Effects of endosulfan in freshwater pond communities

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Abstract: Pesticide use has led to ubiquitous contamination of natural habitats that can cause direct and indirect effects on nontarget organisms. Laboratory toxicity tests are valuable for evaluating the direct lethal effects of pesticides, but whether species differences in sensitivity identified from such tests are representative of more natural conditions is unknown. Studies of pesticide effects on communities are needed to understand the indirect effects of pesticides, but many such studies are focused on simplified communities and overlook the contribution of higher trophic levels (i.e., lethal predators), which can have interactive effects with pesticides and may play a large role in influencing community dynamics in contaminated habitats. Much of the research investigating pesticides in communities has focused on organophosphates, carbamates, and pyrethroids, whereas organochlorines are understudied, despite the fact that they can be highly toxic and persist in the environment. We investigated the effect of the organochlorine insecticide endosulfan on aquatic food webs composed of 3 tadpole species, vertebrate and invertebrate predators, zooplankton, and algae. We manipulated endosulfan concentrations (0, 0.2, 3.1, and 27.3 $\mu$g/L) and free-ranging predators (adult red-spotted newts [\textit{Notophthalmus viridescens}] and dragonfly larvae [\textit{Anax junius}]). Endosulfan caused direct lethal effects on tadpoles, red-spotted newts, and copepods. Patterns of species sensitivity were consistent with past laboratory experiments. Free-ranging predators caused additive, negative effects on tadpole survival, and affected anuran time to and size at metamorphosis. Our study demonstrated that endosulfan can initiate a wide range of direct and indirect effects on nontarget organisms and interacts additively with lethal predators.

Keywords: community ecotoxicology, trophic cascade, \textit{Lithobates sylvaticus}, \textit{Pseudacris crucifer}, \textit{Anaxyrus americanus}

Pesticide use to control pest species and disease vectors has increased dramatically since the 1940s (Grube et al. 2011) and has led to the ubiquitous contamination of natural systems (Gilliom 2007, Smalling et al. 2012, Stone et al. 2014, Stehle and Schulz 2015). Stone et al. (2014) found $\geq$1 pesticide concentrations that exceeded chronic aquatic-life benchmarks in 61 and 90% of all water samples from agricultural and urban streams, respectively, over the past decade (2002–2011). Pesticides negatively influence target and nontarget species through direct lethal effects (Johnson and Finley 1980, Mayer and Ellersieck 1986), and can cause indirect effects that can be transmitted through a food web (Relyea and Diecks 2008). Given their widespread use and occurrence within ecosystems, investigating the direct and indirect effects of pesticides on communities is imperative.

The direct lethal effects of pesticides are commonly evaluated using short-term, single-species toxicity tests under laboratory conditions (ASTM 2008). These tests are useful for estimating the direct toxic effects of numerous chemicals over short time periods and for quantifying differences in pesticide sensitivity among species (Jones et al. 2009, Relyea and Jones 2009, Hammond et al. 2012). When these tests reveal differences in species sensitivities, a key question is whether the patterns in sensitivity occur under more natural conditions. For example, the organochlorine insecticide endosulfan is very highly toxic to numerous nontarget species under laboratory settings (Berrill et al. 1998, Key et al. 2003, Rohr et al. 2003, Wan et al. 2005, Jones et al. 2009, Hammond et al. 2012). When conducting toxicity tests in seminatural, outdoor mesocosm experiments using lethal endosulfan concentrations, differences in mortality of community members have been consistent with the short-term, laboratory-based median lethal concentration (LC$_{50}$) studies (Relyea 2009, Hua and Relyea 2014). However, when considering how nontarget species are affected by the direct toxic effects of pesticides, the other species with which nontarget species interact must be considered (Rohr et al. 2006, Boone et al. 2007). To this extent, many investigators have focused on members of a single trophic level (e.g., herbivores) or used simplified communities (e.g., 2 trophic levels) to investigate the effects of toxic pesticides on community dynamics (Relyea and Hoverman 2006). If we are to better understand how pesticides influence natural communities, more work is needed to investigate both the direct lethal and indirect sublethal effects of pesticides.
using communities that are more representative of natural ecosystems.

Predators are a major force in ecological communities (Paine 1969, Sih et al. 1985, Carson and Root 2000). Lethal predators can both directly and indirectly influence community dynamics by decreasing prey density and inducing trait-mediated responses in prey species (Peacor and Werner 2004). However, much of the research investigating the interactive effects of pesticides and predators has involved nonlethal, caged predators (but see Relyea et al. 2005, Cothran et al. 2011, Trekels et al. 2013). One can imagine 3 scenarios in which pesticides and lethal predators could interact in a contaminated food web to affect the abundance of nontarget prey species. If pesticide tolerance of the prey were lower than that of the predators at a given concentration, then the pesticide would have no direct lethal effect on the predators, and prey survival would be reduced by both the pesticide and the predator (Boone and Smlitsch 2001, Chang et al. 2005, Hanlon and Relyea 2013). In this scenario, predation could cause additive, substitutive, or synergistic effects on prey survival in contaminated systems. If the pesticide tolerance of the predators were lower than that of the prey, then the pesticide would have a direct lethal effect on predators that would indirectly cause an increase in prey survival (Relyea et al. 2005, Relyea and Hoverman 2008). Last, if the pesticide tolerance of the predators and the prey were similar, then the increased prey survival subsequent to predator death by pesticide would be offset by the decreased prey death from the direct toxic effects of the pesticide (Boone and Smlitsch 2003, Chang et al. 2005). Given the diverse ways that predators can influence community dynamics in contaminated habitats, we need studies that consider the effects of pesticides in more complex communities that include lethal predators.

Natural systems are often affected by a number of pesticides (Gilliom 2007, Smalling et al. 2012, Sparling et al. 2014, Stone et al. 2014, Stehle and Schulz 2015), but much of our knowledge of the effects of pesticides in freshwater ecosystems comes from investigating responses to organophosphates, carbamates, and glyphosate in simplified communities (Fleeger et al. 2003, Stehle and Schulz 2015). For example, Relyea and Diecks (2008) investigated the effects of the insecticide malathion (an organophosphate) on aquatic communities and found negative indirect effects on leopard frog (Lithobates pipiens) survival caused by a trophic cascade initiated by direct toxic effects on sensitive zooplankton species. Moreover, the toxicity of the malathion, carbaryl (a carbamate), and glyphosate increases when combined with predator cues for a number of amphibian species (Relyea and Mills 2001, Relyea 2003, 2004, 2005). In contrast, much less is known about the effects caused by organochlorine pesticides (9% of freshwater pelagic studies; Fleeger et al. 2003), which can be orders of magnitude more toxic than organophosphates and carbamates (Relyea 2009, Jones et al. 2009, Hammond et al. 2012) and can be persistent in ecosystems (Sparling et al. 2001, 2014, Quinete et al. 2013, Potter et al. 2014). To fully understand the direct and indirect effects of pesticides, we need to conduct toxicity tests under natural conditions that simulate realistic communities with the pesticides that pose the greatest threat to ecosystems (Wang et al. 2009).

We sought to investigate the direct and indirect effects of the organochlorine insecticide endosulfan on complex aquatic communities with different lethal predators. We asked the following questions: 1) Do toxicity tests under laboratory conditions predict endosulfan toxicity to both prey and predator species under more natural conditions? 2) Are the direct toxic effects of endosulfan additive, compensatory, or synergistic with predation under natural conditions? 3) Do the direct toxic effects of endosulfan cause indirect effects in aquatic food webs?

**METHODS**

**Pesticide background**

We chose to use the organochlorine insecticide endosulfan (CAS 115-29-7; >98% purity; Chem Service, West Chester, Pennsylvania). Endosulfan is a C1-channel antagonist consisting of the 2 isomers α- and β-endosulfan, which degrade into endosulfan sulfate. It is a broad-spectrum insecticide applied at an average of 1.09 kg/ha on crops, such as grapes, tomatoes, and potatoes (California, USA, pesticide records; http://www.pesticideinfo.org/Detail_ChemUse.jsp?Rec_Id=PC35085). The US Environmental Protection Agency (EPA) has initiated a voluntary cancellation program and phase-out of all endosulfan in the USA by 2016. As a result, endosulfan is still being used on crops such as pineapples, strawberries, kale, and cabbage. Moreover, concentrations remain elevated in the environment (Quinete et al. 2013, Potter et al. 2014, Sparling et al. 2014), and endosulfan has been found in tissues of organisms inhabiting contaminated ecosystems (Sparling et al. 2001, 2014, Quinete et al. 2013). Endosulfan is an endocrine disruptor (Colborn et al. 1993, Rossi 2002) and is highly toxic to numerous aquatic organisms (Ernst et al. 1991, Berrill et al. 1998, Broomhall 2002, Wan et al. 2005, Capkin et al. 2006, Bernabò et al. 2008, Jones et al. 2009, Sparling and Fellers 2009, Hua and Relyea 2014). As a result, it has been identified as posing serious environmental risk in freshwater ecosystems (Wang et al. 2009).

**Experimental design**

We examined the separate and combined effects of the insecticide endosulfan and free-ranging predators on seminatural aquatic communities in outdoor mesocosms at the University of Pittsburgh’s Pymatuning Laboratory of Ecology. Outdoor mesocosm experiments are ideal to investigate the effects of stressors on communities in...
more natural contexts (Rowe and Dunson 1994, Preston 2002, Boone and James 2005), and have revealed numerous interactions among natural and anthropogenic stressors that are otherwise absent in simplified laboratory experiments (Boone and James 2003, Fleeger et al. 2003, Rohr and Crumrine 2005, Relyea and Hoverman 2006, Relyea and Diecks 2008, Relyea 2009, Jones et al. 2011). We used a completely randomized design with a factorial combination of 4 concentrations of endosulfan (0, 1.0, 10, and 100 μg/L) crossed with 3 lethal predator treatments (no predator, larval dragonflies [Anax junius], and adult red-spotted newts [Notophthalmus viridescens]). The 12 treatment combinations were replicated 4 times for a total of 48 experimental units.

**Experimental setup**

The experimental units were 1200-L cattle tanks filled with ~790 L of well water. To each mesocosm, we added 25 g of commercial rabbit chow to serve as an initial nutrient source and equal aliquots of pond water and zooplankton (screened for invertebrate predators) from 4 local ponds to provide a natural source of algae and to introduce an invertebrate community. We also added 300 g of dry leaf litter (primarily Quercus spp.) to each mesocosm to serve as a slow-releasing nutrient source and as structure for predators and prey. We placed 4 unglazed tiles (15 × 15 cm) on the north side of each mesocosm to serve as periphyton samplers during the experiment. We covered the mesocosms with 60% shade cloth for the duration of the experiment to prevent colonization and emigration.

We collected our 3 amphibian species as newly oviposited eggs. We collected 10 American toad (Anaxyrus americanus) egg masses, 20 wood frog (Lithobates sylvaticus) egg masses, and 26 spring peeper (Pseudacris crucifer) clutches to ensure genetic diversity. We placed egg masses in 200-L plastic wading pools containing 165 L of aged well water and allowed them to develop under ambient temperature and light conditions. Once hatched, we fed tadpoles an ad libitum supply of rabbit chow (Bunny 16; Blue Seal, Muscatine, Iowa). On 25 May (day 0), we added 20 individuals (ind) (Gosner [1960]-stage 25) of each species (total n = 60) drawn from a mixture of all clutches from each of the 3 species to each mesocosm (American toads = 33 ± 5 mg, wood frogs = 140 ± 10 mg, spring peepers = 20 ± 2 mg). This density (8 ind/m²) is within natural densities (Werner et al. 2007, DKJ, JH, RAR, personal observation).

On day 7, we collected 2 species of predators (larval dragonflies and adult red-spotted newts) from nearby ponds and added 1 predator to each assigned mesocosm. Free-ranging dragonfly larvae pose a significant threat to amphibian larvae in aquatic communities and are capable of reducing tadpole biomass by >80% in a 15-d period (Relyea 2002). In contrast, adult red-spotted newts pose a moderate threat (Relyea 2001a, b). We monitored predator survival using spot checks throughout the experiment at various points in time. We made a final assessment of predator survival when we terminated the experiment.

We also applied the pesticide treatments on day 7 of the experiment. We created a stock solution of endosulfan by dissolving 1500 mg of technical grade endosulfan (endosulfan I + endosulfan II = 99.2% purity; Chem Service) in 150 mL of ethanol. We added 0, 0.079, 0.792, or 7.921 mL of endosulfan stock solution to mesocosms assigned the nominal endosulfan concentrations of 0, 1, 10, and 100 μg/L, respectively. No-endosulfan control mesocosms (0 μg/L) received a sham injection of 7.921 mL of water. We did not include a vehicle treatment because it was applied at a concentration known to be nontoxic to amphibians (Jones et al. 2009), and has been shown to cause little change in previous mesocosm experiments (Relyea 2009). We gently agitated all mesocosms to homogenize endosulfan dispersal after pesticide application.

We collected water samples from all mesocosms 1 h after pesticide application, and pooled all samples of a given endosulfan treatment into a single sample. We sent water samples overnight on ice to be tested at an independent laboratory (Mississippi State Chemical Lab, Mississippi State, Mississippi) by high-pressure liquid chromatography. Chemical analysis confirmed that the no-endosulfan control contained no detectable levels of endosulfan. Actual concentrations of the remaining 3 endosulfan treatments were ~¼ of the nominal concentrations but still reflected a range of concentrations that spanned 2 orders of magnitude: 0.2, 3.1, and 27.3 μg/L (endosulfan concentrations = endosulfan I + endosulfan II + endosulfan sulfate). It is not uncommon for actual concentrations to be lower than nominal concentrations in mesocosm studies (Relyea and Diecks 2008, Relyea and Hoverman 2008, Jones et al. 2010). This effect can be caused by physiochemical breakdown after application, stratification, adherence to organic and inorganic particles, or sorption by algae and macrophytes (van Wijngaarden et al. 2005). In the remainder of the manuscript, we refer to the actual endosulfan concentrations.

**Response variables**

Between day 15 and 20 of the experiment, we measured the water quality in all mesocosms. We assessed water quality using calibrated, digital water meters (YSI Incorporated, Yellow Springs, Ohio). Temperature, dissolved O₂, and pH were measured in the middle of the water column in each mesocosm moving from the lowest (no-endosulfan control) to highest endosulfan concentration over 2.5 h. We measured light decay with a light meter that quantified the number of photons at 10 and 30 cm under the water surface. We calculated the log-linear rate of light decay as:
\[ k = \ln \left( \frac{L_{10}}{L_{30}} \right) / d \]  
(Eq. 1)

where \( L_{10} \) is the intensity of sunlight at a depth of 10 cm, \( L_{30} \) is the intensity of sunlight at a depth of 30 cm, and \( d \) is the difference between the 2 depths.

We then sampled important components of the community including zooplankton, phytoplankton, and periphyton. To sample zooplankton abundance, we took five 200-mL samples of water at the 4 cardinal directions and in the center of each mesocosm. We filtered this water through a 62-μm Nitex® screen, and preserved collected zooplankton in 70% ethanol. Previous work has demonstrated that although cladocerans and copepods vary in their sensitivity to endosulfan, different species within each group have similar sensitivities (Barry and Logan 1998, Relyea 2009). Hence, we identified the zooplankton as either cladocerans or copepods.

To sample phytoplankton abundance, we vacuum-filtered 500 mL of water collected from each mesocosm through Whatman GF/C glass filters, which were then wrapped in aluminum foil to reduce light exposure and placed in a freezer (−4°C) to prevent decomposition. The frozen samples were subsequently assayed for chlorophyll \( a \) using a fluorometer (Model TD-700; Turner Instruments, Sunnyvale, California) and the protocol by Arar and Collins (1997) including the acidification modification.

To sample periphyton abundance, we removed and scrubbed 1 unglazed tile from each mesocosm. We rinsed the brush and unglazed tile with C-filtered, UV-treated water. We vacuum-filtered the periphyton slurry through a Whatman GF/C filter that had been predried at 80°C for 24 h and weighed prior to filtration. After filtration, we dried the filter at 80°C for 24 h and weighed it to calculate periphyton biomass.

The first amphibian metamorph appeared on day 18. From this day forward, we checked all mesocosms daily and collected any metamorphs that had ≥1 forelimb emerged. We held the metamorphs in the laboratory in 1-L plastic tubs containing moistened sphagnum moss until fully metamorphosed (i.e., when their resorbed tails were <2 mm long) under a 15:9 light:dark cycle. We euthanized fully metamorphosed animals (MS-222 overdose), and preserved them in 10% formalin. The last metamorph emerged on day 58. Mesocosms were checked for metamorphs until day 62, at which time we drained the mesocosms and removed, enumerated, and weighed all amphibians. At the same time, we recorded all surviving predators.

Statistical analysis
We analyzed light decay with an analysis of variance (ANOVA). We analyzed amphibian survival with generalized linear models with binary response variables and a logit-link function. We analyzed each anuran species separately, and pared full models down to main effects when the interaction term was not significant. We determined significance with Wald \( \chi^2 \), and described effect size of treatments with odds ratios (exp[B]). Odds ratios describe the likelihood of a specific outcome occurring in a particular treatment compared to the outcome of control treatment (i.e., predator treatments) and are applied after each change in treatment level (i.e., endosulfan concentration). We analyzed amphibian response variables (mass at metamorphosis and time to metamorphosis) with multivariate analyses of variance (MANOVAs). We measured phytoplankton, periphyton, and cladoceran and copepod abundance within 3 d of each other and, therefore, evaluated them with a single multivariate analysis of variance (MANOVA). We followed significant MANOVAs with univariate analyses of variance (ANOVAs) to investigate each response variable. We log (x) amphibian mass, periphyton and phytoplankton abundance) or rank-transformed (time to metamorphosis, cladoceran and copepod abundance, and light decay) data when the assumption of homogeneous errors was not met. The highest endosulfan concentration caused complete amphibian mortality in all replicates. Therefore, analyses on amphibian mass at metamorphosis and time to metamorphosis were conducted with only the 3 lowest endosulfan concentrations (0, 0.2, and 3.1 μg/L). For mean comparisons, we used Fisher’s Least Significant Difference (LSD), which is appropriate when the number of mean comparisons after an ANOVA is relatively small, which was the case in our study.

We analyzed the effect of endosulfan concentration on predator survival (red-spotted newts) with Spearman’s rank correlation coefficient on rank-transformed data. Dragonfly survival by the end of the experiment was low, so we were unable to analyze the effect of endosulfan concentration on dragonfly larvae survival.

RESULTS
Water quality
Average temperature, pH, and dissolved \( O_2 \) across all treatments ranged from 21.5 to 23.3 C (±0.1 to 0.2), 8.2 to 8.3 (±0.01 to 0.03), and 7.5 to 8.6 (±0.1 to 0.2) mg/L, respectively. We found no effect of endosulfan concentration or predator treatment (\( p \geq 0.140 \)) on light decay rate.

Zooplankton, periphyton, and phytoplankton
The MANOVA on cladoceran, copepod, phytoplankton, and periphyton abundances revealed no effect of predator treatment or predator \( \times \) endosulfan interaction, but a multivariate effect of endosulfan was present (Table 1). Therefore, we conducted ANOVAs for only the main effects of endosulfan. We did not find an effect of endosulfan concentration on the abundance of cladocerans, periphy-
ton (Fig. S1), or phytoplankton (Fig. S2), but we did find an effect on copepod abundance \( (p < 0.0001; \text{Fig. 1}) \). Mesocosms exposed to any amount of endosulfan experienced a sharp decrease in copepod abundance compared to the no-endosulfan control \( (p < 0.0001) \), but copepod abundance did not differ among the 3 treatments containing endosulfan \( (p \geq 0.06) \).

**Amphibians**

Our analysis of amphibian survival included all endosulfan concentrations, whereas the MANOVA on amphibian mass at metamorphosis and time to metamorphosis excluded the highest endosulfan concentration in which all amphibians died. The MANOVA on the 3 amphibian species revealed multivariate effects of predator treatment, but not endosulfan concentration or the predator \( \times \) endosulfan interaction \( (\text{Table 1}) \). Separate ANOVAs were conducted only on the main effect of predators on mass at and time to metamorphosis.

**Wood frogs** Increasing endosulfan concentration caused significant wood frog mortality \( (\text{Table 2, Fig. 2A}) \). The odds of wood frog mortality increased \( 3.04^\times \) as endosulfan concentration increased. Predator treatment affected wood frog survival and mass at metamorphosis, but not time to metamorphosis \( (\text{Table 2; Figs 2A, 3A, B}) \). Compared to the no-predator treatment, newts had no effect on tadpole survival \( (p = 0.111) \), whereas dragonflies substantially reduced tadpole survival by a mean of \( 25.8\% \) \( (p = 0.033; \text{Fig. 2A}) \). The 2 predators differed in their effect \( (p = 0.049) \). The odds of wood frog mortality were \( 7.054^\times \) higher with dragonflies compared to the no-predator treatment. Similarly, compared to the no-predator treatment, newts caused no effect on tadpole mass at metamorphosis \( (p = 0.365; \text{Fig. 3A}) \), whereas dragonflies reduced mass at metamorphosis by \( 10\% \) \( (p = 0.021) \). The 2 predators differed in their effect \( (p = 0.002) \).

**Spring peepers** Increasing endosulfan concentration caused increased mortality in spring peeper tadpoles \( (\text{Table 2, Fig. 2B}) \). The odds of spring peeper tadpole mortality were \( 1.25^\times \) higher with increasing endosulfan concentrations compared to the no-endosulfan treatment. Predator treatment did not influence mass at metamorphosis of spring peepers, but did influence their survival and time to metamorphosis \( (\text{Table 2, Figs 2B, 3A, B}) \). Compared to the no-predator treatment, newts and dragonflies reduced spring peeper survival \( (p \leq 0.031) \). The 2 predator treatments differed in their effect \( (p < 0.0001) \). Exposure to dragonflies and newts increased the odds of spring peeper mortality by 9.4 and \( 1.7^\times \), respectively, compared to the no-predator treatment. Newts and dragonflies increased spring peeper time to metamorphosis by 15% and 17%, respectively \( (p \leq 0.023; \text{Fig. 3B}) \). Compared to the no-predator treatment \( (34.4 \text{ days}) \). The 2 predators did not differ in their effect \( (p = 0.950) \).

**American toads** Increasing endosulfan concentration also caused significant declines in the survival of American toads \( (\text{Table 2, Fig. 2C}) \), and American toad survival was negatively influenced by the interaction between endosulfan concentration and predator treatment, probably because of the large impact of newt predation \( (p = 0.008) \). The odds of mortality increased by \( 1.5^\times \) with increasing endosulfan concentration. Predator treatments affected toad survival and both metamorphic responses

<table>
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<th>Multivariate test (Wilks’ lambda)</th>
<th>df</th>
<th>F</th>
<th>p</th>
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<tr>
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<td>Endosulfan ( \times ) predator</td>
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<td>0.706</td>
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</table>

Figure 1. Response curves showing mean \( (\pm 1 \text{ SE}) \) rank-transformed copepod abundance in 3 lethal predator treatments in field mesocosms treated with endosulfan. Note the log-scale on the \( x \)-axis.
Table 2. Results of generalized linear models (Wald \( \chi^2 \)) and analyses of variance examining the effects of endosulfan concentration and predator treatment on amphibian response variables. Data are \( p \)-values (degrees of freedom). Multivariate analysis of variance results indicated no effect of endosulfan concentration on amphibian mass at metamorphosis and time to metamorphosis. As a result, endosulfan concentration was removed from the model.

<table>
<thead>
<tr>
<th>Amphibian response</th>
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<th>Time to metamorphosis</th>
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<tr>
<td>Endosulfan concen-</td>
<td>0.0001 (1)</td>
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<td>Predator treatment</td>
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<td>Endosulfan ( \times )</td>
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<td>–</td>
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(Table 2, Figs 2C, 3A, B). Compared to the no-predator treatment, toad survival was reduced by \( \sim \frac{1}{2} \) when newts and dragonflies were present \( (p \leq 0.0001) \). The 2 predators differed in their effects \( (p = 0.015) \). Toad mass at metamorphosis in the no-predator treatment reached 189 mg, but was reduced by 29% and 21% with newts and dragonflies, respectively \( (p \leq 0.005; \text{Fig. } 3A) \). The 2 predators did not differ in their effect \( (p = 0.230) \). Time to metamorphosis did not differ between the no-predator and dragonfly treatments \( (p = 0.158; \text{Fig. } 3B) \), but time to metamorphosis declined in the newt treatment relative to the no-predator treatment \( (p = 0.002) \). The 2 predators did not differ in their effects \( (p = 0.051) \).

**Predator survival**

Adult newt survival was negatively correlated with endosulfan concentration \( (p = -0.81, p \leq 0.0001) \). Newt survival was high in mesocosms exposed to 0 and 0.2 \( \mu \)g/L endosulfan (100% survival), but declined to 50% and 0% when exposed to 3.1 and 27.3 \( \mu \)g/L, respectively (Fig. 4). We were unable to examine the effects of endosulfan exposure on dragonfly survival because of complete mortality in all treatments by the end of the experiment.

**DISCUSSION**

Our goal was to examine the direct and indirect effects of the organochlorine insecticide endosulfan in aquatic communities containing different lethal predators under seminatural conditions. We discovered direct toxic effects of endosulfan at several trophic levels within our aquatic communities, including a decline in the survival of all 3 tadpole species, newts, and copepods with increasing endosulfan concentration. Among the amphobians, wood frogs were more sensitive to endosulfan than spring peepers and American toads. Free-ranging predators caused negative effects on the survival of all 3 anuran species, but dragonflies caused greater mortality of wood frogs than newts, whereas the 2 predators were equally deadly to spring peepers and American toads. The predators also decreased the mass at metamorphosis of wood frogs and American toads, decreased the time to metamorphosis of American toads, and increased the time to metamorphosis of spring peepers. American toad survival was affected by the interaction of endosulfan concentration and free-ranging predators. We did not observe any endosulfan-induced trophic cascades in our communities.

The direct lethal effects of endosulfan on amphibians have been investigated primarily in laboratory toxicity tests, which have shown that LC\(_{50}\) values can differ by orders of magnitude within a taxonomic group (Berrill et al. 1998, Leight and Van Dolah 1999, Jones et al. 2009, Hammond et al. 2012). For example, Hammond et al. (2012) reported laboratory LC\(_{50(4-d)}\) values of wood frogs, spring peepers, and American toads that were 31.4, 112, and 55.5 \( \mu \)g/L, respectively. Endosulfan is found in natural systems (Muschal 1997, Leonard et al. 2001, Sparling et al. 2001, 2014), but the few investigators who have studied its direct toxic effects to anurans under seminatural conditions in a community context have found varying results (Rohr and Crumrine 2005, Relyea 2009, Hua and Relyea 2014). Rohr and Crumrine (2005) reported no effect of 20 \( \mu \)g/L endosulfan on wood frog survival in outdoor containers, whereas Relyea (2009) and Hua and Relyea (2014) reported complete mortality of leopard frog \( (Lithobates pipiens) \) and wood frog tadpoles exposed to 6.4 and 5.2 \( \mu \)g/L, respectively, in outdoor mesocosms. Anuran survival decreased with endosulfan concentration in aerated containers, whereas in our experiment, newt survival was high in mesocosms exposed to 0 and 0.2 \( \mu \)g/L endosulfan (100% survival), but declined to 50% and 0% when exposed to 3.1 and 27.3 \( \mu \)g/L, respectively (Fig. 4). We were unable to examine the effects of endosulfan exposure on dragonfly survival because of complete mortality in all treatments by the end of the experiment. 

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TABLE 2. Results of generalized linear models (Wald \( \chi^2 \)) and analyses of variance examining the effects of endosulfan concentration and predator treatment on amphibian response variables. Data are \( p \)-values (degrees of freedom). Multivariate analysis of variance results indicated no effect of endosulfan concentration on amphibian mass at metamorphosis and time to metamorphosis. As a result, endosulfan concentration was removed from the model.

<table>
<thead>
<tr>
<th>Amphibian response</th>
<th>Survival ( (\chi^2) )</th>
<th>Mass at metamorphosis</th>
<th>Time to metamorphosis</th>
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</thead>
<tbody>
<tr>
<td>Wood frog</td>
<td></td>
<td></td>
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<tr>
<td>Endosulfan concen-</td>
<td>0.0001 (1)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Predator treatment</td>
<td>0.0001 (2)</td>
<td>0.007 (2,32)</td>
<td>0.715 (2,33)</td>
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<tr>
<td>Spring peeper</td>
<td></td>
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<td></td>
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<tr>
<td>Endosulfan concen-</td>
<td>0.0001 (1)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Predator treatment</td>
<td>0.0001 (2)</td>
<td>0.333 (2,32)</td>
<td>0.030 (2,33)</td>
</tr>
<tr>
<td>American toad</td>
<td></td>
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<tr>
<td>Endosulfan concen-</td>
<td>0.0001 (1)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Predator treatment</td>
<td>0.0001 (2)</td>
<td>0.0001 (2,31)</td>
<td>0.007 (2,31)</td>
</tr>
<tr>
<td>Endosulfan ( \times )</td>
<td>0.008 (2)</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>
In our study, and mortality reached 100% when anurans were exposed to 27.3 \( \mu \)g/L endosulfan. Furthermore, wood frogs were the most sensitive species. Thus, even with different community compositions, we found that the pattern of endosulfan sensitivity under more natural conditions is consistent with the results of toxicity tests conducted under laboratory conditions (Jones et al. 2009, Hammond et al. 2012). These findings suggest that laboratory toxicity tests can be useful tools for predicting which species are at greater risk to endosulfan contamination in more complex community scenarios. Toxicity tests conducted under laboratory conditions may provide relative patterns of species-level sensitivities, but our results show that anuran sensitivity to endosulfan can increase under more natural conditions. Concentrations of endosulfan can reach 9 \( \mu \)g/L in surface waters after rainfall (Muschal 1997), so many anuran species may be at risk in contaminated natural systems (Sparling et al. 2001, 2014, Hua and Relyea 2014). Future work...
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sensitivity reported from toxicity tests conducted under labo-
range of concentrations are consistent with patterns in sen-
tive e...under natural conditions.

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Newt survival declined with increasing endosulfan concentration. Previous investigators
of the effects of endosulfan on newts have reported disruption of pheromonal systems and decreased mating success in newts exposed to a range (0.5–10 μg/L) of endosulfan concentrations (Park et al. 2001, Park and Propper 2002). In a laboratory study, Hanlon and Relyea (2013) found no direct toxic effect of 0.15 to 3.6 μg/L of endosulfan on newt survival. Rohr et al. (2003) reported significant declines in the behavior, physiology, and morphology of newts, and it thins prey populations, influences competitive interactions, and may initiate trophic cascades (Rohr et al. 2006, Relyea 2007). Both dragonflies and newts can reduce tadpole survival, but previous investigators have found that dragonflies consistently initiate higher tadpole mortality than do newts (Caldwell et al. 1980, Smith 1983, Van Buskirk 1988, Relyea 2002). We found that dragonflies significantly reduced wood frog abundance, whereas both dragonflies and newts equally reduced American toad and spring peeper abundances. Wilbur and Fauth (1990) also found that American toad tadpoles were more affected by red-spotted newts than tadpoles of the cricket frog (Rana palustris). American toads are distasteful to many aquatic predators (Brodie et al. 1978, Relyea 2001b), but red-spotted newts find them only moderately distasteful, whereas larval dragonflies immediately reject captured individuals (Relyea 2001b). Furthermore, we hypothesize that the gape-limited newts preferentially preyed upon the American toad and spring peeper tadpoles because of their small size and slow growth (see Brodie and Formanowicz 1983). Our data also indicate an additive effect of free-ranging predators on amphibian survival as the communities became more contaminated with endosulfan. In other words, the lethal effect of free-ranging predators was consistent across endosulfan concentrations in our experiment, and total tadpole survival within a treatment could be predicted by summing the mortality caused by a given endosulfan concentration and the mortality caused by predators in no-endosulfan treatments. Despite

Figure 4. Mean (±1 SE) % survival of red-spotted newts in field mesocosms treated with endosulfan. Note the log-scale on the x-axis.

investigating the changes in community composition, including density and species identity, over time caused by the direct toxic effects of endosulfan will improve prediction of at-risk species and guide conservation efforts in contaminated ecosystems.

Endosulfan also caused direct toxic effects on other species in the community. Newt survival declined with increasing endosulfan concentration. Previous investigators of the effects of endosulfan on newts have reported disruption of pheromonal systems and decreased mating success in newts exposed to a range (0.5–10 μg/L) of endosulfan concentrations (Park et al. 2001, Park and Propper 2002). In a laboratory study, Hanlon and Relyea (2013) found no direct toxic effect of 0.15 to 3.6 μg/L of endosulfan on newt survival. Rohr et al. (2003) reported significant declines in the behavior, physiology, and morphology of newts, and it thins prey populations, influences competitive interactions, and may initiate trophic cascades (Rohr et al. 2006, Relyea 2007). Both dragonflies and newts can reduce tadpole survival, but previous investigators have found that dragonflies consistently initiate higher tadpole mortality than do newts (Caldwell et al. 1980, Smith 1983, Van Buskirk 1988, Relyea 2002). We found that dragonflies significantly reduced wood frog abundance, whereas both dragonflies and newts equally reduced American toad and spring peeper abundances. Wilbur and Fauth (1990) also found that American toad tadpoles were more affected by red-spotted newts than tadpoles of the cricket frog (Rana palustris). American toads are distasteful to many aquatic predators (Bro...
finding an additive effect of free-ranging predators in the 3 lowest endosulfan treatments, this effect was no longer present when the concentration of endosulfan became high enough to kill all of the amphibians (i.e., 27.3 µg/L). Thus, the effects of free-ranging predators in contaminated systems may be observed only at lower, more environmentally realistic concentrations. We report an additive effect of predation and endosulfan concentration on anuran survival, but this pattern may change with pesticide identity or mode-of-action (i.e., the pathway in which a pesticide is designed to kill). For example, the toxicity of the commonly applied carbamate insecticide carbaryl is magnified in environments inundated with predator cues (Relyea and Mills 2001, Relyea 2003). Although predator identity did not influence endosulfan toxicity, future work investigating this interaction would benefit from use of a variety of pesticides at environmentally realistic concentrations and expansion of the number of aquatic predators, including multiple predators within a single treatment.

Lethal predation also may cause sublethal effects in the behavior, physiology, and morphology of surviving individuals through the release of prey alarm cues and kairomones (Benard 2004, Schoeppner and Relyea 2005). The presence of predatory larval dragonflies in our mesocosms caused decreases in the mass at metamorphosis of wood frogs and American toads by 10 and 21%, respectively; predatory newts decreased American toad mass at metamorphosis and time to metamorphosis by 29% and ~2 d, respectively; and both predators increased the time to metamorphosis of spring peepers by 5 to 6 d. The presence of dragonflies can induce less movement and foraging by wood frogs, spring peepers, and American toads under laboratory conditions (Skelly 1995, Petranika and Hayes 1998, Relyea 2001a). Decreased foraging caused by fear of predation can lead to smaller size at metamorphosis and can lengthen time to metamorphosis by decreasing resource acquisition. Our results support previous work showing that spring peepers decrease activity in response to dragonfly larval predation, which can lead to decreased growth rates and extended time to metamorphosis (Skelly 1995). In contrast, some anuran species may limit mortality risk by metamorphosing sooner and at smaller sizes (see Relyea 2007). For example, red-spotted newts selectively foraged on American toads in comparison to the other anuran species, which may have caused their early time to metamorphosis. We report significant decreases in anuran survival when exposed to endosulfan and lethal predators, but this effect did not lead to a competitive release among conspecifics (i.e., no evidence was found that remaining individuals grew to larger sizes on the abundant resources). Moreover, conditions experienced in the larval environment may also influence responses at later life stages (Pechenik 2006) that were not measured during our study. Thus, investigation of the effects of multiple predators and pesticide combinations on anuran life-history traits beyond metamorphosis (e.g., age at maturity, fecundity, etc.) will help illuminate both immediate and lasting effects of these contaminated larval environments.

Contaminated systems also may experience indirect effects that result from changes in species interactions and community composition caused by both sublethal and direct lethal effects on sensitive species. For example, in outdoor mesocosm experiments, the direct lethal effects on zooplankton species can cause an indirect effect of pesticide exposure on phytoplankton abundance (Relyea and Diecks 2008, Relyea 2009, Hua and Relyea 2014). However, experiments investigating the indirect effects of endosulfan have had contrasting outcomes. Phytoplankton abundance in outdoor mesocosms increased 2 wk after exposure to 6.4 µg/L endosulfan, which caused a decrease in copepod abundance because of direct toxic effects (Relyea 2009). Endosulfan (5.2 µg/L) caused a similar decrease in copepod abundance in a study by Hua and Relyea (2014), but phytoplankton abundance was reduced 4 wk after exposure to endosulfan, a result the authors attributed to increased cladoceran abundance. We observed decreases in copepod abundance with endosulfan concentration, but did not observe any trophic cascades following the direct toxic effects on copepods. However, we sampled zooplankton and phytoplankton abundance 8 d after endosulfan exposure, so we may have observed only the immediate, direct lethal effects on copepod abundance. Such a short time frame would preclude any observation of trophic cascades involving cladocerans, phytoplankton, and periphyton, which take several weeks to manifest. Future research that uses a diverse array of zooplankton species will highlight the importance in the sensitivity of key consumer species and those that may be functionally redundant.

According to US EPA guidelines, for amphibians, the organochlorine insecticide endosulfan falls into the “very highly toxic” category (i.e., LC50 < 100 µg/L; Jones et al. 2009, Hammond et al. 2012), and has been identified as posing serious environmental risk in freshwater ecosystems (Wang et al. 2009). Our study adds to the sparse group of studies of the effects of organochlorine pesticides in natural communities. We discovered that relative species-level patterns of endosulfan sensitivity found under laboratory conditions are representative of patterns under more natural conditions, but species sensitivity to endosulfan increases under more natural conditions. In our seminatural communities, free-ranging predators caused an additive effect on tadpole survival at environmentally realistic concentrations of endosulfan, which would have been missed had we used caged, nonlethal predators. Past investigators have observed indirect effects on phytoplankton abundance resulting from the direct toxic effects of endosulfan on zooplankton abundance, but we did not observe any trophic cascade within our aquatic communities. Future studies of the effects of combined free-ranging predators, pesticide mixtures, and the sublethal effects of pesticides on amphib-
ian morphology, physiology, and behavior will improve our understanding of these complex interactions under natural conditions.

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LITERATURE CITED


