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Are we underestimating the impact?

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Running Title: PESTICIDE MIXTURES AND AMPHIBIANS

Key Words: amphibians, atrazine, mixtures, amphibian declines, endocrine disruption, growth, development, immunosuppression, corticosterone

Abbreviations:
a.m. = antemeridian
ANOVA = analysis of variance
Atr = atrazine
C = celcius
CA = California
Co = company
e.g. = exemplia gratia
GnRH = gonadotropin releasing hormone
Inc = incorporated
IO = Iowa
L = liter
MA = Massachusetts
MO = Missouri
NC = North Carolina
NE = Nebraska
PA = Pennsylvania
PBS-g = phosphate-buffered saline with gelatin
ppb = parts per billion
ppm = parts per million
RIA = radioimmunoassay
R. pipiens = Rana pipiens
S-met = S-metolachlor
St. = Saint
TN = Tenessee
TX = Texas
X. laevis = Xenopus laevis
μ = micron

Section Headers

Abstract

Amphibian populations are declining globally at an alarming rate. Pesticides are among a number of proposed causes for these declines. Though a sizable data-base 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 (ppm). Very few studies focus on effects such as endocrine disruption at low concentrations. Further, the majority of studies examine exposures to single chemicals only. The current study examined nine pesticides (four herbicides, two fungicides, and three insecticides) used on cornfields in the mid-western US. Effects of each pesticide alone (0.1 ppb) or in combination were examined. In addition, we examined atrazine and S-metolachlor (0.1 or 10 ppb each) or 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*).

Though some of the pesticides 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 flavo-bacterial

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. Though it cannot be determined whether all of the pesticides in the mixture contribute to these adverse effects or whether some pesticides are “effectors”, some are “enhancers”, and some are “neutral”, the current study revealed that estimating ecological risk and the impact of pesticides on amphibians using studies that examine single pesticides at high concentrations, only, may lead to gross underestimations of the role of pesticides in amphibian declines.

Introduction

Increasing evidence demonstrates that chemical environmental contaminants (including many pesticides) can act as endocrine disruptors in humans and wildlife (Cavieres et al. 2002, Choi et al. 2002, Colborn 1994, Diel et al. 2004, Duft et al. 2003, Fenner-Crisp 1997, Harvey et al. 2004, Harvey et al. 2002, Hayes 1997b, 1998, 2000, 2005a, Hayes et al., 1997, Noriega and Hayes, 2000, Hofmeister et al. 2004, Kirby et al. 2004, Kleinkauf et al. 2004, Lutz et al. 1999, Masutomi et al. 2004, Michallet-Ferrier et al. 2004, Mueller 2004, Nejaty et al. 2001, Palmer et al. 1998, Rawlings et al. 1998, Sohoni et al. 1998, Sonnenschein et al. 1998, Uzumcu et al. 2004, and references in this issue). The impact of endocrine-disrupting chemicals in the environment is of special concern in amphibians, which are declining globally (Adams 1999, Alford et al. 1999, Berger et al. 1998, Bishop et al. 1999, Blaustein et al. 2002, Blaustein et al. 1990, Bridges et al. 2005, Burrowes et al. 2004, Carey et al., Daniels 2003, Davidson et al. 2001, Davidson et al. 2002, Delis et al. 1996, Green 2003, Hayes 1997a, Kiesecker et al. 2001, LeNoir et al. 1999, Licht et al. 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 et al. 2005, Daniels 2003, Davidson et al. 2001, Davidson et al. 2002, Gendron et al. 2003, Hayes et al. 1997, Hayes et al. 2005, Hayes 1997a, 1998, 1999, Hayes 2004, 2005a, b, Hayes et al. 2002a, Hayes et al. 2002b, Hayes et al. 2002c, Kiesecker et al. 2001, LeNoir et al. 1999, Pickford et al. 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, atrazine is of special concern because it is a ubiquitous, persistent contaminant of ground and surface water that is active at low, ecologically relevant concentrations (Hayes et al. 2005). As a result, atrazine is also the most well-examined endocrine disruptor in amphibians. Atrazine is rarely applied alone in its agricultural use, however, but is used rather 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 current study, we examined the effects of a realistic pesticide mixture comprised 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 tebupirimphos), and two fungicides (metalaxyl and propiconizole) 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 northern leopard frogs (*Rana pipiens*) a species that is exposed to this mixture in the wild. Because of an unexpected increase in disease contraction, we examined thymus histology as a measure of immuno-competence 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, tebupirimphos, metalaxyl, and propiconizole were purchased from Chemservice (Chester, PA) and were ≥ 98% pure (except tebupirimphos which was 97%). S-metolachlor, and the commercial atrazine-metolachlor preparation (Bicep II Magnum) were gifts from Syngenta Crop Protection (Research Triangle Park, NC; see below for purity). Except where indicated, all other reagents were obtain from Sigma Chemicals (St. Louis, MO).

Experiment 1: Effects of pesticides on larval Northern leopard frogs (*Rana pipiens*)

Animal care for laboratory studies. Adult northern leopard frogs (*Rana pipiens*) for breeding were purchased from Charles D. Sullivan Co., Inc. (Nashville, TN). Frogs were reportedly obtained from populations within 200 miles of Boston, MA (Charles Sullivan, personal communication). Fertilized eggs were obtained by injecting three males and three females with a gonadotropin releasing hormone agonist (GnRH; des-Gly^{10} [D-His (Bzl)^{6} LHRH ethylamide) as described previously (Hayes et al. 1993) and allowing paired animals to breed. Eggs were maintained in 0.1 × Holtfreter's (e.g. 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 (*5*) 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 carried out at 22-23°C with a 12-hour light-12 hour dark cycle (lights on at 6 a.m.).

Larval laboratory exposures. Larvae were treated by immersion with 0.1 ppb each atrazine, S-metolachlor, alachlor, nicosulfuron, cyfluthrin, λ-cyhalothrin, tebupirimphos, metalaxyl, or propiconizole. These pesticides represented pesticides used on a cornfield in York County, NE (97°22.38 N, 40°55.88W, (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 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 July 22-23, 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 pre-dissolved 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 three times (30 larvae per replicate). Cages were cleaned, water changed, and treatments renewed every three days. All treatments were systematically relocated every three days to ensure that no treatments or tanks experienced position effects. Animals were exposed throughout the larval period from two days post-hatching 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. 2002c). Water samples were extracted in organic solvent and subjected to gas chromatography using a nitrogen phosphorous detector. Analysis was provided for alachlor, atrazine, S-metolachlor, metylaxyl, 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 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 Chemicals, St. Louis MO), 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 SMZ 10A dissecting scope fitted with a 0.5 × lens (Technical Instruments, Burlingame, CA). Tissues were embedded in paraffin and histological analysis conducted as described in Hayes (1995b). In brief, dissected tissues were dehydrated in graded alcohols and infiltrated with histoclear (National Diagnostic, Atlanta, GA) followed by paraffin. Serial histological sections were cut at 8 μ 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 gonads thymus. Following our discovery 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 immuno-competence. The head was dissected (just anterior to the forelegs). Tissues were embedded in paraffin and histological analysis conducted as described for gonads except that sections were cut at 4 μ. The number of thymic plaques and maximum transverse cross-sectional area and cell density were determined using Metamorph software (Universal Imaging, Buckinghamshire, UK). The effects of all single pesticides were examined (20 animal each) as well as effects of 0.1 ppb Bicep II Magnum and the nine compound mixture. Animals exposed to .1 or 10 ppb atrazine + S-metolachlor were not examined because this analysis was not planned and tissues from these animals was prepared for other analyses.

Experiment 2: Effects of the pesticide mixture on corticosterone levels in adult *Xenopus laevis*

Following our discovery 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 African clawed frogs (*Xenopus laevis*) as a surrogate for this experiment, because metamorphic leopard frogs (*Rana 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 house individually in covered tanks. Animals were acclimated in 0.1 × Holtfretter's solution for five 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, St. Louis MO) daily, and solutions were changed and treatments renewed every three days. Animals were treated for 27 days. Blood was collected by cardiac puncture in non-anesthetized animals between 18:00 and 20:00. 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 et al. 1992). Corticosterone antiserum was purchased from Endocrine Sciences (Calabasas, CA) and has been validated for several species including *Xenopus laevis* (Hayes et al. 1995a, Hayes et al. 1995b, Hayes et al. 1992, Hayes et al. 2002c). Plasma from controls and treated animals was assayed in the same assay at three doses and the assay was repeated three times. Intra-assay variation was 1.0%, and inter-assay 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 replicate (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 et al. 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 to 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 (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, metylaxyl at 0.16 ppb, and propiconazole at 0.23 ppb in the nominal 0.1 ppb mixture. No pesticides were detected in the Holfreter'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% to 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 (≤ 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 (Fig. 1). Otherwise, no single compound affected development and the rest of our 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 in animals treated with pesticide mixtures ($F = 37.55$, $df = 3$, $P < 0.005$; Fig. 2). Tail reabsorption (TR) was similarly delayed ($F = 29.84$, $df = 3$, $P < 0.0001$; Fig. 2) and larvae exposed to 10 ppb atrazine + S-metolachlor experienced a slight delay in both FLE ($P = 0.055$) and TR (though 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, 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 snout-vent length (SVL) at metamorphosis ($F = 2.1$, $df = 10$, $P < 0.05$; Fig. 3). The smallest animals to metamorphose were those exposed to cyfluthrin, tebupirimphos, or atrazine. There was also a significant effect on body weight (BW; $F = 2.07$, $df = 10$, $P < 0.05$; Fig. 3).

All of the mixtures (0.1 ppb each pesticide) retarded growth, resulting in smaller animals (SVL) at metamorphosis ($F = 4.03$, $df = 3$, $P = 0.008$; Fig. 4). The mixtures also affected body weight ($F = 3.86$, $df = 3$, $P = 0.01$; Fig. 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. Snout-vent length at metamorphosis was positively correlated with time to complete metamorphosis for controls (Fig. 5). With regards to single chemical exposures, all of the treatments showed a similar positive significant relationship ($P < 0.05$, see Table 1) with the following exceptions: Larvae exposed to metalaxyl showed a positive but non-significant relationship between time to complete metamorphosis (TR) and SVL, whereas larvae exposed to atrazine, cyhalothrin or propiconazole showed a negative, but non-significant relationship between TR and SVL (Fig. 5).

For the pesticide mixtures, 0.1 and 10 ppb atrazine + S-metolachlor resulted in a negative but non-significant 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 (Fig.

5). The nine compound pesticide mixture resulted in a negative, but non-significant relationship (see Table 1; Fig. 5).

For body weight, controls also showed a significant positive correlation between time to complete metamorphosis and body weight. All single treatment exposures showed a similar positive significant relationship ($P < 0.002$ in all cases, see Table 2 and Fig. 6) except nicosulfuron, atrazine, and cyhalothrin, which showed a positive but non-significant relationship between TR and BW at metamorphosis (Table 2; Fig. 6). With regards to 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 non-significant for both 0.1 and 10 ppb atrazine + S-metolachlor, and negative and significant for the nine-pesticide mixture (see Table 2; Fig. 6).

Gonadal development. In the population used in the current study, gonadal development was delayed (even in controls) relative to other populations of *Rana pipiens* that have been examined by our laboratory (Hayes et al. 2002a, Hayes et al. 2002c) or other published accounts (Merchant-Larios et al. 1981, Richards et al. 1978). Histologically, presumptive males maintained both a cortex and a medulla separated by connective tissue without clear formation of testicular lobules (e.g. were 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 (Fig. 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 or not 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) menigosepticum*. Diagnosis was based on signalment, clinical signs, necropsy results, histopathologic examination of internal organs with special staining in select cases, and the ante-mortem isolation of organism from the internal lesions of affected animals. Manifestations of disease included anorexia, head tilt, circling, loss of righting response, aniscoria, and death (Fig. 8). Morbidity and mortality rates in animals treated with the nine-pesticide mixture was significant ($G = 100.12$, $df = 4$, $P < 0.001$) compared to 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. *Chryseobacterium (Flavobacterium) menigosepticum* 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. Following our discovery that animals exposed to the nine-compound mixture contracted flavo-bacterial meningitis (see above), 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 ($G = 9.4$, $df = 4$, $P < 0.05$; Fig. 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 (not reported here).

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 four fold in pesticide-exposed males (ANOVA; $P < 0.05$; Figure 10).

Discussion

Though a sizable data-base 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, though brief episodes of high-concentration exposure may occur, prolonged exposure to low concentrations of pesticide mixtures are more common (Battaglin et al. 1999, Burkhart et al. 2003, Capel et al. 2001, Fischer et al. 1995, Frank et al. 1979, Frank et al. 1988, Frank et al. 1991, Frank et al. 1987, Hennion et al. 2004, Insensee et al. 1990, Kolpin et al. 1998, Kucklick et al. 1994a, b, 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 current chemical mixture was not examined. Further, fewer than 20 published laboratory studies (Boegi et al. 2002, Hayes et al. 1997, Hayes 1997a, 1998, 1999, 2000, Hayes 2004, 2005a, Hayes et al. 2002a, Hayes et al. 2002b, Hayes et al. 2002c, Lutz et al. 1999, Noriega et al. 2000, Palmer et al. 1995, Palmer et al. 1998, Tavera-Mendoza et al. 2002a, Tavera-Mendoza et al. 2002b) and four field studies (Du Preez et al. 2005, Hayes et al. 2002a, Hayes et al. 2002b, 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, Harris et al. 1998b, Ouellet et al. 1997, Sparling et al. 2001), though establishing cause and effect such studies is difficult. The current study is the first to address endocrine-disrupting effects of low-concentration pesticide mixtures 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. 2002c) 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.

Effects of the majority of the nine pesticides used in the current study have not been examined in amphibians at all: No published studies have addressed the effects of cyfluthrin, cyhalothrin, tebupirimphos, metalaxyl, or propiconizole on amphibians. Here, we show that one of these compounds (propiconazole) retards larval development and delays metamorphosis, while two other previously unexamined pesticides (tebupirimphos and cyfluthrin) retard larval growth. In addition to these new data, the current study confirms the retardation of amphibian development (Carr et al. 2003, Rohr et al. 2005, Rohr et al. 2004) and growth (Boone et al. 2003, Britson et al. 1998, Carr et al. 2003, Diana et al. 2000) already reported for atrazine. In fact, Carr et al. also showed a reversal of the relationship between time to metamorphosis and size at metamorphosis in their studies (Carr et al. 2003) as we show here.

The current study is also important because all of 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 (Manzanti 1999, Manzanti 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 the concentrations used in our current study. All but three previous studies (Howe et al. 1998, Manzanti 1999 and Manzanti 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 of these previous studies were conducted at concentrations 10,000 times higher than concentrations used in our current study. The exception is atrazine, for which sub-lethal developmental effects at low concentrations (in

the ppb range) have been examined by multiple laboratories (Allran et al. 2001, Boone et al. 2003, Britson et al. 1998, Carr et al. 2003, Coady et al. 2004, Coady et al. 2005, Diana et al. 2000, Du Preez et al. 2005, Goulet et al. 2003, Gross et al. 2003, Hayes et al. 2005, Hayes 2004, 2005a, b, Hayes et al. 2002a, Hayes et al. 2002b, Hayes et al. 2002c, Hecker et al. 2004, Howe et al. 1998, Jooste et al. 2005a, Jooste et al. 2005b, Miyahara et al. 2003, Reeder et al. 1998, Rohr et al. 2005, Rohr et al. 2004, Sullivan et al. 2003, Tavera-Mendoza et al. 2002a, Tavera-Mendoza et al. 2002b).

Atrazine has a number of well-documented adverse effects on amphibian larvae. Atrazine is a potent endocrine disruptor that both chemically castrates and feminizes exposed male amphibian larvae (Carr et al. 2003, Du Preez et al. 2005, Hayes et al. 2005, Hayes 2004, 2005a, Hayes et al. 2002a, Hayes et al. 2002b, Hayes et al. 2002c, Reeder et al. 1998, Tavera-Mendoza et al. 2002a, Tavera-Mendoza et al. 2002b). In addition, atrazine retards larval development and growth (see references cited above), induces edema (Carr et al. 2003), erratic swimming (Carr et al. 2003), irregular behavioral activity (Rohr et al. 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, but also because atrazine is a ubiquitous, persistent environmental contaminant (Hayes et al. 2005, Solomon et al. 1996): As one of the world's most commonly applied pesticides, it is the most common contaminant of ground and surface water (Hayes et al. 2005). Up to 0.5 million pounds per year are deposited in precipitation in the US (Miller et al. 2000, Nations et al. 1992, Thurman et al. 2000, Van Dijk et al. 1999) and contamination can

spread more than 600 miles from the point of application (Miller et al. 2000, Müller et al. 1997, Thurman et al. 2000).

The current study demonstrates that one of the most well-documented effects of atrazine, demasculinization and feminization of male larvae, can vary between populations. In the current population, effects of atrazine on the gonads were not detectable because individuals from the current population do not complete sexual differentiation of the gonads prior to metamorphosis. In Ranids, atrazine induces testicular oogenesis (Hayes et al. 2002a, Hayes et al. 2002b), but in the population used in the current study, male gonads were not well-differentiated (testicular lobules were not yet developed) and even females lacked significant numbers of oocytes. Though other differences in study design exist (Hayes 2004), this variation in susceptibility may explain some of the disparate findings in the published literature with regards to the effects of atrazine on gonadal development in amphibians (Coady et al. 2004, Coady et al. 2005, Du Preez et al. 2005, Hayes 2005b, Hecker et al. 2005, Hecker et al. 2004, Jooste et al. 2005a, Jooste et al. 2005b). This finding highlights the importance of understanding population variation when assessing the risk of pesticides to amphibians.

Retardation of growth and development has been demonstrated for atrazine in previous studies (see references above). Here, 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 is 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 current 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 metamorphosis 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 (Fig. 12A). Further, smaller individuals are more susceptible to predators, which may also themselves be gape-limited predators (e.g. snakes, Fig. 12B) (De Vito et al. 1999, Kiesecker et al. 1998, Lardner 1998, Lawler et al. 1999, Nicieza 2000, O'Dwyer et al. 2000, Puttlitz et al. 1999, Relyea 2001a, b, 2003, Skelly 1994, Werner 1986, Wilbur et al. 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 post-metamorphic growth decreases the chances that amphibians will survive over-wintering (Berven et al. 1983, Smith 1987). Reduced size at metamorphosis also delays reproductive maturity and decreases fecundity (Berven et al. 1983, Smith 1987, Wilbur et al. 1973). This negative

effect is especially true for exposed females for which size is directly proportional to fecundity (Howard 1981, Shine 1979, 1989), but 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 et al. 1954, Howard 1981, Shine 1979, 1989).

The alteration of the relationship between time to metamorphosis and size at metamorphosis is even more significant. In amphibians, the larval stage is a period of growth. As non-amniotes, 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 as well as to escape predation. As shown in the current study, there is a positive relationship between time to metamorphosis and size at metamorphosis: Larvae that take longer to metamorphosis are larger. With exposure to pesticide mixtures, larvae take longer to metamorphosis, 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.

The current 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. Though the relative roles of all of the individual pesticides in the mixture cannot be discerned from the current study, however, S-metolachlor does appear to be an "effector". In the current study and others (see references above), atrazine retarded larval development and growth. Though S-metolachlor had no effect on its own in the current study, the negative effects of atrazine were 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) (though the S-metolachlor is 22% lower in the Bicep II magnum mixture compared to the atrazine + S-metolachlor). 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 flavo-bacteria but did not display symptoms of disease, the pathogen was otherwise non-virulent under our 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 current 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.

With regards 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 earlier. The immunosuppressive effects are likely even more relevant. Most significantly, the nine-pesticide mixture increased plasma corticosterone levels. Corticosterone can produce all of the effects observed with the pesticide mixtures, including retarded growth (Hayes 1995a, Hayes et al. 1995a, Hayes et al. 1997, Hayes 1995b, Hayes et al. 1993), retarded development (Glennemeier et al. 2002a, b, Hayes 1995a, Hayes et al. 1995a, Hayes et al. 1997, Hayes 1995b, Hayes 1997b, Hayes et al. 1993), and immunosuppression (Belden et al. 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 et al. 2005, Jancovich et al. 2005, Pearman et al. 2004) and chytrid (Berger et al. 1998, Green et al. 2005, McCallum 2005, Ouellet et al. 2005, Rollins-Smith et al. 2002, Weldon et al. 2004) are considered major contributors to amphibian declines. Given our findings with the flavo-bacteria in the current 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" (page 4, Sparling et al., 2003). This approach has to change if problems are to be identified and solutions formulated.

With regards to pesticides, the current 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 necessary to characterize the interactions here as "concentration additive" or "response additive" (Burkhart et al., 2003), to identify the role of individual chemicals in mixtures as "effectors" or "enhancers", and to examine multiple combinations of pesticides at multiple concentrations are not only more difficult to design and carry out than studies that examine exposure to single pesticides, but the outcomes present new challenges to regulators. Nevertheless, full-factoral studies that identify "effectors", "enhancers" and

"neutral" chemicals are necessary 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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Table 1. Statistics for correlational analysis of time to complete tail reabsorption (TR) and snout-vent length (SVL).

Treatment[a]	n[b]	r	chi square	df	*P*	Figure
Ethanol	86	+0.23	4.365	1	0.040	5.A
Alachlor	86	+0.29	7.123	1	0.008	5.B
Atrazine	86	- 0.19	3.061	1	0.080	5.J
Cyfluthrin	86	+0.24	4.872	1	0.027	5.C
λ-Cyhalothrin	84	- 0.00	0.001	1	0.980	5.I
S-Metolachlor	83	+0.29	7.140	1	0.008	5.F
Metalaxyl	58	+0.25	3.210	1	0.073	5.G
Nicolsulfuron	85	+0.29	6.785	1	0.009	5.D
Propiconazole	88	- 0.14	1.581	1	0.209	5.H
Tebupirimphos	85	+0.35	10.59	1	0.001	5.E
.1 ppb Atr + S-Met	89	- 0.19	3.018	1	0.082	5.K
10 ppb Atr + S-Met	85	- 0.14	1.780	1	0.182	5.L
.1 ppb Bicep	81	+0.39	11.50	1	0.001	5.M
10 ppb Bicep	84	+0.19	2.732	1	0.098	5.N
.1 Mix	59	- 0.23	2.100	1	0.147	5.O

a. 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.

b. Sample size (n) represents the number of animals surviving to metamorphosis (out of the original 90; 30 animals in each of three replicates).

Table 2. Statistics for correlational analysis of time to complete tail reabsorption (TR) and body weight (BW).

Treatment[a]	n[b]	r	chi square	df	*P*	Figure
Ethanol	86	+0.41	14.61	1	0.000	6.A
Alachlor	86	+0.46	18.92	1	0.000	6.B
Atrazine	86	+0.07	3.061	1	0.080	6.J
Cyfluthrin	86	+0.34	10.03	1	0.002	6.C
λ-Cyhalothrin	84	+0.13	1.376	1	0.241	6.I
S-Metolachlor	83	+0.47	20.15	1	0.000	6.F
Metalaxyl	58	+0.40	9.038	1	0.003	6.G
Nicolsulfuron	85	+0.49	20.99	1	0.000	6.D
Propiconazole	88	+0.20	3.130	1	0.077	6.H
Tebupirimphos	85	+0.58	32.77	1	0.000	6.E
.1 ppb Atr + S-met	89	- 0.05	0.251	1	0.616	6.K
10 ppb Atr + S-met	85	- 0.01	0.000	1	0.960	6.L
.1 ppb Bicep	81	+0.51	20.69	1	0.000	6.M
10 ppb Bicep	84	+0.33	8.143	1	0.004	6.N
.1 Mix	59	- 0.32	4.164	1	0.041	6.O

a. 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.

b. Sample size (n) represents the number of animals surviving to metamorphosis (out of the original 90; 30 animals in each of three replicates).

Figure legends

Figure 1. Effect of single pesticides (0.1 ppb each) on time to initiate metamorphosis (Gosner stage 42; foreleg emergence; FLE) (top panel) and time to complete metamorphosis (Gosner stage 46, tail reabsorption, TR) (bottom panel). Asterisks show statistically significant groups (ANOVA, $P < 0.05$).

Figure 2. Effect of pesticide mixtures on time to initiate metamorphosis (Gosner stage 42; foreleg emergence; FLE) (top panel) and time to complete metamorphosis (Gosner stage 46, tail reabsorption, TR) (bottom panel). Asterisks show statistically significant groups (Anova, $P < 0.05$). Atr = atrazine, Bicep = Bicep II Magnum, S-Met = S metaolachlor, Mix = nine-chemical mixture (0.1 ppb each pesticides. Letters above bars indicate statistical groupings.

Figure 3. Effect of single pesticides (0.1 ppb each) on snout-vent length (SVL; top panel) and body weight (BW; bottom panel). Asterisks show statistically significant groups (ANOVA, $P < 0.05$).

Figure 4. Effect of pesticide mixtures on snout-vent length (SVL; top panel) and body weight (BW; bottom panel). Atr = atrazine, Bicep = Bicep II Magnum, S-Met = S metaolachlor, Mix = nine-chemical mixture (0.1 ppb each pesticides. Letters above bars show statistical groupings.

Figure 5. Correlational analysis of time to complete tail reabsoprtion (TR) and snout-vent length (SVL). Results are shown for single pesticides (0.1 ppb) in first two rows and for mixtures (bottom two rows). 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), Tebupirimphos (E), S-metolachlor (F), Metylaxyl (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). Elipses show Gaussian bivariate confidence limits. Ellipses are color-coded: Black = a positive and significant correlation, Blue = a positive but non-significant correlation, Yellow = a negative but non-significant correlation, Red = a negative and significant correlation. Lines through the long axes of ellipses show the orientation of the moment of correlation as determined by the covariance between TR and SVL. Significance ($P \leq 0.05$) was determined by Bonferroni probabilities and based on Spearmen rank order correlation coefficients. Sample size (as reported in Table 1) cannot be determined from the number of data points due to overlapping data.

Figure 6. Correlational analysis of time to complete tail reabsoprtion (TR) and body weight (BW). Results are shown for single pesticides (0.1 ppb) in first two rows and for mixtures (bottom two rows). 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), Tebupirimphos (E), S-metolachlor (F), Metylaxyl (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). Elipses show Gaussian bivariate confidence limits. Ellipses are color-coded: Black = a positive and significant correlation, Blue = a positive but non-significant correlation, Yellow = a negative but non-significant correlation, Red = a negative and significant correlation. Lines through the long axes of ellipses show the orientation of the moment of correlation as determined by the covariance between TR and BW. Significance ($P \leq 0.05$) was determined by Bonferroni probabilities and based on Spearmen rank order correlation coefficients. Sample size (as reported in Table 2) cannot be determined from the number of data points due to overlapping data.

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

Figure 8. Dorsal (A,C) and frontal (B,D) view of newly metamorphosed (Gosner stage 46) control (A,B) *Rana pipiens* and similar-aged animal exposed to the nine-pesticide mixture. Control animal is in good body condition with normal (A,B). The pesticide-

treated animal (C,D) is in poor body condition due to a generalized gram negative bacterial infection (C,D). The pathogen was identified in control and treated frogs, but only pesticide-exposed animals show the disease. As displayed here, the predominant signs associated with outbreak included head tilt, unilateral extensor muscle rigidity, anisocoria, and intermittent recumbency due to a severe *otitis interna* and meningitis. This presentation is consistent with *Chyrseobacterium menigosepticum* infection, a stress induced disease of frogs.

Figure 9. Representative transverse cross-section through the thymus of a control (A) and an animal treated with the nine-compound pesticide mixture (B). The frequency of animals with detectable damage to the thymus is shown in panel C. No control animals showed damage to the thymus. Atrazine and S-metolachlor exposed animals (0.1 ppb) showed damage as shown, with increasing frequencies of damage with exposure to atrazine + S-metolachlor and Bicep II magnum (atrazine + metolachlor, given to provide 0.1 ppb atrazine) and maximum damage with exposure to the pesticide mixture. Histological sections were conducted at 4 μ and stained in Mallory's trichrome stain. Bar (in A) = 0.5 mm.

Figure 10. Effect of the pesticide mixture on plasma corticosterone levels in adult male African clawed frogs (*Xenopus laevis*). Asterisk show statistical significance ($P< 0.05$).

Figure 11. Field 413 (40°55.88N, 97°22.38W), York County, NE (Panels A-F). In 2001 frogs bred in the irrigation ditch (A) and were present by the 1000s. Even at this time, the water level (and thus temperature and pesticide levels) fluctuated drastically from one

day to the next: The photo in panel B was taken on July 22, 2001 and photos in A and C taken on July 23, 2001. In 2004, seven pairs of frogs bred at the same site when the irrigation ditch filled with rainwater and snowmelt (Panel D, arrow indicates where frog clutches were found). Panel E shows one of the seven clutches. 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 our initial collection. Panel G similarly shows a breeding pond (arrow) in Hitchcock County, Nebraska (40°08.433N, 101°13.804W) along the Republican River. All of 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. Newly metamorphosed leopard frog (*Rana pipiens*) attempting to ingest a cricket that is too large (panel A; frog = 3.3 cm SVL and cricket = 2.1 cm long) and (B) a 67 cm long garter snake (*Thamnophis sirtalis*) feeding on a newly metamorphosed 3.2 cm long leopard frog (*Rana 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 and smaller frogs are more vulnerable to predation.

Figure 1

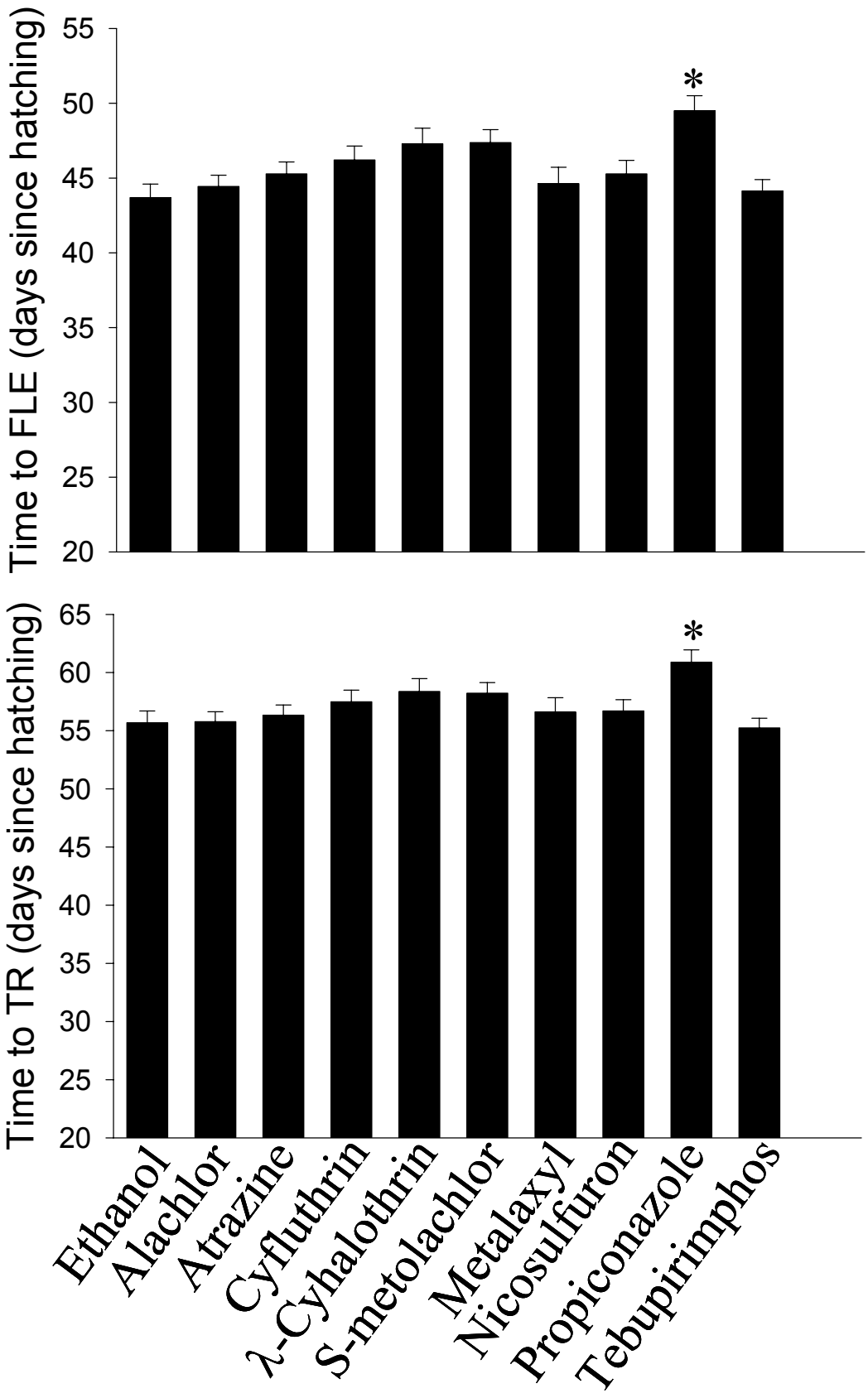
Time to FLE (days since hatching)
55
50
45
40
35
30
25
20
*
Time to TR (days since hatching)
65
60
55
50
45
40
35
30
25
20
*
Ethanol
Alachlor
Atrazine
Cyfluthrin
λ-Cyhalothrin
S-metolachlor
Metalaxyl
Nicosulfuron
Propiconazole
Tebupirimphos

Figure 2

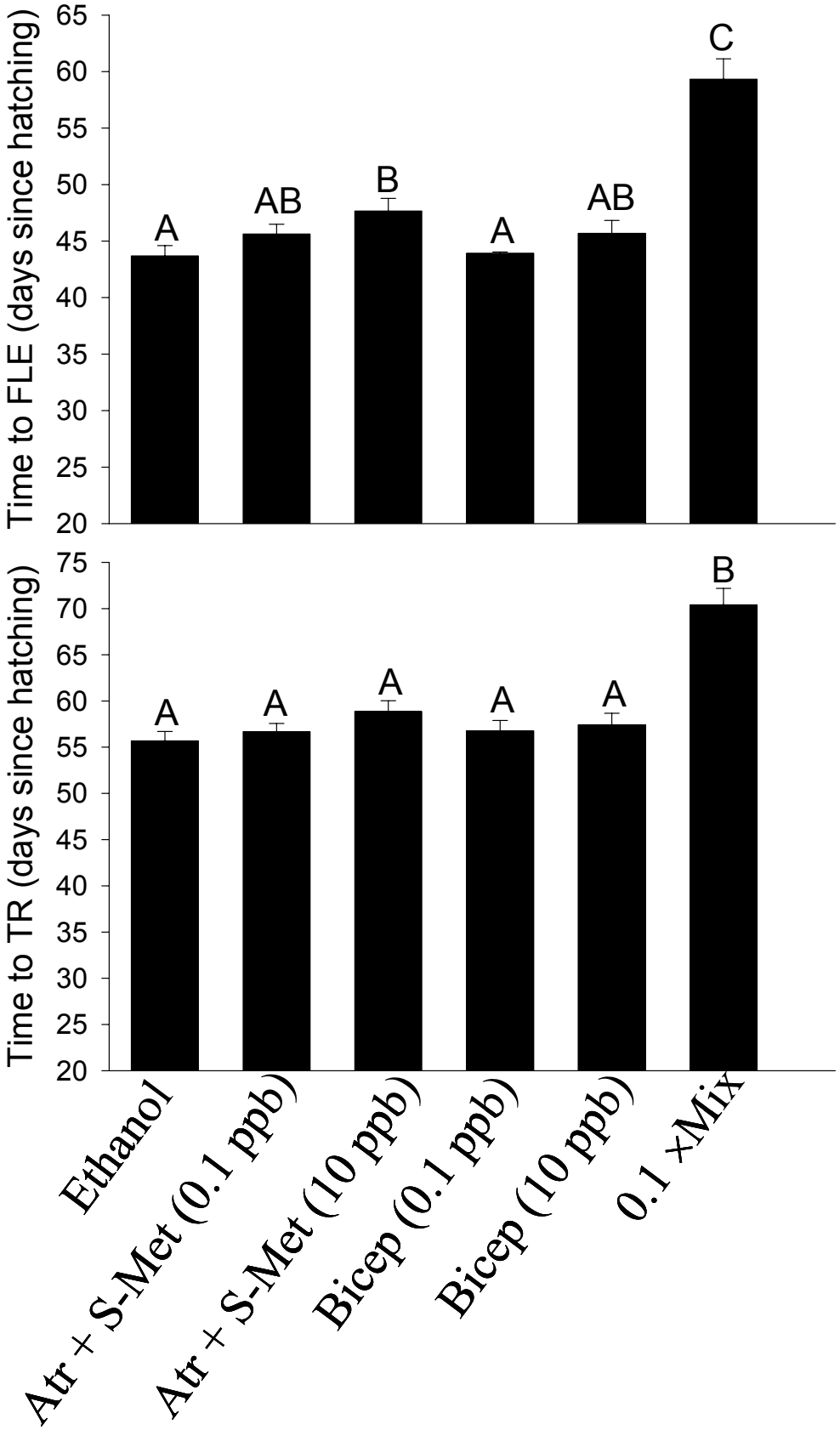
Time to FLE (days since hatching)
65
60
55
50
45
40
35
30
25
20
A
AB
B
A
AB
C
Time to TR (days since hatching)
75
70
65
60
55
50
45
40
35
30
25
20
A
A
A
A
A
B
Ethanol
Atr + S-Met (0.1 ppb)
Atr + S-Met (10 ppb)
Bicep (0.1 ppb)
Bicep (10 ppb)
0.1 ×Mix

Figure 3

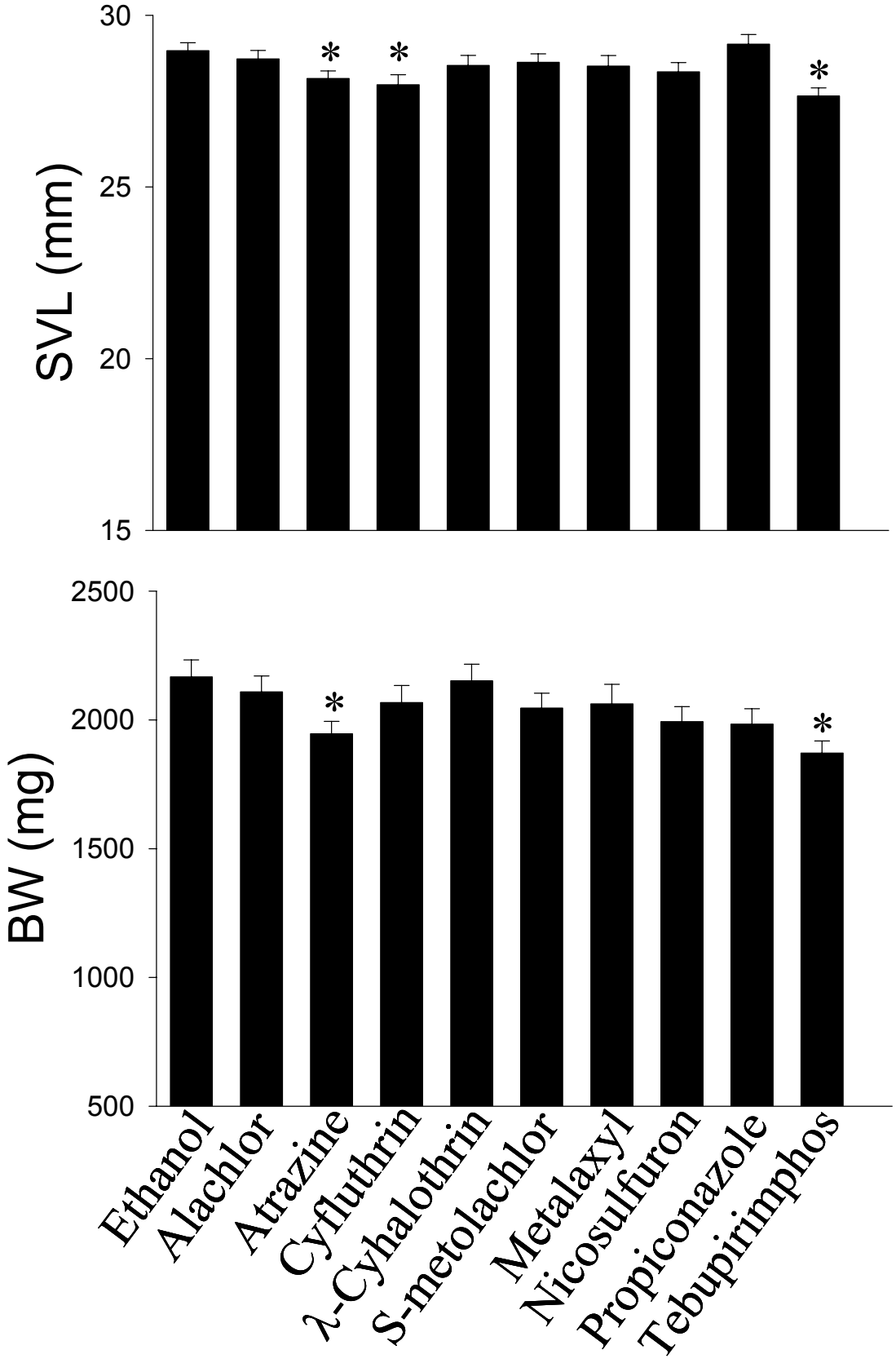
SVL (mm)
30
25
20
15
*
*
*
BW (mg)
2500
2000
1500
1000
500
*
*
Ethanol
Alachlor
Atrazine
Cyfluthrin
λ-Cyhalothrin
S-metolachlor
Metalaxyl
Nicosulfuron
Propiconazole
Tebupirimphos

Figure 4

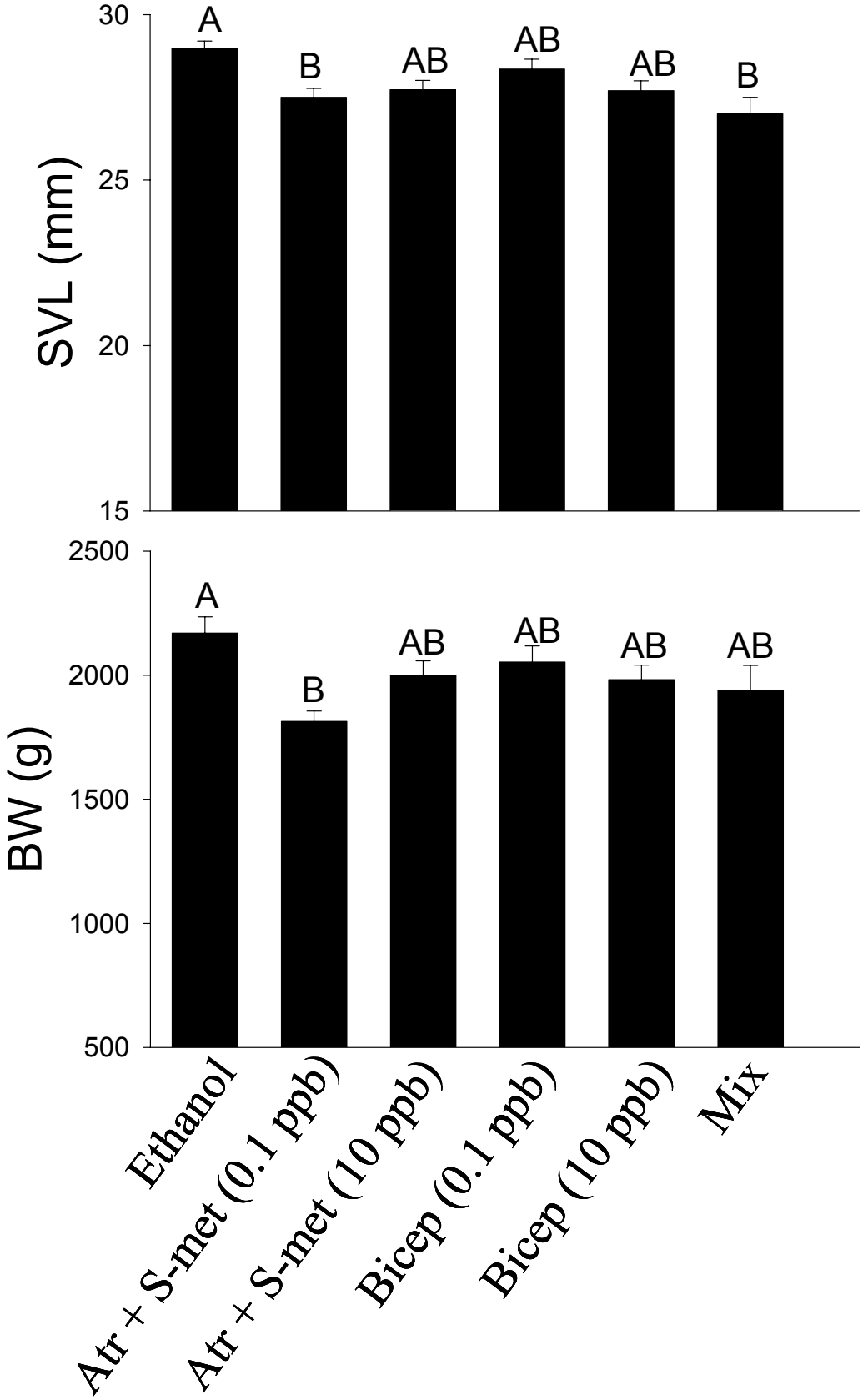
SVL (mm)
30
25
20
15
A
B
AB
AB
AB
B
BW (g)
2500
2000
1500
1000
500
A
B
AB
AB
AB
AB
Ethanol
Atr + S-met (0.1 ppb)
Atr + S-met (10 ppb)
Bicep (0.1 ppb)
Bicep (10 ppb)
Mix

Figure 5

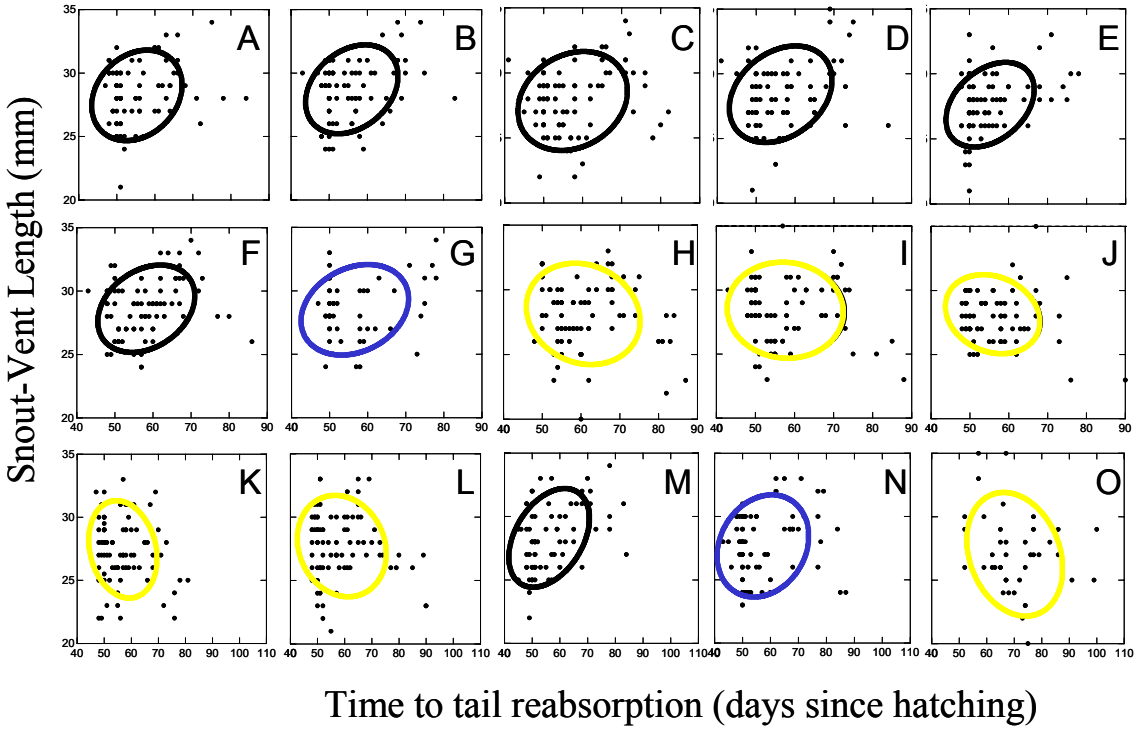
Snout-Vent Length (mm)
Time to tail reabsorption (days since hatching)
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O

Figure 6

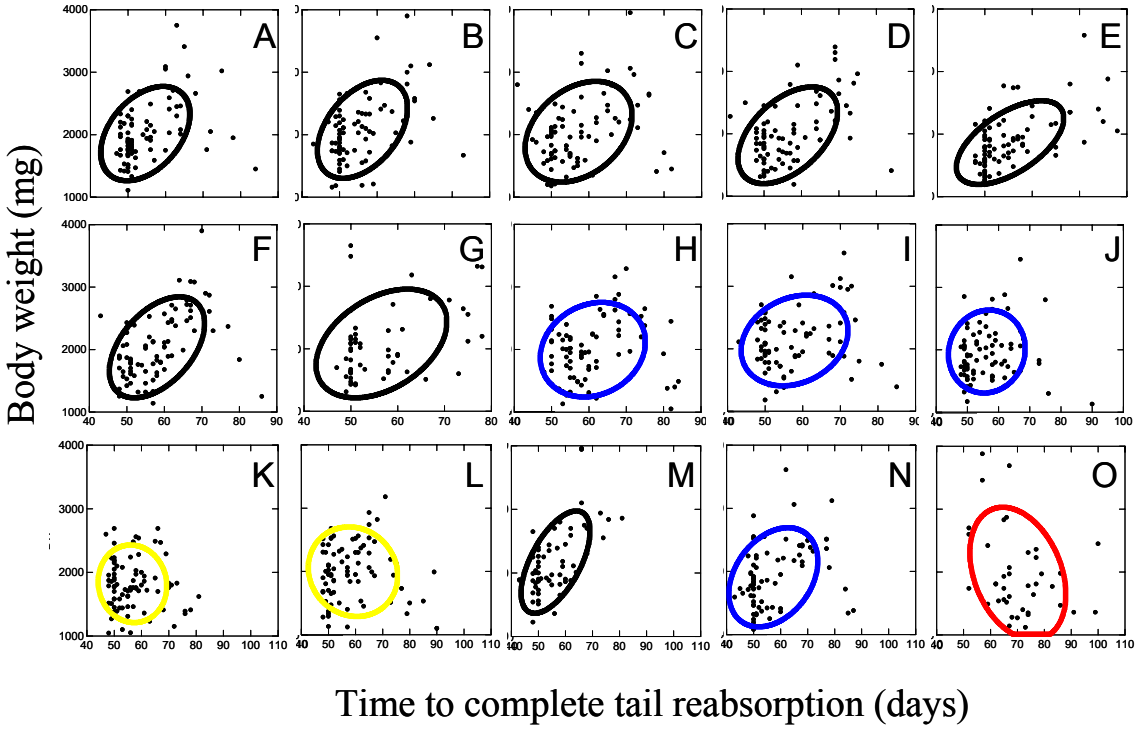
Body weight (mg)
Time to complete tail reabsorption (days)
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O

Figure 7

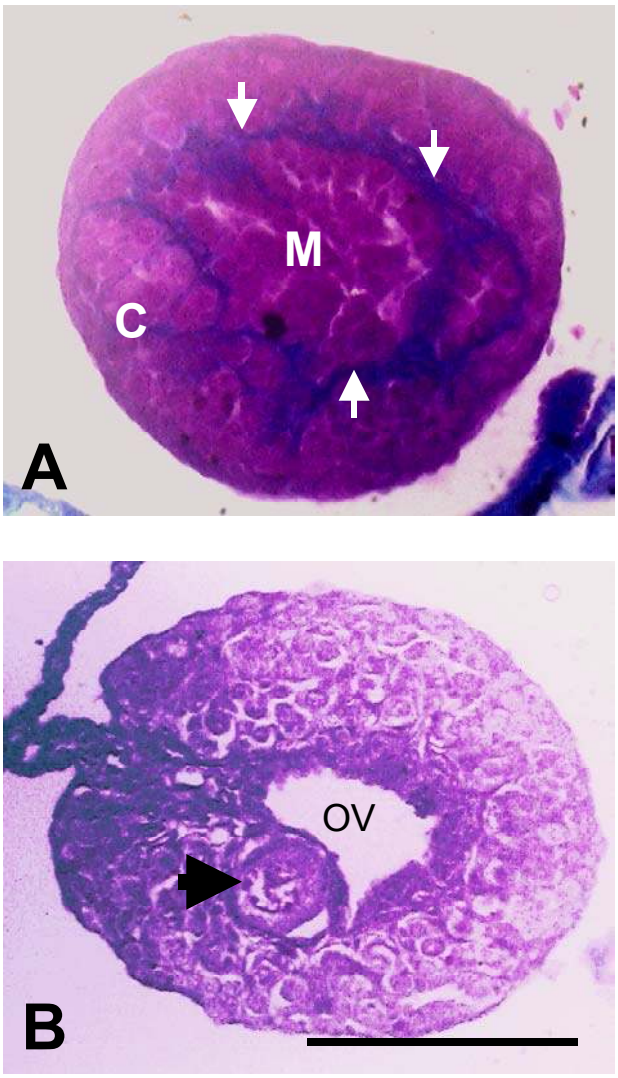

Figure 8

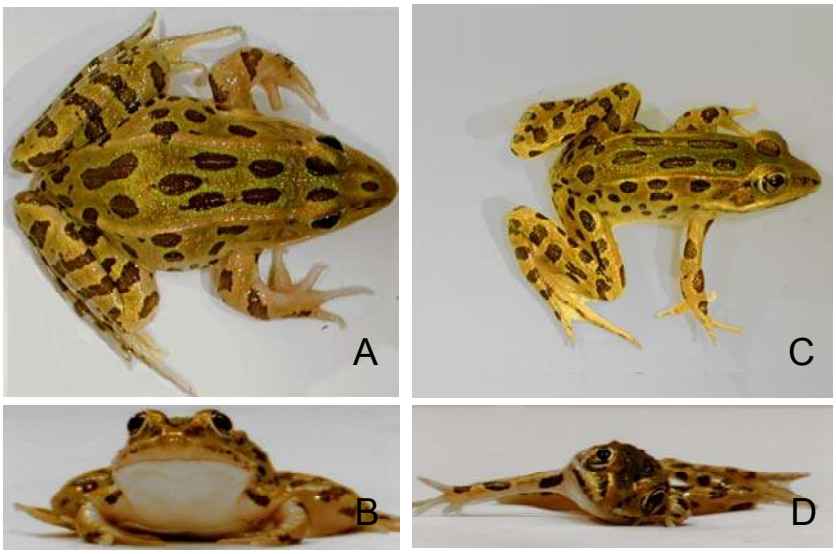

Figure 9

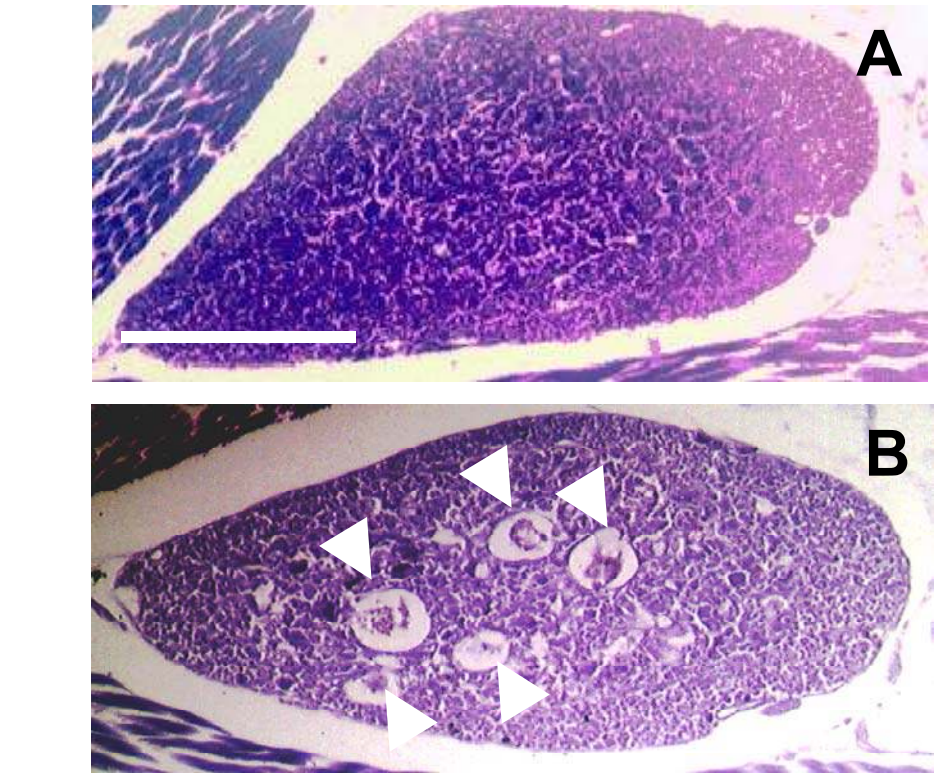
A
B
C
Animals with Thymic plaques (%)
30
25
20
15
10
5
0
0
con
atzrazine
S-met
Bicep
Mix

Figure 10

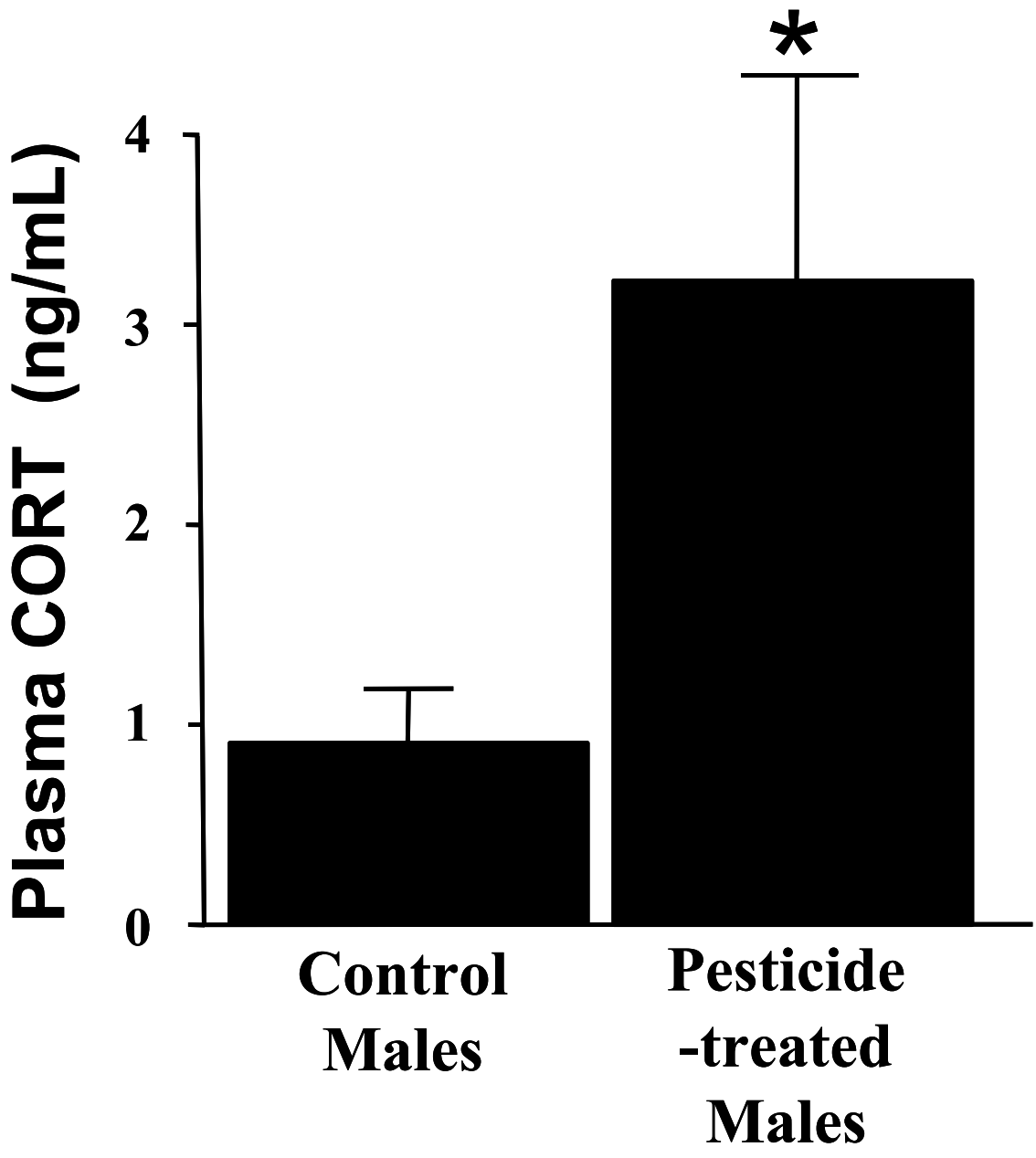
*
Plasma CORT (ng/mL)
4
3
2
1
0
Control Males
Pesticide -treated Males

Figure 11

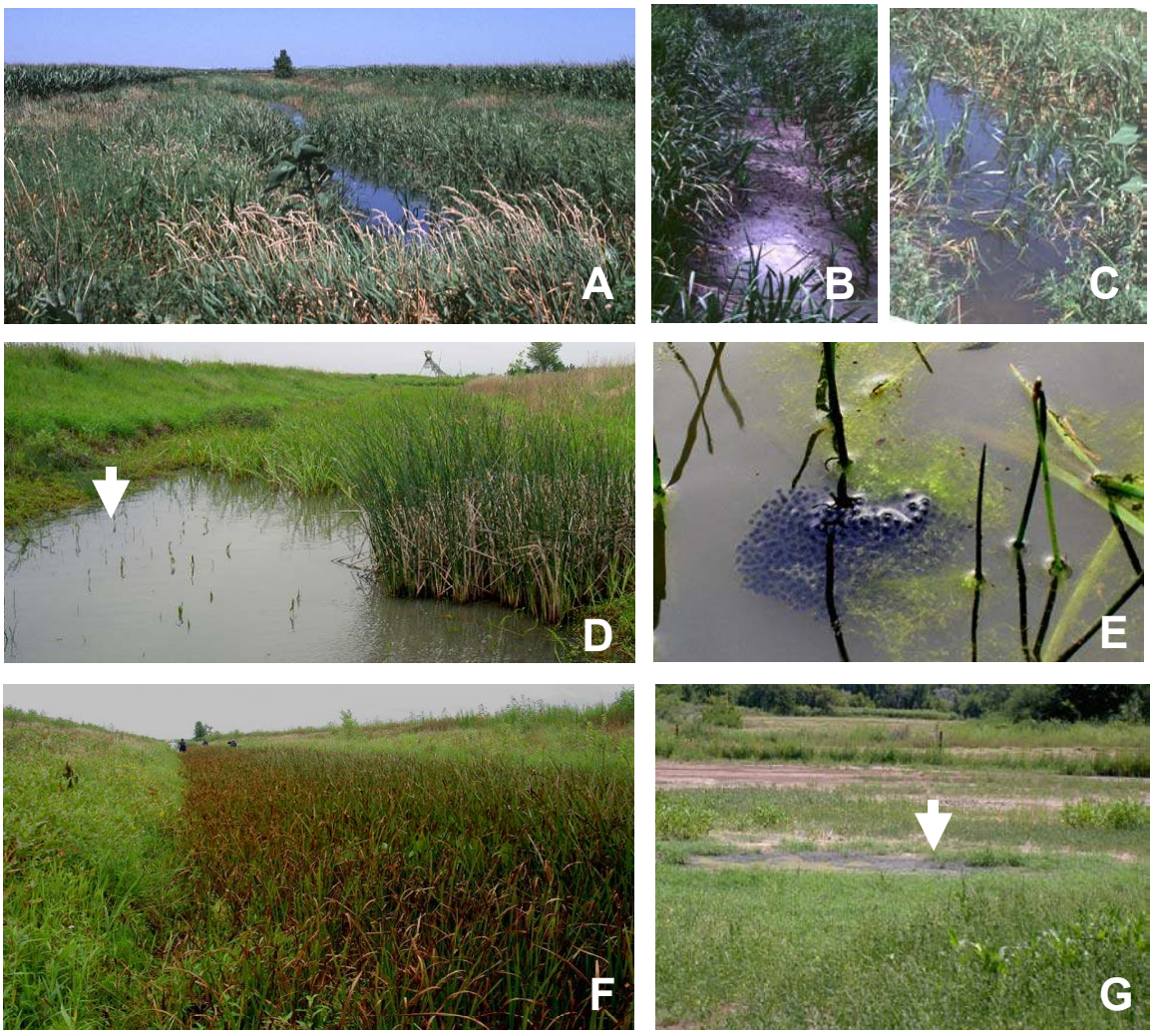

Figure 12