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Pesticide Mixtures, Endocrine Disruption, and Amphibian Declines: Are We Underestimating the Impact?

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Amphibian populations are declining globally at an alarming rate. Pesticides are among a number of proposed causes for these declines. Although a sizable database examining effects of pesticides on amphibians exists, the vast majority of these studies focus on toxicological effects (lethality, external malformations, etc.) at relatively high doses (parts per million). Very few studies focus on effects such as endocrine disruption at low concentrations. Further, most studies examine exposures to single chemicals only. The present study examined nine pesticides (four herbicides, two fungicides, and three insecticides) used on cornfields in the midwestern United States. Effects of each pesticide alone (0.1 ppb) or in combination were examined. In addition, we also examined atrazine and *S*-metolachlor combined (0.1 or 10 ppb each) and the commercial formulation Bicep II Magnum, which contains both of these herbicides. These two pesticides were examined in combination because they are persistent throughout the year in the wild. We examined larval growth and development, sex differentiation, and immune function in leopard frogs (*Rana pipiens*). In a follow-up study, we also examined the effects of the nine-compound mixture on plasma corticosterone levels in male African clawed frogs (*Xenopus laevis*). Although some of the pesticides individually inhibited larval growth and development, the pesticide mixtures had much greater effects. Larval growth and development were retarded, but most significantly, pesticide mixtures negated or reversed the typically positive correlation between time to metamorphosis and size at metamorphosis observed in controls: exposed larvae that took longer to metamorphose were smaller than their counterparts that metamorphosed earlier. The nine-pesticide mixture also induced damage to the thymus, resulting in immunosuppression and contraction of flavobacterial meningitis. The study in *X. laevis* revealed that these adverse effects may be due to an increase in plasma levels of the stress hormone corticosterone. Although it cannot be determined whether all the pesticides in the mixture contribute to these adverse effects or whether some pesticides are effectors, some are enhancers, and some are neutral, the present study revealed that estimating ecological risk and the impact of pesticides on amphibians using studies that examine only single pesticides at high concentrations may lead to gross underestimations of the role of pesticides in amphibian declines. **Key words:** amphibian declines, amphibians, atrazine, corticosterone, development, endocrine disruption, growth, immunosuppression, mixtures. *Environ Health Perspect* 114(suppl 1):40–50 (2006). doi:10.1289/ehp.8051 available via <http://dx.doi.org/> [Online 24 January 2006]

Increasing evidence demonstrates that chemical environmental contaminants (including many pesticides) can act as endocrine disruptors in humans and wildlife (Cavieres et al. 2002; Choi and Jeung 2002; Colborn 1994; Diel et al. 2004; Duft et al. 2003; Fenner-Crisp 1997; Harvey et al. 2002, 2004; Hayes 1997b, 1998, 2000b, 2005; Hayes et al. 1997; Hofmeister et al. 2004; Kirby et al. 2004; Kleinkauf et al. 2004; Lutz and Kloas 1999; Masutomi et al. 2004; Michallet-Ferrier et al. 2004; Mueller 2004; Nejaty et al. 2001; Noriega and Hayes 2000; Palmer et al. 1998; Rawlings et al. 1998; Sohoni and Sumpter 1998; Sonnenschein and Soto 1998; Uzumcu et al. 2004). The impact of endocrine-disrupting chemicals in the environment is of special concern in amphibians, which are declining globally (Adams 1999; Alford and Richards 1999; Berger et al. 1998; Bishop et al. 1999; Blaustein and Kiesecker 2002; Blaustein and Wake 1990; Bridges and Semlitsch 2005; Burrowes and Green 2004; Carey et al. 1999;

Daniels 2003; Davidson et al. 2001, 2002; Delis et al. 1996; Green 2003; Hayes 1997a; Kiesecker et al. 2001; LeNoir et al. 1999; Licht and Grant 1997; Lips et al. 2004; Mazzoni et al. 2003; Muths et al. 2003; Renner 2002; Rollins-Smith et al. 2002; Schmidt 2003; Storfer 2003; Stuart et al. 2004; Wang et al. 2004), but the role of pesticides in this decline is not clear (Bishop et al. 1999; Bridges and Semlitsch 2005; Daniels 2003; Davidson et al. 2001, 2002; Gendron et al. 2003; Hayes 1997a, 1998, 2004, 2005; Hayes et al. 1997, 2002a, 2002b, 2002c, 2006; Kiesecker et al. 2001; LeNoir et al. 1999; Pickford and Morris 1999; Withgott 2002). In addition to having highly permeable skin (which makes amphibians particularly vulnerable to chemical contaminants), amphibians also typically reproduce and pass through critical hormone-regulated developmental stages while in the aquatic environment. Performing these important aspects of the life cycle in habitats contaminated with

endocrine-disrupting chemicals may have significant effects on individuals and populations.

Of the known pesticides that act as endocrine disruptors in wildlife, the herbicide atrazine is of special concern because it is a ubiquitous, persistent contaminant of groundwater and surface water that is active at low, ecologically relevant concentrations. As a result, atrazine is also the best-examined endocrine disruptor in amphibians. Atrazine is rarely applied alone in its agricultural use, but rather is used in combination with a number of other pesticides (herbicides, insecticides, and fungicides) that may have their own effects and that may interact in various ways with atrazine.

In the present study, we examined the effects of a realistic pesticide mixture composed of chemicals applied to cornfields in York County, Nebraska. We examined the effects of four herbicides (atrazine, metolachlor, alachlor, and nicosulfuron), three insecticides (cyfluthrin, cyhalothrin, and tebufipirimphos), and two fungicides (metaxyl and propiconazole) alone or in two combinations observed in the wild (Hayes et al. 2002b). We examined size at metamorphosis, time to metamorphosis, and gonadal differentiation in the northern leopard frog (*Rana pipiens*), a species that is exposed to this mixture in the wild. Because of an unexpected increase

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in disease contraction, we examined thymus histology as a measure of immunocompetence in *R. pipiens*. We also examined plasma levels of corticosterone in adult African clawed frogs (*Xenopus laevis*) exposed to this same mixture to explore a possible mechanism underlying the observed immunosuppressive effects.

Materials and Methods

Materials

Atrazine, alachlor, nicosulfuron, cyfluthrin, λ -cyhalothrin, tebufipirimphos, metalaxyl, and propiconazole were purchased from Chem Service, Inc. (Chester, PA) and were \geq 98% pure (except tebufipirimphos, which was 97%). *S*-metolachlor and the commercial atrazine–metolachlor preparation Bicep II Magnum were gifts from Syngenta Crop Protection U.S. (Research Triangle Park, NC; see below for purity). Except where indicated, all other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

Experiment 1: Effects of Pesticides on Larval *Rana pipiens*

Animal care for laboratory studies. Adult *R. pipiens* for breeding were purchased from Charles D. Sullivan Co. Inc. (Nashville, TN). Frogs were reportedly obtained from populations within 200 miles of Boston, Massachusetts (Sullivan C, personal communication). Fertilized eggs were obtained by injecting three males and three females with a gonadotropin-releasing hormone agonist des-Gly¹⁰ [D-His (Bzl)⁶ luteninizing hormone releasing–hormone ethylamide] as described previously (Hayes et al. 1993) and allowing paired animals to breed. Eggs were maintained in 0.1× Holtfreter's (i.e., 10%) solution (Holtfreter 1931), and after hatching, the free-swimming larvae (Gosner stage 21; Gosner 1960) were apportioned into rearing tanks (plastic mouse boxes). Larvae (30 per tank) were reared in 4 L of aerated 0.1× Holtfreter's solution and fed Purina rabbit chow (Purina Mills, St. Louis, MO). Tanks were covered throughout the experiment, and food levels were adjusted as larvae grew to maximize growth. Experiments were conducted at 22–23°C with a 12/12-hr light/dark cycle (lights on at 0600 hr).

Larval laboratory exposures. Larvae were treated by immersion with 0.1 ppb each atrazine, *S*-metolachlor, alachlor, nicosulfuron, cyfluthrin, λ -cyhalothrin, tebufipirimphos, metalaxyl, or propiconazole. These pesticides represented pesticides used on a cornfield in York County, NE (97°22.38 N, 40°55.88 W) (Hayes et al. 2002b) in the years 2000–2001. We also tested a mixture of all nine pesticides (0.1 ppb each or 10 ppb each), which represented the mixture applied to the cornfield in 2000–2001. Further, we examined a

two-compound mixture (atrazine + *S*-metolachlor; tested at 0.1 ppb or 10 ppb each; technical-grade *S*-metolachlor obtained from Syngenta Crop Protection Inc. was reportedly 88% *S*-metolachlor/12% *R*-metolachlor), which represented ecologically relevant concentrations of the two persistent compounds identified in water at the breeding site on 22–23 July 2001 (Hayes et al. 2002b). We also tested the commercial preparation Bicep II Magnum [reported as 33% atrazine, 0.7% atrazine-related products, 26.1% technical-grade *S*-metolachlor (18.57% *S*-metolachlor, 2.5% *R*-metolachlor), 40.2% inert ingredients], a commercial preparation applied to the field twice in 2001. Bicep II Magnum was delivered to tadpoles at two concentrations, one to provide an equivalent of 0.1 ppb atrazine and one to provide 10 ppb atrazine. Comparisons between Bicep II Magnum and the atrazine + *S*-metolachlor mixture were examined to estimate the potential effects of the solvent used in the Bicep II Magnum mixture.

All pesticides were predissolved in ethanol for a final concentration of 0.0036% ethanol and 0.1 ppb of each pesticide. Controls received an equal amount of ethanol only. Each treatment was replicated 3 times (30 larvae per replicate). Cages were cleaned, water changed, and treatments renewed every 3 days. All treatments were systematically relocated every 3 days to ensure that no treatments or tanks experienced position effects. Animals were exposed throughout the larval period from 2 days posthatching until complete tail reabsorption (TR; Gosner stage 46). In all experiments, all dosing and analyses were conducted blindly in color-coded tanks, and animals were number coded when fixed for examination.

Confirmation of pesticide concentrations. Confirmation of nominal concentrations was conducted by the Iowa Hygienics Laboratory (University of Iowa, Iowa City, Iowa) as described previously (Hayes et al. 2002a). Water samples were extracted in organic solvent and subjected to gas chromatography using a nitrogen phosphorus detector. Analysis was provided for alachlor, atrazine, *S*-metolachlor, metalaxyl, and propiconazole. Samples were collected just after making the solution (before tadpoles were added) and shipped frozen in chemical-free glassware (Fisher Scientific Co., Houston, TX). Water samples were color coded, and analyses were conducted blindly. The negative control consisted of Holtfreter's solution only.

General measurements. Upon completion of metamorphosis (TR), each animal was weighed and measured. Animals were euthanized in 0.2% benzocaine (Sigma), assigned a unique identification number, fixed in Bouin's fixative, and preserved in 70% ethanol until further analysis.

Histological analysis of gonads. All analyses were conducted blindly. Gonads and attached kidneys were dissected under a Nikon SMZ10A dissecting scope fitted with a 0.5× lens (Technical Instruments, Burlingame, CA). Tissues were embedded in paraffin, and histological analysis was conducted as described in Hayes (1995b). Briefly, dissected tissues were dehydrated in graded alcohols and infiltrated with Histoclear (National Diagnostics, Atlanta, GA) followed by paraffin. Serial histological sections were cut at 8 μ m using a rotary microtome. Slides were stained in Mallory's trichrome stain and analyzed using a Nikon Optiphot 2 microscope. Digital images were recorded using a Sony DKC-5000 digital camera.

Histological analysis of thymus. After noting that animals exposed to the pesticide mixture experienced increased disease rates due to the pathogen *Chryseobacterium (Flavobacterium) menigosepticum* (see "Results"), we examined the thymus histologically as a measure of immunocompetence. The head was dissected (just anterior to the forelegs). Tissues were embedded in paraffin, and histological analysis was conducted as described for gonads except that sections were cut at 4 μ m. The number of thymic plaques, maximum transverse cross-sectional area, and cell density were determined using Metamorph software (version 2.75; Universal Imaging Ltd, Buckinghamshire, UK). The effects of all single pesticides were examined (20 animals each), as well as effects of 0.1 ppb Bicep II Magnum and the nine-compound mixture. Animals exposed to 0.1 or 10 ppb atrazine + *S*-metolachlor were not examined because this analysis was not planned and tissues from these animals were prepared for other analyses.

Experiment 2: Effects of the Pesticide Mixture on Corticosterone Levels in Adult *Xenopus laevis*

After noting that animals exposed to the nine-compound mixture suffered from thymic damage (increased thymic plaques) and increased disease rates (see "Results"), we examined the effects of the same pesticide mixture on plasma corticosterone levels. We used male *X. laevis* as a surrogate for this experiment because metamorphic *R. pipiens* are too small to obtain repeated blood samples and because *X. laevis* adults are available year-round for such studies.

Adult treatments. Adult males were obtained from a long-term captive colony at University of California, Berkeley. Adults were maintained under the same light and temperature cycles as described for *R. pipiens* larvae, but animals were housed individually in covered tanks. Animals were acclimated in 0.1× Holtfreter's solution for 5 days and then exposed to the pesticide mixture (0.1 ppb each

compound) or an equal amount of ethanol (0.0036%). Five males were treated with the pesticide mixture and five with ethanol only. Holtfreter's solution was not aerated, animals were fed Purina trout chow (Purina Mills) daily, and solutions were changed and treatments renewed every 3 days. Animals were treated for 27 days. Blood was collected by cardiac puncture in nonanesthetized animals between 1800 and 2000 hr. Plasma was collected by aspiration after low-speed centrifugation and stored frozen (-80°C) until analysis.

Radioimmunoassay. For corticosterone analysis, plasma was thawed and extracted with diethyl ether and evaporated under nitrogen. All samples were reconstituted in phosphate-buffered saline with gelatin. Hormone radioimmunoassays were conducted as described previously (Hayes and Licht 1992). Corticosterone antiserum was purchased from Endocrine Sciences (Calabasas, CA) and has been validated for several species including *X. laevis* (Hayes and Licht 1992, 1995; Hayes and Wu 1995; Hayes et al 2002a). Plasma from controls and treated animals was assayed in the same assay at three doses, and the assay was repeated 3 times. Intraassay variation was 1.0%, and interassay variation was 1.4%.

Statistical analyses. Metamorphosis (initiation, total time, and completion), size at metamorphosis [snout-vent length (SVL) and body weight (BW)], and maximum cross-sectional area of the thymus (experiment 1), and hormone levels (experiment 2) were examined using analysis of variance (ANOVA) with replicates (tank) nested within treatment. Statistical groupings were determined using a Tukey post-hoc test. The relationship between time to metamorphosis and size (SVL or BW) at metamorphosis was analyzed by correlational analysis using Spearman's rank order correlation coefficients followed by Bonferroni probability post-hoc tests. The frequency of

thymic plaques, frequency of disease transmission, and mortality were analyzed using a G-test (Sokal and Rohlf 1981) with treatment and tanks as independent variables. Once significant effects were identified, the total G was apportioned into contributions by the individual treatments, first using the results from controls to establish expected frequencies, and then by using the results of the individual chemicals to establish expected frequencies to test if the pesticide mixtures differed from the observed effects of the individual chemicals. In all cases, once statistical significance was obtained using the entire data set, single chemical exposures were compared with ethanol-treated controls without considering the mixtures. All mixtures were then analyzed against ethanol-treated controls. ANOVA and correlational analysis were conducted with the aid of SYSTAT software (version 7; Systat Software Inc., Point Richmond, CA).

Results

Confirmation of pesticide concentrations. The limit of detection for all pesticides analyzed was 0.1 ppb. Alachlor was detected at 0.15 ppb, atrazine at 0.19 ppb, S-metolachlor at 0.22 ppb, metalaxyl at 0.16 ppb, and propiconazole at 0.23 ppb in the nominal 0.1 ppb mixture. No pesticides were detected in the Holtfreter's control (< 0.1 ppb).

Mortality. With the exception of metalaxyl, no single compound affected mortality ($p > 0.05$; see sample sizes in Table 1, which reflect the number of larvae surviving to metamorphosis for each treatment). On average, mortality was 4% for animals exposed to single pesticides (range = 0–7.8%), with the highest mortality (7.8%) in S-metolachlor-treated larvae. Larvae treated with metalaxyl, which experienced 35% mortality before reaching metamorphosis, are not included in this analysis because one replicate suffered high mortality

(90%) all on a single day, most likely as a result of a mechanical failure in the aeration system. Larvae exposed to the atrazine + S-metolachlor mixture or Bicep II Magnum experienced very low mortality ($\leq 10\%$), but only 65% of the larvae exposed to the 0.1-ppb nine-compound mixture survived to the initiation of metamorphosis [foreleg emergence (FLE), Gosner stage 42]. Animals treated with the nine-compound mixture at 10 ppb all died after the first day of exposure.

Time to metamorphosis. Propiconazole significantly delayed time to initiate metamorphosis (FLE; $F = 2.72$, $df = 10$, $p = 0.003$) and time to complete metamorphosis (TR; $F = 2.81$, $df = 10$, $p = 0.002$) relative to controls (Figure 1). Otherwise, no single compound affected this measure of development, and the rest of the analyses focused on the effects of the three mixtures (atrazine + S-metolachlor, Bicep II Magnum, and the nine-compound mixture).

Animals exposed to pesticide mixtures at 0.1 ppb had significantly longer larval periods: initiation of metamorphosis (days to FLE) was delayed ($F = 37.55$, $df = 3$, $p < 0.005$; Figure 2), and TR was similarly delayed ($F = 29.84$, $df = 3$, $p < 0.0001$; Figure 2). Larvae exposed to 10 ppb atrazine + S-metolachlor experienced a slight delay in both FLE ($p = 0.055$) and TR (although not statistically significant, $p > 0.05$). Larvae exposed to the nine-compound mixture experienced an even greater delay ($p < 0.0001$). There was no difference in the interval between FLE and TR,

Table 1. Statistics for correlational analysis of time to complete TR and SVL.

Treatment ^a	n	r	Chi-squared	df	p-Value	Figure ^a
Ethanol	86	+0.23	4.365	1	0.040	5A
Alachlor	86	+0.29	7.123	1	0.008	5B
Atrazine	86	-0.19	3.061	1	0.080	5J
Cyfluthrin	86	+0.24	4.872	1	0.027	5C
λ-Cyhalothrin	84	-0.00	0.001	1	0.980	5I
S-Metolachlor	83	+0.29	7.140	1	0.008	5F
Metalaxyl	58	+0.25	3.210	1	0.073	5G
Nicosulfuron	85	+0.29	6.785	1	0.009	5D
Propiconazole	88	-0.14	1.581	1	0.209	5H
Tebuipirimphos	85	+0.35	10.59	1	0.001	5E
0.1 ppb atrazine + S-metolachlor	89	-0.19	3.018	1	0.082	5K
10 ppb atrazine + S-metolachlor	85	-0.14	1.780	1	0.182	5L
0.1 ppb Bicep II	81	+0.39	11.50	1	0.001	5M
10 ppb Bicep II	84	+0.19	2.732	1	0.098	5N
0.1 ppb mix	59	-0.23	2.100	1	0.147	5O

All treatments were at 0.1 ppb except where indicated for mixtures as described in "Materials and Methods." Bicep II Magnum (Bicep II) was administered to provide 0.1 ppb atrazine. The nine-compound mixture (mix) was administered to provide 0.1 ppb of all nine pesticides. Sample size (n) represents the number of animals surviving to metamorphosis (of the original 90; 30 animals in each of three replicates). ^aIndicates the figure number in this article where data are depicted.

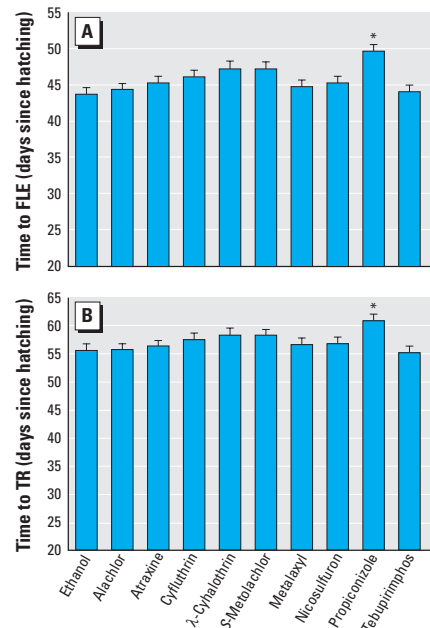


Figure 1. Effect of single pesticides (0.1 ppb each) on time to initiate metamorphosis (FLE; A) and time to complete metamorphosis (TR; B). Error bars show SEM.

*Statistically significant groups (ANOVA, $p < 0.05$).

however, for any treatment ($F = 1.15$, $df = 3$, $p = 0.33$). No position or tank effects were observed in any of the analyses of the effects of mixtures on time to metamorphosis ($p > 0.05$).

Size at metamorphosis. For the individual chemicals, there was a significant effect on SVL at metamorphosis ($F = 2.1$, $df = 10$, $p < 0.05$; Figure 3). The smallest animals to metamorphose were those exposed to cyfluthrin, tebupirimphos, or atrazine. There was also a significant effect on BW ($F = 2.07$, $df = 10$, $p < 0.05$; Figure 3).

All the mixtures (0.1 ppb each pesticide) retarded growth, resulting in smaller animals (SVL) at metamorphosis ($F = 4.03$, $df = 3$, $p = 0.008$; Figure 4). The mixtures also affected BW ($F = 3.86$, $df = 3$, $p = 0.01$; Figure 4), and in this regard, the atrazine + *S*-metolachlor mixture had the greatest negative effect, followed by the nine-compound mixture. No tank or position effects were observed ($p > 0.05$).

Relationship between time to complete metamorphosis and size at metamorphosis. SVL at metamorphosis was positively correlated with time to complete metamorphosis for controls (Figure 5). Regarding single chemical exposures, all the treatments showed a similar positive significant relationship ($p < 0.05$; Table 1) with the following exceptions: larvae exposed to metalaxyl showed a positive but nonsignificant relationship between time to complete

metamorphosis (TR) and SVL, whereas larvae exposed to atrazine, cyhalothrin or propiconazole showed a negative but nonsignificant relationship between TR and SVL (Figure 5).

For the pesticide mixtures, 0.1 and 10 ppb atrazine + *S*-metolachlor resulted in a negative but nonsignificant relationship between TR and SVL, whereas 0.1 and 10 ppb Bicep II Magnum exposure resulted in maintenance of the positive relationship between TR and SVL, but the relationship was significant for the 0.1 ppb concentration only (Figure 5). The nine-compound pesticide mixture resulted in a negative but nonsignificant relationship (Table 1, Figure 5).

For BW, controls also showed a significant positive correlation between time to complete metamorphosis and BW. All single-treatment exposures showed a similar positive significant relationship ($p < 0.002$ in all cases; Table 2, Figure 6) except nicosulfuron, atrazine, and cyhalothrin, which showed a positive but nonsignificant relationship between TR and BW at metamorphosis. Regarding pesticide mixtures, a positive relationship was observed with Bicep II Magnum but was significant only for the 0.1 ppb concentration. The relationship was negative but nonsignificant for both 0.1 and 10 ppb atrazine + *S*-metolachlor and negative and significant for the nine-pesticide mixture (Table 2, Figure 6).

Gonadal development. In the population used in the present study, gonadal development was delayed (even in controls) relative to

other populations of *R. pipiens* that have been examined by our laboratory (Hayes et al. 2002a, 2002c) or documented in other published accounts (Merchant-Larios and Villapando 1981; Richards and Nace 1978). Histologically, presumptive males maintained both a cortex and a medulla separated by connective tissue without clear formation of testicular lobules (e.g., undifferentiated), whereas females showed regression of the gonadal medulla and an ovarian vesicle but lacked significant numbers of developing oocytes in the cortical regions of the gonad (Figure 7). Because of the underdeveloped state of the gonads and gametes in this population, assessing the effects of atrazine or pesticide mixtures containing atrazine on sex differentiation of the gonads and gametogenesis (e.g., whether testicular oogenesis occurred) was not possible.

Flavobacterial response. Seventy percent of the animals exposed to the nine-compound mixture were unable to sit upright. Exposure to the nine-compound pesticide mixture was associated with meningitis, otitis interna, and septicemia due to the gram-negative, water-borne bacteria *Chryseobacterium (Flavobacterium) meningosepticum*. Diagnosis was based on signalment, clinical signs, necropsy results, histopathologic examination of internal organs with special staining in select cases, and the antemortem isolation of organism from the internal lesions of affected animals. Manifestations of disease included anorexia, head tilt, circling, loss of righting response, anisocoria, and death

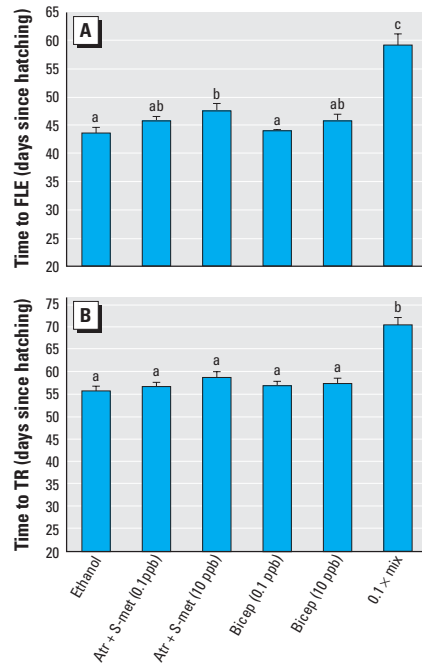


Figure 2. Effect of pesticide mixtures on time to initiate metamorphosis (FLE; A) and time to complete metamorphosis (TR; B). Abbreviations: Atr, atrazine; Bicep, Bicep II Magnum; S-Met, *S*-metolachlor; Mix, nine-chemical mixture (0.1 ppb each pesticide). Letters above bars indicate statistical groupings. Error bars show SEM.

*Statistically significant groups (ANOVA, $p < 0.05$).

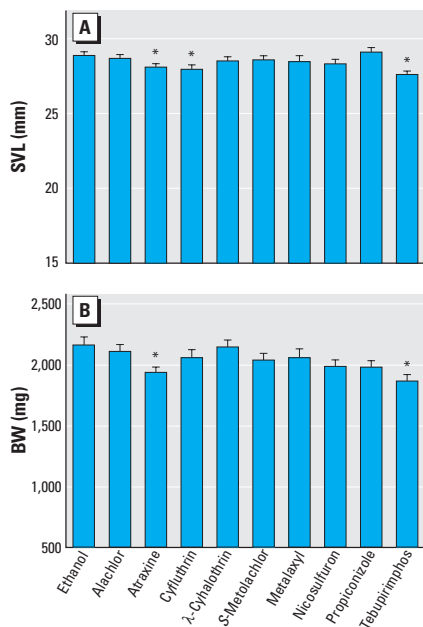


Figure 3. Effect of single pesticides (0.1 ppb each) on SVL (A) and BW (B). Error bars show SEM.

*Statistically significant groups (ANOVA, $p < 0.05$).

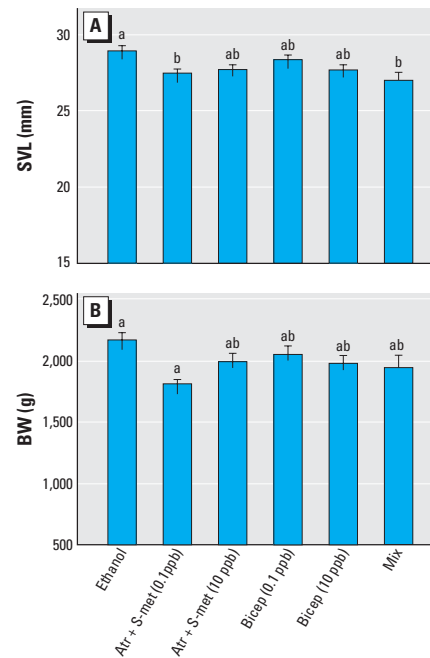


Figure 4. Effect of pesticide mixtures on SVL (A) and BW (B). Abbreviations: Atr, atrazine; Bicep, Bicep II Magnum; S-met, *S*-metolachlor; Mix, nine-chemical mixture (0.1 ppb each pesticide). Letters above bars show statistical groupings. Error bars show SEM.

(Figure 8). Morbidity and mortality rates in animals treated with the nine-pesticide mixture were significant ($G = 100.12$, $df = 4$, $p < 0.001$) compared with those in controls or the other mixtures (all of which showed a 0% incidence) and reached 70% of the 59 animals that survived to complete metamorphosis in animals exposed to the pesticide mixture. *C. (Flavobacterium) meningosepticum* was successfully cultured from animals from all groups (including controls), but no animals from other treatments contracted the disease or suffered the symptoms described above.

Thymus characteristics. After noting that animals exposed to the nine-compound mixture contracted flavobacterial meningitis (see above “Flavobacterial response”), we examined the condition of the thymus as an estimate of immune function. Exposure to atrazine and *S*-metolachlor resulted in damage to the thymus as measured by thymic plaques. No other single pesticide produced this effect. The frequency of animals with plaques increased in animals treated with the mixtures (Bicep II Magnum), followed by larvae treated with the nine-compound pesticide mixture ($G = 9.4$, $df = 4$, $p < 0.05$; Figure 9). Larvae treated with atrazine + *S*-metolachlor (0.1 or 10 ppb) were not examined because this analysis was not planned, and these samples were preserved for another analysis (data not shown).

Effects of the pesticide mixture on plasma corticosterone. The nine-pesticide mixture had a clear effect on corticosterone levels in male African clawed frogs (*X. laevis*). Corticosterone levels increased 4-fold in pesticide-exposed males (ANOVA, $p < 0.05$; Figure 10).

Discussion

Although a sizable database examining the toxicological effects of pesticides on amphibians exists (Pauli 2004), most of these studies examine acute toxicity, morbidity, and mortality only. Few studies have examined low-concentration effects (especially endocrine disruption) or the effects of pesticide mixtures. In reality, amphibians in the wild (especially in agricultural areas) are exposed to mixtures of pesticides. Further, although brief episodes of high-concentration exposure may occur, prolonged exposures to low concentrations of pesticide mixtures are more common (Battaglin and Goolsby 1999; Burkhart et al. 2003; Capel and Larson 2001; Fischer et al. 1995; Frank and Logan 1988; Frank and Sirons 1979; Frank et al. 1987, 1991; Insensee et al. 1990; Kolpin et al. 1998; Kucklick and Bidleman 1994a, 1994b; Pennington et al. 2001; Solomon et al. 1996; Thurman et al. 1992). Only a few studies have examined the effects of pesticide mixtures on amphibians (Christin et al. 2003; Dawson and Wilke 1991; Gendron et al. 2003; Howe et al. 1998; Mazanti 1999;

Mazanti et al. 2003; Relyea 2004), but chemicals were examined at much higher concentrations than those examined here, only toxicity was examined, and the chemical mixture in the present analysis was not examined. Further, fewer than 20 published laboratory studies (Boegi et al. 2002; Hayes 1997a, 1998, 2000, 2004, 2005; Hayes et al. 1997, 2002a, 2002b, 2002c; Lutz and Kloas 1999; Noriega and Hayes 2000; Palmer and Palmer

1995; Palmer et al. 1998; Tavera-Mendoza et al. 2002a, 2002b) and four field studies (du Preez et al. 2005; Hayes et al. 2002b, 2002c; Reeder et al. 1998) have addressed low-concentration, endocrine-disrupting effects of pesticides (single compounds only) on amphibians. A few studies have examined amphibians in the wild (Harris et al. 1998a, 1998b; Ouellet et al. 1997; Sparling et al. 2001), although establishing cause and effect

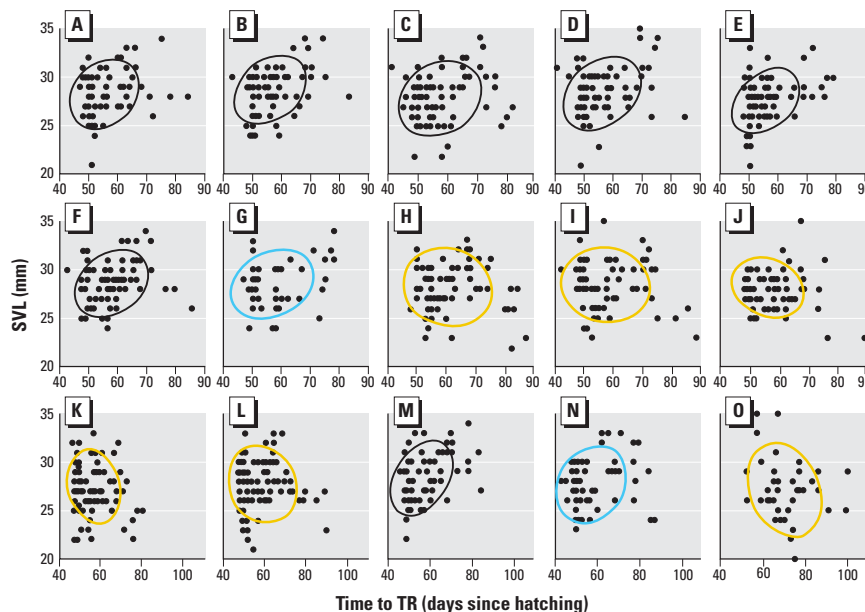


Figure 5. Correlational analysis of time to complete TR and SVL. Results are shown for single pesticides (0.1 ppb) in A–J and for mixtures (K–O). For the x-axis, the scales for A–J are identical, but note the difference in scale for the mixtures as a result of the delay in metamorphosis in the animals treated with the nine-compound mixture (O). Results are shown for ethanol (A), alachlor (B), cyfluthrin (C), nicosulfuron (D), tebuiripimphos (E), *S*-metolachlor (F), metalaxyl (G), propiconazole (H), λ -cyhalothrin (I), atrazine (J), 0.1 ppb atrazine + *S*-metolachlor (K), 10 ppb atrazine + *S*-metolachlor (L), 0.1 ppb Bicep II Magnum (M), 10 ppb Bicep II Magnum (N), and the nine-compound mixture (O). Ellipses show Gaussian bivariate confidence limits. Ellipses are color-coded: black, a positive and significant correlation; blue, a positive but nonsignificant correlation; yellow, a negative but nonsignificant correlation. Significance ($p \leq 0.05$) was determined by Bonferroni probabilities and based on Spearman rank order correlation coefficients. Sample size (as reported in Table 1) cannot be determined from the number of data points due to overlapping data.

Table 2. Statistics for correlational analysis of time to complete TR and BW.

Treatment	<i>n</i>	<i>r</i>	Chi-squared	<i>df</i>	<i>p</i> -Value	Figure ^a
Ethanol	86	+0.41	14.61	1	0.000	6A
Alachlor	86	+0.46	18.92	1	0.000	6B
Atrazine	86	+0.07	3.061	1	0.080	6J
Cyfluthrin	86	+0.34	10.03	1	0.002	6C
λ -Cyhalothrin	84	+0.13	1.376	1	0.241	6I
<i>S</i> -Metolachlor	83	+0.47	20.15	1	0.000	6F
Metalaxyl	58	+0.40	9.038	1	0.003	6G
Nicosulfuron	85	+0.49	20.99	1	0.000	6D
Propiconazole	88	+0.20	3.130	1	0.077	6H
Tebuiprimphos	85	+0.58	32.77	1	0.000	6E
0.1 ppb atrazine + <i>S</i> -metolachlor	89	-0.05	0.251	1	0.616	6K
10 ppb atrazine + <i>S</i> -metolachlor	85	-0.01	0.000	1	0.960	6L
0.1 ppb Bicep	81	+0.51	20.69	1	0.000	6M
10 ppb Bicep	84	+0.33	8.143	1	0.004	6N
0.1 ppb mix	59	-0.32	4.164	1	0.041	6O

All treatments were at 0.1 ppb except where indicated for mixtures as described in “Materials and Methods.” Bicep II Magnum (Bicep) was administered to provide 0.1 ppb atrazine. The nine-compound mixture (mix) was administered to provide 0.1 ppb of all nine pesticides. Sample size (*n*) represents the number of animals surviving to metamorphosis (out of the original 90; 30 animals in each of three replicates). ^aIndicates the figure number in this article where data are depicted.

in such studies is difficult. The present study is the first to address endocrine-disrupting effects of low-concentration pesticide mixtures on amphibians in the laboratory.

We demonstrated that a realistic pesticide mixture (based on a mixture applied to an actual field) at low ecologically relevant concentrations can have dramatic effects on amphibian development and growth, and ultimately (we predict) survivorship. We propose here that the lack of

examinations of endocrine-disrupting effects of low concentrations of pesticides in amphibians has resulted in underestimates of the impacts of pesticides on wildlife (Hayes et al. 2002a), as similarly suggested by Burkhart et al. (2003). The absence of studies that examine low-concentration effects of pesticide mixtures makes this underestimation even more severe.

Most of the nine pesticides used in the present study have not been examined in

amphibians at all: no published studies have addressed the effects of cyfluthrin, cyhalothrin, tebufipirimphos, metalaxyl, or propiconazole on amphibians. Here, we show that one of these compounds (propiconazole) retards larval development and delays metamorphosis, and two others (tebufipirimphos and cyfluthrin) retard larval growth. In addition to these new data, the present study confirms the retardation of amphibian development (Carr et al. 2003; Rohr and Palmer 2005; Rohr et al. 2004) and growth (Boone and James 2003; Britson and Threlkeld 1998; Carr et al. 2003; Diana et al. 2000) already reported for atrazine. Carr et al. (2003) also showed a reversal of the relationship between time to metamorphosis and size at metamorphosis in their studies, consistent with present results.

The present study is also important because all the pesticides were examined at low ecologically relevant concentrations (0.1 ppb). The few studies that have previously examined the effects of nicosulfuron (Fort et al. 1999), metolachlor (Mazanti 1999; Mazanti et al. 2003; Osano et al. 2002), and alachlor (Howe et al. 1998; Osano et al. 2002) on amphibians have examined concentrations 10,000 times higher than those used in the present study. All but four previous studies (Howe et al. 1998; Mazanti 1999; Mazanti et al. 2003; Relyea 2004) examined single pesticide exposures, and most examined mortality and teratogenesis, with only two studies addressing effects of these pesticide mixtures on larval growth and development (Howe et al. 2004; Relyea 2004). Again, all these previous studies were conducted at concentrations 10,000 times higher than those used in the present study. The exception is atrazine, for which sublethal developmental effects at low concentrations (in the parts per billion range) have been examined by multiple laboratories (Allran and Karasov 2001; Boone and James 2003; Britson and Threlkeld 1998; Carr et al. 2003; Coady et al. 2004, 2005; Diana et al. 2000; du Preez et al. 2005; Goulet and Hontela 2003; Gross et al. 2003; Hayes 2004, 2005; Hayes et al. 2002a, 2002b, 2002c, 2006; Hecker et al. 2004; Howe et al. 1998; Jooste et al. 2005a, 2005b; Miyahara et al. 2003; Reeder et al. 1998; Rohr and Palmer 2005; Rohr et al. 2004; Sullivan and Spence 2003; Tavera-Mendoza et al. 2002a, 2002b).

Atrazine has a number of well-documented adverse effects on amphibian larvae. It is a potent endocrine disruptor that both chemically castrates and feminizes exposed male amphibian larvae and also retards larval development and growth (Carr et al. 2003; du Preez et al. 2005; Hayes 2004, 2005; Hayes et al. 2002a, 2002b, 2002c, 2006; Reeder et al. 1998; Tavera-Mendoza et al. 2002a, 2002b). It also induces edema (Carr et al. 2003), erratic swimming (Carr et al. 2003), and irregular

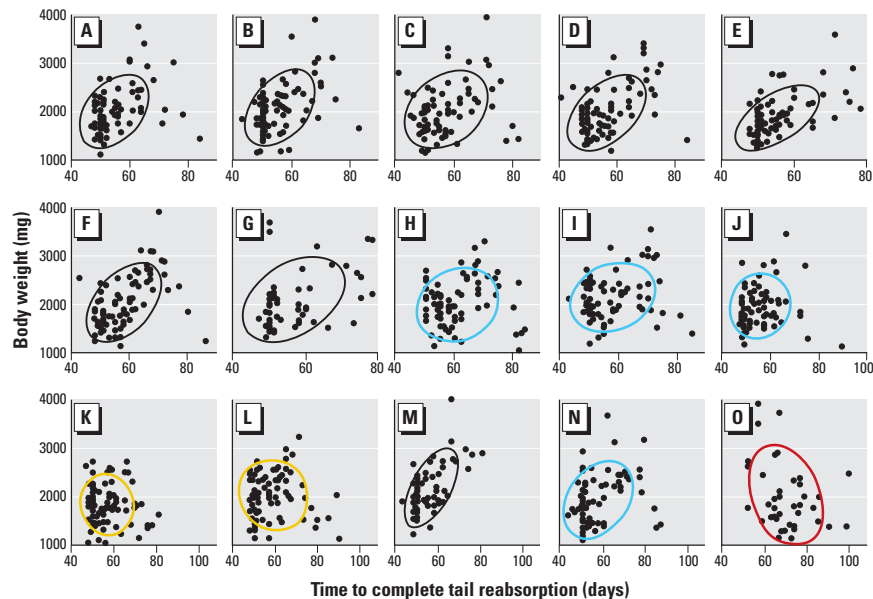


Figure 6. Correlational analysis of time to complete TR and BW. Results are shown for single pesticides (0.1 ppb) in A–J and for mixtures (K–O). For the x-axis, the scales for the top two rows are identical, but note the difference in scale for the mixtures as a result of the delay in metamorphosis in the animals treated with the nine-compound mixture (O). Results are shown for ethanol (A), alachlor (B), cyfluthrin (C), nicosulfuron (D), tebufipirimphos (E), *S*-metolachlor (F), metalaxyl (G), propiconazole (H), λ -cyhalothrin (I), and atrazine (J), 0.1 ppb atrazine + *S*-metolachlor (K), 10 ppb atrazine + *S*-metolachlor (L), 0.1 ppb Bicep II Magnum (M), 10 ppb Bicep II Magnum (N), and the nine-compound mixture (O). Ellipses show Gaussian bivariate confidence limits. Ellipses are color-coded: black, a positive and significant correlation; blue, a positive but nonsignificant correlation; yellow, a negative but nonsignificant correlation; red, a negative and significant correlation. Significance ($p \leq 0.05$) was determined by Bonferroni probabilities and based on Spearman rank order correlation coefficients. Sample size (as reported in Table 2) cannot be determined from the number of data points because of overlapping data.

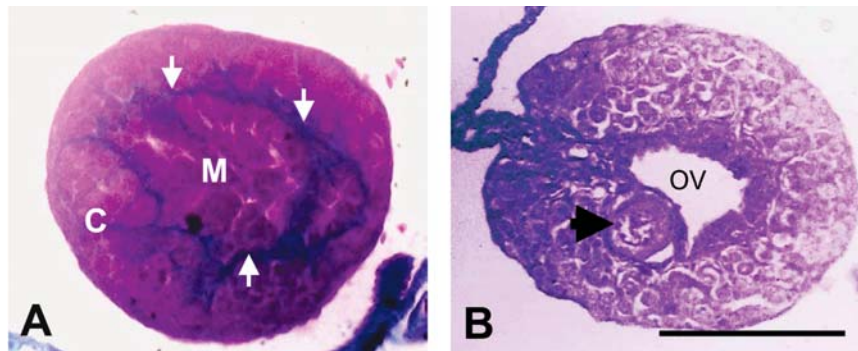


Figure 7. Histological transverse cross-section (8 μ m) of presumptive male (A) and female (B) leopard frog (*R. pipiens*) at metamorphosis (Gosner stage 46). Gonads are not completely differentiated. Note the intact cortex (C) and medulla (M) separated by blue connective tissue (arrows in A). Also note medullary regression and ovarian vesicle (OV) but absence of significant oogenesis in the female (B). A single oocyte (arrow) is noted in the female. Scale bar = 125 μ m.

behavioral activity (Rohr and Palmer 2005) and is an immunosuppressant (Christin et al. 2003; Gendron et al. 2003; Kiesecker 2002) in amphibians. The impact of atrazine on amphibian larvae is important both because of the number of documented adverse effects in amphibians and also because atrazine is a ubiquitous, persistent environmental contaminant (Hayes et al. 2006; Solomon et al. 1996): As one of the world's most commonly applied pesticides, it is the most common contaminant of groundwater and surface water (Hayes et al. 2006). Up to 0.5 million pounds per year are deposited in precipitation in the United States (Miller et al. 2000; Nations and Hallberg 1992; Thurman and Cromwell 2000; Van Dijk and Guicherit 1999), and contamination can spread more than 600 miles from the point of application (Miller et al. 2000; Müller et al. 1997; Thurman and Cromwell 2000).

The present study demonstrates that one of the best-documented effects of atrazine, demasculinization and feminization of male larvae, can vary across populations. In the present population, effects of atrazine on the gonads were not detectable because individuals from the present population do not complete sexual differentiation of the gonads before metamorphosis. In ranids, atrazine induces testicular oogenesis (Hayes et al. 2002b, 2002c), but in the population used in the present study, male gonads were not well differentiated (testicular lobules were not yet developed), and even females lacked significant numbers of oocytes. Although other differences in study

design exist (Hayes 2004), this variation in susceptibility may explain some of the disparate findings in the published literature regarding the effects of atrazine on gonadal development in amphibians (Coady et al. 2004, 2005; du Preez et al. 2005; Hayes 2005; Hecker et al. 2004, 2005; Jooste et al. 2005a, 2005b). This finding highlights the importance of understanding population variation when assessing the risk of pesticides to amphibians.

As noted above, retardation of growth and development has been demonstrated for atrazine in previous studies. In the present study, retardation of growth and development was more severe when atrazine was combined with other pesticides (e.g., *S*-metolachlor), and the nine-pesticide mixture had the most severe impact. The delay in time to initiate and complete metamorphosis was significant. Many amphibians (including leopard frogs) often breed in temporary water sources. In particular, in agricultural areas, where water is manipulated for agricultural purposes, aquatic habitats can be unpredictable from year to year and even day to day (Figure 11). In these habitats, it is important for survivorship that larvae respond to desiccation by metamorphosing rapidly (Denver 1993; Denver et al. 1998). It has already been shown that atrazine alone retards development and prevents the acceleration of metamorphosis induced by pond drying as well as reduces size at metamorphosis (Rohr et al. 2004). As demonstrated by the present study, developing in water sources contaminated with pesticide mixtures (even

simply 0.1 ppb atrazine + *S*-metolachlor) will decrease survivorship because these mixtures delay metamorphosis. Further, as water sources desiccate, pesticide concentrations will increase. Even if larvae metamorphose and escape desiccation, delayed metamorphosis along with decreased size at metamorphosis reduces adult recruitment and the likelihood of reproduction in amphibians (Smith 1987).

Retardation of growth is also detrimental. Smaller size at metamorphosis limits food availability for newly metamorphosed frogs, which are gape-limited predators (Figure 12A). Further, smaller individuals are more susceptible to predators, which may also themselves be gape-limited predators (e.g., snakes; Figure 12B) (De Vito et al. 1999; Kiesecker and Blaustein 1998; Lardner 1998; Lawler et al. 1999; Niecieza 2000; O'Dwyer et al. 2000; Puttlitz et al. 1999; Relyea 2001a, 2001b, 2003; Skelly 1994; Werner 1986; Wilbur and Collins 1973). Because pesticide mixtures retard growth and size at metamorphosis, exposed amphibians are less likely to find food and more likely to be preyed upon. Also, decreased size at metamorphosis combined with subsequent decreased postmetamorphic growth

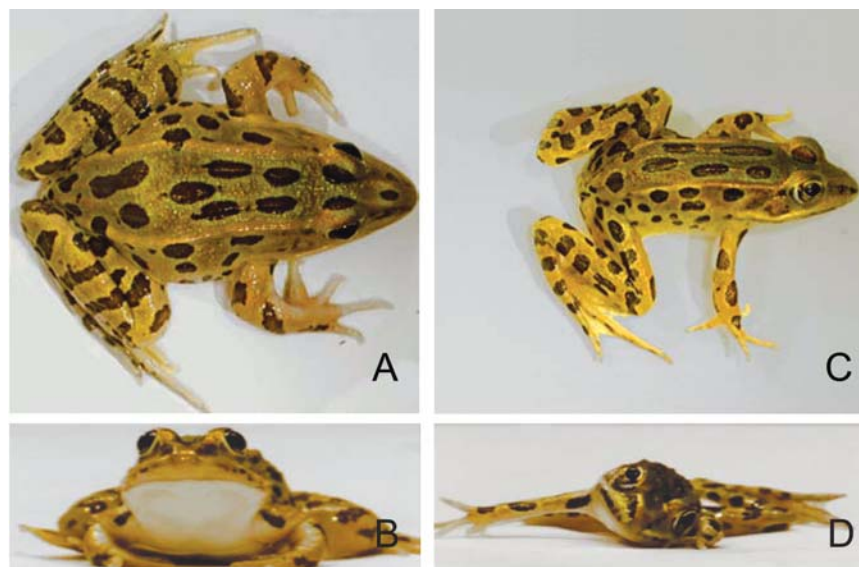


Figure 8. Dorsal (A,C) and frontal (B,D) view of newly metamorphosed (Gosner stage 46) control (A,B) *R. pipiens* and similar-aged animal exposed to the nine-pesticide mixture (C,D). Control animal is in good body condition as expected. The pesticide-treated animal is in poor body condition because of a generalized gram-negative bacterial infection. The pathogen was identified in control and treated frogs, but only pesticide-exposed animals show signs of disease: head tilt, unilateral extensor muscle rigidity, anisocoria, and intermittent recumbency due to a severe otitis interna and meningitis. This presentation is consistent with *C. meningosepticum* infection, a stress-induced disease of frogs.

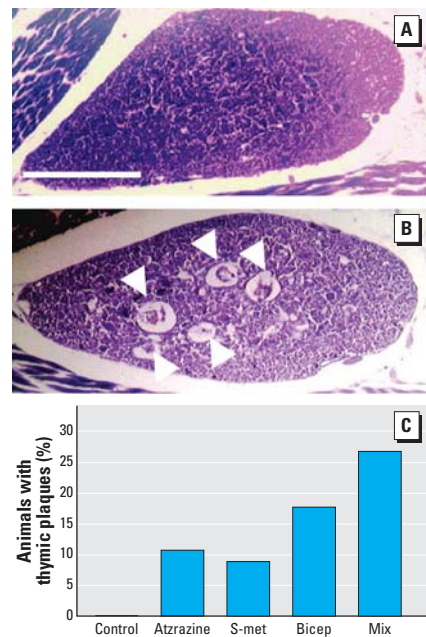


Figure 9. Representative transverse cross-section through the thymus of a control animal (A) and (B) an animal treated with the nine-compound pesticide mixture. (C) The frequency of animals with detectable damage to the thymus. No control animals showed damage to the thymus. Animals exposed to 0.1 ppb atrazine or to *S*-metolachlor (*S*-met) had damage as shown in B, with increasing frequencies of damage with exposure to Bicep II Magnum (atrazine + metolachlor, given to concentration of 0.1 ppb atrazine), and maximum damage with exposure to the pesticide mixture (Mix). Histological sections were 4 μ m stained in Mallory's trichrome stain. Scale bar = 0.5 mm for A and B.

decreases the chances that amphibians will survive overwintering (Berven and Gill 1983; Smith 1987). Reduced size at metamorphosis also delays reproductive maturity and decreases fecundity (Berven and Gill 1983; Smith 1987; Wilbur et al. 1973). This negative effect is especially true for females, for which size is directly proportional to fecundity (Howard 1981; Shine 1979, 1989), but this is true for males as well. In many species, females prefer larger males as mates, and increased size may be necessary for maintaining territories and fending off rival male suitors during copulation (Balinsky and Balinsky 1954; Howard 1981; Shine 1979, 1989).

The alteration of the relationship between time to metamorphosis and size at metamorphosis is even more significant than either measured alone. In amphibians, the larval stage is a period of growth. As nonamniotes, the size of hatching amphibians is limited. The larval stage, during which time amphibians are typically herbivorous, provides a period of growth that allows individuals to both become large enough to be effective predators and to escape predation. As shown in the present study, there is a positive relationship between time to metamorphosis and size at metamorphosis: among controls, larvae that take longer to metamorphose are larger. However, with exposure to pesticide mixtures, larvae take longer to metamorphose but do not obtain a size advantage and, in fact, are smaller at metamorphosis than larvae that metamorphose earlier. Interestingly, at least three of the single pesticides tested (propiconazole, λ -cyhalothrin, and atrazine) and potentially metalaxyl had a slight effect on the relationship between time to metamorphosis and size at metamorphosis. Potentially, only these three (or four) pesticides in the mixture produce the additive effects observed with the nine-compound mixture, with no contribution from the other pesticides. Alternatively, pesticides that produce no effects alone may act as “enhancers” that worsen the effects of pesticides that act as “effectors” when the two groups of chemicals are combined.

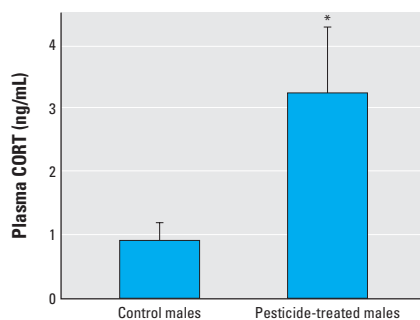


Figure 10. Effect of the pesticide mixture on plasma corticosterone levels in adult male African clawed frogs (*X. laevis*). Error bars show SEM. *Statistical significance ($p < 0.05$).

The present effects of mixtures cannot be assigned to the categories of concentration additive or response additive, as described in Burkhart et al. (2003), and further, the roles of each of the individual pesticides in the effects of the mixture cannot be identified. Although the relative roles of all the individual

pesticides in the mixture cannot be discerned from the present study, *S*-metolachlor does appear to be an effector. In the present study and others cited above, atrazine retarded larval development and growth. Although *S*-metolachlor had no effect on its own in the present study, the negative effects of atrazine were

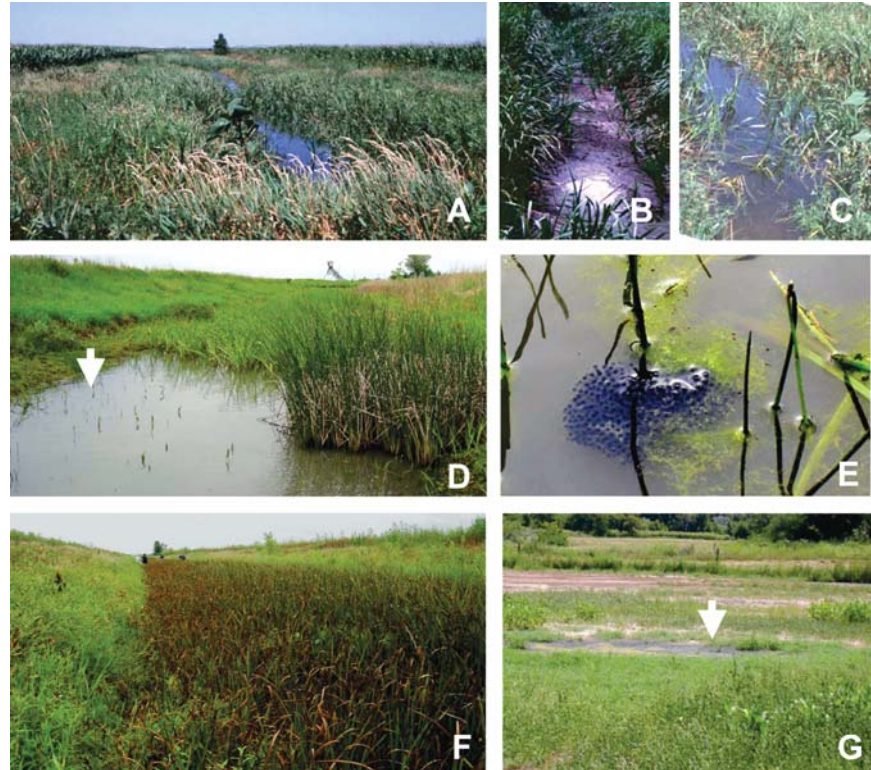


Figure 11. Field 413 (40°55.88 N, 97°22.38 W), York County, Nebraska (A–F). In 2001 frogs bred in the irrigation ditch (A) and were present by the thousands. Even at this time, the water level (and thus temperature and pesticide levels) fluctuated drastically from one day to the next: the photo in B was taken on 22 July 2001 and photos in A and C were taken on 23 July 2001. (B) All the water has evaporated and only the small pool (white glare) remains. In 2004 seven pairs of frogs bred at the same site when the irrigation ditch filled with rainwater and snowmelt (D; arrow indicates where frog clutches were found). (E) One of the seven clutches. (F) The fields around the irrigation ditch were not planted in 2003 or 2004, and the ditch dried up, resulting in 100% failure of the population at this site for the second year since initial collection. (G) A breeding pond (arrow) in Hitchcock County, Nebraska (40°08.433 N, 101°13.804 W) along the Republican River. All the tadpoles died of desiccation at this site, further illustrating the interaction between pesticides that slow development and delay metamorphosis and the impact of agriculture (and irrigation practices) on amphibian populations.

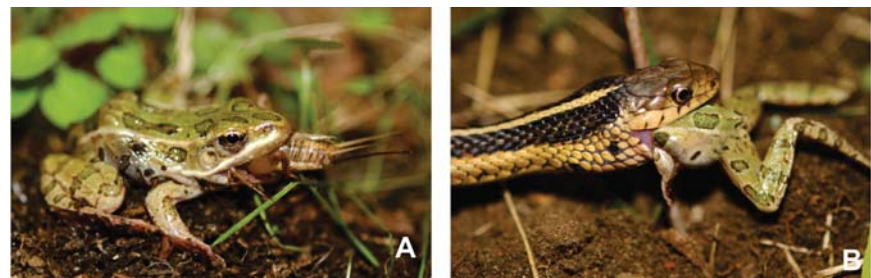


Figure 12. Demonstration of the effects of decreased size at metamorphosis on amphibians. (A) Newly metamorphosed leopard frog (*R. pipiens*) attempting to ingest a cricket that is too large (frog is 3.3 cm SVL; cricket is 2.1 cm long). (B) A 67-cm-long garter snake (*Thamnophis sirtalis*) feeding on a newly metamorphosed 3.2-cm-long leopard frog (*R. pipiens*). Frogs are gape-limited predators and less likely to find appropriate food when size at metamorphosis is reduced by pesticides. Similarly, garter snakes (like all snakes) are gape-limited predators, so smaller frogs are more vulnerable to snake predation.

increased when combined with *S*-metolachlor. Interestingly, the commercial mixture (Bicep II Magnum) appeared more benign than the pure mixture of atrazine and *S*-metolachlor. Bicep II Magnum at 0.1 ppb resulted in a positive relationship between time to metamorphosis and size at metamorphosis. There was a concentration effect, however: 10 ppb Bicep II Magnum eliminated the positive relationship. The atrazine + *S*-metolachlor mixture and Bicep II Magnum differ primarily in the surfactant (inert ingredients) (although the concentration of *S*-metolachlor was 22% lower in the Bicep II Magnum mixture than in the atrazine + *S*-metolachlor mixture). These data suggest that the surfactant used in this mixture reduces the effect of the two pesticides. Previous studies have shown that surfactant used in pesticides can have biological activity in amphibian larvae (Relyea 2005).

In addition to the adverse effects on the relationship between time to metamorphosis and size at metamorphosis, the pesticide mixture unexpectedly increased disease rates. Based on the observation that controls tested positive for the flavobacteria but did not display symptoms of disease, the pathogen was otherwise nonvirulent under the present experimental conditions. The increased disease rates were associated with an increased frequency of animals with damage to the thymus. Of all the pesticides tested alone, only atrazine and *S*-metolachlor increased the frequency of damage to the thymus. Further, atrazine has been shown to increase disease rates and parasite loads in amphibians by several pathogens (Christin et al. 2003; Gendron et al. 2003), including the trematode associated with development of limb deformities (Kiesecker 2002). In the present study, atrazine and *S*-metolachlor combined (Bicep II Magnum) increased the frequency of animals with thymic plaques relative to either herbicide alone, but disease rates were not increased unless animals were exposed to the nine-pesticide mixture.

Regarding to adverse effects that contribute to amphibian declines, the effects of atrazine on sex differentiation can negatively affect amphibian populations. The effects of the pesticide mixture on growth can have an even more rapid negative effect on populations, as described above. The immunosuppressive effects are likely even more relevant. Most significantly, the nine-pesticide mixture increased plasma corticosterone levels. Corticosterone can produce all the effects observed with the pesticide mixtures, including retarded growth (Hayes 1995a, 1995b; Hayes and Wu 1995; Hayes et al. 1993, 1997), retarded development (Glennemeier et al. 2002a, 2002b; Hayes 1995a, 1995b, 1997b; Hayes and Wu 1995; Hayes et al. 1993, 1997), and immunosuppression (Belden and Kiesecker 2005; Hayes 1995b). Given these adverse effects and the

continued increase and use of pesticides in agriculture over the last 50 years, it is likely that pesticides have played and will continue to play a role in amphibian declines. In particular, the effects described here are very important. Pesticide-induced declines in populations as a result of decreased prey availability and increased susceptibility to predators (as a result of decreased size and the negation or reversal of the relationship between time to metamorphosis and size at metamorphosis) may be difficult to discern in the wild. Perhaps more important, emergent diseases caused by agents such as ranavirus (Brunner et al. 2005; Green and Muths 2005; Jancovich et al. 2005; Pearman et al. 2004) and chytrid (Berger et al. 1998; Green and Muths 2005; McCallum 2005; Ouellet et al. 2005; Rollins-Smith et al. 2002; Weldon et al. 2004) are considered major contributors to amphibian declines. Given the present findings with the flavobacteria in the present study, perhaps these diseases are not emergent at all. As suggested by Burkhart et al. (2003), perhaps what is emergent is the inability to mount proper immune responses as a result of pesticide exposure. As Sparling et al. (2003) pointed out, "Unfortunately, almost all research on amphibian population declines has focused on single factors or multiple factors considered individually with little consideration for interactions." This approach has to change if problems are to be identified and solutions formulated.

Regarding pesticides, the present study demonstrates that examinations of effects of single pesticides are inadequate to assess adverse impacts on amphibian development or to address the role of pesticides in amphibian declines. The examinations needed to characterize pesticide interactions as concentration additive or response additive (Burkhart et al. 2003), to distinguish between effectors and enhancers, and to examine multiple combinations of pesticides at multiple concentrations are difficult to design and carry out and present new challenges to regulators. Such studies are necessary, however, to determine if the net effects of pesticide mixtures are truly due to the sum of the parts or simply some of the parts.

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