal uptake of pesticides from both the aquatic and terrestrial environments are important routes of exposure for frogs (Van Meter et al. 2014, 2015) that are often ignored when assessing only the quality of the aquatic habitat. Pesticide exposure may not necessarily result in direct mortality, but repeated exposure and accumulation through food and the dermis, throughout the lifetime of an animal, may induce sub-lethal effects that can exacerbate exposure or susceptibility to disease. Several pesticides, including the herbicide atrazine and the insecticides, dieldrin and DDT, have the potential to cause immunosuppression in amphibians (Hayes et al. 2006; Albert et al. 2007; Brodkin et al. 2007) but limited information is available on the relationship between pesticide exposure and *Bd* infection. Paetow et al. (2012) reported no relationship between *Bd* infections in Northern Leopard Frogs after exposure to the herbicides, atrazine and glyphosate, both observed in the current study. We did observe a negative relationship between total dissolved insecticide concentrations and *Bd* zoospore abundance in water. The neonicotoinoids, clothianidin and thiamethoxam, were the only two insecticides observed frequently in surface water during the months of April and June (Smalling et al. 2015). Neonicotinoids are receiving increased scrutiny because they have the potential to adversely affect pollinators and other wildlife and have been linked to colony collapse disorder in bees (Spivak et al. 2011; vanEngelsdorp et al. 2009). The potential effects of neonicotinoids on amphibians or their interaction with fungal pathogens are largely unknown. This negative relationship does not support our hypothesis and interactions between neonicotinoids and fungal pathogens such as *Bd* should be explored further.

The amphibian cutaneous microbiome (Belden and Harris 2007) and antimicrobial peptides (Rollins-Smith et al. 2005) found within natural secretions in the skin of the host are influencial in host suspetibility to pathogens. Currently, limited information is available on the impacts of environmental stressors such as pesticides on the skin microbiome or antimicrobial peptides. Davidson

et al (2007) noted a reduction in skin peptide defenses after exposure to the insecticide, carbaryl, suggesting that certain pesticides may have the potential to inhibit immune defenses thereby increasing suseptibility to pathogens. Other water quality characteristics such as pH and conductance have been shown to influence both the antimicrobial peptides and the community structure of the skin microbiome (Krynak et al. 2016). Future studies are needed to assess the influence of environmental stressors such as pesticides on skin defenses to improve our understanding of disease resistance across populations and land-use types.

Global demands for agriculture are predicted to increase (Ray et al. 2013), as are changes in climate (Wuebbles and Hayhoe 2004), circumstances that have the potential to foster an increase in the application of agrochemicals. Understanding relationships among pesticides used on these landscapes, pathogens, and the impact of these stressors on resident organisms such as amphibians, will facilitate sound conservation decisions aimed at enhancing the persistence of native wildlife in agricultural systems.

Acknowledgments.—This project was funded by the Fort Collins Science Center and USGS Amphibian Research and Monitoring Initiative as a part of ongoing technical assistance to the Farm Service Agency to assess Iowa Conservation Reserve Enhancement Program wetlands. The authors thank Shawn Richmond, Matthew Lechtenberg, the field technicians, and the army of volunteers and that made this study possible. We also thank Hladik, Michael Meyer, Megan McWayne-Holmes and Corey Sanders of the USGS for the pesticide analysis, and the Amphibian Disease Diagnostic Laboratory at Washington State University for the swab analysis. We especially thank the landowners and public land managers who allowed access to wetlands (written permission prior to sampling). Exact wetland locations are proprietary. This study was performed under the auspices of Iowa State University Institutional Animal Care and Use Committee (IACUC) protocol # 3-12-7324-D, and animals were collected under Iowa state permit #SC699. This is contribution no. 572 of the USGS Amphibian Research and Monitoring Initiative. Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the US Government.

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Rebecca Reeves is a Fish and Wildlife Biologist with the U.S. Fish & Wildlife Service in Carlsbad, California, USA. She received her B.S. (2010) in Environmental Science from the University of Maryland, Baltimore County (UMBC), USA, and her M.S. (2014) in Wildlife Ecology from Iowa State University, Ames, Iowa. She is broadly interested in stream and wetland restoration, aquatic ecology, and herpetology. Before moving to the West Coast of North America, Rebecca, a Maryland native, spent time restoring streams and marshes at the Edwin B. Forsythe National Wildlife Refuge in Galloway, New Jersey. (Photographed by Adrian Rice).

Clay Pierce is the Assistant Leader for Fisheries of the Iowa Cooperative Fish and Wildlife Research Unit of the U.S. Geological Survey at Iowa State University, Ames, Iowa, USA. He supervises graduate students and his research is aquatic ecology, aquatic vertebrate conservation, and fisheries research in the wetlands, lakes, rivers, and streams of Iowa. He teaches courses in Stream Ecology and Fisheries Science. Clay is a native of Minnesota and received his B.S. (1980) at Mankato State University, his M.S. (1982) at the University of Kentucky, and his Ph.D. (1987) at the University of Maryland. He was a Postdoctoral Researcher at McGill University and an Assistant Professor at Eastern Illinois University before assuming his present position in 1993. (Photographed by Ann Pierce).

Mark Vandever is a Rangeland Management Specialist for the U.S. Geological Survey at the Fort Collins Science Center, Colorado, USA. He received his B.S. (1997) and M.Ag. (2007) from Colorado State University, Fort Collins, Colorado. Mark's broad research interests include understanding pollinator-plant relationships, amphibian and native bee exposure to pesticides, and how land management practices can produce quality wildlife and pollinator habitat in Conservation Reserve Program grasslands. (Photographed by Erin Muths).

Erin Muths has studied declining amphibians for more than 20 y, focusing on demography and disease in endangered species in mountain ecosystems. She has served as Co-Editor for the Journal of Herpetology and is a Principal Investigator for the Amphibian Research and Monitoring Initiative (ARMI) of the U.S. Geological Survey. She received her B.S. (1986) at the University of Wisconsin-Madison, her M.S. (1990) at Kansas State University, and her Ph.D. (1997) at the University of Queensland, Australia. (Photographed by Don Campbell).

Kelly Smalling is an Environmental Organic Chemist with the New Jersey Water Science Center of the U.S. Geological Survey. She is a principal investigator for the Amphibian Research and Monitoring Initiative (ARMI) of the U.S. Geological Survey whose research focuses on the exposure and effects of current-use pesticides and other contaminants on amphibians. She received her B.S. (1999) at the University of Alabama in Huntsville and her M.S.P.H. (2003) at the University of South Carolina. (Photographed by John Bunnell).