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

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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 least 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 nonagricultural 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 place 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-napthbyl-*N*-methylcarbamate) is an acetylcholinesterase-inhibiting insecticide commonly used in agricultural and forestry practices throughout the United States and Canada [6]. Endosulfan (6,7,8,9,10,10-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

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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 shuffled 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 non-target 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.

Hypotheses

We expected standard dose-dependent responses for survival, growth, and behavior for all chemicals. We predicted that atrazine would have the least adverse effects of the four chemicals, because numerous studies have demonstrated that it has minimal impact on amphibian survival [7,21,23]. All other chemicals tested were expected to alter behavior and decrease growth and survival relative to controls, but were not necessarily expected to differ from one another in these response variables. We predicted a greater percentage of larvae with asymmetries when exposed to chemicals than in controls, because pesticides have been implicated in amphibian deformities [6,7]. Finally, because increased activity and decreased refuge use can increase amphibian feeding rates [24], larvae were expected to be in refuge more and be less active when sated (food present) than when hungry (food absent). This presumed adaptive response to hunger was expected to be inhibited by carbaryl, endosulfan, and octylphenol via induction of lethargy.

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 no evidence 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
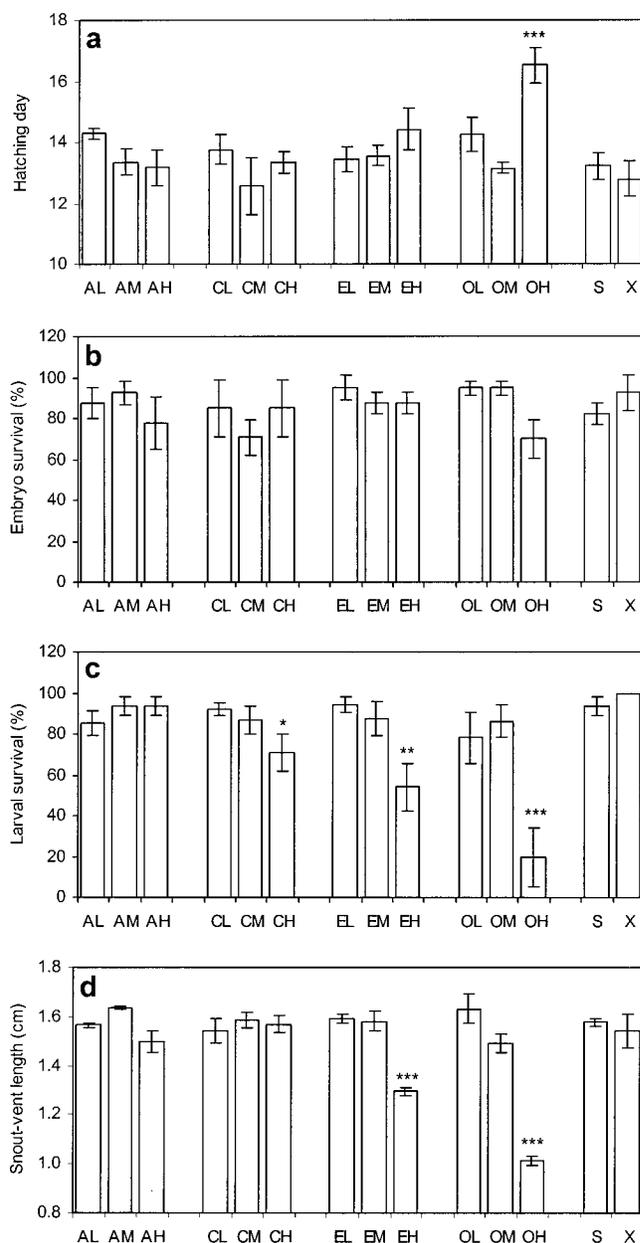

Fig. 1. The effects of three concentrations (L = low; M = medium; H = high) of atrazine (A), carbaryl (C), endosulfan (E), and octylphenol (O), and solvent (S) and water (X) controls on (**a**) hatching day, (**b**) embryo, and (**c**) larval survival, and (**d**) larval snout–vent length at experimental day 50. Larval mass was quantified but is not shown, because it was highly correlated with snout–vent length ($r = 0.939$, $t_{1,52} = 19.621$, $p < 0.001$). 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 (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$).

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

Hatching

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).
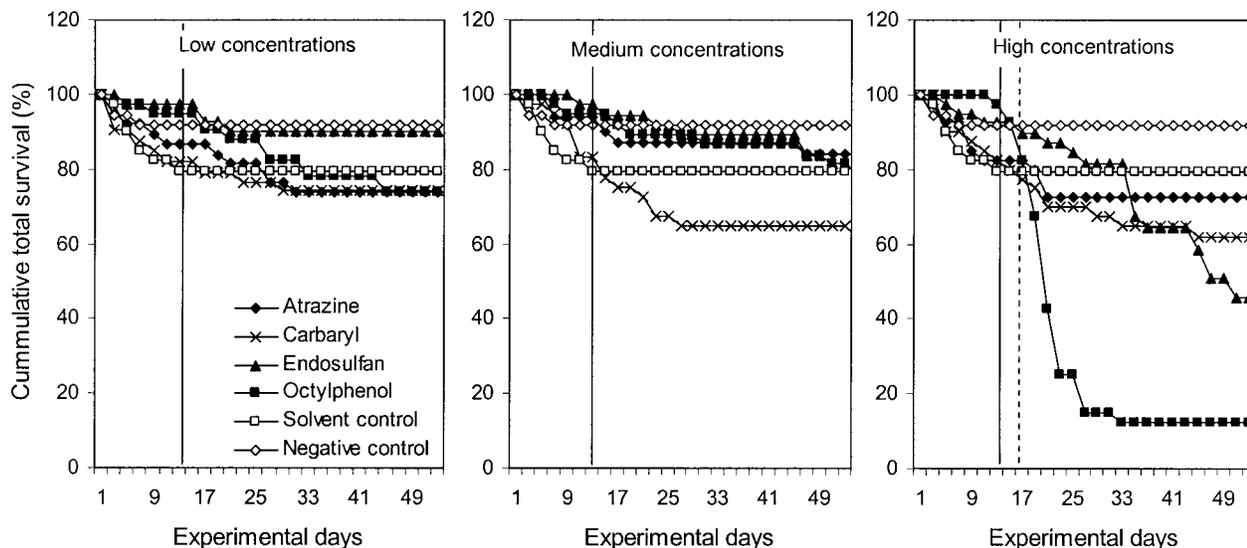
Fig. 2. The effects of three concentrations (low, medium, and high) of atrazine, carbaryl, endosulfan, and octylphenol, and solvent and water controls on total survival (embryo + larval) through time. Shown are the means of the four replicates of each treatment. Vertical solid lines represent the mean 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.

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 × 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, $F_{2,36} = 11.16$, $p < 0.001$; chemical × 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 × 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, octylephenol, and solvent + negative control tanks. The ANOVA revealed a significant difference in the percent of larvae with asymmetries among these treatments ($F_{3,44} = 2.570$, $p = 0.049$), 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
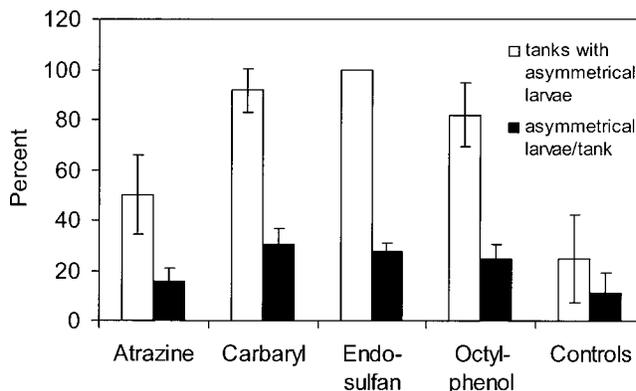

Fig. 3. The effects of atrazine, carbaryl, endosulfan, octylphenol, and controls on mean (± standard error) larval asymmetries (missing limbs or digits).
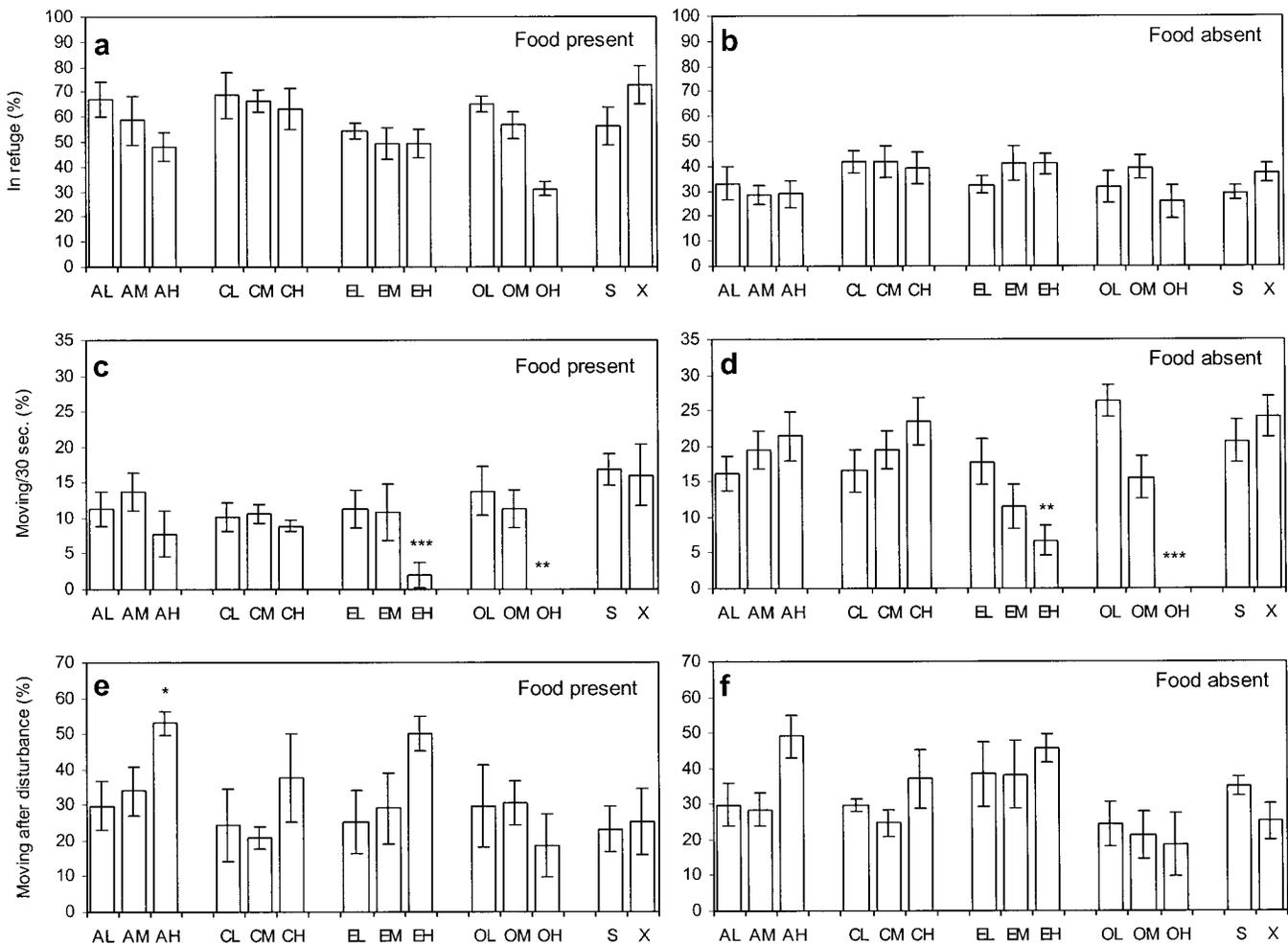
Fig. 4. The effects of food presence or absence, and three concentrations (L = low; M = medium; H = high) of atrazine (A), carbaryl (C), endosulfan (E), and octylphenol (O), and solvent (S) and water (X) controls on mean (± standard error) larval refuge use (**a, b**) and activity when undisturbed (**c, d**) and disturbed (**e, f**). Asterisks above bars represent significant *p* values for comparisons to solvent controls when using Dunnett's multiple comparison tests (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$).

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 ($F_{3,32} = 143.42$, $p < 0.001$), 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 ($F_{3,34} = 5.30$, $p = 0.005$) and concentration ($F_{2,34} = 5.37$, $p < 0.001$; Fig. 4e and f). Reflex activity was lower in octylphenol than in atrazine (HSD, $p = 0.011$) or endosulfan (HSD, $p < 0.009$, other $p > 0.12$) and was higher in high concentrations than in medium (HSD, $p = 0.012$) or low (HSD, $p = 0.018$, other $p = 0.985$) concentrations. Considerable variation was found in reflex activity, which made it difficult to detect differences between treatments and solvent controls (DT, high atrazine, $p = 0.035$, other $p > 0.070$; 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 (moving when disturbed − when undisturbed). Disturbance resulted in a greater increase in larval activity in endosulfan than in carbaryl (HSD, $p = 0.013$) or octylphenol (HSD, $p < 0.007$, other $p > 0.11$), in high concentrations relative to medium (HSD, $p < 0.001$) or low (HSD, $p < 0.001$, other $p = 0.977$) concentrations, and in the presence rather than in the absence of food ($F_{1,34} = 5.45$, $p = 0.026$; 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 (DT, $p = 0.007$, $p = 0.004$, respectively; Fig. 4c to f).

Change in larval refuge use and activity after food removal (behavior in presence − in absence of food) did not differ between solvent control and any other treatment (DT, all $p > 0.12$), indicating that these agrichemicals 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 (Dunnett's tests) were reconducted to exclude observations on endosulfan larvae after concentrations increased (the last 3 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 µg/L of endosulfan was sufficient to alter larval activity. However, on day 34, approximately one half of the larvae exposed to 100 µg/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 agrichemicals 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 agrichemicals 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-

phibian populations than direct morality [32]. The mass and length of larvae in endosulfan and octylphenol were less than for larvae in solvent controls, consistent with growth reductions reported for fish [13] and other amphibians [27] exposed to these agrichemicals. Reduced larval growth, or the delayed hatching induced by octylphenol, could delay *A. barbouri* metamorphosis or result in metamorphosis at a smaller size. A major source of mortality of larval *A. barbouri* is desiccation from stream drying [14], which could be exacerbated by delayed metamorphosis. Reduced growth also could contribute to declines in *A. barbouri* by decreasing the rate of recruitment to the breeding adult population [10]. Because *A. barbouri* seems to lack a sufficiently linked metapopulation structure to adequately compensate for declines [15], decreased growth rates or increased mortality may lead to local extinctions.

The increase in limb deformities attributable to carbaryl, endosulfan, and octylphenol exposure could also contribute to declines in *A. barbouri* because metamorphs with missing or deformed limbs should be less effective at foraging and avoiding predators. Missing limbs and digits were likely the result of impaired development rather than cannibalism, because cannibalism in salamanders has been repeatedly demonstrated to be size-dependent [33], but asymmetrical and symmetrical larvae were not different in size, and no larvae were fully cannibalized (missing). However, we cannot rule out the possibility that these agrichemicals stimulated cannibalism that was responsible for some of the missing digits and limbs. Increased developmental deformities in anurans have been attributed to atrazine [7], carbaryl [6], and endosulfan [34], but at much higher concentrations than the doses used in our study. This appears to be the first report of deformities linked to octylphenol. Pesticide exposure can compromise amphibian immune response, which has been shown to intensify trematode parasite infections that increase limb deformities [35]. Thus, these chemicals may have greater detrimental effects in nature, where they can act synergistically with parasites and other stressors.

Sublethal effects of these agrichemicals also included altered larval behavior. When food was present, increasing concentrations of all chemicals increased the percentage of larvae found out of refuge, which would increase larval exposure to predators. Larvae in high concentrations of endosulfan or octylphenol displayed lower ambient activity than controls, but this was not attributable to motor impairment, because they displayed reflex activity. This lower activity may have reduced feeding rates, and could account for the lower mass and length of larvae exposed to endosulfan and octylphenol.

Larval activity increased more after disturbance in high concentrations of atrazine and endosulfan than in controls, indicating skittishness and suggesting underlying nervous system malfunction. Motor disorders in animals exposed to atrazine and endosulfan have been linked to their toxic effects on the central nervous system [27,36,37]. Endosulfan was found concentrated in tissues of frogs with depressed cholinesterase activity [38] and was shown to induce hyperactivity in frogs followed by paralysis [27]. Atrazine decreased acetylcholinesterase activity in fish [36], and, similar to the response of *A. barbouri*, increased burst swimming reactions [37,39]. Collectively, these results suggest that atrazine and endosulfan can affect the central nervous system, producing a variety of modifications to motor activity. Because the consequences of subtle changes in activity are poorly understood, and because atrazine is the most commonly used herbicide in

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.

REFERENCES

1. Blaustein AR, Kiesecker JM. 2002. Complexity in conservation: Lessons from the global decline of amphibian populations. *Ecol Lett* 5:597–608.

2. Bonin J, Des Granges J, Rodrigue J, Oullet M. 1997. Anuran species richness in agricultural landscapes of Quebec: Foreseeing long-term results of road call surveys. *Herpetol Conserv* 1:141–149.

3. Berger L. 1989. Disappearance of amphibian larvae in the agricultural landscape. *Ecol Int Bull* 17:65–73.

4. Boone MD, Bridges CM, Rothermel BB. 2001. Growth and development of larval green frogs (*Rana clamitans*) exposed to multiple doses of an insecticide. *Oecologia* 129:518–524.

5. Semlitsch RD, Bridges CM, Welch AM. 2000. Genetic variation and a fitness tradeoff in the tolerance of gray treefrog (*Hyla versicolor*) tadpoles to the insecticide carbaryl. *Oecologia* 125:179–185.

6. Bridges CM. 2000. Long-term effects of pesticide exposure at various life stages of the southern leopard frog (*Rana sphenocephala*). *Arch Environ Contam Toxicol* 39:91–96.

7. Allran JW, Karasov WH. 2001. Effects of atrazine on embryos, larvae, and adults of anuran amphibians. *Environ Toxicol Chem* 20:769–775.

8. Hayes TB, Collins A, Lee M, Mendoza M, Noriega N, Stuart AA, Vonk A. 2002. Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. *Proc Natl Acad Sci USA* 99:5476–5480.

9. Larson DL, McDonald S, Fivizzani AJ, Newton WE, Hamilton SJ. 1998. Effects of the herbicide atrazine on *Ambystoma tigrinum* metamorphosis: Duration, larval growth, and hormonal response. *Physiol Zool* 71:671–679.

10. Rehage JS, Lynn SG, Hammond JI, Palmer BD, Sih A. 2002. Effects of larval exposure to triphenyltin on the survival, growth, and behavior of larval and juvenile *Ambystoma barbouri* salamanders. *Environ Toxicol Chem* 21:807–815.

11. Solomon KR, Baker DB, Richards RP, Dixon DR, Klaine SJ, LaPoint TW, Kendall RJ, Weisskopf CP, Giddings JM, Giesy JP, Hall LW, Williams WM. 1996. Ecological risk assessment of atrazine in North American surface waters. *Environ Toxicol Chem* 15:31–74.

12. Ernst WR, Jonah P, Doe K, Julien G, Hennigar P. 1991. Toxicity to aquatic organisms of off-target deposition of endosulfan applied by aircraft. *Environ Toxicol Chem* 10:103–114.

13. Ashfield LA, Pottinger TG, Sumpter JP. 1998. Exposure of female juvenile rainbow trout to alkylphenolic compounds results in modifications to growth and ovosomatic index. *Environ Toxicol Chem* 17:679–686.

14. Petranka JW. 1998. *Salamanders of the United States and Canada.* Smithsonian Institution Press, Washington, DC.

15. Storfer A. 1999. Gene flow and population subdivision in the streamside salamander, *Ambystoma barbouri*. Copeia 1999:174–181.

16. Fioramonti E, Semlitsch RD, Reyer HU, Fent K. 1997. Effects of triphenyltin and pH on the growth and development of *Rana lessonae* and *Rana esculenta* tadpoles. *Environ Toxicol Chem* 16:1940–1947.

17. Norris LA, Lorz HW, Gregory SV. 1983. Influence of forest and rangeland management on anadromous fish habitat in western North America: Forest chemicals. PNW-149. General Technical Report. U.S. Department of Agriculture Forest Service, Portland, OR.

18. Battaglin WA, Furlong ET, Burkhardt MR, Peter CJ. 2000. Occurrence of sulfonylurea, sulfonamide, imidazolinone, and other herbicides in rivers, reservoirs and ground water in the midwestern United States, 1998. *Sci Total Environ* 248:123–133.

19. Gruber SJ, Munn MD. 1998. Organophosphate and carbamate insecticides in agricultural waters and cholinesterase (ChE) inhibition in common carp (*Cyprinus carpio*). *Arch Environ Contam Toxicol* 35:391–396.

20. Miles JRW, Moy P. 1979. Degradation of endosulfan and its metabolites by a mixed culture of soil microorganisms. *Bull Environ Contam Toxicol* 23:13–19.

21. Diana SG, Resetarits WJ Jr, Schaeffer DJ, Beckmen KB, Beasley VR. 2000. Effects of atrazine on amphibian growth and survival in artificial aquatic communities. *Environ Toxicol Chem* 19:2961–2967.

22. Gibbs KE, Mingo TM, Courtemanch DL. 1984. Persistence of carbaryl (Sevin-4-oil) in woodland ponds and its effects on pond macroinvertebrates following forest spraying. *Can Entomol* 116:203–213.

23. Allran JW, Karasov WH. 2000. Effects of atrazine and nitrate on northern leopard frog (*Rana pipiens*) larvae exposed in the laboratory from posthatch through metamorphosis. *Environ Toxicol Chem* 19:2850–2855.

24. Horat P, Semlitsch RD. 1994. Effects of predation risk and hunger on the behavior of two species of tadpoles. *Behav Ecol Sociobiol* 34:393–401.

25. Siegel S, Castellans NJ Jr. 1988. *Nonparametric Statistics for the Behavioral Sciences,* 2nd ed. McGraw-Hill, New York, NY, USA.

26. Relyea RA, Mills N. 2001. Predator-induced stress makes the pesticide carbaryl more deadly to gray treefrog tadpoles (*Hyla versicolor*). *Proc Natl Acad Sci USA* 98:2491–2496.

27. Berrill M, Coulson D, McGillivray L, Pauli B. 1998. Toxicity of endosulfan to aquatic stages of anuran amphibians. *Environ Toxicol Chem* 17:1738–1744.

28. Naqvi SM, Vaishnavi C. 1993. Bioaccumulative potential and toxicity of endosulfan insecticide to nontarget animals. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol* 105:347–361.

29. Mora BR, Martinez-Tabche L, Sanchez-Hidalgo E, Hernandez GC, Ruiz MCG, Murrieta FF. 2000. Relationship between toxicokinetics of carbaryl and effect on acetylcholinesterase activity in *Pomacea patula* snail. *Ecotoxicol Environ Saf* 46:234–239.

30. Ferreira-Leach AMR, Hill EM. 2001. Bioconcentration and distribution of 4-*tert*-octylphenol residues in tissues of the rainbow trout (*Oncorhynchus mykiss*). *Mar Environ Res* 51:75–89.

31. Little EE, Archeski RD, Flerov BA, Kozlovskaya VI. 1990. Behavioral indicators of sublethal toxicity in rainbow trout. *Arch Environ Contam Toxicol* 19:380–385.

32. Peacor SD, Werner EE. 2001. The contribution of trait-mediated indirect effects to the net effects of a predator. *Proc Natl Acad Sci USA* 98:3904–3908.

33. Ziemba RE, Collins JP. 1999. Development of size structure in tiger salamanders: The role of intraspecific interference. *Oecologia* 120:524–529.

34. Harris ML, Chora L, Bishop CA, Bogart JP. 2000. Species- and age-related differences in susceptibility to pesticide exposure for two amphibians, *Rana pipiens*, and *Bufo americanus*. *Bull Environ Contam Toxicol* 64:263–270.

35. Kiesecker JM. 2002. Synergism between trematode infection and pesticide exposure: A link to amphibian limb deformities in nature? *Proc Natl Acad Sci USA* 99:9900–9904.

36. Davies PE, Cook LSJ, Goenarso D. 1994. Sublethal responses to pesticides of several species of Australian fresh water fish and crustaceans and rainbow trout. *Environ Toxicol Chem* 13:1341–1354.

37. Saglio P, Trijasse S. 1998. Behavioral responses to atrazine and diuron in goldfish. *Arch Environ Contam Toxicol* 35:484–491.

38. Sparling DW, Fellers GM, McConnell LL. 2001. Pesticides and amphibian population declines in California, USA. *Environ Toxicol Chem* 20:1591–1595.

39. Macek KJ, Buxton KS, Sauter S, Gnilka S, Dean JW. 1976. Chronic toxicity of atrazine to selected aquatic invertebrates and fishes. EPA 600/3-76-047. U.S. Environmental Protection Agency, Duluth, MN.

40. Jonsson CM, Toledo MCF. 1993. Acute toxicity of endosulfan to the fish *Hyphessobrycon bifasciatus* and *Brachydanio rerio*. *Arch Environ Contam Toxicol* 24:151–155.