LETHAL AND SUBLETHAL EFFECTS OF ATRAZINE, CARBARYL, ENDOUSULFAN, AND OCTYLPHENOL ON THE STREAMSIDE SALAMANDER (AMBYSTOMA BARBOURI)

JASON R. ROHR,* ADRIA A. ELSKUS, BRIAN S. SHEPHERD, PHILIP H. CROWLEY, THOMAS M. MCCARTHY, JOHN H. NIEDZIECKI, TYLER SAGER, ANDREW SIH, and BRENT D. PALMER
Department of Biology, University of Kentucky, Lexington, Kentucky 40506-0225, USA

(Received 31 October 2002; Accepted 7 April 2003)

Abstract—Agricultural contaminants may be contributing to worldwide amphibian declines, but little is known about which agrichemicals pose the greatest threat to particular species. One reason for this is that tests of multiple contaminants under ecologically relevant conditions are rarely conducted concurrently. In this study, we examined the effects of 37-d exposure to the agrichemicals atrazine (4, 40, and 400 µg/L), carbaryl (0.5, 5, and 50 µg/L), endosulfan (0.1, 1, and 10 µg/L for 31 d and 0.1, 10, and 100 µg/L for the last 6 d), and octylphenol (5, 50, and 500 µg/L) and to a solvent control on streamside salamanders (Ambystoma barbouri) in the presence and absence of food. We found that none of the agrichemicals significantly affected embryo survival, but that hatching was delayed by the highest concentration of octylphenol. In contrast to embryos, larval survival was reduced by the highest concentrations of carbaryl, endosulfan, and octylphenol. Growth rates were lower in the highest concentrations of endosulfan and octylphenol than in all other treatments, and the highest concentration of endosulfan caused respiratory distress. Significantly more carbaryl, endosulfan, and octylphenol tanks had larvae with limb deformities than did control tanks. Refuge use was independent of chemical exposure, but 10 µg/L of endosulfan and 500 µg/L of octylphenol decreased larval activity. Systematically tapping tanks caused a greater activity increase in larvae exposed to 400 µg/L of atrazine and 10 µg/L of endosulfan relative to solvent controls, suggesting underlying nervous system malfunction. Hunger stimulated a decrease in refuge use and an increase in activity, but this response was less pronounced in larvae exposed to the highest concentration of any of the four agrichemicals, possibly because these larvae were the most lethargic. More studies are needed that concurrently examine the effect of multiple contaminants on amphibians so we can better identify effective mitigating measures.

Keywords—Pesticide Amphibian Survival Growth Behavior

INTRODUCTION

Evidence suggests that many species of amphibians have experienced substantial declines in number and distribution, and that exposure to environmental contaminants, such as agrichemicals, may be contributing to these losses [1]. Agricultural sites where pesticides are often used have lower amphibian species richness and abundance than at adjacent non-agricultural sites [2], in some cases resulting in the disappearance of amphibians from agricultural landscapes [3]. Amphibians are especially at risk from agricultural contaminants because they have permeable skin and eggs that readily absorb chemicals from the environment. Moreover, many species complete their life cycles in ponds and streams adjacent to agricultural fields where agrichemicals are applied, and these applications often coincide with breeding and larval development [1].

Assessing which of the many agricultural contaminants pose particular species at greatest risk is essential for designing appropriate mitigating measures. However, it is not possible to reliably evaluate the tolerance of amphibian species to various contaminants by comparing across studies, because amphibian susceptibility to agrichemicals can differ among populations, individuals, environmental conditions, times in development, and exposure concentrations [4–6 and references therein]. Most studies have only concurrently tested the effects of one or two agrichemicals on anurans [7,8], so little is known about the sensitivity of any one amphibian species to several contaminants or the effect of agricultural contaminants on caudate amphibians [9,10].

In this study, we simultaneously evaluate the behavioral, developmental, growth, and survival effects of chronic exposure to the agrichemicals atrazine, carbaryl, endosulfan, and octylphenol in the streamside salamander (Ambystoma barbouri). Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) is an herbicide used predominantly in corn and sorghum production, and is one of the most prevalent herbicides found in the environment [11]. Carbaryl (1-naphthyl-N-methylcarbamate) is an acetylcholinesterase-inhibiting insecticide commonly used in agricultural and forestry practices throughout the United States and Canada [6]. Endosulfan (6,7,8,9,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepin-3-oxide) is an organochlorine insecticide used on a variety of crops and it can be quite toxic to aquatic organisms [12]. Octylphenol is one of the major degradation products of alkylphenol polyethoxylates, a large group of surfactants used in pesticide formulations [13].

Ambystoma barbouri is found in ephemeral streams that run through the agricultural landscape of central Kentucky, southern Indiana, and southern Ohio (USA). Eggs are laid under submerged rocks from January to April, hatching is from April to May, and larval metamorphosis is from May to July [14]. Egg and larval development coincide with the heaviest periods of pesticide application on neighboring cropland [10]. Larvae are generally restricted to the upper portion of streams...
and can suffer heavy mortality from stream drying or from drifting into pools containing fish [14].

*Ambystoma barbouri* is of conservation concern for several reasons. Its restricted range consists of only a small number of populations [14] with very little gene flow among many populations [15]. Thus, many populations likely contain little variation on which selection can act and are likely within a tenuous metapopulation structure, incapable of compensating for local declines. Furthermore, examination of available data suggests that species with limited ranges may be narrowly adapted, and thus more susceptible to contaminants [16].

**MATERIALS AND METHODS**

**Materials**

Atrazine (80% pure) was obtained from ICN Laboratories (Aurora, OH, USA); carbaryl (99% pure), endosulfan (I and II isomer, 99% pure), and octylphenol (no purity given, assumed to be 100% pure for nominal concentration calculations) were purchased from ChemService (West Chester, PA, USA). Dimethylsulfoxide (DMSO) was obtained from Fisher Scientific [www.fishersci.com], and acetone was high purity, Burdick and Jackson pesticide-grade obtained from VWR [www.vwrsp.com].

**Experimental design and treatments**

Twelve egg clutches of *A. barbouri* were collected from Fossil Creek (Jessamine County, KY, USA) in February 2002. Eggs were carefully separated from one another, placed into a 37-L aquarium filled with carbon-filtered tap water, and mixed thoroughly. Ten arbitrarily chosen eggs were placed into each of 56 glass bowls (3.7 L) located in an environmental chamber maintaining 15°C and a 12:12 h light:dark photoperiod. The bowls were placed on light brown shelving and isolated with cardboard partitions. Each bowl contained a submerged, translucent, gray semicircular glass refuge plate (9-cm radius, 1 cm from the bottom) and 2 L of charcoal-filtered tap water (pH ~ 8, 15°C) that was constantly aerated.

Separate stock solutions were prepared for each agrichemical (atrazine: 1.6 and 0.16 mg/ml of DMSO, carbaryl: 0.153 and 0.015 mg/ml of acetone, endosulfan: 0.04 and 0.004 mg/ml of acetone, octylphenol: 1.6 and 0.16 mg/ml of acetone). All stock solutions were kept in amber glass bottles to minimize photodegradation and stored at −20°C. Standard range-finding conditions were used to select exposure doses. The highest concentration reflects a sublethal concentration based on estimated lethal concentrations to aquatic organisms as published in laboratory and field exposure studies. Ten- and 100-fold lower doses were used to encompass the full range of possible environmental concentrations. The lower concentrations represent typical exposure from contaminated surface waters, whereas the higher concentrations may represent levels from runoff events near application sites or the increased concentration of these compounds as streams dry (see other studies [11–13,17,18] for concentrations found in wild). We do not have data from our collection site documenting the extent of exposure of *A. barbouri* to the four agrichemicals, but *A. barbouri* likely is exposed to these compounds because of the agricultural practices surrounding the collection site. Furthermore, *A. barbouri* may be a useful indicator of the potential susceptibility of other amphibians to these widely distributed compounds.

Exposure solutions were made just before water changes by spiking 4 L of charcoal-filtered tap water (15°C) with either 1.25, 0.125, or 0.0125 ml of atrazine, carbaryl, endosulfan, or octylphenol stock solutions, 1.25 ml of acetone and DMSO (solvent control), or 1.25 ml of charcoal-filtered tap water (negative control). This produced nominal concentrations (adjusted for purity) of 4, 40, and 400 µg/L of atrazine; 0.5, 5, and 50 µg/L of carbaryl; 0.1, 1, and 10 µg/L of endosulfan; 5, 50, and 500 µg/L of octylphenol; and a 0.03% acetone + 0.03% DMSO solvent control. As a result, there were three concentrations of each test substance and two control treatments (solvent and water), and four replicate bowls per treatment (10 animals per bowl), for a total of 40 animals for each test concentration and each control.

Water changes were conducted on each bowl every other day by removing 1 L with a vacuum pump and replacing it with 1 L of exposure solution containing the same chemical and concentration. Nominal and actual concentrations were expected to be similar and remain constant for 48-h between water changes because of the stability of the four compounds (see below). During water changes, we recorded the number of live embryos and larvae, removed and preserved dead embryos and larvae (in neutral buffered formalin), fed larvae live blackworms (*Lumbriculus variegates*) ad libitum, and randomly shuffling bowls within the environmental chamber to control for potential location effects.

Exposure to agrichemicals began 2 d after egg collection and lasted for 37 d, but survival was tracked for 53 d. A 37-d exposure is considered ecologically realistic, because nontarget biota in agricultural regions can be exposed to even rapidly degrading pesticides for often several consecutive months [19], and because the agrichemicals tested, or their degradation products, are quite persistent in nature. Atrazine and the degradation products of endosulfan have long half-lives in freshwater [20,21], and octylphenol is resistant to degradation [13]. The half-life of carbaryl in laboratory settings is relatively short [6], but it can persist for more than a year in aquatic ecosystems [22].

**Behavior, growth, and development**

Larval behavior was recorded in the presence and absence of food. On experimental day 16 and 28, all blackworms were removed. Four and 6 d later at 0900, 1200, and 1500 h, we recorded the number of larvae under refuge and moving in 30 s, with the latter referred to as ambient activity. Four days without food was presumed to be long enough to stimulate hunger. After the trial at 1500 h, we placed a standard spring-loaded mousetrap against the top of each bowl, released the lever from a height of 4 cm, and recorded the number of larvae moving immediately after this disturbance, which is referred to as reflex activity. Blackworms were provided after reflex activity trials on days 22 and 34, and the same behaviors were recorded on days 24, 26, 28, and 36 in the presence of blackworms.

On day 50, we measured mass and snout–vent length of all surviving larvae, and recorded the number of live and preserved larvae with missing or additional limbs and digits, which are referred to as asymmetries (only one animal had symmetric deformities).

**Statistical analyses**

All statistical analyses were conducted with Statistica® 5.5a (Statsoft, Tulsa, OK, USA). Data presented as percentages were arcsine–square-root transformed before analyses. We
used a repeated-measures analysis of variance (ANOVA) to evaluate the effects of the four chemicals and three concentrations on total mortality (embryo + larva) through time. We then conducted a two-way ANOVA to evaluate the effects of agrichemicals and concentration on hatching day, percent embryo and larval survival, and on larval mass and length at day 50. For behavioral data, we averaged observations within a day and conducted multivariate analysis of variance (MANOVA) to test the effects of chemical, concentration, and food (presence, absence; a repeated-measures factor) on the percent of larvae in refuge and moving when undisturbed and disturbed. After MANOVA, ANOVA was conducted on each response variable. Tukey’s honestly significant difference tests (HSDs) were used to compare treatment means where significant ($p < 0.05$). We used Dunnett’s multiple comparison tests (DTs) to compare hatching day, survival, mass, length, and behavior in solvent controls to all other treatments. To test for an interaction between hunger stress and agrichemical, we used Dunnett’s tests to compare behavior in the presence of food minus behavior in the absence of food. Because several tanks had no larvae with asymmetries, we added one to the arcsine of the percent of larvae with asymmetries, took the natural log, and then conducted ANOVA. Any $A. barbouri$ accidentally killed during water changes were removed from the starting number in that tank for survival analyses.

**Results**

**Exposure concentrations**

Concentrations of dosing stock solutions declined no more than 4.8% for any of the test compounds over the course of the 37-d exposures, with none of chemical degradation, as evaluated by flame ionization detection and electron-capture gas chromatography (instrument detection limits were 0.5 pg, 5 ng, 0.2 ng, and 0.2 ng for endosulfan, carbaryl, octylphenol, and atrazine, respectively [Elskus et al., unpublished data]). Single stock solutions were used throughout the experiment for all atrazine, carbaryl, and octylphenol doses and for low-dose endosulfan, and exposure concentrations for these chemicals were consistent throughout the study. For high and medium doses of endosulfan, a single stock solution was used for the first approximately 30 d of the 37-d exposures. During the final week of exposures, a fresh endosulfan stock was incorrectly prepared and high and medium nominal doses were

![Fig. 1. The effects of three concentrations (L = low; M = medium; H = high) of atrazine (A), carbaryl (C), endosulfan (E), and octylphenol (O), and solvent (S) and water (X) controls on (a) hatching day, (b) embryo, and (c) larval survival, and (d) larval snout-vent length at experimental day 50. Larval mass was quantified but is not shown, because it was highly correlated with snout-vent length ($r = 0.939$, $t_{152} = 19.621$, $p < 0.001$). Shown are the means ($\pm$ standard error) of the four replicates for each treatment. Asterisks above bars represent significant $p$ values for comparisons to solvent controls when using Dunnett’s multiple comparison tests (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$).](image)

10-fold higher (100 $\mu$g/L, 10 $\mu$g/L) than the previous four weeks (10 $\mu$g/L, 1 $\mu$g/L); this error was identified during chemical analysis of the dosing solutions.

**Hatching day**

Hatching day was affected by chemical ($F_{3,36} = 5.34$, $p = 0.004$) and concentration ($F_{2,36} = 7.38$, $p = 0.002$), with embryos hatching later in octylphenol than in atrazine or carbaryl (HSD, $p < 0.037$, other $p > 0.127$), and in high than in medium concentrations (HSD, $p = 0.001$, other $p > 0.052$; Fig. 1a).
These results were strongly influenced by the delayed hatching of embryos in the highest concentration of octylphenol, which were the only embryos to hatch later than those in solvent control (Fig. 1a).

Survival

Total survival did not differ among chemicals ($F_{3,36} = 2.44$, $p = 0.080$), and high concentrations induced greater mortality than medium (HSD, $p < 0.003$) and low (HSD, $p < 0.002$, other $p = 0.952$) concentrations, but this concentration difference was not consistent through time across chemicals (chemical $\times$ time, $F_{18,216} = 4.19$, $p < 0.001$; Fig. 2). The high concentration of octylphenol caused a significantly greater rate of mortality than all other chemicals at the high concentration (HSD, $p < 0.001$), and induced greater total mortality than the solvent control (DT, $p = 0.004$; Fig. 2). Larval, but not embryo, survival differed across chemical treatments and concentrations (larvae: chemical, $F_{3,36} = 4.37$, $p = 0.010$; concentration, $F_{3,36} = 11.16$, $p < 0.001$; chemical $\times$ concentration, $F_{6,36} = 2.80$, $p = 0.024$; embryo: $F < 1.96$, $p > 0.155$; Fig. 1b and c). Fewer larvae survived in octylphenol than in atrazine (HSD, $p < 0.006$, other $p > 0.16$), and in high than in medium (HSD, $p < 0.001$) and low (HSD, $p < 0.002$, other $p = 0.958$) concentrations. Embryo survival did not differ significantly between solvent control and all other treatments ($p > 0.464$), but a greater percentage of larvae survived in solvent control than in high concentrations of carbaryl, endosulfan, and octylphenol (Fig. 1c).

Growth and development

Despite high mortality in some treatments, significant effects on growth and development were evident. Only two of the four high-concentration octylphenol tanks had surviving larvae (two in each) on day 50, but larval mass (data not shown) and length (Fig. 1d) in these and in high-concentration endosulfan tanks were significantly lower than in all other tanks, including controls (DT, $p < 0.003$, $p < 0.001$, respectively), producing significant main effects and an interaction for larval mass and length (chemical, $F_{3,34} > 8.84$, $p < 0.001$; concentration, $F_{2,34} > 27.58$, $p < 0.001$; chemical $\times$ concentration, $F_{6,34} > 9.41$, $p < 0.001$). Larval mass and length were less in octylphenol than in atrazine (HSD, $p < 0.001$) or carbaryl (HSD, $p < 0.001$, other $p > 0.06$), and in high than in medium (HSD, $p < 0.001$) or low (HSD, $p < 0.001$, other $p > 0.856$) concentrations.

Many larvae had missing and deformed limbs and digits, but none had additional limbs or digits. No difference was found in the percent of larvae with asymmetries among concentrations ($F_{2,36} = 1.379$, $p = 0.265$) and no chemical-by-concentration interaction was found ($F_{6,36} = 1.888$, $p = 0.111$), so to conserve degrees of freedom, we conducted a one-way ANOVA to compare asymmetries in atrazine, endosulfan, carbaryl, octylphenol, and solvent + negative control tanks. The ANOVA revealed a significant difference in the percent of larvae with asymmetries among these treatments ($F_{5,44} = 2.570$, $p = 0.049$), but a Dunnett’s test detected greater asymmetries only in carbaryl tanks relative to controls ($p = 0.044$, other $p > 0.08$; Fig. 3). The low power of these comparisons is attributable to a single control tank containing the highest percentage of larvae with asymmetries out of all tanks, greatly

![Fig. 2. The effects of three concentrations (low, medium, and high) of atrazine, carbaryl, endosulfan, and octylphenol, and solvent and water controls on total survival (embryo + larval) through time. Shown are the means of the four replicates of each treatment. Vertical solid lines represent the mean hatching day for that concentration (all standard errors < 0.031). The dashed vertical line is the mean hatching day for embryos in the high concentration of octylphenol (standard error = 0.59), which hatched later than embryos in all other treatments.](image1)

![Fig. 3. The effects of atrazine, carbaryl, endosulfan, octylphenol, and controls on mean (± standard error) larval asymmetries (missing limbs or digits).](image2)
increasing the mean and variance for controls. To eliminate the skewing effect of this tank, we used an independent samples chi-square test with a Yates correction [25] to consider the number of tanks with asymmetrical larvae. Fewer control tanks had asymmetrical larvae than did carbaryl ($\chi^2 = 6.68$, $p = 0.009$), endosulfan ($\chi^2 = 9.53$, $p = 0.002$), and octylphenol ($\chi^2 = 4.02$, $p = 0.045$) tanks, but not atrazine tanks ($\chi^2 = 0.43$, $p = 0.514$; Fig. 3). Asymmetrical and symmetrical larvae did not significantly differ in mass (167.39 ± 8.34, 166.34 ± 4.09 mg, respectively; $F_{1,357} = 0.015$, $p = 0.903$) or length (mean ± standard error: 1.537 ± 0.025, 1.548 ± 0.012 cm, respectively; $F_{1,357} = 0.208$, $p = 0.649$).

Behavior

The MANOVA revealed that larval behaviors differed when exposed to different chemicals ($F_{9,78} = 4.53$, $p < 0.001$), concentrations ($F_{6,64} = 5.59$, $p < 0.001$), and food levels ($F_{3,34} = 143.42$, $p < 0.001$), and that responses to food removal were not consistent across chemicals (chemical × food, $F_{9,78} = 2.77$, $p = 0.007$; Fig. 4). More specifically, refuge use differed among chemicals, concentrations, and food levels ($F_{3,34} = 3.93$, $p < 0.016$; $F_{2,34} = 3.56$, $p = 0.039$; $F_{1,34} = 154.44$, $p < 0.001$, respectively; Fig. 4a and b). Larvae were in refuge more when exposed to carbaryl than when exposed to any of the other chemicals (HSD, $p = 0.019$ for octylphenol), although this was marginally nonsignificant for endosulfan and atrazine (HSD, $p = 0.06$ for each). Refuge use was greater in low than in high concentrations (HSD, $p = 0.032$, other $p > 0.11$), and when food was present versus when absent. Larvae also remained in refuge more frequently in endosulfan than in atrazine (HSD, $p = 0.025$) and carbaryl (HSD, $p = 0.037$, other $p > 0.36$) tanks after food was removed. Despite these differences among chemicals, no differences were found in refuge use between larvae exposed to any treatment and solvent control regardless of whether food was present or absent (DT, $p > 0.244$).

Larval activity also was significantly affected by chemical treatment. Larvae exhibited lower ambient activity in endosulfan than in atrazine (HSD, $p = 0.017$) and carbaryl (HSD, $p = 0.020$, other $p > 0.11$), in high relative to medium (HSD, $p < 0.003$) and low (HSD, $p < 0.001$, other $p = 0.580$) concentrations, and in the presence versus absence of food ($F_{1,34} = 74.20$, $p < 0.001$; Fig. 4c and d). Larvae in the highest concentrations of atrazine or carbaryl had a higher ambient activity and a greater increase in activity after food removal than in the highest concentrations of endosulfan or octylphenol. Solvent control larvae were only more active than...
larvae in high concentrations of endosulfan and octylphenol (Fig. 4c and d).

Reflex activity was independent of hunger \((F_{1.34} < 0.01, p = 0.984)\) but was influenced by chemical \((F_{1.34} = 5.30, p = 0.005)\) and concentration \((F_{1.34} = 5.37, p < 0.001)\; \text{(Fig. 4e and f)}.\) Reflex activity was lower in octylphenol than in atrazine \((\text{HSD,} \; p < 0.01)\) or endosulfan \((\text{HSD,} \; p < 0.009, \; \text{other} \; p > 0.12)\) and was higher in high concentrations than in medium \((\text{HSD,} \; p = 0.012)\) or low \((\text{HSD,} \; p = 0.018, \; \text{other} \; p = 0.985)\) concentrations. Considerable variation was found in reflex activity, which made it difficult to detect differences between treatments and solvent controls \((\text{DT, high atrazine,} \; p = 0.035, \; \text{other} \; p > 0.070); \; \text{Fig. 4e and f).}\)

Dramatic treatment differences were found in how strongly larvae responded to disturbance as measured by the change in their activity between ambient and reflex states \((\text{moving when disturbed} - \text{when undisturbed}).\) Disturbance resulted in a greater increase in larval activity in endosulfan than in carbaryl \((\text{HSD,} \; p = 0.013)\) or octylphenol \((\text{HSD,} \; p < 0.007, \; \text{other} \; p > 0.11),\) in high concentrations relative to medium \((\text{HSD,} \; p < 0.001)\) or low \((\text{HSD,} \; p < 0.001, \; \text{other} \; p = 0.977)\) concentrations, and in the presence rather than in the absence of food \((F_{1.34} = 5.45, \; p = 0.026; \; \text{Fig. 4c to f}).\) When food was present, larvae in high concentrations of atrazine or endosulfan had a greater increase in activity after disturbance than did larvae in solvent controls \((\text{DT,} \; p = 0.007, \; p = 0.004, \; \text{respectively}; \; \text{Fig. 4c to f}).\)

Change in larval refuge use and activity after food removal \((\text{behavior in presence} - \text{in absence of food})\) did not differ between solvent control and any other treatment \((\text{DT, all} \; p > 0.12),\) indicating that these agrochemicals did not significantly alter standard refuge and activity responses to hunger relative to solvent control. However, larvae exposed to the highest concentration of all chemicals had the lowest ambient activity. Also, when food was removed, ambient activity was correlated with change in activity \((r = 0.284, \; t_{1.52} = 2.138, \; p = 0.037)\) and nearly correlated with change in refuge use \((r = 0.249, \; t_{1.52} = 1.854, \; p = 0.069).\) This indicates that lethargic larvae were least likely to display standard responses to hunger. Caution should be taken in interpreting the behavior of larvae exposed to the high concentration of octylphenol, because most of these observations were for only four larvae in two tanks.

Various observations and analyses provide insight into the effects of increasing endosulfan concentrations during the end of the dosing period. Larvae exposed to the highest concentration of endosulfan were visibly smaller than control larvae before the increase in concentration, suggesting that the relatively brief exposure to the higher concentration cannot account for the growth differences between endosulfan and control larvae. Behavioral analyses \((\text{Dunnnett’s tests)}\) were reconducted to exclude observations on endosulfan larvae after concentrations increased \((\text{the last} \; 3 \; \text{d of observations}).\) No nonsignificant probability values from the statistical model that included all observations became significant when these observations were removed, nor did any significant values become nonsignificant, indicating that exposure to only 10 \(\mu g/L\) of endosulfan was sufficient to alter larval activity. However, on day 34, approximately one half of the larvae exposed to 100 \(\mu g/L\) of endosulfan accumulated gas in their abdomens, and most of these larvae were observed floating at the surface of the water, incapable of returning to the bottom of the tanks. Some released air bubbles which facilitated swimming down in the water column. Several of these larvae were trapped under the refuge and drowned, apparently because they could not return to the surface for air. Because this respiratory distress and associated mortality occurred soon after exposure concentrations increased, it is probable that the increase in endosulfan concentration was the cause.

**DISCUSSION**

Examination of our results indicates that atrazine, carbaryl, endosulfan, and octylphenol varied greatly in their effects on survival, growth, development, and behavior of *A. barbouri*. But for all chemicals, the least growth and greatest mortality were found at the highest concentrations, suggesting dose-dependent growth and survival. As predicted, carbaryl, endosulfan, and octylphenol did not significantly differ in induced mortality, and atrazine had the fewest adverse lethal and sublethal effects. Growth was significantly inhibited only in high concentrations of endosulfan and octylphenol, but missing limbs and digits, probable developmental abnormalities, were observed more frequently in larvae exposed to carbaryl, endosulfan, and octylphenol. Although refuge use did not differ dramatically among treatments, ambient activity did, with apparent lethargy observed in high concentrations of endosulfan and octylphenol.

Mortality rates were comparable to those reported for amphibians exposed to similar concentrations of atrazine, but not carbaryl or endosulfan. Atrazine did not affect larval survival in our experiment, nor did it affect larval survival for gray treefrogs (*Hyla versicolor*) [21], northern leopard frogs (*Rana pipiens*) [7,23], or American toads (*Bufo americanus*) [7]. Significant larval mortality was induced in gray treefrogs [5,26] and various ranid species [4–6] at or above carbaryl concentrations of 160 \(μg/L\), and endosulfan concentrations at or above 138 \(μg/L\) caused significant larval mortality in American toads, green frogs (*Rana clamitans*), and wood frogs (*Rana sylvatica*) [27]. In contrast, 50 and 100 \(μg/L\) of carbaryl and endosulfan, respectively, produced significant mortality in our study. The greater sensitivity of *A. barbouri* to carbaryl and endosulfan may be due to species differences in susceptibility, which have been reported for amphibians exposed to both of these insecticides [5,27]. The restricted range of *A. barbouri* may make it a narrowly adapted species, that is, one that highly sensitive to contaminants [16]. Octylphenol caused the greatest *A. barbouri* mortality, but we were unable to find any other studies that documented mortality rates for amphibians exposed to octylphenol. Larval death occurred most rapidly in the highest concentration of octylphenol, indicating that short-term exposure to this concentration would be more detrimental than for any other chemical or concentration we tested.

The greater mortality of larvae than embryos could be explained by bioconcentration of these agrochemicals or by susceptibility differences between the life-history stages. Each of the four focal compounds in our study has been reported to bioconcentrate [23,28–30], which could pose greater risk to larvae than embryos, because the older larvae would have had more time to accumulate chemicals. However, the jelly coating of eggs may protect embryos from direct exposure to pesticides [27], so we cannot discount greater susceptibility of the larval life-history stage.

All agrochemicals tested also had numerous sublethal effects, which may be more valuable in assessing sensitivity to contaminants than lethal effects [31], can have important impacts on communities, and can be more detrimental to am-
Atrazine and endosulfan have been linked to their toxic effects on the United States and probably the world [8], the long-term fitness costs of activity alterations induced by atrazine should be investigated.

In addition to altered activity, endosulfan caused respiratory distress, consistent with that reported for rats [28] and fish [40] exposed to endosulfan. Larvae showing respiratory distress were observed ventral side up at the surface of the water, which would make them easy prey in nature. This suggests that we may be underestimating the effects of endosulfan, and perhaps the other agrichemicals, by conducting our study in the absence of predators [26].

In the absence of food, larvae increased activity and decreased refuge use, which are typical responses to hunger [24]. These presumed adaptive response changes did not significantly differ between treatments and controls, meaning that similar percentages of larvae in control and in chemical-treated tanks left refuge and increased activity when hungry. However, concurrently examining multiple toxicants typically implies that fewer animals can be exposed to any one chemical or concentration, reducing statistical power. Consequently, it is possible that the lack of difference between treatments and solvent control was due to a type 2 error. Support for a type 2 error and a hunger-by-pesticide interaction comes from comparing across chemicals and concentrations. These comparisons suggest that agrichemicals did induce lethargy, and as predicted, lethargic larvae were least likely to exhibit standard responses to hunger. Larvae in high concentrations of all chemicals had the lowest ambient activity, increasing activity and leaving refuge the least when hungry. Furthermore, larvae in high concentrations of endosulfan or octylphenol had a lower ambient activity than did larvae in high concentrations of atrazine or carbaryl, increasing activity less when food was removed. Because fluctuations in food are ubiquitous, because our results suggest an interaction between food availability and agricultural contaminants, and because commonly used short-term lethal concentration and median lethal dose tests are usually conducted in the absence of food, the interaction of hunger stress and pesticides deserves further study.

CONCLUSIONS

Under our ecologically relevant test conditions, octylphenol seemed to have the greatest detrimental effects on A. barbouri. Chronic exposure to octylphenol induced the greatest mortality, delay in hatching, growth reduction, and lethargy. Endosulfan also had deleterious effects, including increased mortality, reduced growth rates, respiratory distress, limb deformities, and altered behavior. Carbaryl caused significant larval mortality at the highest concentration, and produced the greatest percent of malformed larvae, but did not significantly affect behavior relative to controls. Although atrazine did not induce significant mortality, it did seem to affect motor function. Recently, concentrations of atrazine as low as 1 µg/L, one third the allowable amount in drinking water, produced hermaphroditic male frogs [8]. Consequently, the effect of these agrichemicals on reproductive adults is needed to fully understand their impact on the population dynamics of A. barbouri and other amphibians. More studies are needed that concurrently examine the effect of multiple stressors and multiple agrichemicals on amphibians so we can better identify and mitigate the effects of the agrichemicals that pose the greatest threat.
Acknowledgement—We thank Bert Lynn for help with chemical analyses; Randal Voss for improving the manuscript; and Debasish Ghosh, Brandon Stiff, Ian Struwing, and Sylvia Palmer for laboratory assistance. This research was funded by a U.S. Environmental Protection Agency STAR grant (R829086) to B.D. Palmer (primary investigator), P.H. Crowley, A.A. Elskus, B.S. Shepherd, and A. Sih; a Department of the Interior, U.S. Geological Survey, and Kentucky Water Resources Institute grant (01HQGR0133) to B.S. Shepherd; and funding from the University of Kentucky Biology Department. All experiments conducted in this study complied with the University of Kentucky’s animal care protocols. The views and conclusions contained within this document are those of the authors and should not be interpreted as necessarily representing the official policies, either expressed or implied, of the U.S. government.

REFERENCES


