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The effects of multiple stressors on wetland communities: pesticides, pathogens and competing amphibians

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SUMMARY

1. Anthropogenic effects have propelled us into what many have described as the sixth mass extinction, and amphibians are among the most affected groups. The causes of global amphibian population declines and extinctions are varied, complex and context-dependent and may involve multiple stressors. However, experimental studies examining multiple factors contributing to amphibian population declines are rare.

2. Using outdoor mesocosms containing zooplankton, phytoplankton, periphyton and tadpoles, we conducted a 2 × 2 × 3 factorial experiment that examined the separate and combined effects of an insecticide and the fungal pathogen *Batrachochytrium dendrobatidis* (Bd) on three different assemblages of larval pacific treefrogs (*Pseudacris regilla*) and Cascades frogs (*Rana cascadae*).

3. Larval amphibian growth and development were affected by carbaryl and the amphibian assemblage treatment, but only minimally by Bd. Carbaryl delayed metamorphosis in both amphibian species and increased the growth rate of *P. regilla*. Carbaryl also reduced cladoceran abundance, which, in turn, had positive effects on phytoplankton abundance but no effect on periphyton biomass. Substituting 20 intraspecific competitors with 20 interspecific competitors decreased the larval period but not the growth rate of *P. regilla*. In contrast, substituting 20 intraspecific competitors with 20 interspecific competitors had no effect on *R. cascadae*. Results of real-time quantitative polymerase chain reaction (qPCR) analysis confirmed infection of Bd-exposed animals, but exposure to Bd had no effects on either species in univariate analyses, although it had significant or nearly significant effects in several multivariate analyses. In short, we found no interactive effects among the treatments on amphibian growth and development.

4. We encourage future research on the interactive effects of pesticides and pathogens on amphibian communities.

Keywords: amphibian population decline, *Batrachochytrium dendrobatidis*, carbaryl, competition, mesocosm

Introduction

A current challenge in ecology is to identify multiple and complex causes of species extinctions (May, 2010). At the forefront of the biodiversity crisis are amphibians (Blaustein & Kiesecker, 2002; Wake & Vredenburg, 2008), with recent estimates suggesting that more than 40% of amphibian species are experiencing population declines and extinctions (Stuart *et al.*, 2004). The causes of global amphibian population declines are varied and complex. Stressors may operate independently of one another or synergistically (Blaustein & Kiesecker, 2002; Hayes *et al.*, 2010; Blaustein *et al.*, 2011). Identifying interactions between multiple stressors through experimental investigations may suggest how future declines can be minimised.

With increasing land-use change, contaminants are a ubiquitous threat in amphibian breeding habitats. One recent survey determined that 30–60% of shallow ground water and 60–95% of streams across different land-use categories in the U.S.A. are contaminated with at least one pesticide (Gilliom, 2007). Contaminants in high doses can have direct lethal effects on amphibians. More often, however, concentrations of contaminants in natural

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breeding sites are not lethal to amphibians (e.g. Davidson, 2004). Sublethal concentrations can cause changes in immune response, reproduction, physiology, morphology and behaviour (reviewed in Relyea & Hoverman, 2006) and can also affect amphibians indirectly, often through effects on the food web (Boone & Semlitsch, 2001; Relyea, Schoeppner & Hoverman, 2005; Relyea & Diecks, 2008). When presented in a community context (i.e. in the presence of predators and competitors) or in the presence of other anthropogenic stressors including different contaminants, cofactors can become more deadly to amphibians than when presented alone (Relyea, 2003, 2009).

Carbaryl (1-naphthyl-*N*- methylcarbamate; commercial name, Sevin) is a broad-spectrum insecticide used to control various arthropods in a variety of agricultural and urban settings across the U.S.A. It is an acetylcholinesterase inhibitor, interfering with the nervous system of exposed insects. In the Pacific Northwest, U.S.A., carbaryl is applied to apples, cherries and a variety of other agricultural crops (A.B.J. Williams, unpubl. data) and is also applied to forests at higher elevations against bark beetles (*Scolytidae*) (Hastings *et al.*, 2001). Cholinesterase-inhibiting pesticides may enter amphibian habitats through direct aerial spraying, surface runoff and erosion, and can enter high-elevation habitats through aerial drift from agricultural areas at lower elevations (e.g. LeNoir *et al.*, 1999). For example, in California, U.S.A., pesticides, presumably applied in the San Joaquin Valley lowlands, have been discovered in frogs inhabiting high-altitude areas in the Sierra Nevada (e.g. Cory, Fjerd & Serat, 1970), and depressed cholinesterase activity in tadpoles has been documented in this area (Sparling, Fellers & McConnell, 2001). Davidson, Shaffer & Jennings (2001, 2002) and Davidson (2004) found correlative evidence for population declines of several species of the genus *Rana* at sites downwind of pesticide application in the Sierra Nevada Mountain Range of California, U.S.A. Of 64 classes of pesticides considered, the cholinesterase-inhibiting pesticides (including most organophosphates and carbamates) were most strongly implicated in population declines. Although fewer studies have examined atmospheric transport of pesticides to the Cascade Range of Oregon, U.S.A., predominant wind direction patterns indicate that pesticides applied in the Central Valley of California and in the Willamette Valley of Oregon are likely to be transported there (Stanley, 2009). Despite compelling evidence that pesticide application is associated with downwind amphibian population declines, pesticide concentrations measured at these sites are orders of magnitude below lethal concentrations determined through laboratory experiments (Davidson, 2004), suggesting a role for cofactors in these population declines.

A growing body of literature suggests that pathogens such as water moulds, several viruses and the fungus *Batrachochytrium dendrobatidis* (Longcore, Pessier & Nichols) have contributed significantly to global amphibian population declines (e.g. Daszak *et al.*, 1999; Blaustein & Kiesecker, 2002; Daszak, Cunningham & Hyatt, 2003). Chytridiomycosis, an emerging infectious disease of amphibians caused by the fungus *B. dendrobatidis* (hereafter Bd), is considered to be among the greatest disease threats to global amphibian biodiversity (Skerratt *et al.*, 2007; Rohr *et al.*, 2008; Wake & Vredenburg, 2008). The pathogen occurs on every continent inhabited by amphibians and is implicated in the recent decline of several hundred species (Skerratt *et al.*, 2007). Aquatic zoospores of the fungus infect keratinised tissue of larval, juvenile and adult amphibians. Susceptibility varies with species (e.g. Blaustein *et al.*, 2005), and although larval mortality is rare, presumably because the infection is restricted to the keratinised mouthparts (Longcore, Pessier & Nichols, 1999), Bd can cause a variety of sublethal effects on growth, development and behaviour (Parris & Cornelius, 2004; Blaustein *et al.*, 2005; Parris, Reese & Storfer, 2006). Although Bd is associated with numerous population declines and extinctions, some populations persist in spite of pathogen presence (e.g. Retallick, McCallum & Speare, 2004; Briggs *et al.*, 2005; Daszak *et al.*, 2005; Briggs, Knapp & Vredenburg, 2010). Furthermore, surveys of museum specimens show that individuals in many populations were infected for years preceding population declines (e.g. Ouellet *et al.*, 2005). Taken together, this evidence suggests that cofactors may have played a role in population declines for which Bd is the only identifiable proximate cause.

Many recent studies have called for an examination of possible interactive effects of multiple stressors on amphibians (agricultural contaminants and Bd in particular; Collins & Storfer, 2003; Davidson, 2004; Rollins-Smith *et al.*, 2006). One study looked for interactive effects between carbaryl and Bd (Davidson *et al.*, 2007). It has been suggested that sublethal concentrations of contaminants could negatively affect the immune system of larval amphibians, making them more susceptible to infection (Taylor, Williams & Mills, 1999; Kiesecker, 2002; Gilbertson *et al.*, 2003; Christin *et al.*, 2004). For example, exposure to the contaminants chlorpyrifos and atrazine increases larval mortality and susceptibility of salamanders to *Ambystoma tigrinum* virus (Kerby & Storfer, 2009). Skin peptide defences thought to inhibit the growth of Bd are negatively affected by the pesticide carbaryl

(Rollins-Smith *et al.*, 2006; Davidson *et al.*, 2007). The limited research has thus far not detected a synergism between any pesticide and Bd, although interactive effects between pesticides and pathogens have not been investigated under more natural conditions.

Natural biotic stressors such as competition may also have negative effects on amphibians. At high densities of interspecific and intraspecific competitors, for example, amphibians may experience delayed metamorphosis, as well as reduced survival and growth owing to competition for resources (Skelly, 1997; Skelly & Kiesecker, 2001). Recent research has focused on interactions between natural biotic stressors and anthropogenic abiotic stressors, especially pesticides, in amphibian habitats (e.g. Boone & Semlitsch, 2001; Relyea, 2003). A growing body of literature focuses on interactions between natural biotic stressors and diseases (e.g. Kiesecker & Blaustein, 1999; Parris & Cornelius, 2004). Among other possibilities, the strength and direction of pairwise competitive interactions between species may be context-dependent; they may change in the presence of other stressors, especially if species are differentially affected by direct effects of the other stressors (Kiesecker & Blaustein, 1999; Boone & Semlitsch, 2001).

We examined the interactive effects of the pesticide carbaryl, Bd and amphibian assemblage through a $2 \times 2 \times 3$ factorial experiment. We used technical-grade carbaryl as a representative contaminant in this experiment because its direct toxic effects on amphibians have been established (Relyea & Mills, 2001); LC50 estimates vary from 1 to 18 mg L^{-1} (Marian, Arul & Pandian, 1983; Bridges, 1997; Zaga *et al.*, 1998). Furthermore, this broad-spectrum insecticide is widely applied owing to its rapid breakdown and low toxicity to mammals, and upwind application of cholinesterase-inhibiting pesticides including carbamates was identified as being associated with amphibian population declines (Davidson *et al.*, 2001, 2002; Davidson, 2004).

We used Bd as a representative pathogen in this experiment because chytridiomycosis is the foremost disease threatening amphibian populations worldwide (Rohr *et al.*, 2008), and it occurs on the west coast of the U.S.A., in populations of the Pacific treefrog *Pseudacris regilla* (Baird and Girard) (Padgett-Flohr & Hopkins, 2010) and the Cascades frog *Rana cascadae* (Slater) (Pearl *et al.*, 2009), among other species. Its direct effects on larval amphibians are increasingly known from laboratory studies. Infected larvae usually experience reduced growth and delayed metamorphosis, although effects vary widely by species (Parris & Baud, 2004; Parris & Beaudoin, 2004; Parris & Cornelius, 2004; Blaustein *et al.*, 2005).

We used larvae of the *P. regilla* and *R. cascadae* because they commonly co-occur at breeding sites in the Cascade Range of Oregon (Nussbaum, Brodie & Storm, 1983). *Pseudacris regilla* is a habitat generalist (Nussbaum *et al.*, 1983) and is one of the most common amphibian species across most of its range. In contrast, *R. cascadae* inhabits moderate-to-high-elevation sites throughout the Cascade Range. Recently, population declines have been reported in the southern portion of the species' range (e.g. Fellers *et al.*, 2007) and may be associated with upwind pesticide application (Davidson, 2004). Its IUCN (Red List) status is 'near-threatened'. These two species compete for periphyton, phytoplankton and detrital resources, and they have larval periods of similar duration (Nussbaum *et al.*, 1983; Kiesecker & Blaustein, 1999). Furthermore, they exhibit differential susceptibility to Bd: *P. regilla* is less susceptible than *R. cascadae* (Blaustein *et al.*, 2005; Garcia, Romansic & Blaustein, 2006), and *R. cascadae* larvae are more likely to exhibit mouthpart abnormalities (Blaustein *et al.*, 2005). Padgett-Flohr & Hopkins (2009, 2010) suggest that *P. regilla* may be a resistant carrier of Bd, effectively vectoring it to ephemeral habitats where it may infect more susceptible species.

We predicted that carbaryl would reduce the abundance of zooplankton (Mills & Semlitsch, 2004; Relyea, 2005, 2009), increase the abundance of phytoplankton (Hanazato & Yasuno, 1987; Boone & James, 2003), reduce the biomass of periphyton (Distel & Boone, 2009) and have a negative impact on the growth and development of larval amphibians (Mills & Semlitsch, 2004). We predicted that Bd would extend the larval period and reduce growth rate of amphibians but would not affect survival (Parris & Baud, 2004; Parris & Beaudoin, 2004; Parris & Cornelius, 2004; Blaustein *et al.*, 2005). We predicted that at the highest density of *R. cascadae*, the superior competitor (Kiesecker & Blaustein, 1999), periphyton biomass would be reduced, which would extend the larval period and reduce survival and growth rate of larval amphibians (Skelly, 1997; Skelly & Kiesecker, 2001). Furthermore, we hypothesised that carbaryl, Bd and amphibian assemblage would have interactive effects on larval period, survival and growth rate of larval amphibians.

Methods

We manipulated the presence of carbaryl, Bd and the amphibian assemblage in artificial ponds. The experiment took place at the Lewis-Brown Horticulture Research Farm near Corvallis, Oregon, U.S.A., and ran from 24 July to 13 August 2009. Experimental units consisted of plastic wading pools 1.5 m in diameter filled with about 120 L of

tap water (pH = 8) on 19 April and covered with screen lids. On 20 April, 36 g of leaf litter, which created habitat heterogeneity, and 3 g of rabbit food (Purina, St. Louis, MO, U.S.A.), which served as a nutrient source, were added to each pool. On 24 April, all pools were inoculated with zooplankton, phytoplankton and periphyton collected from 10 natural ponds in the area. This experiment employed a completely randomised $2 \times 2 \times 3$ factorial design. We crossed carbaryl (absent or present at a nominal concentration = 10 ppb) with Bd (absent or present) and three different assemblages of tadpoles using a substitutive design (*P. regilla* alone, *R. cascadae* alone, or *P. regilla* and *R. cascadae* combined). The resulting 12 treatments were replicated four times each for a total of 48 experimental units. We controlled for density between single-species and combined-species treatments.

Egg masses of *P. regilla* (25 egg masses) and *R. cascadae* (five egg masses) were collected within 48 h after oviposition between 15 May and 17 May from site 1, a natural pond in the Cascade Range (elevation = 1140 m). Eggs were hatched, and tadpoles were reared in outdoor holding tanks adjacent to the experimental site. Remnants of egg masses including any unhatched eggs were removed from the holding tanks on 28 May. Tadpoles in holding tanks were fed rabbit food ad libitum. On 14 June, 40 tadpoles of Gosner stage 25–27 were added to each experimental pool (Gosner, 1960) in three different assemblages: 40 *P. regilla*, 40 *R. cascadae* or 20 of each species. The initial mass of the tadpoles (mean ± 1 SE) was 47 ± 22 mg for *P. regilla* and 88 ± 14 mg for *R. cascadae*.

Ten days later, on day 1 of the experiment and every week thereafter for 7 weeks, pools assigned to the carbaryl treatment received an application of technical-grade carbaryl to achieve a nominal concentration of 10 ppb, which is 0.06–1% of LC50 estimates for tadpoles (Marian *et al*., 1983; Bridges, 1997; Zaga *et al*., 1998) and is well within ecologically relevant levels (maximum expected concentration in wetlands = 4.8 mg L^{-1}; Norris, Lorz & Gregory, 1983; Peterson *et al*., 1994). This type of 'press' application probably approximates real-world pesticide applications more closely than 'single-pulse' applications, especially for high-elevation amphibian habitats where aerial drift of pesticides from distant agricultural areas represents the greatest source. The frequency of application of the pesticide was within the range of other studies examining press disturbances of pesticides (Hanazato & Yasuno, 1990; Boone, Bridges & Rothermel, 2001; Relyea & Diecks, 2008). Additionally, the frequency of application of the pesticide in this study (7 days) was within the recommended application rate for the insecticide Sevin (7–10 days, or as needed).

We created a carbaryl stock solution by dissolving 30 mg of technical-grade carbaryl (Chem Service, West Chester, PA, U.S.A.) into 75 mL of 100% ethanol. We added 3 mL of this solution to each pool in the carbaryl treatment. To ensure that any effects observed were attributable to carbaryl rather than to ethanol, 3 mL of 100% ethanol was added to each pool not dosed with carbaryl as a vehicle control. After the chemicals were applied, all pools were thoroughly stirred to ensure uniform exposure of all animals. One hour after dosing, a water sample of 10 mL was collected from each experimental pool. Water samples were pooled by pesticide treatment (carbaryl or no carbaryl) and frozen in precleaned amber glass jars, and the samples from the first and last weeks were shipped to Mississippi State Chemical Laboratory (Mississippi State, MS, U.S.A.) for independent analysis of contaminant concentration using high-pressure liquid chromatography. Results of these analyses indicated that actual concentrations were 5 and 3 ppb, respectively. Thus, the actual concentrations were, on average, 40% of the nominal concentration and did not accumulate with multiple applications. The half-life of carbaryl is 3.2 h at pH = 9 and 12.1 days at pH = 7. pH in our mesocosms averaged 7.9, but because carbaryl was reapplied on a weekly basis, observed effects are likely to be due to the insecticide itself, rather than its breakdown product, 1-naphthol.

On day 1 of the experiment (24 June) and every 2 weeks thereafter for a total of four inoculations, Bd inoculate was added to pools assigned to the Bd treatment. The fungus was grown in pure culture on plastic Petri plates (10 cm diameter; Fisherbrand, Santa Clara, CA, U.S.A.) with standard TGhL nutrient agar medium (Becton, Dickinson and Company, Sparks, MD, U.S.A.; Longcore *et al*., 1999). Plates were inoculated with liquid culture of Bd isolate JEL 274, originally isolated from *Anaxyrus boreas* (Baird & Girard) from Colorado and incubated at 22 °C for 8 days prior to use. A broth containing Bd scraped from 50 flooded plates was diluted to 800, and 30 mL of this broth was added to each pool in the Bd treatment. A small sample of this broth was examined in the laboratory with the use of a hemocytometer to determine zoospore concentration. Average zoospore concentration in mesocosms following Bd inoculation was 20 000 zoospores L^{-1}. A broth containing water from 50 flooded control plates was diluted to 800, and 30 mL of this broth was added to each pool in the Bd control treatment.

To measure how the treatments affected the other members of the pond community, we sampled the zooplankton, phytoplankton and periphyton from each

mesocosm on days 11–12 and on day 32. To measure the abundance of zooplankton, a 1.5-cm tube sampler that held *c.* 30 mL of water was plunged vertically through the water column and sealed near the bottom of the pool. Three samples were taken on opposite sides of each pool and in the centre and pooled. This procedure was repeated three times for each pool. Water samples were then filtered through 150-μm mesh (Florida Aquatic Nurseries, Ft. Lauderdale, FL, U.S.A.), and zooplankton from each sample were pooled and preserved in 30% ethanol for later quantification. Zooplankton were identified to the level of copepods and cladocerans because past research has shown that the two groups differ in their susceptibility to insecticides, but species within each group are similar in their sensitivities (Relyea, 2005; Relyea & Diecks, 2008). Furthermore, cladocerans are relatively indiscriminant filter feeders, consuming smaller food particles than the more selective copepods (Sommer & Sommer, 2006), so shifts in the relative composition of the zooplankton could change the size distribution of the phytoplankton.

To measure the abundance of phytoplankton, the three water samples from each mesocosm (25 mL each) described earlier were filtered through a Type A/E 25 mm GF/F filter (Pall Corporation, Port Washington, NY, U.S.A.). Filtering was conducted under full shade to minimise chlorophyll breakdown, and filters were stored in 25-mL centrifuge tubes on ice. Samples were stored at −20 °C for 4 days before chlorophyll extraction. Following the Welschmeyer method, chlorophyll-*a* was extracted with 10 mL of 90% acetone, agitated and incubated for 24 h at −20 °C (Welschmeyer, 1994). A Turner Designs fluorometer (model TD-700; Sunnyvale, CA, U.S.A.) was used to take fluorescence measurements, and chlorophyll-*a* concentration was calculated as the mean value of the three replicates from each mesocosm.

Two periphyton samplers, consisting of glass microscope slides mounted vertically on a small Styrofoam block, were deployed in each pool on day 1 of the experiment. The periphyton on both sides of one slide was scraped into a Petri plate using a straight-edge razor blade on each community sampling day. Contents of the Petri plate were filtered through a Type A/E 25 mm GF/F filter that had been previously dried for 24 h at 60 °C and weighed. To determine periphyton biomass, filters were dried again for 24 h at 60 °C and reweighed.

iButton temperature probes (Maxim, Sunnyvale, CA, U.S.A.) were deployed in ten pools on 24 May. Each probe logged temperature every hour over the course of the experiment. Dissolved oxygen and pH measurements were taken using digital meters (Oakton Instruments, Vernon Hills, IL, U.S.A.) on days 5 and 51 of the experiment.

On day 31, ten individuals from each pool (10 *P. regilla* or 10 *R. cascadae* from single-species pools or five of each species from combined-species pools, hereafter termed 'tadpoles') were haphazardly chosen, euthanised using an overdose of MS-222, preserved in 90% ethanol and subsequently weighed and staged (Gosner, 1960). Measures of performance for tadpoles from each mesocosm included mean daily growth rate (mass divided by 31 days), mean Gosner stage and mean infection level.

All remaining individuals were euthanised and preserved upon emergence from the pools at stage 45–46 (hereafter termed 'metamorphs') and were subsequently weighed (Gosner, 1960). The first *P. regilla* metamorph was observed on day 28 of the experiment, and the first *R. cascadae* metamorph was observed 2 days later. Following the initial observation of a metamorph, pools were checked daily for metamorphs until the end of the experiment on day 51. At that time, all remaining individuals (<2% of all individuals added to mesocosms) were preserved, regardless of Gosner stage; these individuals were excluded from statistical analyses. Measures of performance for metamorphs from each mesocosm were larval period, mean daily growth rate (mass at metamorphosis divided by the number of days from the addition of tadpoles to the mesocosms until metamorphosis) and survival to metamorphosis (the proportion of larvae surviving to metamorphosis from those initially added to each tank, excluding the 10 individuals sampled as tadpoles from each tank).

We used real-time quantitative polymerase chain reaction (qPCR) following the methods of Boyle *et al.* (2004) to confirm the infection status of five of the individuals sampled as tadpoles from single-species pools and all ten of the individuals sampled as tadpoles from multiple-species pools. The mouthparts of preserved tadpoles were dissected, and each sample was run in triplicate against a Bd standard titration from 10^{-1} to 10^{2} zoospores on an Applied Biosystems StepOne Plus real-time PCR machine (Applied Biosystems, Inc., CA, U.S.A.). The experimenter was unaware of the treatment from which each sample originated at the time of qPCR analysis. A tadpole was considered infected if two of three replicates tested positive, and replicates were averaged for each sample.

We recognise the risk of releasing contaminants into the environment when conducting manipulative studies involving pesticides and pathogens in outdoor experimental tanks (Parris & Beaudoin, 2004), and precautions were taken to minimise the risk of environmental contamination (see supporting information, Appendix S1).

Statistical analyses

We conducted statistical analyses in R and S-plus to test for effects of treatments. Because of non-independence of individuals within tanks, mean values per tank were used as the unit of analysis for all variables. Most response variables met the parametric assumptions although survival to metamorphosis was arcsine-transformed.

The response variables were analysed using a series of multivariate analysis of variance (MANOVA) tests. Separate MANOVAS were conducted for each amphibian species. For tadpoles, the response variables were daily growth rate and developmental stage (Gosner, 1960). For metamorphs, the response variables were daily growth rate, larval period and survival. Whenever a multivariate effect was significant, we conducted subsequent univariate analyses of variance (ANOVAS).

We also examined the effects of the treatments on the other members of the community (copepods, cladocerans, phytoplankton and periphyton). For all four groups, we log-transformed the data to meet parametric assumptions. We conducted separate MANOVAS for each of the two samples. Whenever a multivariate effect was significant, we conducted subsequent ANOVAS on each of the response variables.

Finally, we examined the effects of the treatments on abiotic variables (temperature, dissolved oxygen and pH). Temperature measurements were averaged within pools for each week, and we used separate ANOVAS to test for differences between treatments. We tested for effects of the treatments on dissolved oxygen and pH using separate ANOVAS for each time point on which the variables were measured.

Results

Our first analysis examined the growth and development of *P. regilla* tadpoles (Table 1, Figs 1 & 2). There were significant multivariate effects of amphibian assemblage, Bd and carbaryl as well as significant assemblage-by-carbaryl and assemblage-by-Bd-by-carbaryl interactions. Subsequent univariate analyses indicated that carbaryl increased tadpole growth by *c.* 0.006 mg days^{-1}. The remaining main effects and interactions were not significant at the univariate level.

The second analysis examined the growth rate, development and survival of *P. regilla* metamorphs (Table 2, Figs 1 & 2). There were significant multivariate effects of assemblage and carbaryl; there was no effect of Bd, and there were no significant interactions. Subsequent univariate tests indicated that substituting in 20 *R. cascadae* tadpoles

Table 1 Results of a MANOVA on the effects of amphibian assemblage, Bd and carbaryl on the daily growth rate and developmental stage (Gosner, 1960) of *Pseudacris regilla* tadpoles

(a) Multivariate	d.f.	Wilks' *F*	*P*
Assemblage	2.14	8.49	**<0.001**
Bd	2.14	8.17	**0.01**
Carbaryl	2.14	22.0	**<0.001**
Assemblage*Bd	2.14	2.93	**0.027**
Assemblage*carbaryl	2.14	5.85	**0.003**
Bd*carbaryl	2.14	0.92	0.815
Assemblage*Bd*carbaryl	2.14	3.43	**0.018**

(b) Univariate	Growth rate	Gosner stage
Assemblage	0.072	0.191
Bd	0.366	0.549
Carbaryl	**<0.001**	0.107
Assemblage*Bd	0.690	0.536
Assemblage*carbaryl	0.141	0.743
Assemblage*Bd*carbaryl	0.917	0.586

Subsequent univariate tests (*P*-values) were conducted for all significant multivariate effects. Bold *P*-values are significant at $P < 0.05$.

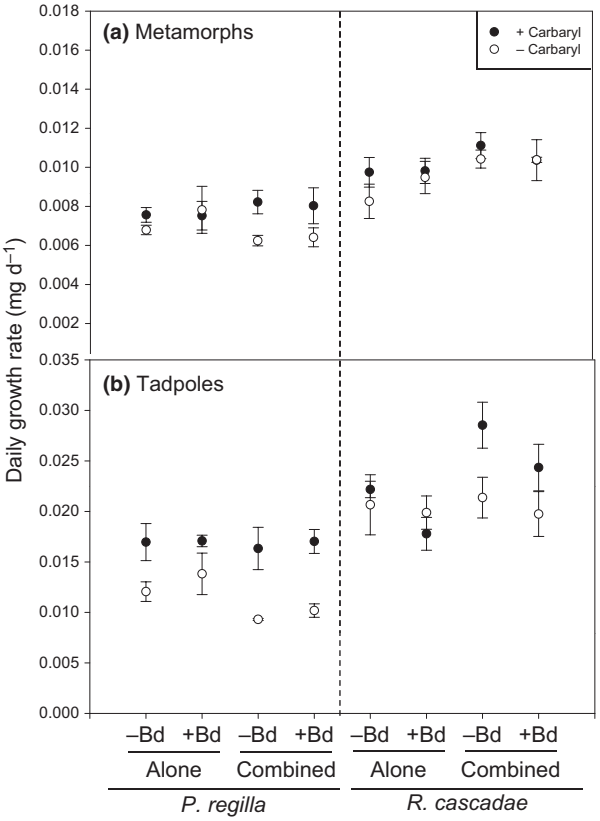

Fig. 1 The effects of amphibian assemblage, Bd and carbaryl on daily growth rate of metamorphs (panel a) and tadpoles (panel b) for *Pseudacris regilla* alone, *P. regilla* when combined with *Rana cascadae*, *R. cascadae* alone and *R. cascadae* when combined with *P. regilla*. Values plotted are means ± 1 SE.

Table 2 Results of a MANOVA on the effects of amphibian assemblage, Bd and carbaryl on the daily growth rate, larval period and survival of *Pseudacris regilla* metamorphs

(a) Multivariate	d.f.	Wilks' *F*	*P*
Assemblage	3.14	5.38	**0.013**
Bd	3.14	2.87	0.077
Carbaryl	3.14	14.3	**<0.001**
Assemblage*Bd	3.14	0.31	0.816
Assemblage*carbaryl	3.14	1.90	0.179
Bd*carbaryl	3.14	0.47	0.706
Assemblage*Bd*carbaryl	3.14	3.18	0.060

(b) Univariate	Growth rate	Larval period	Survival
Assemblage	0.677	**0.035**	0.625
Carbaryl	**0.039**	**<0.001**	0.145

Subsequent univariate tests (*P*-values) were conducted for all significant multivariate effects. Bold *P*-values are significant at $P < 0.05$.

Table 3 Results of a MANOVA on the effects of amphibian assemblage, Bd and carbaryl on the daily growth rate and developmental stage (Gosner, 1960) of *Rana cascadae* tadpoles

Multivariate	d.f.	Wilks' *F*	*P*
Assemblage	2.14	1.27	0.310
Bd	2.14	0.96	0.406
Carbaryl	2.14	2.70	0.100
Assemblage*Bd	2.14	0.58	0.571
Assemblage*carbaryl	2.14	2.22	0.143
Bd*carbaryl	2.14	0.61	0.556
Assemblage*Bd*carbaryl	2.14	0.12	0.884

Bold *P*-values are significant at $P < 0.05$.

decreased the larval period of *P. regilla* by *c.* 0.7 days. Carbaryl increased growth rate of *P. regilla* by *c.* 0.001 mg days^{-1} and increased the larval period by *c.* 2 days.

The third analysis examined the growth and development of *R. cascadae* tadpoles (Table 3, Figs 1 & 2). The MANOVA found no significant main effects or interactions. As a result, we did not conduct any subsequent univariate analyses.

The final amphibian analysis examined the growth, development and survival of *R. cascadae* metamorphs (Table 4, Figs 1 & 2). There was a multivariate effect of carbaryl but no other main effects or interactions. The subsequent univariate analysis indicated that carbaryl increased the larval period of *R. cascadae* by *c.* 0.0006 mg days^{-1}, but there were no effects on growth or survival.

The qPCR analysis on the dissected mouthparts of tadpoles revealed that the majority of individuals exposed to Bd harboured infections by day 31, while none of the unexposed individuals showed infection, thus confirming infection of Bd-exposed animals. Species exhibited similar

Table 4 Results of a MANOVA on the effects of amphibian assemblage, Bd and carbaryl on the daily growth rate, larval period and survival of *Rana cascadae* metamorphs

(a) Multivariate	d.f.	Wilks' *F*	*P*
Assemblage	3.14	2.12	0.143
Bd	3.14	0.71	0.560
Carbaryl	3.14	5.24	**0.012**
Assemblage*Bd	3.14	0.69	0.576
Assemblage*carbaryl	3.14	3.25	0.054
Bd*carbaryl	3.14	0.36	0.786
Assemblage*Bd*carbaryl	3.14	0.19	0.903

(b) Univariate	Growth rate	Larval period	Survival
Carbaryl	0.249	**0.021**	0.097

Subsequent univariate tests (*P*-values) were conducted for all significant multivariate effects. Bold *P*-values are significant at $P < 0.05$.

Table 5 Results of a MANOVA on the effects of amphibian assemblage, Bd and carbaryl on the abundance of cladocerans, copepods, phytoplankton and periphyton early in the experiment (day 11–12)

(a) Multivariate	d.f.	Wilks' *F*	*P*
Assemblage	8.66	0.65	0.062
Bd	4.33	0.82	0.154
Carbaryl	4.33	0.20	**<0.001**
Assemblage*Bd	8.66	0.63	**0.042**
Assemblage*carbaryl	8.66	0.78	0.361
Bd*carbaryl	4.33	0.98	0.965
Assemblage*Bd*carbaryl	8.66	0.89	0.845

(b) Univariate	Cladocerans	Copepods	Phytoplankton	Periphyton
Carbaryl	**<0.001**	0.095	**0.001**	0.388
Assemblage*Bd	0.132	0.193	0.056	0.063

Subsequent univariate tests (*P*-values) were conducted for all significant multivariate effects. Bold *P*-values are significant at $P < 0.05$.

levels of infection. A univariate ANOVA indicated that neither carbaryl, amphibian assemblage nor their interaction had an effect on infection levels of either species (supporting information, Table S1).

We also tested for effects of treatment variables on the other trophic groups in the community. Our first analysis examined the effects of amphibian assemblage, Bd, carbaryl and their interactions on cladocerans, copepods, phytoplankton and periphyton during the first sample (days 11–12; Table 5, Fig. 3). We found a multivariate effect of carbaryl and an assemblage-by-Bd interaction. Subsequent univariate analyses indicated that carbaryl decreased cladoceran abundance by about 50 times, resulting in more than twice the concentration of phytoplankton. There was a nearly significant assemblage-by-Bd interaction on both phytoplankton abundance and periphyton biomass in the univariate analyses: presum-

ably, Bd-exposed *R. cascadae* consumed less periphyton and more phytoplankton than unexposed counterparts, but the opposite was true for *P. regilla*. Together, these nearly significant effects probably drove the significant multivariate effect. The remaining main effects and interactions were not significant at the univariate level.

Our second analysis examined the same response variables during the second sample (day 32; Table 6, Fig. 3). We found a significant multivariate effect of carbaryl. Subsequent univariate analyses indicated that carbaryl continued to cause a decrease in the abundance of cladocerans, by about 14 times. The increase in phytoplankton was no longer significant because phytoplankton in the carbaryl-free mesocosms had increased by the second sample to be similar in abundance. The remaining main effects and interactions were not significant at the univariate level.

Lastly, we examined the effects of treatment variables on abiotic parameters including temperature, dissolved oxygen and pH (supporting information, Table S2). Average weekly temperatures ranged from 15.01 to 18.38 °C, which is suitable for growth of Bd (Piotrowski, Annis & Longcore, 2004). Average dissolved oxygen and pH were 16.8 ppm and 7.94, respectively, on day 5 and 6.5 ppm and 7.97, respectively, on day 51. Separate univariate analyses indicated that amphibian assemblage, Bd and carbaryl treatments did not affect temperature (averaged over each week of the experiment), dissolved oxygen (measured on two occasions) or pH (measured on two occasions).

Discussion

We demonstrated effects of amphibian assemblage, carbaryl and Bd on larval period, growth rate and survival of amphibians. Treatments differentially affected *P. regilla* and *R. cascadae*, and effects were dependent upon developmental stage. We also demonstrated effects of treatments on the aquatic community, including zooplankton and algae.

We hypothesised that manipulating the amphibian assemblage would affect growth rate and larval period of both amphibian species. We controlled for density by adding 40 larvae to each pool (40 *P. regilla*, 40 *R. cascadae* or 20 of each species). Kiesecker & Blaustein (1999) established that *R. cascadae* is a superior competitor to *P. regilla*. In our experiment, *P. regilla* was negatively affected by the replacement of 20 intraspecific competitors with 20 superior *R. cascadae* competitors. When *R. cascadae* were added, the larval period of *P. regilla* increased. In contrast, *R. cascadae* was unaffected by the replacement of 20 intraspecific competitors with 20 inferior *P. regilla* competitors. The amphibian assemblage did not affect the other members of the aquatic community (zooplankton, algae), except for a nearly significant assemblage-by-Bd interaction on phytoplankton, discussed below.

The insecticide carbaryl increased the growth rate and larval period of *P. regilla* and increased the larval period of *R. cascadae*. Effects of carbaryl on growth rate and larval period of tadpoles in mesocosms are quite mixed (Boone & Semlitsch, 2001; Boone & Bridges, 2003; Boone *et al.*, 2004; Relyea, 2006), but cases of increased growth rate are hypothesised to be caused by a short-term increase in periphyton biomass caused by a pesticide-induced reduction in tadpole foraging activity (Boone *et al.*, 2005; Distel & Boone, 2009). As carbaryl breaks down and its negative effects on tadpole foraging diminish, tadpoles may benefit from the overabundance of food resources.

We hypothesised that growth and development of tadpoles would be negatively affected by exposure to carbaryl owing to a trophic cascade involving zooplankton: carbaryl is known to reduce the abundance of herbivorous cladocerans (Mills & Semlitsch, 2004; Relyea, 2005, 2009), thus increasing phytoplankton concentration (Hanazato & Yasuno, 1987; Boone & James, 2003). Given enough time, the bloom of phytoplankton can lead to a reduction in periphyton biomass owing to competition for nutrients and light, which could negatively impact tadpole growth and development (Mills & Semlitsch, 2004; Distel & Boone, 2009). We confirmed that carbaryl reduced cladoceran abundance by about 50 times and that a phytoplankton bloom (more than twice the concentration) was detected in carbaryl mesocosms at the first sample. However, we did not detect changes in periphyton biomass, probably because the tadpoles metamorphosed before the trophic cascade could fully develop (i.e. it

Table 6 Results of a MANOVA on the effects of amphibian assemblage, Bd and carbaryl on the abundance of cladocerans, copepods, phytoplankton and periphyton late in the experiment (day 32)

(a) Multivariate	d.f.	Wilks' *F*	*P*
Assemblage	8.66	0.44	0.892
Bd	4.33	2.51	0.060
Carbaryl	4.33	8.52	**<0.001**
Assemblage*Bd	8.66	0.47	0.872
Assemblage*carbaryl	8.66	0.85	0.560
Bd*carbaryl	4.33	0.06	0.994
Assemblage*Bd*carbaryl	8.66	1.09	0.380

(b) Univariate	Cladocerans	Copepods	Phytoplankton	Periphyton
Carbaryl	**<0.001**	0.236	0.073	0.617

Subsequent univariate tests (*P*-values) were conducted for all significant multivariate effects. Bold *P*-values are significant at $P < 0.05$.

appears to require more than 30 days; Relyea & Diecks, 2008), and *P. regilla* tadpoles grew larger, not smaller, in the presence of carbaryl. One possible explanation for the observed pattern is that *P. regilla* were able to utilise the abundant phytoplankton resources after cladoceran abundance was severely decreased by carbaryl. Whiles *et al.* (2010) determined via fatty acid analysis that rasping tadpoles exhibit high dietary plasticity and that phytoplankton may contribute significantly to their diet, a suggestion put forth previously by Altig, Whiles & Taylor (2007).

Interestingly, previous studies that documented increased growth and delayed metamorphosis of amphibians in response to carbaryl applied the insecticide at concentrations that were 100–700 times greater than those used in our study. This suggests that even at much lower concentrations (0.06–1% of the LC50 estimate for tadpoles), tadpoles may be affected (Relyea & Edwards, 2010).

We found no effect of Bd on the growth and development of amphibians, although qPCR analysis confirmed the infection of the majority of Bd-exposed individuals. We did, however, detect a significant multivariate effect of Bd on *P. regilla* tadpoles. Our results confirm previous findings that the effects of Bd on certain species of infected larval amphibians may be minimal (Blaustein *et al.*, 2005). Previous studies have shown varied effects on larvae of different species after exposure to Bd. These include effects on growth and development, as well as mortality in some species, and the magnitude of these effects may vary among species (Parris & Baud, 2004; Parris & Beaudoin, 2004; Parris & Cornelius, 2004; Blaustein *et al.*, 2005). One possible mechanism that has been suggested is that keratinised mouthparts of infected larvae may become disfigured by Bd, thus inhibiting normal feeding behaviour (Fellers, Green & Longcore, 2001; Rachowicz & Vredenburg, 2004; Venesky, Parris & Storfer, 2009). In our experiment, abundant algal resources and weak competition among the tadpoles may have provided excellent growth conditions for tadpoles, thereby allowing them to avoid clinical signs of infection (extended larval period, reduced growth rate, reduced survival) in spite of their infection status. Although larvae may fail to exhibit clinical signs of infection, infected tadpoles in natural populations may serve as important reservoirs for the pathogen, thereby increasing the infection risk of conspecifics and heterospecifics of all developmental stages (Briggs *et al.*, 2010).

A nearly significant interaction between amphibian assemblage and Bd on phytoplankton concentration ($P = 0.056$) and periphyton biomass ($P = 0.063$) during the first sample was unexpected and suggests that under certain circumstances, Bd can somehow reduce phytoplankton abundance and increase periphyton biomass. One hypothesis is that infected tadpoles may be less effective at scraping periphyton, owing to degradation of keratinised tissues of the mouthparts, thereby forcing a switch to a diet composed largely of phytoplankton. Indeed, tadpoles of both species were observed to spend a significant amount of time in the water column, presumably foraging on suspended algal particles. The significant assemblage-by-Bd interaction in the multivariate analysis could be explained if this switch was more pronounced in *R. cascadae* than in *P. regilla*, as would be suggested by previously reported patterns of keratin loss in infected individuals of these species (Blaustein *et al.*, 2005). Interestingly, our data on tadpole growth rate and larval period support the idea that Bd infection may influence consumption of phytoplankton and periphyton. Figure 2 shows that in the absence of Bd and the presence of *R. cascadae* competitors, the effect of carbaryl extending the larval period of *P. regilla* disappeared. One interpretation is that Bd-exposed *R. cascadae* competitors are hampered by their infections, thus allowing *P. regilla* to capitalise on the carbaryl-induced abundance of phytoplankton and metamorphose earlier. Indeed, Fig. 1 shows that *R. cascadae* achieved the fastest growth in the absence of Bd and the presence of carbaryl and *P. regilla*.

Although we demonstrated main effects of carbaryl and amphibian assemblage, main effects of Bd and interactive effects between the treatments were not detected. Despite the differences between immune function of larval and metamorphic amphibians, both the current study and

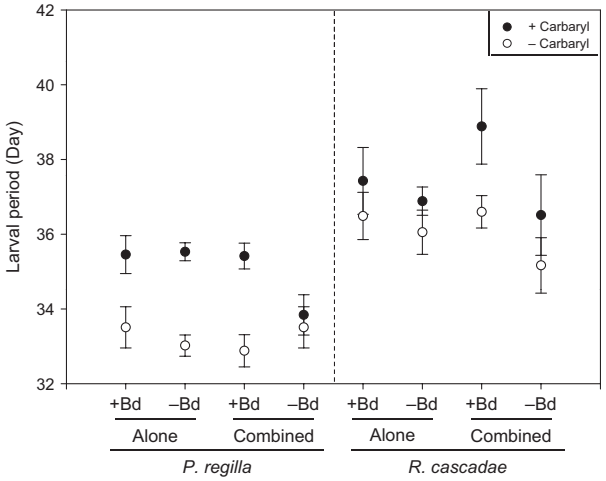

Fig. 2 The effects of amphibian assemblage, Bd and carbaryl on the larval period of amphibians for *Pseudacris regilla* alone, *P. regilla* when combined with *Rana cascadae*, *R. cascadae* alone and *R. cascadae* when combined with *P. regilla*. Values plotted are means ± 1 SE.

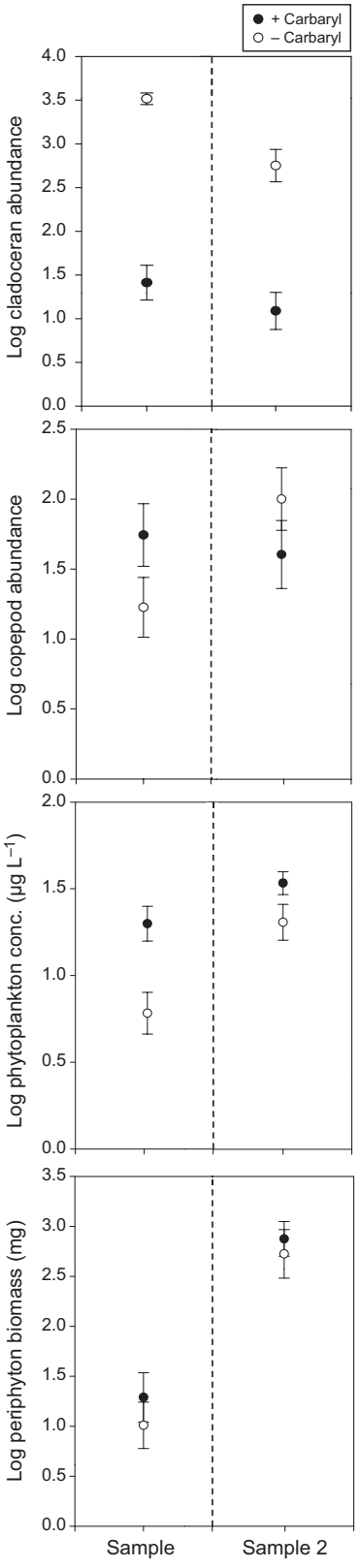

Fig. 3 The effect of carbaryl on log cladoceran abundance, log copepod abundance, log phytoplankton concentration and log periphyton biomass at the time of first sampling (days 11–12) and second sampling (day 32). Values plotted are means ± 1 SE.

Davidson *et al.*'s (2007) failed to demonstrate interactive effects between carbaryl and Bd. Davidson *et al.*'s (2007) study differed from ours in that carbaryl was applied as a 'pulse treatment' (a single high-dose application) rather than a 'press treatment' (multiple applications of lower concentration) (Relyea & Diecks, 2008). To explain the absence of interactive effects of carbaryl and Bd, Davidson *et al.* (2007) hypothesised that (1) while immune function may have been reduced by carbaryl, it may not have been reduced to the extent that susceptibility to Bd increased, (2) immune function may have recovered after a one-time application of carbaryl or (3) carbaryl and Bd may have affected different aspects of immune function. Hypotheses 1 and 3 seem plausible in terms of the results of our study, but carbaryl was applied on a weekly basis in our experiment, so recovery of immune function after a carbaryl dose seems unlikely. Previous studies have documented interactive effects between pesticides and interspecific competition (Mackey & Boone, 2009) and Bd and interspecific competition (Parris & Cornelius, 2004; Han, 2008), although not at the low concentrations of carbaryl and Bd tested in our experiment.

We found evidence suggesting that phytoplankton contributed to the diet of tadpoles in this experiment. *P. regilla* appeared to benefit from the overabundance of phytoplankton caused by carbaryl-induced mortality of zooplankton, and Bd-infected *R. cascadae* may have switched from a diet composed primarily of periphyton to one rich in phytoplankton. Although inconclusive, both results suggest that a closer examination of phytoplankton as an important component of the larval anuran diet is warranted, as has been previously suggested (e.g. Altig *et al.*, 2007; Whiles *et al.*, 2010).

We encourage future studies examining possible interactive effects between Bd and other pesticides. Furthermore, we find mesocosms to be an ecologically realistic and tractable venue for studies examining the interaction between community structure and host–pathogen dynamics.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Risk mitigation procedures.

Table S1. Infection level

Table S2. Abiotic response variables

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