AQUATIC AND TERRESTRIAL EXPOSURE OF AMPHIBIANS TO ESTROGENIC ENDOCRINE DISRUPTING CONTAMINANTS

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This work is dedicated in the memory of

LINDSAY ANNE GRAYGO

1978-2008

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ABSTRACT

Hormones play an important role in shaping growth and development. Endocrine disrupting contaminants (EDCs) come from the external environment and can mimic or block internal hormone pathways. Many amphibians begin to develop major body systems, both reproductive and non-reproductive, while they are aquatic larvae. Because this critical period of development occurs in water, amphibian larvae can also be exposed to and surrounded by EDCs that find their way into the water via direct spraying, surface water runoff, and even rainfall. Further, as they metamorphose into terrestrial juveniles, they are subject to exposure on land, especially in agricultural landscapes. The main objective of my research was to investigate how low, ecologically relevant concentrations of EDCs (atrazine, estradiol) could impact both reproductive and non-reproductive endpoints, and further, how these effects might impact amphibian population persistence.

Two experiments examined the effects of exposure of aquatic larval amphibians on their reproductive development. Three species – American toads (*Bufo americanus*), gray treefrogs (*Hyla versicolor*), and Southern leopard frogs (*Rana sphenocephala*) – were selected for exposure due to important differences in their length of larval period, and therefore length of exposure, as well as suspected differences in the rate of reproductive development. Together these experiments confirmed that 1) they displayed different rates of gonad development, 2) the faster the rate of development (reproductive or non-reproductive), the more susceptible that development was to interruption by EDC exposure, 3) Southern leopard frogs, and possibly all three species, encounter a normal

phase of intersex under control conditions *en route* to fully developed testes or ovaries, and 4) exposure to EDCs appears to result in a persistence intersex phase beyond that observed under control conditions.

Two other experiments examined the effects of exposure on non-reproductive endpoints in American toads. The first investigated the possible lag effect of exposure to EDCs during the aquatic larval period on the dehydration risk and bladder morphology of terrestrial juveniles. Although no effect was found in the rate of dehydration, bladder structure indicated deleterious effects of aquatic exposure to estradiol highlighting that non-reproductive endpoints should not be ignored in EDC investigations. The second non-reproductive experiment examined the possibility of dermal exposure in the terrestrial environment. Using radio-labeled atrazine, we found that not only can EDC exposure occur across the skin, but that once inside, radio-labeled atrazine is rapidly taken up by the bloodstream. We found the highest concentration of radio-labeled atrazine in the gall bladder but the largest amounts in the intestines, after which it is eliminated.

The results presented in my thesis make important contributions to the literature in both reproductive and non-reproductive chemical exposure assessments. Moreover, this research emphasizes the importance of examining both aquatic and terrestrial exposure as well as reproductive and non-reproductive endpoints when investigating the impact of EDC exposure on individuals and on population persistence.

CHAPTER 1

THE EXPERIMENTAL FRAMEWORK

The sex of a vertebrate organism is often determined by the presence or absence of specific genes (e.g. XX or XY in humans) that lay down a blueprint for the construction of the appropriate gonad. The execution of those genetic instructions, or sexual differentiation, occurs as a result of a hormonal cascade of events, positive and negative feedback pathways, and signaling events that result in an individual with either male or female characteristics. The length and timing of sexual differentiation varies depending on the species. In mammals, sex differentiation of the gonads occurs primarily during prenatal life (vom Saal et al. 1992). For birds and turtles, sex differentiation occurs prior to hatching (Clinton 1998, Pieau et al. 1998), while teleost fish begin sex differentiation after hatching (Nakamura et al. 1998). In amphibians, sex differentiation can occur before or after metamorphosis (Ogielska and Kotusz 2004).

As gonad development proceeds, exogenous hormones can alter the endogenous cascade of events in many non-mammalian vertebrates (Short 1998). Laboratory experiments in amphibians, reptiles and birds have demonstrated that exposure to exogenous hormones can results in sex reversal (Hayes 1998, Pieau et al. 1998, Clinton 1998). However recently, scientists recognized that outside of the laboratory exposure to chemical contaminants in nature was altering the development of gonads; they published a consensus statement, the Wingspread Consensus Statement, on the importance of

investigating these occurrences (Colborn and Clement 1992). Contaminants that can mimic or block endogenous hormones are referred to as endocrine disrupting chemicals (EDCs). Following the Wingspread Consensus Statement, the USEPA established a program to screen for and test possible EDCs (Hotchkiss et al. 2008).

Endocrine disrupting chemicals come from the external environment and alter the internal hormonal environment in individual organisms. They can be effective in disrupting a normal hormonal pathway at very low levels (e.g. parts per billon, parts per trillion). Because many developmental processes, such as sexual differentiation, involve a cascade of hormonal events, exposure to EDCs during critical periods of development can be especially harmful. EDC exposure can produce permanent changes to developmental mechanisms, thus resulting in a change in phenotype (the characteristics it displays). These EDC-induced changes in phenotype can ultimately be subject to selection and therefore may impact the evolutionary process. (reviewed in Crews and McLachlan 2006)

Exposure to EDCs can have lasting effects on individuals and, as a result of their interactions, on populations and communities. Many of these incidences are highlighted in the popular book *Our Stolen Future* (Colborn et al. 1996), a modern sequel to Rachel Carson's 1962 classic, *Silent Spring*. Several accounts mentioned in *Our Stolen Future* tell of maternal EDC exposure which then affected the viability of their offspring. The reports involve reptiles, birds, and mammals including humans. In many cases, offspring

of exposed mothers could not successfully reproduce and some didn't even survive to reproductive age which negatively impacted populations.

Population declines are occurring worldwide for many groups of organisms with habitat loss usually linked to the majority of these declines. Amphibian populations are no exception to the threat of habitat loss because they require both aquatic and terrestrial habitats (Dodd and Smith 2003). In fact, amphibians are declining faster than mammals or birds (Stuart et al. 2004). Several causes for amphibian decline have been proposed including exposure to contaminants. Amphibians are thought to be especially sensitive to contaminants in general due to their semipermiable skin and biphasic life cycle exposing them to both aquatic and terrestrial threats (Hall and Henry 1992).

The impact of contaminant exposure on amphibian populations has been studied largely in terms of larval exposure. Experimentation on aquatic larvae is relatively economical and easy to do under controlled laboratory conditions. Pesticides have been a major focus of these studies because they are applied deliberately, as opposed to byproduct contaminants from industry or mining, thus allowing for a greater opportunity to manage their use (Boone and Bridges 2003). Amphibians can be exposed to pesticides via direct spraying in agricultural fields, golf courses, or forests as well as indirect surface water runoff (Semlitsch 2003) and aerial transport (Davidson et al. 2001). Pesticide exposure can directly affect amphibian physiology and behavior. For example, exposure of a tadpole to carbaryl, a commonly used insecticide, can disrupt its ability to eliminate acetylcholineesterase from the nervous system, which in turn can cause a reduction in

activity and swimming performance (Bridges 1997). Exposure can also indirectly affect amphibians by disrupting community interactions and immune system function. For example, exposure to atrazine, a commonly used herbicide, can decrease the amount of algae available for tadpoles, which in turn can cause an increase in the number of snails that harbor larval trematodes (parasitic flatworms). The exposure to atrazine also decreased the ability of the tadpoles to fight off infestations of trematodes in their bodies (Rohr et al. 2008).

Pesticides can also act as EDCs in amphibians and have even been linked to amphibian declines (McCoy et al. 2008). Several pesticides have been investigated as EDCs in amphibians but most studies have focused on atrazine. Atrazine is a widespread herbicide used primarily with corn and sorghum. Since Hayes and colleagues (2002) published a hypothesis that atrazine induced aromatase, the enzyme that converts testosterone to estrogen, investigations of atrazine's impact on reproductive development have flooded the literature. Most reports focus on anuran amphibians (frogs and toads) with *Xenopus laevis* (African clawed frog) and *Rana pipiens* (Northern leopard frog). Endpoints of several amphibian investigations include plasma hormone levels, gonad aromatase, larynx size, and gonad morphology and histology (Hayes et al. 2002, Hayes et al. 2003, Carr et al. 2003, Hecker et al. 2004, Coady et al. 2005, Hecker et al. 2005, Murphy et al. 2006).

There are conflicting reports regarding the potential for atrazine to act as an estrogenic EDC. Hayes and colleagues (2002, 2003) and Carr and colleagues (2003) have

demonstrated that exposure of larval amphibians to atrazine can result in abnormal gonad development (e.g. higher incidence of intersex in atrazine-treated larvae). However, others have reported larval exposures to atrazine without any abnormal gonad development (Coady et al. 2004, Coady et al. 2005, Hecker et al. 2005, Murphy et al. 2006). The debate has centered on the incidence of ovarian cells in the testes (testicular oocytes; TO) and whether their presence is part of normal development. Nearly a century ago, Emil Witschi (1921) reported that a hermaphroditic phase with TO was a normal part of development. Since then, reports of TO during normal development have been sporadic (Gallien 1974, Gramapurohit et al. 2000, Mackenzie et al. 2003).

The work presented in my thesis attempts to offer some clarification in the atrazine debate as well as to examine EDC exposure effects beyond larval life. First, we examine the development of females from three species of anuran amphibians under control, estradiol, and atrazine larval exposure conditions. We examined both somatic and reproductive development from the free-swimming stage to metamorphosis in this study to better understand the timing and rates of female development and how these rates influence anuran susceptibility to possibly estrogenic EDCs (i.e. atrazine) during the aquatic portion of life. In a second study, we examined male and female reproductive development of the same exposure conditions and the same three species from the free-swimming stage, through metamorphosis, to a terrestrial, juvenile stage. This study was conducted in order to examine the time-course of development for both sexes, under control and contaminant-exposed conditions. In addition, this study investigated the long-term influence of possibly estrogenic EDCs on anuran reproductive development

through the transition from aquatic to terrestrial habitats for the three rates of gonad development found among anurans.

Then we turned our attention to the effects of EDCs solely on the terrestrial stage of anuran life. In the third study, we examined the influence of larval exposure to the same control, estradiol, and atrazine conditions on rate of dehydration and bladder morphology, water balance being a critical factor in amphibian survival. The third study examined these effects on one species to expand investigations of EDC to include non-reproductive endpoints. And finally, we investigated the possibility of exposure to atrazine solely in the terrestrial environment via uptake across the skin. We monitored both uptake and elimination in one species in order to more fully understand all pathways of EDC exposure in anuran amphibians all of which should be considered for the health of a population.

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CHAPTER 2

VARIATION IN SOMATIC AND OVARIAN DEVELOPMENT: PREDICTING SUSCPTIBILITY OF AMPHIBIANS TO ESTROGENIC CONTAMINANTS

ABSTRACT

Although amphibian sex determination is genetic, it can be manipulated by exogenous hormone exposure during sexual differentiation. The timing of sexual differentiation varies among anuran amphibians such that species may or may not be a tadpole during this period, and therefore, may or may not be exposed to aquatic contaminants. Estrogenic contamination is present in amphibian habitats worldwide. We examined three species with varying somatic and ovarian developmental rates to assess their susceptibility to estrogenic contaminants. American toads (*Bufo americanus*), gray treefrogs (*Hyla versicolor*), and Southern leopard frogs (*Rana sphenocephala*) were exposed as larvae to 17-ß-estradiol (10^{-7}M), three concentrations of a widespread estrogenic herbicide (1, 3, 30 ppb atrazine), or a solvent control (ethanol). Somatic and ovarian developmental stages as well as time to metamorphosis were recorded. Toads and treefrogs were examined at three weeks and metamorphosis, while leopard frogs were examined at three, six, and nine weeks as well as at metamorphosis. Our results demonstrate that each species displays heterochronic somatic and ovarian development. Further, the more rapid of the two rates determines the susceptibility to estrogenic contaminants. These results suggests that amphibians with shorter larval periods, and

therefore quicker somatic developmental rates (i.e. American toads, gray treefrogs), are more susceptible to somatic treatment effects (i.e. prolonged time to metamorphosis) due to estrogenic contaminants. Moreover, the results suggest that amphibians with relatively rapid ovarian development (i.e. Southern leopard frogs) are more susceptible to gonadal treatment effects caused by estrogenic contaminants.

INTRODUCTION

While some lower vertebrates display temperature-dependent sex determination, the sex of amphibians is determined genetically. However exogenous exposure of amphibians to sex steroids during sexual differentiation can influence their ultimate sex. Many species exposed to estrogen during sexual differentiation display ovaries; however the results differ between species (Hayes 1998). Estrogenic chemicals in the environment are prevalent due to the widespread use of pesticides and industrial chemicals. These contaminants can be distributed worldwide through surface water runoff, sewage effluent, aerial drift, and even rainfall (Colborn et al. 1993). Amphibians are thought to be especially sensitive to contaminants in general due to their semipermiable skin and biphasic life cycle exposing them to both aquatic and terrestrial threats (Hall and Henry 1992). Although these estrogenic chemicals are found across amphibian habitats, not all species appear equally affected by estrogenic contaminants. When exposed to exogenous estrogens during the larval stage, several species from the genus *Rana* demonstrate altered gonadal development (Hayes et al. 2003, MacKenzie et al. 2003, Pettersson and Berg 2007).

The disparity in effects may be linked to the variety of life-history traits among amphibians. Many amphibians have a complex life cycle with aquatic larvae and terrestrial adults (Wilbur 1980). Among those species with aquatic larvae, the larval stage varies anywhere from two weeks to several months and some even overwinter as larvae (Just et al. 1981). The time spent in the aquatic environment determines the time semipermeable skin is in contact with aquatic contaminants. The length of the larval period may be critical to the sensitivity of a species to contaminants (Sondgrass et al. 2005). The majority of hydrophilic and hydrophobic contaminants that enter an organism pass through the skin via simple diffusion (Rozman and Klaassen 2001). Moreover, the differentiation and development of cells, organs and systems during this aquatic portion of the life-cycle makes the larval stage a critical period of development.

The length of the larval period is associated with several characteristics. Duration of the larval period generally correlates with the size of metamorphosing individuals with shorter larval periods resulting in smaller metamorphs (Emerson et al. 1988, Blouin 1992, Gomez-Mestre and Buchholz 2007). Size at metamorphosis is important for survival (Berven 1990), reproduction (Semlitsch et al. 1988), and may be an important life history characteristic in amphibian decline (Murray and Hose 2005). Further, the length of larval period and the amount of nuclear DNA are generally positively correlated, with shorter larval periods having smaller amounts of nuclear DNA (Goin et al. 1968). In addition, those with shorter larval periods have higher metabolic activity (Beck and Congdon 2000). Thus a characterization of anurans by the length of larval period encompasses several other physiological and developmental processes.

In addition to being characterized by length of larval period, anurans can also be distinguished by their rate of ovarian development which could affect their susceptibility to estrogenic contaminants. Ogielska and Kotusz (2004) described three rates of ovarian development – basic, retarded, and accelerated – in relation to somatic development, with ten stages to a fully differentiated ovary. Species with the basic rate reach metamorphosis with a Stage V or VI ovary and finally reach Stage X only a few weeks later. For species following the retarded rate, a Stage V ovary is attained about 3.5 weeks after metamorphosis. Those species with the accelerated rate of differentiation reach a Stage X ovary at or days after metamorphosis. In general, most species undergo the basic rate, while the retarded rate is followed by many toads (*Bufo*; Ogielska and Kotusz 2004). Some members of the genus *Rana* appear to follow the accelerated rate (*R. lessonae, R. ridibunda, R. catesbeiana, R. pipiens*), while other follow the basic rate (*R. temporaria, R. arvalis*; Ogielska and Kotusz 2004).

Therefore, although species with aquatic larvae develop somatically from Gosner Stage 25 (free-swimming larvae; Gosner 1960) to Gosner Stage 46 (complete tail resorption) in the aquatic environment, not all reach the same ovarian stage by Gosner Stage 46 – they experience varying rates of ovary differentiation. The rates of somatic and ovary differentiation are not only heterochronus, the timing of that heterochrony is different for species following different rates of ovary differentiation. Further, the rate of somatic differentiation varies with the length of larval period. In the context of estrogenic contaminants, species experience and may be influenced by estrogenic contaminants differently depending on their rate of somatic and ovarian development.

We examined three species of anurans with varying somatic developmental rates (*Bufo americanus, Hyla versicolor, Rana sphenocephala*). We predicted that the rates of ovarian development varied based on Ogielska and Kotusz (2004) – retarded rate for *B. americanus*, basic rate for *H. versicolor*, and accelerated rate for *R. sphenocephala.* In addition, we assessed their susceptibility to estrogenic contaminants by exposing larvae to atrazine, a widespread estrogenic endocrine disruptor (Hayes et al. 2003), and estradiol (10^{-7}M).

MATERIALS AND METHODS

Experimental Design

Three laboratory experiments – one for each species (American toads, *Bufo americanus*; gray treefrogs, *Hyla versicolor*; Southern leopard frogs, *Rana sphenocephala*) – were conducted at the same research facility of the University of Missouri. Each experiment incorporated four levels of atrazine exposure (0, 1, 3, 30 ppb, parts per billion) and a solvent control (ethanol). In addition, we also included a positive control [17-ß-estradiol (10^{-7}M)] because atrazine acts as an estrogenic endocrine disruptor (reviewed in Fan et al. 2007). A sufficient number of animals were included such that 10 animals from each treatment could be removed at three-week intervals during the larval period of each species to assay gonad and somatic development. Individual *R. sphenocephala* were sacrificed at three, six, and nine weeks and at metamorphosis, while *B. americanus* and *H. versicolor* were only sacrificed at three weeks and metamorphosis due to their shorter larval period.

Animals for each experiment were raised individually in one-liter glass canning jars filled with 800 ml of UV-sterilized, carbon-filtered water. Jars were replicated on shelves with each vertical level acting as a spatial block. Five treatments were replicated ten times in three blocks for *B. americanus* (n = 150), six times in five blocks for *H. versicolor* (n = 150), and sixteen times in three blocks for *R. sphenocephala* (n = 240).

Animal Collection and Maintenance

All species were obtained from Thomas Baskett Wildlife Area, Boone County, MO. Three amplexed pairs of *H. versicolor* were collected from a pond on 20 April 2005. The pairs were transported to a laboratory at the University of Missouri within two hours of capture. Each pair was placed in a plastic container with approximately 2 cm of UV-sterilized, carbon-filtered water where eggs could be laid. The following morning, pairs were returned to the collection site. Two *R. sphenocephala* egg masses were collected 16 & 18 August 2005 respectively, while three *B. americanus* egg masses were collected 13 April 2006. For all species, eggs developed into free-swimming hatchlings during the next week. During this time, jelly from egg masses and dead individuals were removed and fresh water was added. Free-swimming tadpoles (Gosner stage 25; Gosner 1960) were placed in cups and randomly assigned to jars. Experiments were started 28 April 2005, 22 August 2005, and 17 April 2006 for *H. versicolor, R. sphenocephala* and *B. americanus* respectively. For each experiment, water was completely changed every third day to remove waste, replenish oxygen levels, renew chemical concentrations, and to monitor mortality. When the first metamorph was found, jars were checked every day. Individuals with at least one forelimb (Gosner stage 42 – 46) were removed from the jars

and allowed to fully metamorphose (Gosner stage 46, complete tail resorption) in a plastic container with about 1 cm of water and tilted to allow both wet and dry areas. Once fully metamorphosed, individual mass (to the nearest 0.0001 g) and time to metamorphosis (days from the start of the experiment) were recorded for each individual. Each animal was then anesthetized in MS-222 and fixed in 10% neutral buffered formalin. Subsequently, the gonad-kidney complex was dissected for histological analysis.

In addition to preserving animals at metamorphosis, animals from each treatment were removed and preserved during the experiments to assay somatic and gonadal development. For *B. americanus* and *H. versicolor*, 10 individuals per treatment were preserved at week three; any individuals remaining at metamorphosis were preserved. For *R. sphenocephala,* ten individuals per treatment were preserved at week three, six and nine (n = 10 for each time) and any remaining at metamorphosis were preserved. Note that sample sizes for each time period include only females. At each time period, each animal was weighed, anesthetized in MS-222 and fixed in 10% neutral buffered formalin. Later, individual somatic stage (Gosner 1960) was recorded and gonad-kidney complex dissected for histological analysis.

Dosing

Stock solutions of analytical grade atrazine (99.2% atrazine; Sigma-Aldrich, Allentown PA) were created with an ethanol solvent. During water changes, jars were dosed with 100µl of stock solution to yield either 0, 1, 3, or 30 ppb atrazine. Controls included both

a solvent control (ethanol) and a positive control (10^{-7}M 17-β-estradiol). This concentration of estradiol was chosen because exposure of tadpoles results in a significant number of females (Kloas et al. 1999).

Water samples

Water samples (n = 5) for each treatment were collected from jars from each experiment to assess atrazine concentration. An atrazine ELISA kit (antibody-coupled paramagnetic particles, Abraxis, Philadelphia PA) was used to analyze the samples. The minimum reportable concentration for this kit was 0.05 ppb atrazine. Anything below this concentration was reported as nondetectable. Concentrations are displayed as mean ± standard deviation in parts per billion.

Gonad morphology & histology

Ovaries were assessed using the Ogielska and Kotusz (2004) ovarian staging system (I – X), while testes were assessed using Ogielska and Bartmańska (1999). Gonads were examined for cell types and presence or absence of an ovarian-like cavity to assign sex and stage of ovary development. Only females that had sexually differentiated (96% of females were Stage IV or above) were included in the analysis. Any unidentifiable samples (10.5%) or any displaying stages less than Stage IV were excluded.

Gonads were dissected and processed using a Shandon Excelsior tissue processor (Thermo Electron Corp., Massachusetts). Gonads were subsequently embedded in paraffin and sliced into 7µm sections (AO Spencer microtome). Sections were placed on

slides and stained with haematoxylin and eosin. Slides were examined using an inverted microscope (Nikon Diaphot).

Statistical analysis

Both somatic and ovarian development were examined in terms of chemical treatment and chemical by time interaction. Response variables for somatic development included Gosner stage (Gosner 1960) and time to metamorphosis. Somatic development was examined regardless of sex. Ovarian development was examined at all time periods in terms of chemical treatment and the interaction between chemical and time. The response variable for ovarian development was the ovarian stage based on Ogielska and Kotusz (2004).

To evaluate *R. sphenocephala* response to chemical treatment at a time scale closer to *B. americanus* and *H. versicolor*, we also examined the effect of chemical and time on both ovarian and somatic development for *R. sphenocephala* for only weeks three and six.

Each of these analyses was performed using a general linear model ANOVA (Minitab 12.1). Somatic stage and time to metamorphosis were log10 transformed. Normality for all ANOVAs was examined using a plot of the residuals. Pairwise comparisons were conducted using Tukey's test for multiple comparisons. Statistical significance was established for all analyses by a P-value < 0.05.

RESULTS

Water samples

For *B. americanus,* actual concentrations for control (nondetectable) and estradiol (nondetectable) agreed with nominal concentrations, however low (2.7 ± 0.75), medium (7.55 ± 2.82), and high (124.87 ± 41.26) concentrations were higher than nominal concentrations (0, 0, 1, 3, 30 ppb, respectively). This experiment was run one year after the *H. versicolor* and *R. sphenocephala* experiments. Although stock solutions were tested prior to the *B. americanus* experiment, it appears they may have experienced some evaporation between tests. For *H.* versicolor, actual concentrations for control (nondetectable), estradiol (one sample 0.05ppb, others nondetectable), low (0.916 ± 0.071), medium (2.808 ± 0.146), and high (25.1 ± 7.063) generally agreed with nominal atrazine concentrations (0, 0, 1, 3, 30 ppb, respectively). For the *R. sphenocephala* experiment, actual concentrations for control (nondetectable), estradiol (nondetectable), low (0.798 ± 0.05), medium (3.126 ± 0.245), and high (30.36 ± 1.226) also agreed with nominal atrazine concentrations (0, 0, 1, 3, 30 ppb, respectively).

Control ovary differentiation rates

The average control *B. americanus* female at three weeks displayed an ovarian stage of 4.167 ± 0.167, while at metamorphosis they demonstrated an ovarian stage of 4.25 ± 0.164 (Figure 1a, 2a). The average time to metamorphosis for control females was 27 ± 1.614 days (Figure 1b). *B. americanus* appeared to display a rate closest to the retarded ovary differentiation rate by displaying ovaries that have not yet reached Stage V by metamorphosis.

Gonad identification and ovarian staging from *H. versicolor* individuals was limited due to histological errors and absence of sexually differentiated features (a few had not reached stage IV by week three). One female and one male *H. versicolor* could be identified as sexually differentiated by week three; all other samples were either histologically deficient or had clear cellular structures but were not able to be identified as sexually differentiated (less than Stage IV). The week three control female displayed ovarian Stage IV. At metamorphosis, the average control female displayed an ovary at Stage 4.667 ± 0.333 (Figure 1a, 2b). The average time to metamorphosis for control females was 38 ± 2.186 days (Figure 1b). *H. versicolor* appeared to display a rate closest to the basic ovary differentiation rate by displaying ovaries near Stage V at metamorphosis.

All sexually differentiated control female *R. sphenocephala* at week three displayed ovarian Stage IV, at week six displayed an ovarian stage of 6.429 ± 0.202, at week nine an ovarian stage of 8.714 ± 0.039, and at metamorphosis an ovarian stage of 9.667 ± 0.333 (Figure 1a. 2c). The average time to metamorphosis for control females was 105 ± 2.028 days (Figure 1b). *R. sphenocephala* appeared to display a rate closest to the accelerated ovary differentiation rate by displaying ovaries near Stage X at metamorphosis.

Chemical effect on somatic development

Somatic development of *B. americanus* displayed a chemical treatment effect ($F_{4,108}$ = 3.30, P = 0.014) and a chemical by time interaction ($F_{4,108}$ = 3.30, P = 0.014) for Gosner

stage as well as a chemical treatment effect on time to metamorphosis ($F_{4,66}$ = 2.90, P = 0.028; Figure 3a). Pairwise comparisons among treatment indicated that tadpoles from controls (P = 0.0136) and the medium treatment (P = 0.0271) developed significantly faster than those from the estradiol treatment (P = 0.0136). At week three, control (P = 0.0027) and the medium treatment (P = 0.0062) larvae were significantly more developed than those from the estradiol treatment (P = 0.0027), while larvae from the low treatment metamorphosed significantly faster than those from the estradiol treatment (P = 0.0099).

For *H. versicolor*, there was a chemical treatment effect ($F_{4,117}$ = 6.32; P < 0.001) and chemical by time interaction ($F_{4,117}$ = 6.32; P < 0.001) for Gosner stage (Figure 3b). There was also a chemical treatment effect on time to metamorphosis ($F_{4,72}$ = 4.80; P = 0.002). Although pairwise comparisons among treatments in general and at week three alone demonstrated that control tadpoles were not significantly different from any other treatment, controls did metamorphose significantly earlier (P = 0.0051) than the estradiol treated tadpoles. The larvae from the estradiol treatment developed significantly slower than medium (3ppb; P = 0.0005) and high (30ppb; P = 0.002) treated larvae. On a finer scale, the tadpoles from the estradiol treatment were significantly less developed than tadpoles from all three atrazine treatments (P < 0.05) at week three and metamorphosed significantly later than the low (1ppb; P = 0.0033) and high (P = 0.0269) treated tadpoles.

Somatic development of *R. sphenocephala* was not affected by chemical treatment in terms of Gosner stage ($F_{4,159}$ = 1.15; P=0.334) or time to metamorphosis ($F_{4,36}$ = 0.04; P =

0.996; Figure 3c). At a time scale more comparable to the other two species (weeks three and six only), there was no chemical treatment effect ($F_{4,82} = 0.87$; $P = 0.487$).

Chemical effect on ovarian development

The ovarian development of *B. americanus* (Figure 4a) and *H. versicolor* (Figure 4b) was not effected by chemical treatment ($F_{4,50} = 1.21$;$P = 0.317$ and $F_{4,42} = 0.28$; $P = 0.891$, respectively). The average ovarian stages for control larvae at week three and metamorphosis were 4.17 and 4.25 for *B. americanus* and 4 and 4.67 for *H. versicolor*, respectively.

For *R. sphenocephala*, there was a significant chemical treatment effect ($F_{4,79}= 16.77$; $P < 0.0001$; Figure 4c) as well as a significant chemical by time interaction ($F_{12,79} = 2.54$; $P = 0.007$) for ovarian development. In general, the control larvae and all atrazine treated larvae developed significantly faster than those from the estradiol treatment ($P < 0.001$). None of the treatments were significantly different at week three ($P > 0.05$). At week six, control tadpoles did not differ significantly from treated tadpoles ($P > 0.05$), however estradiol larvae were significantly slower than medium (3ppb; $P = 0.003$) and high (30 ppb; $P = 0.0087$) treated larvae but not different from low (1ppb; $P = 0.1271$). At week nine, tadpoles from controls were significantly faster than tadpoles from the estradiol ($P = 0.004$) treatment, while those from the estradiol treatment were significantly slower than the medium ($P = 0.0276$) and nearly from the high ($P = 0.0536$) but not the low ($P = 0.1459$) treated tadpoles. Also at week nine, tadpoles from the estradiol treatment are not significantly different from control, low, medium and high treated tadpoles at week six (P

> 0.05). By metamorphosis, the control larvae were significantly faster only from larvae from the estradiol treatment ($P = 0.001$), while the estradiol treated larvae were significantly slower in ovarian development than all atrazine treated larvae ($P > 0.05$). At metamorphosis, tadpoles from the estradiol treatment were not significantly different from any of the treatments at week nine ($P > 0.05$). When compared at weeks three and six only, a statistically significant effect of chemical treatment on ovarian development was demonstrated ($F_{4,52} = 3.51$; $P = 0.013$) as well as a chemical by time interaction ($F_{4,52} = 3.51$; $P = 0.013$). The average ovarian stages for controls at weeks three, six, nine and metamorphosis were 3.88, 6.43, 8.71, and 9.71, respectively.

DISCUSSION

Our results support our predictions for ovary differentiation rates in *Bufo americanus*, *Hyla versicolor*, and *Rana sphenocephala*. Moreover, they indicate that the rates of somatic and ovarian development may act as predictors of anuran susceptibility to estrogenic contaminants. Species following the retarded and basic ovary differentiation rates (*B. americanus* and *H. versicolor*, respectively) had the fastest somatic rates and were susceptible to estrogenic contaminants only in terms of their fastest rate – somatic development. In contrast, a species following the accelerated ovary differentiation rate (*R. sphenocephala*) displayed the slowest somatic developmental rate and was susceptible to estrogenic contaminants only in terms of its fastest rate – ovarian development.

According to Ogielska and Kotusz (2004), the rates of ovarian differentiation are defined by the ovarian stage relative to metamorphosis (Gosner stage 46). We found that *B. americanus* displayed the retarded rate and added support to the hypothesis that most *Bufo* follow a retarded ovary differentiation rate. *H. versicolor*, like most species examined (Ogielska and Kotusz 2004), appeared to exhibit a rate closest to the basic ovary differentiation rate by displaying ovaries near Stage V at metamorphosis. *R. sphenocephala* followed the accelerated rate and enhanced support for the hypothesis that accelerated ovarian rates are displayed by a subset of *Rana*. The ovary differentiation rates of the species examined here generally fit with our predictions based on the findings of Ogielska and Kotusz (2004).

Our results also demonstrate the close relationship between the rate of ovary differentiation, the length of the larval period, and the rate of somatic development. For example, the species with the shortest larval period (*B. americanus*) exhibited the fastest somatic developmental rate and the slowest ovary differentiation rate. The other species followed the expected trends with *H. versicolor* displaying an intermediate larval period and intermediate ovary differentiation rate. *Rana sphenocephala* had the longest larval period, slowest somatic rate, and fastest ovary differentiation rate. Thus, our predictions of ovarian differentiation rates based on larval period were supported.

When exposed to estrogenic contamination during the larval period, only species following the fastest rates demonstrated treatment effects. For somatic development, *B. americanus* and *H. versicolor*, the two species with the fastest somatic rates respectively,

demonstrated significant treatment effects; while *R. sphenocephala*, the species with the slowest somatic rate, did not. Others have found mixed effects in species following the retarded and basic rates (Ogielska and Kotusz 2004). At high concentrations, atrazine decreased growth in a basic rate species, *H. versicolor* (Diana et al. 2000), yet atrazine exposure of a species following the same basic rate, *X. laevis,* yielded mixed results on somatic development (Hayes et al. 2002, Sullivan and Spence 2003). For *B. americanus* exposed to high concentrations of atrazine, no effect was demonstrated on time to metamorphosis (Freeman et al. 2005). Somatic development was not affected in a species following the accelerated rate, *R. pipiens,* exposed to atrazine (Allran and Karasov 2000, Mackenzie et al. 2003).

For ovarian development, *R. sphenocephala*, the species with the fastest ovarian developmental rate, demonstrated significant treatment effects when exposed to estrogenic contamination during the larval period, while *B. americanus* and *H. versicolor*, the two species with the slowest ovarian developmental rates, respectively, did not. Under the same threats of estrogenic contamination, the two species with the slower ovarian rates did not exhibit treatment effects and thus were less susceptible to estrogenic contamination.

Slower ovarian rates may help these species elude exposure during sexual differentiation, which occurs at ovarian Stage IV (Ogielska and Kotusz 2004). At metamorphosis, *B. americanus* and *H. versicolor* were at or near sexual differentiation, while *R. sphenocephala* was well past sexual differentiation. Thus, each rate of ovary

differentiation determines the duration of estrogenic contaminant exposure during sexual differentiation. Therefore, for species with retarded and basic rates of ovary differentiation, estrogenic contamination may not have a chance to influence reproductive development as strongly as for species undergoing an accelerated rate. Accelerated rate species may be more susceptible to estrogenic contamination because all individuals of a population would be exposed during sexual differentiation (Stage IV). For females this may mean a slower rate of gonadal development, as was demonstrated here. Further, others have demonstrated that atrazine and estrogen exposure of males alters their reproductive development (Tavera-Mendoza et al. 2002a, Hayes et al. 2003, Mackenzie et al. 2003).

In terms of both somatic and ovarian development, estrogenic contamination seemed to slow development. The slowing result for somatic development may be due to a toxic effect by such high levels of estradiol (10^{-7}M). Kloas et al. (1999) found higher mortality of *Xenopus laevis* larvae exposed to 10^{-7}M estradiol than 10^{-8}M estradiol treatments. Estrogenic contamination at the level examined (10^{-7}M) slowed ovarian development by three weeks. For ovary development, exogenous estrogens may initiate a negative feedback mechanism (Kloas 2002). In *Xenopus laevis*, a basic rate species (Ogielska and Kotusz 2004), estrogen levels are high in hatchlings leading to the appearance of elevated estrogen receptor mRNAs; these concentrations are transferred maternally and result in the appearance of estrogen receptors (Bögi et al. 2002). Estrogen levels peak again for *X. laevis* around metamorphosis suggesting that individuals produce their own estrogen by this time (Bögi et al. 2002). Exogenous estrogen exposure can also stimulate estrogen

receptor gene expression (Bögi et al. 2002). These data support our findings of exogenous estrogens slowing ovarian development. *X. laevis* exposed to atrazine during sexual differentiation exhibited a reduced frequency of primary germ cells necessary for normal reproduction (Tavera-Mendoza et al. 2002b). The mechanism of estrogenic contamination may vary depending on the rate disrupted (somatic or ovarian).

The overall effect of atrazine was not as strong as that of the estradiol treatment used here (10^{-7}M). However, these data do not rule out the estrogenic potential of atrazine to alter somatic or ovarian development. Tadpoles treated with the lowest level of atrazine (1ppb) were not significantly different from the estradiol treated tadpoles for overall *B. americanus* and *H. versicolor* somatic development. Further, tadpoles treated with the low level of atrazine were not significantly faster in ovarian development than the estradiol treatment at weeks six and nine of *R. sphenocephala.* During weeks six and nine, *R. sphenocephala* reached Stage IV (appearance of first diplotene oocytes) and Stage VI (increase in number and size of diplotene oocytes) respectively. Mackenzie and colleagues (2003) found that anurans exposed to estrogens and antiestrogens exhibited significantly more oocytes in an early vitellogenic phase whereas controls displayed previtellogenic oocytes; vitellogenin is a yolk precursor protein dependent on estrogen for production. These data illustrate that, of the three levels of atrazine, only the tadpoles treated with the lowest level (1ppb) displayed effects suggestive of estrogenic activity examine here (10^{-7}M). Examining somatic and ovarian development under a range of estradiol concentrations and low concentrations of atrazine would be appropriate to clarify the role of atrazine exposure in somatic and ovarian development.

Overall, our data indicate that rates of somatic and ovarian development may be used as predictors of anuran susceptibility to estrogenic contaminants. Species, such as the *Bufo* examined here and by Ogielska and Kotusz (2004), with relatively short larval periods have fast somatic developmental rates and, in turn, relatively slow rates of ovarian development (retarded rate). These species appear to be susceptible to estrogenic contamination only in terms of somatic development. Conversely species, such as a subset of *Rana* (here *R. sphenocephala*), that have relatively long larval periods and therefore relatively slow somatic development are characterized as having an accelerated rate of ovary differentiation. This group appears most susceptible to estrogenic contamination in terms of gonad differentiation. Many other species develop at intermediate somatic and ovarian rates and follow a basic rate of ovary differentiation. Here, *H. versicolor* represented this group and was affected somatically but not in terms of ovary differentiation. Further, it is possible that species following both the retarded and basic rates might exhibit mixed results because many of them are at or near sexual differentiation (Stage IV) at metamorphosis; therefore, if environmental factors lengthen or shorten their larval period, their chance for exposure during sexual differentiation may be altered. Moreover, the data presented here illustrate that susceptibility to estrogenic contamination can be predicted based on the somatic and ovarian differentiation rates of a species and possibly simply by knowing the somatic rate. These data are important for the conservation of species threatened by estrogenic contamination.

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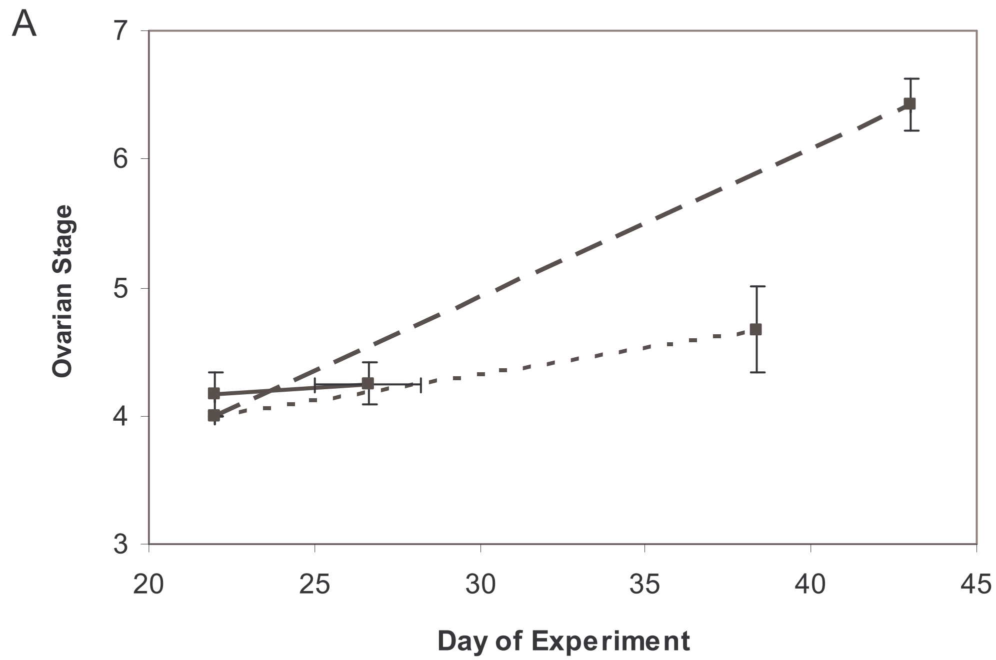
Figure 1. Developmental rates for *Bufo americanus* (solid), *Hyla versicolor* (dotted), and *Rana sphenocephala* (broken) from week three (day 22) to week six (day 43) only. (A.) Ovary differentiation based on Ogielska and Kotusz (2004) staging system (Stage I – X). (B.) Somatic development based on Gosner (1960) staging system (Stage 1-46). Error bars display standard error of the mean.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Ovarian Stage
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3
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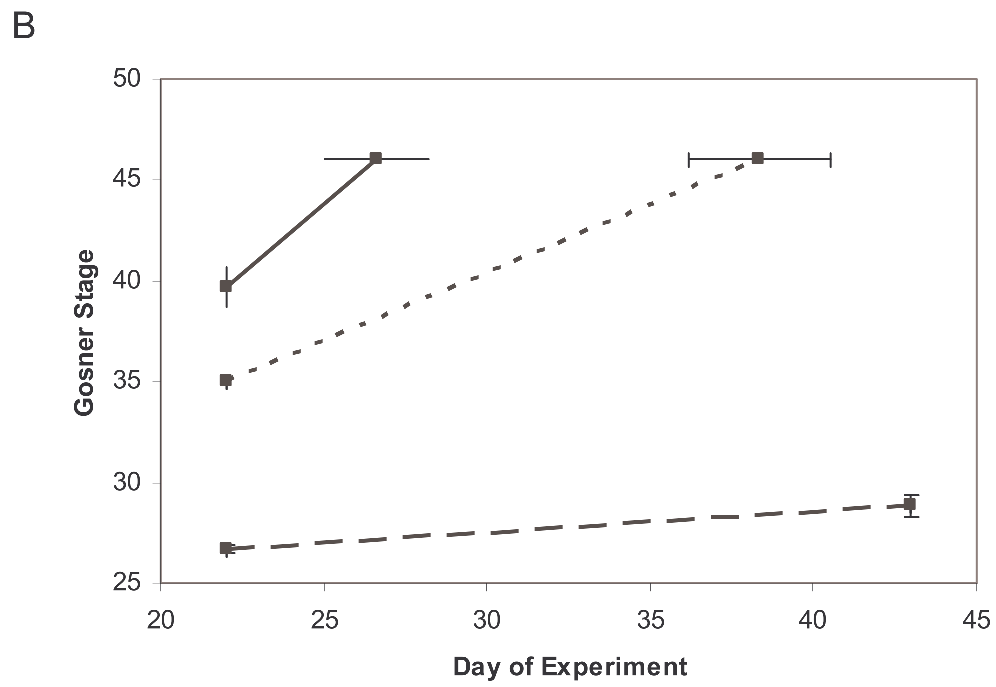
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Figure 2. Representative ovaries at metamorphosis. (A) *Bufo americanus* Stage IV, (B) *Hyla versicolor* Stage IV, (C) *H. versicolor* Stage V, and *Rana sphenocephala* (D) Stage IV, (E) Stage VI, (F) Stage VII, (G) Stage VIII, (H) Stage IX, (I) Stage X. Stage V was so rare in *B. americanus* and *R. sphenocephala*, suitable photographs could not be taken. (oc) = ovarian cavity, (bo) = Bidder's organ, (k) = kidney, (mc) = meiocytes, (do) = diplotene oocytes. The scale bar represents 30 µm.

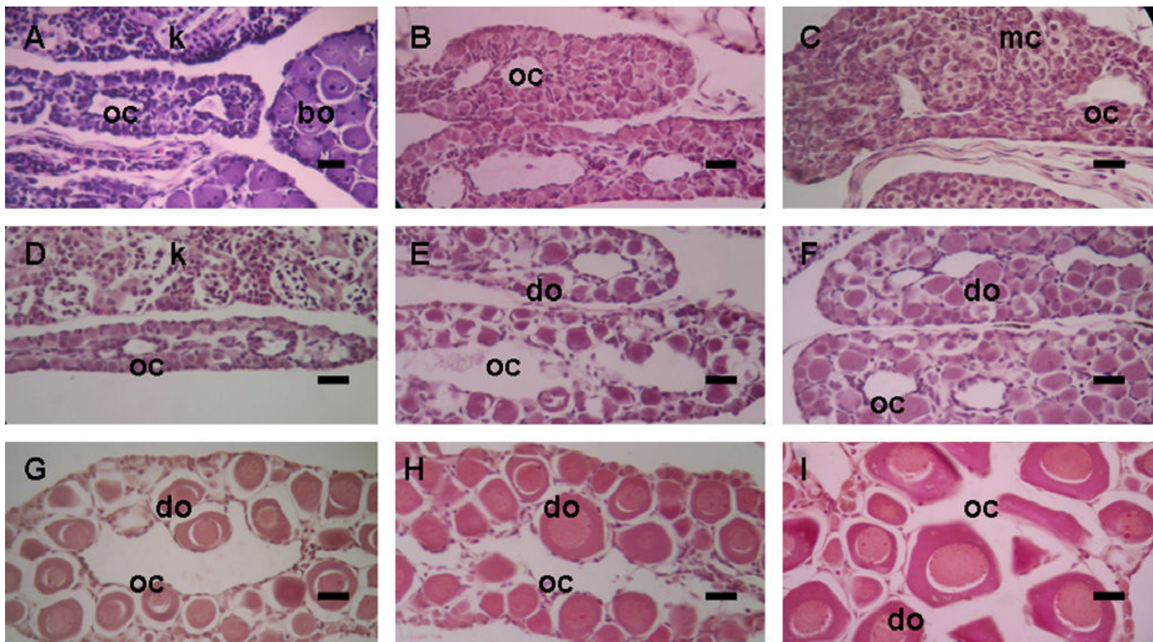
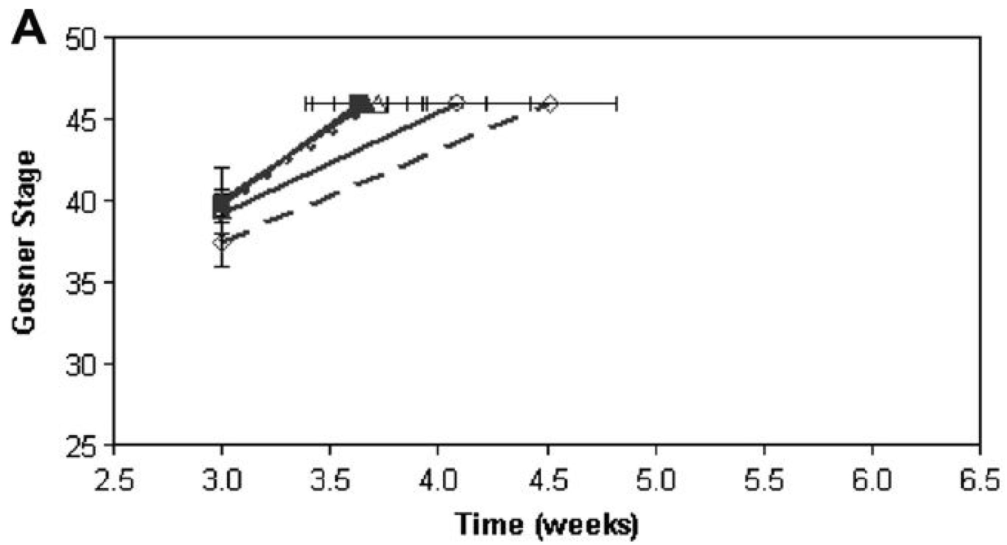
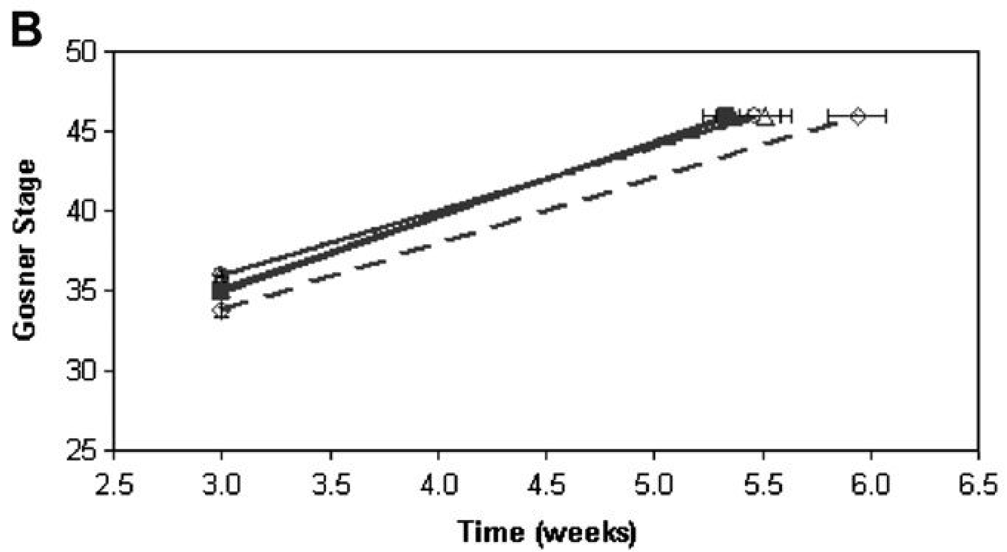


Figure 3. Effect of estrogenic contaminants on somatic development of (A.) *Bufo americanus*, (B.) *Hyla versicolor*, (C.) *Rana sphenocephala*. Solvent control = (solid line, filled square), estradiol = (broken line, open diamond), 1 ppb atrazine = (solid line, filled triangle), 3ppb atrazine = (dotted line, open triangle), 30ppb atrazine = (solid line, open circle). Error bars display standard error of the mean.


A
Gosner Stage
50
45
40
35
30
25
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
Time (weeks)


B
Gosner Stage
50
45
40
35
30
25
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
Time (weeks)

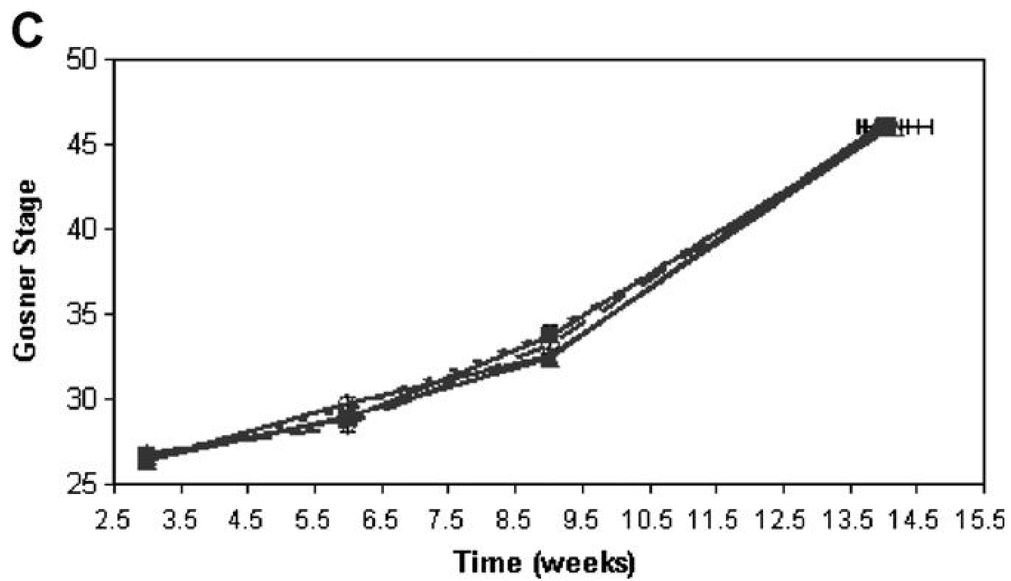
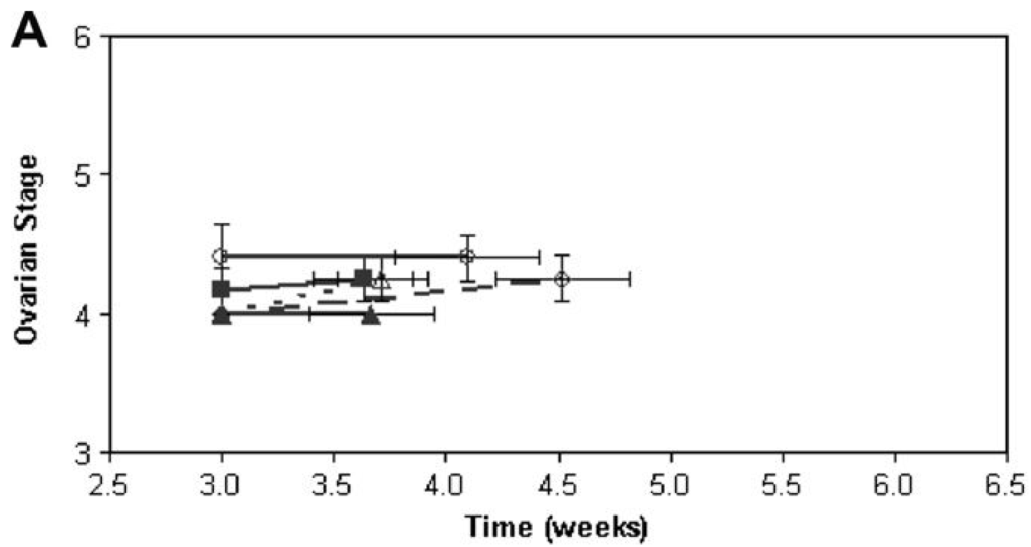
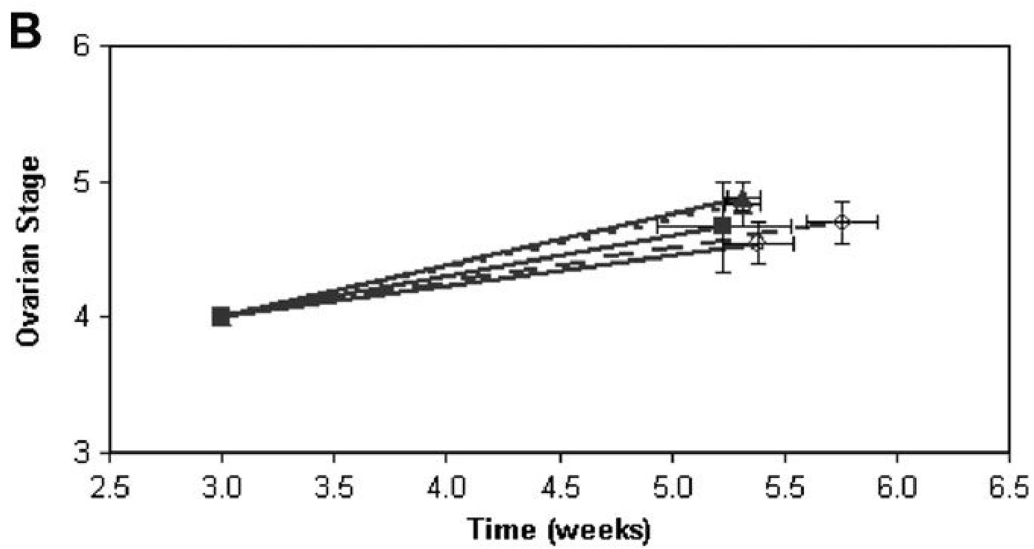
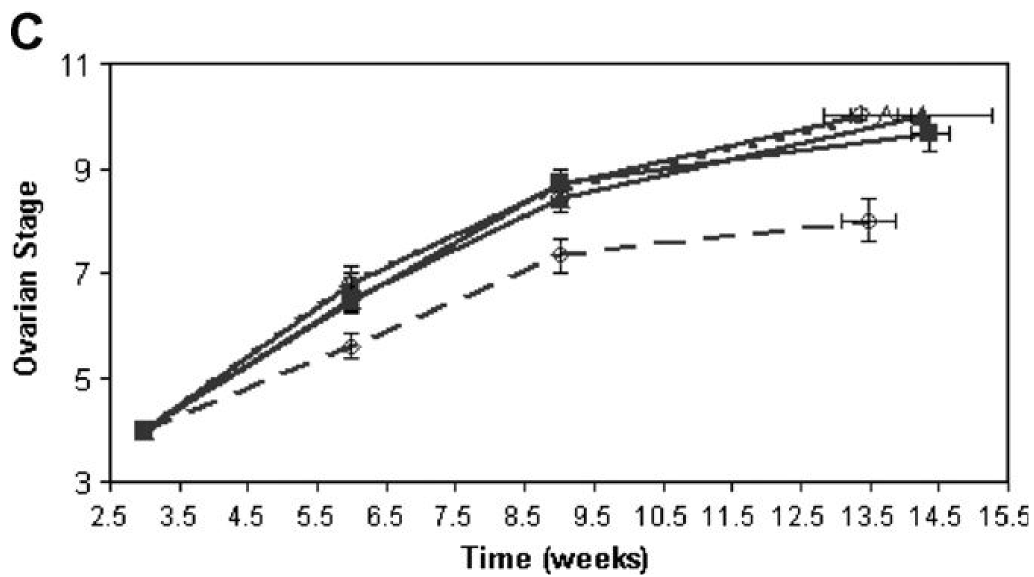

C
Gosner Stage
50
45
40
35
30
25
2.5
3.5
4.5
5.5
6.5
7.5
8.5
9.5
10.5
11.5
12.5
13.5
14.5
15.5
Time (weeks)

Figure 4. Effect of estrogenic contaminants on ovarian development of (A.) *Bufo americanus*, (B.) *Hyla versicolor*, (C.) *Rana sphenocephala*. Solvent control = (solid line, filled square), estradiol = (broken line, open diamond), 1 ppb atrazine = (solid line, filled triangle), 3ppb atrazine = (dotted line, open triangle), 30ppb atrazine = (solid line, open circle). Error bars display standard error of the mean.


A
Ovarian Stage
6
5
4
3
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
Time (weeks)


B
Ovarian Stage
6
5
4
3
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
Time (weeks)


C
Ovarian Stage
11
9
7
5
3
2.5
3.5
4.5
5.5
6.5
7.5
8.5
9.5
10.5
11.5
12.5
13.5
14.5
15.5
Time (weeks)

CHAPTER 3

INTERSEX GONADS IN FROGS: UNDERSTANDING THE TIME COURSE OF NATURAL DEVELOPMENT AND ROLE OF ENDOCRINE DISRUPTORS

ABSTRACT

The paucity of data on sexual development of anuran amphibians has played an important role in the recent controversy over atrazine exposure. While some studies have demonstrated the presence of abnormal gonads in control treatments, others have not, leading to varying interpretations of the effects of atrazine exposure on sexual development. However, the timing of development varies among anuran amphibians such that, at any snapshot in time, different species may exhibit different stages of sexual differentiation. We examined three species representing each of the differentiation rates (*Bufo americanus* = retarded rate; *Hyla versicolor* = basic rate; *Rana sphenocephala* = accelerated rate), to examine the natural time course of sexual development along with the influence of atrazine exposure. For each species, exposure to atrazine (1, 3, 10, 30 parts per billion), 17-β-estradiol or control water occurred throughout larval life. Gonad histology was performed at three-week intervals during the larval period or at a juvenile stage to examine the proportion of males, females, underdeveloped testes, testicular oocytes (TO; testes with 0-30% oocytes), and ovotestes OVT (testes with >30% oocytes). Our results illustrate that a phase of intersex gonads (TO or OVT) is normal during *R. sphenocephala* sexual development, a species representing the accelerated differentiation rate. Further, intersex gonads were found in juvenile stages of *B. americanus* and *H.*

versicolor, representing retarded and basic rates respectively, suggesting that a phase of intersex may be common regardless of differentiation rate. Moreover, these data highlight the importance of longitudinal studies rather than snapshots in time.

INTRODUCTION

Sexual development of anuran amphibians can be altered by exogenous hormone exposure during sexual development (reviewed in Hayes 1998). Contaminants that mimic hormones (endocrine disrupting contaminants; EDCs) are prevalent in the environment due to the widespread use of pesticides and industrial chemicals. EDCs can be distributed worldwide through surface water runoff, sewage effluent, aerial drift, and even rainfall (Colborn et al. 1993). Exposure to such contaminants has resulted in numerous reproductive abnormalities in wildlife (Colborn et al. 1993, Hotchkiss et al. 2008) and has even been linked to amphibian declines (McCoy et al. 2008).

Atrazine is a widespread herbicide used primarily with corn and sorghum. Since Hayes and colleagues (2002) published a hypothesis that atrazine induced aromatase, the enzyme that converts testosterone to estrogen, investigations of atrazine's impact on reproductive development have flooded the literature. The species concerned include amphibians (e.g. Hayes et al. 2003, Carr et al. 2003), fish (Bringolf et al. 2004, Suzawa & Ingraham 2008), and reptiles (de Solla et al. 2006, Stoker et al. 2008); however, most reports focus on anuran amphibians with *Xenopus laevis* (African clawed frog) and *Rana pipiens* (Northern leopard frog) sharing the majority of reports. Endpoints of several amphibian investigations include plasma hormone levels, gonad aromatase, larynx size,

and gonad morphology and histology (Hayes et al. 2002, Hayes et al. 2003, Carr et al. 2003, Hecker et al. 2004, Coady et al. 2005, Hecker et al. 2005, Murphy et al. 2006).

Amphibian gonad histology has come under much scrutiny with the presence of oocytes in testicular tissues being highlighted often (McDaniel et al. 2008). Some authors reported testicular oocytes in controls and contend that it is a normal part of gonad development (e.g. Coady et al. 2004 Jooste et al. 2005), while others reported an absence of testicular oocytes in controls but found them in amphibians exposed to either estradiol or low levels of atrazine (e.g. Hayes et al. 2002, Carr et al. 2003). In an attempt to offer some clarity to these contradicting results, our study examined the incidence of testicular oocytes in three species that exhibit varying rates of gonad development and are differentially susceptible to EDC exposure (Storrs and Semlitsch 2008).

Ogielska and Kotusz (2004) demonstrated that anuran amphibians follow one of three ovary differentiation rates. Most species are slightly past sexual differentiation at metamorphosis, the basic rate. Species near or at sexual differentiation at metamorphosis have the retarded rate, found in most *Bufo*. Species with ovaries that are at or near full sexual differentiation at metamorphosis follow the accelerated rate, which includes many, but not all, *Rana*. Therefore, if a species differentiates before or during metamorphosis, they run the risk of concurrent exposure to aquatic contaminants, while those that sexually differentiate after metamorphosis would not.

Bufo americanus (American toads), *Hyla versicolor* (gray treefrogs), and *Rana sphenocephala* (Southern leopard frogs) are native to central Missouri. They were chosen due to their varying lengths in larval periods (and thus exposure) and rates of ovary differentiation (Storrs and Semlitsch 2008). *B. americanus* have the shortest larval period of the three species and follow a retarded rate of differentiation. *H. versicolor* have an intermediate larval period and follow the basic rate of differentiation. *R. sphenocephala* display the longest larval period and follow the accelerated rate of ovary differentiation.

We have previously demonstrated that *R. sphenocephala*, a species that is at risk of exposure to aquatic contamination throughout sexual differentiation (accelerated rate), is more susceptible to endocrine disruption as larvae and metamorphs than species following slower rates of sexual differentiation (Storrs and Semlitsch 2008). Our goal in the current study was to determine if this susceptibility had an effect on gonad morphology in the terrestrial anurans no longer exposed to aquatic contaminants. To do so we examined gonad morphology over time in control, positive control (estradiol) and atrazine treatments in three species exhibiting the three rates of ovary differentiation (Storrs and Semlitsch 2008). Other atrazine studies have examined amphibian gonad development over time (Jooste et al. 2005, Du Preez et al. 2008), however the species investigated (*Xenopus laevis*) was fully aquatic and therefore may not offer a good model for amphibians with terrestrial life stages.

MATERIALS AND METHODS

Experimental Design

Two sets of laboratory experiments were conducted at the University of Missouri using American toads (*Bufo americanus*), gray treefrogs (*Hyla versicolor*), and Southern leopard frogs (*Rana sphenocephala*). The first set of experiments monitored the development of gonads of each species from free swimming tadpoles (Gosner stage 25; Gosner 1960) to metamorphosis at three-week intervals (larval experiments), and a second set of experiments examined gonads of each species as terrestrial juveniles regardless of somatic development (juvenile experiments).

The larval experiments incorporated three concentrations of atrazine exposure (1, 3, and 30 ppb, parts per billion), a solvent control (ethanol), and 17-ß-estradiol (10^{-7}M). A sufficient number of animals were used in each treatment such that 10 animals from each treatment could be removed at three-week intervals during the larval period of each species to assay gonad development. Any remaining at metamorphosis were sacrificed for examination of gonad morphology. Individual *R. sphenocephala* were sacrificed at three, six, and nine weeks and at metamorphosis, while *B. americanus* and *H. versicolor* were only sacrificed at three weeks and metamorphosis due to their shorter larval period. Tadpoles for each larval experiment were raised individually in one-liter glass canning jars filled with 800 ml of UV-sterilized, carbon-filtered water. Jars were replicated on shelves with each vertical level representing as a spatial block. Five treatments were replicated ten times in three blocks for *B. americanus* (n = 150), six times in five blocks

for *H. versicolor* (n = 150), and sixteen times in three blocks for *R. sphenocephala* (n = 240).

The juvenile experiments incorporated two concentrations of atrazine exposure (1 and 10 ppb), a solvent control (ethanol), and 17-ß-estradiol (10^{-7}M). Tadpoles were raised in glass aquaria filled with 20 liters of UV-sterilized, carbon-filtered water. Each aquarium was initially stocked with 20 tadpoles of only one species for the *H. versicolor* and *R. sphenocephala* experiments, while each aquarium was initially stocked with 12 tadpoles for the *B. americanus* experiment. For each species, four treatments were replicated three times with one replicate on each vertical block.

Dosing

Stock solutions of analytical grade atrazine (99.2% atrazine; Sigma-Aldrich, Allentown PA) were created with an ethanol solvent. During water changes, jars (larval experiment) were dosed with 100 µl of stock solution to yield 1, 3, or 30 ppb atrazine, and aquaria (juvenile experiment) were dosed with 2.5 ml of stock solution to yield 1 or 10 ppb atrazine. Identical amounts of solvent control (ethanol) and estradiol (10^{-7}M 17-β-estradiol) were added to the appropriate test chambers in each experiment. Estradiol solutions were mixed for each water change. The concentration of estradiol was chosen because exposure of tadpoles to this dose results in a significant number of females (Kloas et al 1999).

Water samples for each treatment in the larval experiment (n = 5) and each treatment in the juvenile experiment (n = 4) were collected from all test chambers from each experiment to assess atrazine concentration. An atrazine ELISA kit (antibody-coupled paramagnetic particles, Abraxis, Philadelphia PA) was used to analyze the samples. The minimum reportable concentration for this kit was 0.05 ppb atrazine. Anything below this concentration was reported as nondetectable. Concentrations are displayed as mean ± standard deviation in parts per billion.

Estrogenic activity was tested using an MCF-7 cell proliferation assay as described by Howdeshell and colleagues (2003). Water samples were taken from carbon-filtered, UV-sterilized water (n = 3), carbon-filtered, UV-sterilized water in jars (n = 3), and from water in jars dosed with estrogen (n = 3). Estrogenic activity is displayed as a mean (ng E2 eq/ml) ± standard deviation.

Animal Collection and Maintenance

All species were obtained from Thomas Baskett Wildlife Area, Boone County, MO. Tadpoles from the larval experiments were pooled from egg masses laid by three amplexed pairs of *H. versicolor* in 2005. Tadpoles were pooled from two and three egg masses collected directly from ponds for *R. sphenocephala* in 2005 and *B. americanus* in 2006, respectively. Tadpoles for the juvenile experiment were pooled from egg masses laid by three amplexed pairs of *H. versicolor* in 2006. Tadpoles were pooled from two and three egg masses collected directly from ponds for *R. sphenocephala* in 2006 and *B. americanus* in 2007, respectively.

For all species, eggs developed into free-swimming tadpoles (Gosner stage 25; Gosner 1960) and were randomly assigned to jars or aquaria. For each experiment, water was completely changed every third day to remove waste, replenish oxygen levels, renew chemical concentrations, and to monitor mortality. When the first metamorph was found, jars were checked every day. Individuals with at least one forelimb (Gosner stage 42 – 46) were removed from the test chambers and allowed to fully metamorphose (Gosner stage 46, complete tail resorption) in a plastic container (5cm x 13cm) with about 1 cm of water and tilted to allow both wet and dry areas. Once fully metamorphosed, individual mass (to the nearest 0.0001 g) and time to metamorphosis (days from the start of the experiment) were recorded for each individual. At the appropriate time, each animal was anesthetized in MS-222 and the gonad-kidney complex was dissected for histological analysis. Animals in the larval experiment were fixed whole in 10% neutral buffered formalin and subsequently were dissected, while animals in the juvenile experiment were reared to 4 months of age for *R. sphenocephala* and *H. versicolor* in an animal facility in 2005 while *B. americanus* were reared to nearly 6 months of age in outdoor pens in 2006. Juveniles were anesthetized and the gonad-kidney complex was dissected immediately and fixed in Bouin's fixative. Prior to anesthetization, *B. americanus* were subjected to dehydration tests the results of which will be reported elsewhere. The use of animals in this study was approved by the Institutional Animal Care and Use Committee of the University of Missouri (IACUC # 2774).

Gonad histology & morphology

Gonads were dissected and processed using a Shandon Excelsior tissue processor (Thermo Electron Corp., Massachusetts). Gonads were subsequently embedded in paraffin and sliced longitudinally into 7µm sections (AO Spencer microtome). Sections were placed on slides and stained with haematoxylin and eosin. Slides were examined using an inverted microscope (Nikon Diaphot) and a compound light microscope (Olympus CX21).

Gonads were examined for cell types to assign sex. Sex categories included male and female (no abnormalities), testicular oocytes (TO; testis with 0-30% oocytes [Hecker et al. 2006]), ovotestis (OVT; testis with > 30% testicular oocytes [Hecker et al. 2006]), and underdeveloped testis (UT; poorly structured, closed lobules or no lobules at all, and only a few or the complete absence of germ cells [Hecker et al. 2006]). Because the sex of 49% of *H. versicolor* tadpoles at week three were unidentifiable, all data for week three were excluded for all three species. Of the remaining samples in the larval experiment, 9.3 % were unidentifiable. All samples in the juvenile experiment were identifiable.

Statistical analysis

For all experiments, sex ratios were examined using a randomization test of goodness of fit comparing estradiol and all atrazine treatments to control ratios as well as comparing atrazine treatments to estradiol ratios. Statistical significance was established for all analyses by a P-value < 0.05.

RESULTS

Water samples

Nominal concentrations for the larval experiments were 0 (solvent control), 0 (17-β-estradiol), 1, 3, and 30 ppb of atrazine. For *B. americanus*, actual concentrations were nondetectable for the control and estradiol samples with atrazine treatments at 2.7 ± 0.75, 7.55 ± 2.82, and 124.87 ± 41.26 ppb, respectively. For *H. versicolor*, actual concentrations were nondetectable for the controls with estradiol having one sample 0.05 ppb and all others nondetectable. Atrazine treatments for *H. versicolor* were at 0.92 ± 0.07, 2.81 ± 0.15, and 25.1 ± 7.06 ppb, respectively. For *R. sphenocephala*, actual concentrations were nondetectable for control and estradiol samples with atrazine treatments at 0.80 ± 0.05, 3.13 ± 0.25, and 30.36 ± 1.23 ppb, respectively.

Nominal concentrations for the juvenile experiments were 0, 0, 1, and 10 ppb atrazine. For *B. americanus*, actual concentrations were nondetectable for control with estradiol at 0.07 ± 0.02 and atrazine treatments at 3.02 ± 0.25 and 31.90 ± 1.79 respectively. For *H. versicolor*, actual concentrations were nondetectable for the control and estradiol samples with atrazine treatments at 4.12 ± 0.10 and 29.52 ± 8.74 ppb, respectively. For *R. sphenocephala*, actual concentrations were nondetectable for control and estradiol samples with atrazine treatments at 1.46 ± 0.11 and 24.58 ± 9.60 ppb, respectively.

Estrogenic activity of water samples was 0.0161 ± 0.0016 in filtered water, 0.0007 ± 0.0004 ng E2 eq/ml in filtered water from jars, and 8.615 ± 4.317 ng E2 eq/ml for water in jars dosed with 17-β-estradiol.

Larval experiment sex ratios

All *B. americanus* metamorphs (Fig. 1A; average day to metamorphosis = 28) expressed male and female gonads. Metamorphs in the control treatment of this species displayed nearly a 1:1 sex ratio of males to females (53% males) at metamorphosis. There were no significant differences ($P > 0.05$) between the treatments for *B. americanus* at metamorphosis except for the control vs. 30 ppb ($P = 0.047$). The larvae exposed to 30 ppb expressed significantly more female gonads at metamorphosis than the control. Those exposed to 30 ppb did not differ from those tadpoles exposed to estradiol; both had a higher incidence of females than controls.

H. versicolor metamorphs (Fig. 1B; average day to metamorphosis = 41) expressed male and female gonads with one UT in the 1 ppb atrazine treatment. Metamorphs in the control treatment had a male-biased sex ratio (73% males). All *H. versicolor* treatments had significantly different sex ratios from the control ($P < 0.05$) except the 3 ppb treatment ($P = 0.232$). None of the gray treefrogs exposed to atrazine had significantly different sex ratios than those exposed to estradiol; estradiol- and atrazine-treated treefrogs all had a higher incidence of females than controls.

R. sphenocephala metamorphs (Fig. 2C; average day to metamorphosis = 103) expressed testes, ovaries, TO, and OVT. Control tadpoles at week six (Fig. 2A) and nine (Fig. 2B) had female biased sex ratios with very few males (11 and 0% males respectively), but by metamorphosis, 50% of those in the control expressed male gonads (Fig. 2C). Controls also expressed both TO and OVT. Testicular oocytes were present in control *R.*

sphenocelphala at weeks six and nine and at metamorphosis (11, 10, and 20% respectively), while OVT were only present in controls at week nine (20%). None of the sex ratios for *R. sphenocephala* exposed to atrazine at week six were significantly different (P > 0.05) from the control or estradiol treated larvae. All treatments at week six included TO and contained large proportions of females; OVT were present only among atrazine-treated larvae. However, at week nine sex ratios for nearly all chemical treatments were significantly different from the control larvae (estradiol, P = 0.027; 1 ppb, P = 0.074; 3 ppb, P = 0.014; 30 ppb, P = 0.002) with higher proportions of TO in chemical treatments. At metamorphosis, none of the sex ratios were significantly different (P > 0.05) except in the comparison between estradiol and 30 ppb (P = 0.026); the 30 ppb-treated larvae had a higher incidence of males than the estradiol-treated larvae and no OVT.

Junvenile experiment sex ratios

B. americanus were raised to 166 ± 1 day (average ± standard error mean) after the larval exposure began. Juveniles of this species expressed testes, ovaries, TO, and OVT (Fig. 3A). In TO, testes with 0-30% oocytes, oocytes were often found at the proximal end of the gonad adjacent to the Bidder's organ, a latent ovary found in both sexes of *Bufo*. These oocytes appeared to be "bleeding" into the testis tissue from the Bidders' organ. Toads in the control treatment included testes, ovaries, and TO. Ovotestes were found only in toads of the estradiol treatment. In comparison to toads of the control treatment, only toads exposed to 1 ppb atrazine had a significantly different sex ratio with a higher inicidence of females and no TO. Toads exposed to 1 ppb atrazine during their larval

period expressed a high proportion of females (71%) but did not express any TO. In comparison to toads of the estradiol treatment, only toads exposed to 10 ppb had a significantly different sex ratio with a lower proportion of females, a higher proportion of TO, and no OVT. Toads exposed to 10 ppb atrazine during their larval period expressed testes, ovaries, and TO, whereas toads exposed to estradiol expressed testes, ovaries, and OVT.

H. versicolor were raised to 124 ± 0.15 days after the larval exposure began. Juvenile gray treefrogs expressed testes, ovaries, OVT, and UT (Fig. 3B). Treefrogs in the control treatment expressed testes, ovaries, and OVT with males representing the majority of the sexes (58%). Underdeveloped testes were found only in treefrogs exposed to estradiol along with testes, ovaries, and OVT. None of the treefrogs in the chemical treatments had significantly different sex ratios from the controls, and all treatments had significantly different sex ratios from those exposed to estradiol ($P < 0.05$); estradiol-treated *H. versicolor* expressed a low incidence of males, a high incidence of OVT, and UT. The non-estradiol treatments had greater proportions of males and an absence of UT.

R. sphenocephala were raised to 120 ± 0.05 days after the larval exposure began with the controls expressing nearly a 1:1 (male:female) sex ratio (Fig. 3C). Southern leopard frogs expressed all sex categories (Fig. 4; UT not shown). Similar to *H. versicolor*, none of the leopard frogs in the chemical treatments had significantly different sex ratios from the controls, and all treatments had significantly different sex ratios from those exposed

to estradiol ($P < 0.05$); estradiol-treated *R. sphenocephala* expressed a higher incidence of females than any other treatment. Non-estradiol treated *R. sphenocephala* had a significantly lower proportion of females than those in the estradiol treatment. However, leopard frogs exposed to 10 ppb atrazine displayed a higher proportion (though non-significant) of females than the control ($P = 0.092$). Additionally, only chemical treatments expressed other sex categories in addition to male and female.

Sex ratios over time

Figure 5 illustrates the gonad types for *R. sphenocephala* across both larval and juvenile stages for controls (Fig. 5A) and estradiol-treated (Fig. 5B) larvae as well as those exposed to 1 ppb (Fig. 5C) and 10 ppb (Fig. 5D) atrazine. No juvenile experiment was conducted for 10 ppb atrazine. Data for 3 and 30 ppb are not shown; these exposures were only used during the larval experiment. Females were present across all life stages represented, while males were present in all stages except week nine. Testicular oocytes or OVT were present in controls through metamorphosis but were absent in the juvenile stage. Testicular oocytes and OVT were also present in the estradiol and 1 ppb atrazine treatments through metamorphosis, however OVT persisted into the juvenile stage in these treatments as well as in the 10 ppb atrazine treatment. Underdeveloped testes were only found in juveniles exposed to atrazine.

DISCUSSION

Our data demonstrate the importance of understanding the normal sexual development of a population when examining the effects of endocrine disruptors. These data illustrated

that a phase of intersex in *R. sphenocephala* appeared *en route* to full sexual differentiation. At week six of larval life the majority of controls were female, but that proportion decreased over time. At week nine, the presence of OVT in controls coupled with an absence of males suggests that a portion of females from week six go through week nine in a state of transition *en route* to becoming males shortly after metamorphosis. By the juvenile stage, *R. sphenocephala* control frogs expressed nearly a 1:1 (male:female) sex ratio with no other gonads present. Witschi (1921, 1930) reported a similar event for *R. temporaria* with most larvae developing first as females and then into males after metamorphosis. More recent studies involving EDC exposure have noted the presence of some TO in control treatments of recently metamorphosed amphibians (Mackinzie et al. 2003, Coady et al. 2004, Jooste et al. 2005) and in hatchling turtles (de Solla et al. 2006).

The presence of TO and OVT appeared to be normal in control *R. sphenocephala*. However, it was only normal during larval life and metamorphosis. For this population, the presence of abnormal gonads in the juvenile stage was only found in frogs exposed to estradiol or atrazine during their larval life suggesting the possibility that exposure to aquatic EDCs during sexual differentiation can alter gonad morphology such that it continues into the terrestrial juvenile stage. The persistence of abnormal gonads past EDC exposure has also been demonstrated in *Xenopus tropicalis.* Petterson and colleagues (2006) found that exposure to ethynylestradiol during *X. tropicalis* larval life resulted in persistent phenotypic sex reversal of males to females at nine months.

Abnormal gonads were also present in control toads and treefrogs. For *B. americanus*, gonads with TO were present in control juveniles (at 166 days), while OVT were found in control *H. versicolor* during their juvenile stage (at 124 days). *B. americanus* and *H. versicolor* both exhibit slower rates of ovary differentiation (retarded and basic, respectively) than *R. sphenocephala* (accelerated; Storrs and Semlitsch 2008). If *B. americanus* and *H. versicolor* follow the same developmental pathway as *R. sphenocephala* by going through a phase of intersex, the presence of abnormal gonads in control toads and treefrogs would indicate that neither had completed sexual differentiation at the juvenile stages examined here.

Larvae exposed to estradiol also illustrated some patterned development. Similar to controls, estradiol-treated *R. sphenocephala* exhibited a high proportion of females at week six. However by week nine, when controls went through a transitional phase, estradiol-treated leopard frogs exhibited an exaggerated transitional phase. While control frogs came through the transitional phase to yield a greater proportion of males at metamorphosis, sex ratios for estradiol-treated frogs never fully "recovered" from the transition resulting in only a small proportion of males. Leopard frogs in the estradiol treatment continued to maintain high proportions of abnormal gonads and females. By 120 days, estradiol-treated *R. sphenocephala* exhibited a female-biased sex ratio. *B. americanus* treated with estradiol also demonstrated female-biased sex ratios in general. *H. versicolor* juveniles exhibited very few females and a large proportion of abnormal gonads, a pattern similar to estradiol-treated *R. sphenocephala* at nine weeks.

Results of atrazine exposure in all species established the potential for the herbicide to alter amphibian sex ratios. At metamorphosis, *B. americanus* exposed to 30 ppb atrazine had a significantly higher proportion of females than controls. For *Hyla versicolor* and *Rana sphenocephala* (metamorphosis and nine weeks, respectively), exposure to low levels of atrazine resulted in deviations from the control sex ratio. Moreover, at these time periods *H. versicolor* and R. *sphenocephala* exposed to atrazine had higher incidences of female and abnormal gonads than the control and were similar to those treated with estradiol.

Underdeveloped testes were found only in animals exposed to a chemical treatment. For *H. versicolor,* one UT was found among larvae exposed to 1 ppb atrazine at metamorphosis and two among larvae exposed to estradiol in the juvenile experiment. For *R. sphenocephala,* one was found in each group of larvae exposed to atrazine in the juvenile experiment. These data indicate that UT are not normal, because they are not found in controls, and that this condition is associated with exposure to contaminants.

Our data illustrate the importance of understanding gonad development over time, as opposed to a snapshot in time, when investigating the effects of contaminants. In *R. sphenocephala* for example, gonads with TO were present at metamorphosis in control frogs as well as chemical treatments. However in the juvenile experiment abnormal gonads were found only in frogs exposed to estradiol and atrazine illustrating the potential for these contaminants to affect gonad development for *R. sphenocephala* even after exposure ended.

Our data also highlight the importance of understanding the developmental differences between species in order to understand contaminant effects. We demonstrated that species following the accelerated rate of ovary differentiation, here *R. sphenocephala*, serve as a good model for examining the effects of contaminants in gonad development because 1) they have the longest larval period and are therefore exposed to the contaminant for the longest period of gonad development, and 2) they reach full gonad development fastest. However, the fate of other species may not be represented by such models because their ovarian development is slower. If those exhibiting the retarded or basic ovary differentiation rates, here *B. americanus* and *H. versicolor*, respectively, follow similar pathways as accelerated-rate species, the phase of intersex transition would occur during early terrestrial life. Our data indicate that in the juvenile stage *B. americanus* and *H. versicolor* in the control treatment both exhibit abnormal gonads. *B. americanus*, the species with the slowest rate of differentiation, has the greatest proportion of abnormal gonads in the control treatment, followed by *H. versicolor*, the species with an intermediate rate, while *R. sphenocephala*, the species with the fastest rate of differentiation, do not exhibit any abnormal gonads in the control treatment. Further, although the species with slower ovary differentiation rates would be free from aquatic exposure to EDCs while completing sexual differentiation, the threat of exposure can continue in the terrestrial environment. Storrs-Mendez and coauthors (in press) demonstrated that terrestrial *B. americanus* can take up radiolabeled atrazine across their skin and directly into the bloodstream. The effects of direct exposure to contaminants across all life stages should be considered for gonad development because full sexual differentiation may not occur until early terrestrial life.

Our data illustrate the potential of atrazine to alter gonad development and may offer clarity in the contradicting results in recent atrazine studies. These data demonstrate that, when the rate of ovary differentiation is considered, species undergoing all rates of differentiation express abnormal gonads at some point in their development. However at full sexual differentiation only those exposed to a chemical treatment persist in expressing abnormal gonads. Moreover, our data indicate the importance of longitudinal studies rather than snapshots in investigating the effects of EDCs on gonad development especially in the cases of species exhibiting retarded and basic rates of differentiation.

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Figure 1. Sex ratios of (A) *Bufo americanus* and (B) *Hyla versicolor* at metamorphosis in the larval experiment. C = control; E = estradiol; 1PPB, 3PPB, 30 PPB = concentrations of atrazine. Sample size of each treatment shown in parentheses. Black = female, gray = male, diagonal hatch = underdeveloped testes. * = significantly different from control, + = significantly different from estradiol.

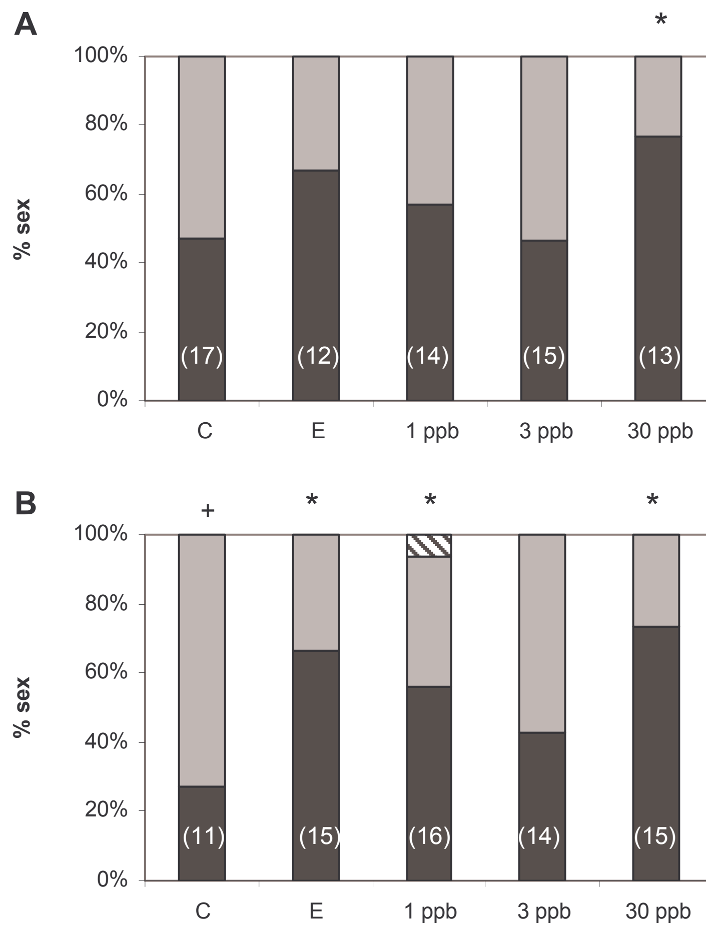
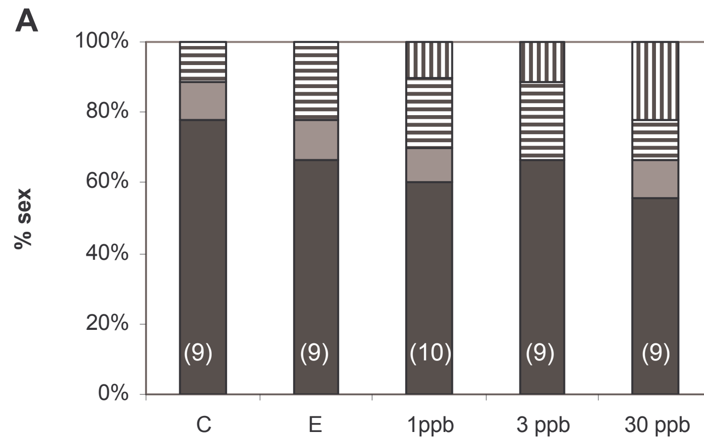


Figure 2. Sex ratios of *Rana sphenocephala* at (A) 6 weeks, (B) 9 weeks, (C) metamorphosis in the larval experiment. Black = female, gray = male, horizontal hatch = testicular oocytes, vertical hatch = ovotestes. * = significantly different from control, + = significantly different from estradiol.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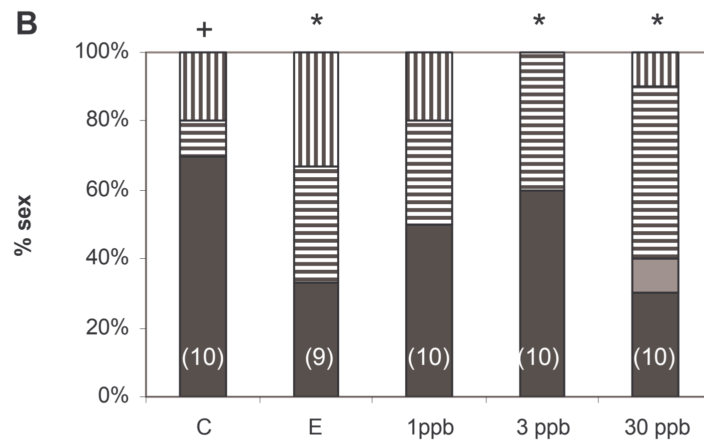
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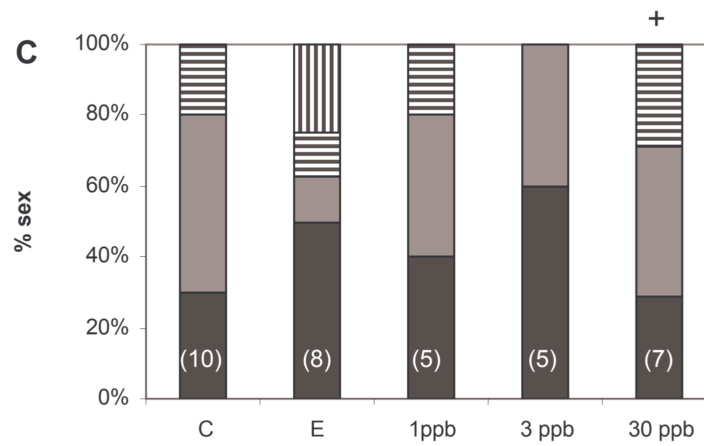
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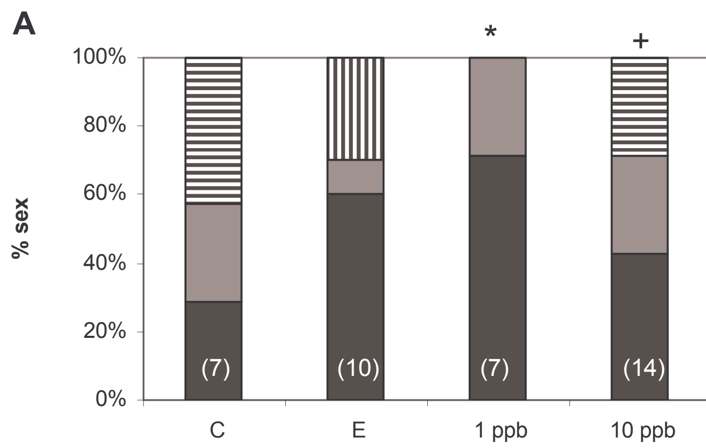
Figure 3. Sex ratios of juvenile (A) *Bufo americanus*, (B) *Hyla versicolor* and (C) *Rana sphenocephala.* C = control; E = estradiol; 1PPB, 3PPB, 30 PPB = concentrations of atrazine. Sample size of each treatment shown in parentheses. Black = female, gray = male, horizontal hatch = testicular oocytes, vertical hatch = ovotestes, diagonal hatch = underdeveloped testes. * = significantly different from control, + = significantly different from estradiol.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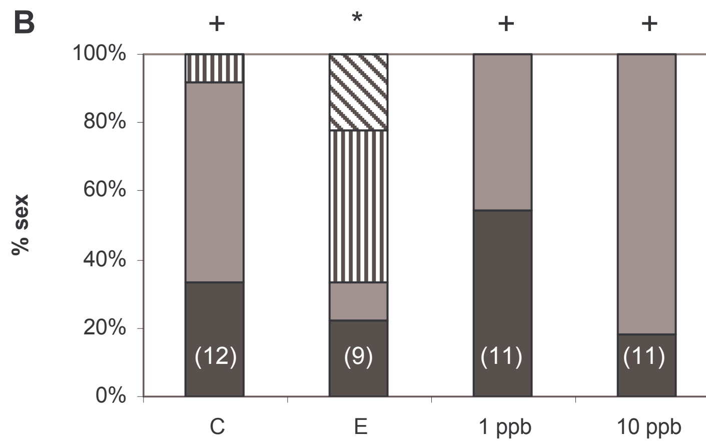
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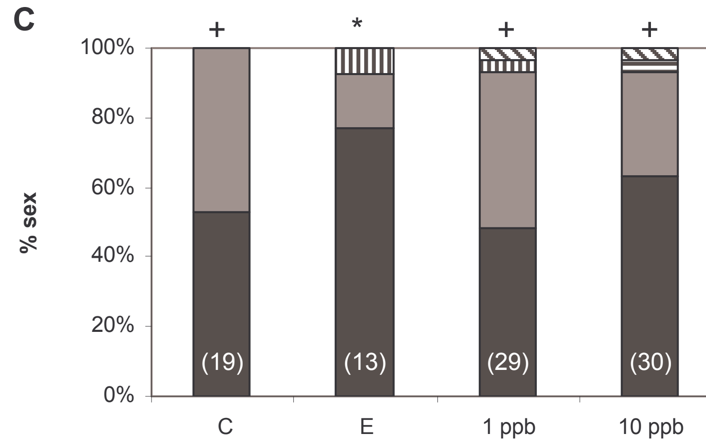
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Figure 4. Histology of juvenile *Rana sphenocephala.* The normal female (A) displays large diplotene oocytes (do) with nests of meiocytes and secondary oogonia near the edge (arrow). The normal male (B) exhibits developing spermatogonia (sg) inside seminiferous tubules. (C) An individual with a singular testicular oocyte. (D) An ovotestis with large diplotene oocytes and disorganized testicular tissue. Bar = 50 μm.

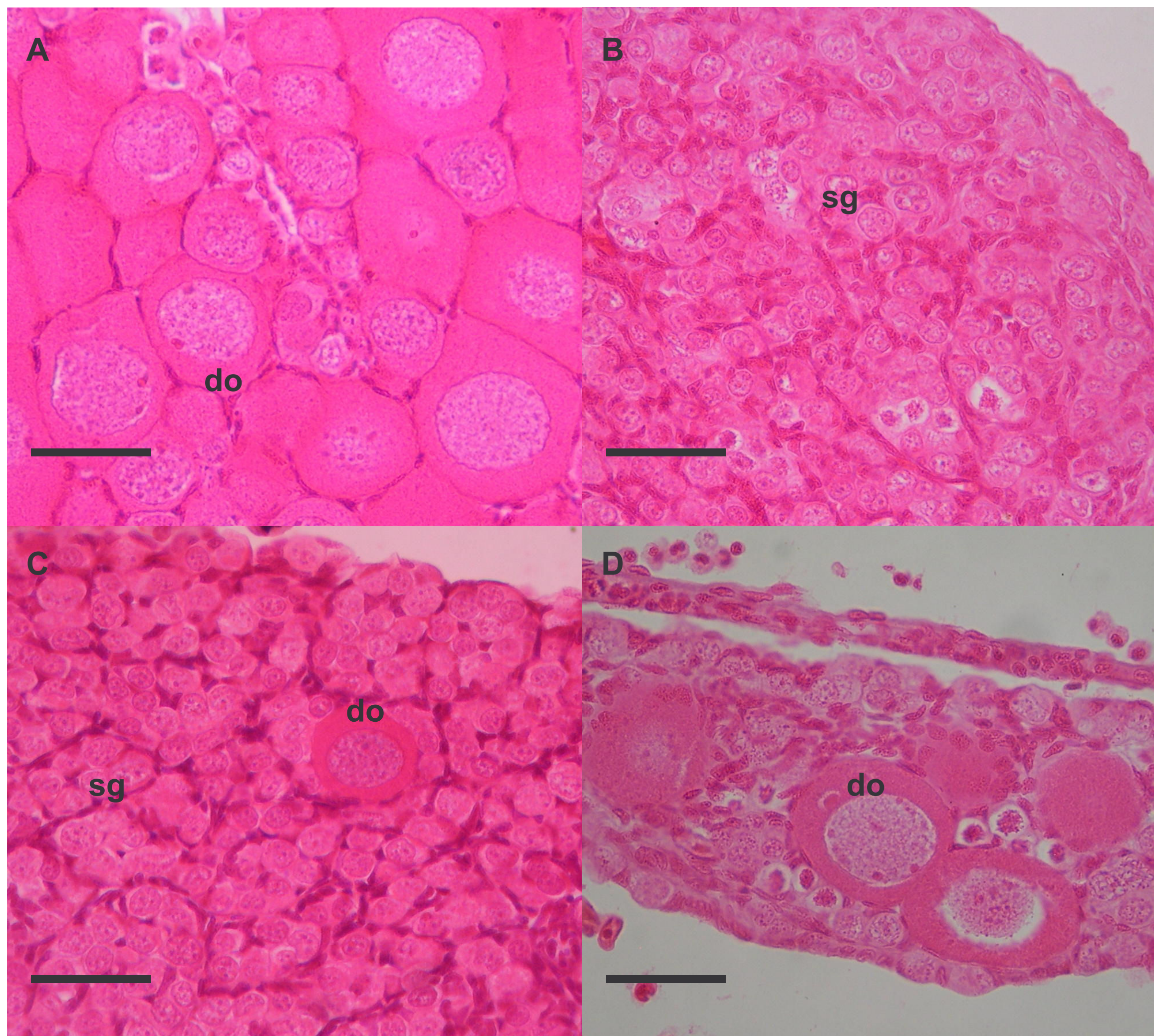
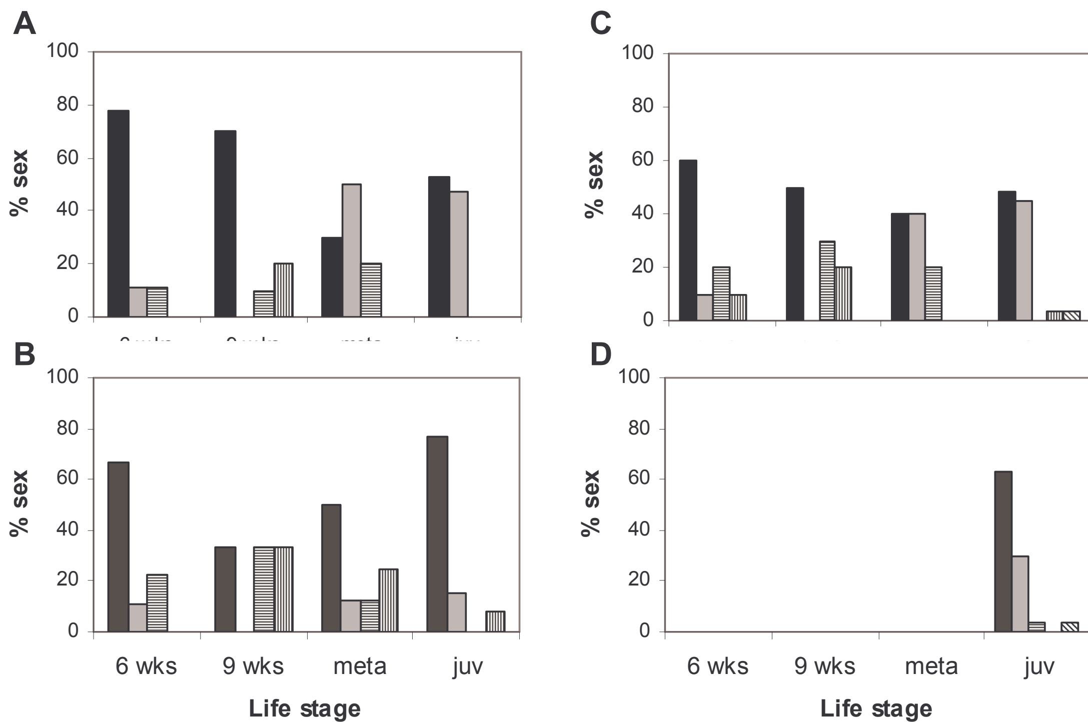


Figure 5. Sex ratios of *Rana sphenocephala* (A) control, (B) estradiol, (C) 1 ppb atrazine, and (D) 10 ppb atrazine treatments from both larval and juvenile experiments. Larvae at 6 and 9 weeks and metamorphs represent data from the larval experiment. No larval experiment was conducted at 10 ppb atrazine. Sample sizes are indicated in parentheses. Black = female, gray = male, horizontal hatch = testicular oocytes, vertical hatch = ovotestes, diagonal hatch = underdeveloped testes.



CHAPTER 4

DEHYDRATION RISK AND BLADDER MORPHOLOGY OF AMERICAN TOADS (*BUFO AMERICANUS*) EXPOSED TO ESTROGENIC CONTAMINANTS

ABSTRACT

Exposure to estrogenic contaminants is often examined in terms of reproductive endpoints, however, other systems should not be ignored. Water loss among amphibians is rapid in open-air environments, thus adequate hydration abilities are a key component of amphibian fitness and are controlled by hormones. The urinary bladder is critical to amphibian water maintenance because it can be used to store dilute urine used for hydration when water is unavailable. We examined the effect of estrogenic contaminant exposure during the aquatic larval period, a time of rapid organogenesis, had on the rate of dehydration and bladder morphology of terrestrial juvenile American toads (*Bufo americanus*). Larvae were exposed from the free-swimming stage to metamorphosis to a solvent control (ethanol), 17-β-estradiol (10^{-7}M), 1 part per billion (ppb) or 10 ppb atrazine. As they metamorphosed, toads were placed in terrestrial pens until they reached approximately four months of age. At that time, toads were collected, subjected to dehydration trials, and sacrificed for histological bladder examination. Our results suggest that larval exposure did not affect the juvenile rate of dehydration, however bladder morphology of those exposed to estradiol was altered such that portions completely lacked smooth muscle and epithelial cells. Our study highlights the

importance of examining non-reproductive endpoints under exposure to estrogenic contaminants.

INTRODUCTION

In the examination of endocrine disrupting contaminants (EDCs), chemicals that can interfere with hormonal pathways, much attention has been paid to the reproductive system. Because several EDCs are (anti)estrogenic and (anti)androgenic, there is good reason for this emphasis on reproductive effects, however other systems should not be ignored. Exposure to EDCs has also been demonstrated to affect the digestive system, cardiovascular system, adipose tissue (WHO 2002), and the immune system (Inadera 2006) of various species.

Atrazine, a widespread herbicide used mainly on corn and sorghum, has been investigated largely as an estrogenic EDC affecting the reproductive development of anuran amphibians with contradictory findings. Some authors report atrazine-induced gonadal abnormalities, while others report that atrazine has no impact on gonad development (Hayes et al. 2002, Hayes et al. 2003, Carr et al. 2003, Hecker et al. 2004, Coady et al. 2005, Hecker et al. 2005, Murphy et al. 2006). However Storrs-Mendez and Semlitsch (in press) have suggested that variation in the rate of anuran sexual differentiation, and therefore the presence or absence of EDC exposure during sexual differentiation, may offer some clarification in the debate. The impact of atrazine exposure on amphibians has also been examined on multiple non-reproductive endpoints including respiratory capability and feeding behavior (Allran and Karasov 2001), time to

metamorphosis and mass at metamorphosis (Sullivan and Spence 2003), survival (Storrs and Kiesecker 2004), and organogenesis (Lenkowski et al. 2008).

As with many vertebrates, amphibians depend on multiple biotic and abiotic factors for their survival and reproductive success. However, amphibians are unique in their hydration requirements. Water is lost from amphibian's thin, semi-permeable skin virtually the same way water evaporates from free-standing water (Jørgensen 1997). They hydrate cutaneously by placing a highly-vascularized section of ventral skin on water or moist soil. Water is rapidly taken up and stored in the bladder; dilute urine can later be absorbed by the body to hinder desiccation (Jørgensen 1997). Ions are also taken up via the skin and bladder. The transport of water and ions is regulated by four hormone systems (Uchiyama and Konno 2006).

Atrazine exposure in amphibians has been linked with higher rates of desiccation. Streamside salamanders (*Ambystoma barbouri*, Order: Caudata) exposed to atrazine (40 and 400 μg/L) during the larval period were found to have greater water loss than controls eight months after exposure (Rohr and Palmer 2005). They also found greater activity and fewer water conservation behaviors in those exposed to the same levels of atrazine (40 and 400 μg/L). To examine this phenomenon further, we measured the rate of dehydration in juvenile American toads (*Bufo americanus,* Order: Anura) exposed to either a solvent control (ethanol), 17-β-estradiol, or atrazine (1 or 10 μg/L) during their larval period. Based on Rohr and Palmer's (2005) data, we expected the rate of dehydration to be faster in estradiol- or atrazine-treated toads. We also examined the basic urinary bladder morphology of the toads. Bladder formation begins during the

larval period just prior to metamorphosis (Viertel and Richter 1999). Exposure to EDCs has been shown to induce ectopic cell death in the formation of other organs (i.e. kidney, brain; Lenkowski et al. 2008). Therefore, because the bladder helps in water conservation, we hypothesized that toads exposed to estradiol or atrazine would have an underdeveloped structure in comparison to control bladders.

MATERIALS AND METHODS

Experimental Design

Tadpoles of *B. americanus* were exposed to one of two concentrations of atrazine (1 or 10 µg/L), a solvent control (ethanol), or 17-ß-estradiol (10^{-7}M) at a research facility at the University of Missouri. The four treatments were replicated three times with one replicate on each shelf; shelves acted as blocks to partition a vertical temperature gradient. Tadpoles were pooled together from three egg masses and then randomly assigned to treatments once they reached the free-swimming stage (Gosner stage 25; Gosner 1960). Tadpoles were raised in glass aquaria filled with 20 liters of UV-sterilized, carbon-filtered water. Each aquarium was initially stocked with 12 tadpoles. Water was completely changed every third day. When the first metamorph was found, aquaria were checked every day. Individuals with at least one forelimb (Gosner stage 42 – 46) were removed from the test chambers and allowed to fully metamorphose (Gosner stage 46, complete tail resorption) in individual plastic containers (5cm x 13cm). Time to and size at metamorphosis were recorded.

Metamorphs were then weighed, given a unique mark using toe-clips, and transported to outdoor pens (1m x 2m) where they were kept with others from the same aquarium until they reached approximately four months of age. Pens were covered with shade cloth and watered as needed; naturally occurring insects provided food, no other food was added. After the terrestrial growth period, toads were collected, identified, weighed, and dehydration trials were conducted.

Dehydration trials were conducted as toads were collected from pens on days 162 (n = 25), 173 (n = 11), and 183 (n = 2) after the free-swimming stage. However only 20 trials could be conducted per day, therefore on day 162, only twenty toads were included. Prior to dehydration trials, each toad (n = 33) was allowed to hydrate for four hours. Dehydration trials were conducted with 20 wire mesh cages (5cm x 5cm) suspended to expose the maximum surface area of each toad to surrounding air. Mass was recorded every 30 minutes for 150 minutes or until toads lost 20% of their initial body mass. After dehydration trials, each toad was returned to individual terraria containing enough water to rehydrate.

Dissection and fixation

After dehydrations trials and subsequent rehydration, all toads obtained from pens were weighed and fatally anesthetized in MS-222. The urinary bladder was dissected and fixed based on Lui and Daneshgari (2006). Briefly, the bladders were incubated for 20 minutes at 23°C in Krebs buffer and aerated with CO_2. Aeration was facilitated by adding an over-the-counter heartburn medication (Alka-Seltzer, Bayer Corp.) in the

buffer. After incubation, the bladders were fixed in neutral-buffered formalin. The gonad-kidney complex was also dissected at this time and fixed in Bouin's fixative; treatment effects on sex are reported elsewhere (Storrs-Méndez and Semlitsch, in press).

Histology and morphology

Organs were dissected and processed using a Shandon Excelsior tissue processor (Thermo Electron Corp., Massachusetts). The first attempt at dissecting a bladder was unsuccessful, therefore only 37 were used to assess bladder morphology. After dissection, they were subsequently embedded in paraffin and sliced longitudinally into 7µm sections (AO Spencer microtome). Sections were placed on slides and stained with haematoxylin and eosin. Slides were examined using a compound light microscope (Olympus CX21).

Bladder tissues were assessed by examining the proportion of bladder wall that was underdeveloped. Fully developed bladders contained epithelial cells on the mucosal side and smooth muscle on the serosal side. Pictures of the widest portion of each bladder (1-2 pictures depending on size) were taken with a Nikon Cool Pix 4300 camera and an Olympus CX21 compound microscope at 4x. A grid was placed over the representative pictures using Microsoft PowerPoint. To obtain the proportion of underdeveloped bladder, quadrats containing underdeveloped bladder were counted and divided by the total number of quadrats that the bladder occupied. This assessment was done blind to treatment.

Dosing

Stock solutions of analytical grade atrazine (99.2% atrazine; Sigma-Aldrich, Allentown PA) were mixed with an ethanol solvent. During water changes, aquaria were dosed with 2.5 ml of stock solution to yield 1 or 10 μg/L atrazine. Identical amounts of solvent control (ethanol) and estradiol (10^{-7}M 17-β-estradiol) were added to the appropriate aquaria. Estradiol solutions were mixed for each water change. The concentration of estradiol was chosen because exposure of tadpoles results in a significant number of females (Kloas et al. 1999).

Water samples from each aquarium were collected from test chambers from each treatment to assess atrazine concentration. An atrazine ELISA kit (antibody-coupled paramagnetic particles, Abraxis, Philadelphia PA) was used to analyze the samples. The minimum reportable concentration for this kit was 0.05 ppb atrazine. Any sample below this concentration was reported as nondetectable. Concentrations are shown as mean ± standard deviation in μg/L.

Statistical analysis

Mass at metamorphosis and time to metamorphosis were analyzed using a generalized linear model analysis of variance (Minitab 12.1) for all toads ($n = 38$). The changes in mass over time (rate of dehydration; $n = 30$) were examined using a repeated measures analysis of variance with toad surface area [surface area = $9.9 * (\text{mass at time zero})^{0.56}$; McClanahan and Baldwin 1969] as a covariate (SAS). Least squared means (LS means) obtained from this analysis were used to compare differences between treatments at each

time period. Because only three out of seven estradiol-treated toads remained in the study by the 150-minute time interval (all others had lost 20% body mass prior to this time interval), masses for all treatments were only examined through the 120-minute time interval to increase sample size for the estradiol treatment. Alpha was set at 0.05 for all analyses.

RESULTS

There were no statistically significant differences between treatments in time to metamorphosis ($F_{3,29} = 2.78$, $P = 0.059$), however estradiol-treated larvae took the longest time to metamorphose with an average of 40 ± 2.57 days (± standard error mean). For all treatments overall, the average time to metamorphosis was 36 ± 0.89 days (± standard error mean) after hatching. There were no significant differences between treatments for mass at metamorphosis ($F_{3,29} = 1.45$, $P = 0.249$). The average mass was 0.093 ± 0.004 g.

Surface area had a significant effect on rate of dehydration (loss of mass from 0 to 120 minutes, $F_{1,27} = 560.16$, $P < 0.001$). With surface area as the covariate, there were no significant differences between treatments in the rate of dehydration ($F_{3,27} = 0.51$, $P = 0.679$). Although they were not significant, there were trends in rate of dehydration among the treatments. The rate of dehydration was highest for estradiol-treated toads and lowest for toads exposed to 1 µg/L atrazine, with controls and toads exposed to 10 µg/L atrazine having intermediate rates (Figure 1).

In the examination of bladder morphology, the only underdeveloped bladders were found among estradiol-treated toads. Underdeveloped bladders lacked any identifiable epithelial cells or muscle fibers and were stained much lighter than other sections of urinary bladder. Four out of nine toads in this treatment (two had not been used in the dehydration trials) had portions of underdeveloped bladder ranging from 17.4% to 61.2% of underdeveloped bladder (Figure 2). All others in this treatment and other treatments had fully developed bladders.

DISCUSSION

Our data do not support the hypothesis that EDC exposure during the larval period impacts the rate of dehydration of American toads. This hypothesis was based on data demonstrating that salamanders exposed to atrazine as larvae had faster rates of dehydration as juveniles (Rohr and Palmer 2005). These authors used higher concentrations of atrazine (40 and 400 μg/L) for their larval exposures which suggests that lower, more ecologically relevant concentrations of atrazine may have no impact on amphibian water loss.

There are some important species differences that may also contribute to the disparity between our study with toads and the Rohr and Palmer study with salamanders. Toads are known to be habitat generalists, can be commonly found in disturbed areas (i.e. agricultural, residential), and are highly mobile compared to salamanders. Salamanders tend to remain closer to ponds or streams suggesting that they may be restricted by water. There is also some data suggesting that salamanders dehydrate faster than either toads or

frogs (reviewed in Jørgensen 1997). In general, salamander bladders are proportionally smaller than anuran bladders (Duellman and Trueb 1986). Thus, toads may be better adapted to maintain their hydration needs than salamanders.

Our data demonstrate that estradiol exposure of toad larvae impacted bladder development. The anuran bladder begins formation just before metamorphosis, while the muscular layer develops during metamorphic climax (Viertel and Richter 1999), a time of continued aquatic exposure. In the underdeveloped sections of bladder identified here, we noted a lack of any muscle tissue. Because anuran bladder muscle allows for distension during urine formation (Duelleman and Trueb 1986), we suspect that these portions of bladder were unable to fill with urine. The lack of any structure (e.g. epithelial cells, muscle tissue) in these portions suggests the possibility that the bladder may have formed and later regressed in these sections, as was seen in other organs of EDC-exposed anurans (Lenkowski et al. 2008), or may have never fully developed.

Although the underdeveloped bladders did not affect the rate of dehydration, we hypothesize that the formation of other important systems may also be compromised by exposure to similar concentrations of estradiol, or EDCs in general, possibly impacting juvenile or adult fitness. For example, Lenkowski and colleagues (2008) reported that exposure to atrazine (10, 25, and 35 mg/L) during early organ morphogenesis (just prior to and during the free-swimming stage; Gosner 25; Gosner 1960) significantly impacted the development of multiple organ systems (i.e. circulatory system, digestive system).

Storrs and Semlitsch (2008) demonstrated that American toad larvae exposed to the same concentration of estradiol used here exhibited significantly slower somatic development as compared to controls and atrazine-treated toad larvae. They characterized the effect as resulting from a high concentration of estradiol (Storrs and Semlitsch 2008). Our current study suggests that American toad juveniles exposed to estradiol as larvae may also experience slower or regressed somatic development in the formation of the urinary bladder.

Our results highlight the importance of investigating non-reproductive endpoints in the examination of EDCs. In addition, these results provide a basis for understanding the impact that EDCs have on anuran urinary bladder development and aid in any further investigation EDC impacts of the development of related systems. Future studies should include the exposure of multiple species, chosen based on varying hydration needs, to the same concentrations of contaminants. These studies might also investigate the more realistic effect of exposure to mixtures of contaminants and/or their interaction with non-chemical stressors on organ development. An underdeveloped or malformed urinary bladder has the potential to impact long term hydration needs which could ultimately affect fitness.

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Figure 1. Rate of dehydration for juvenile *Bufo americanus* exposed during the larval period to a solvent control = solid diamond, solid line; estradiol = open diamond, dashed line; 1 μg/L atrazine = open square, dotted line; 10 μg/L atrazine = solid square, dotted line.

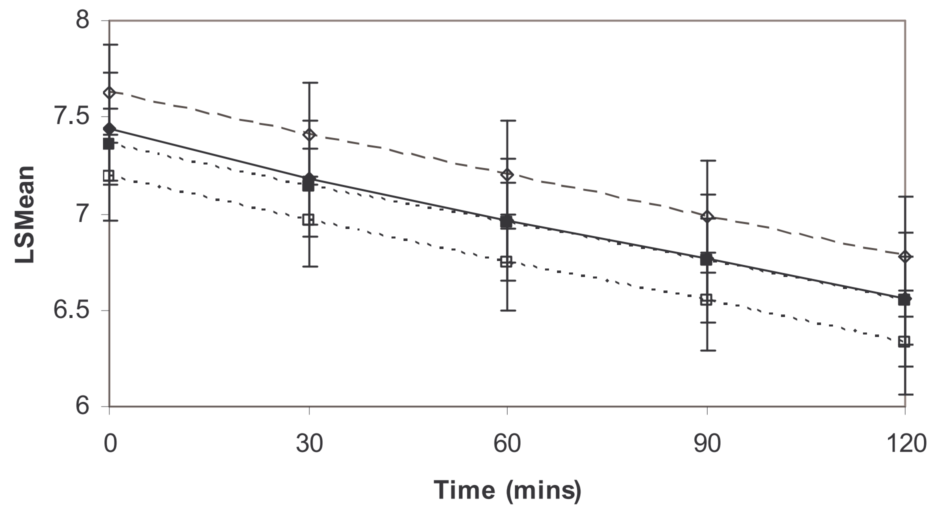
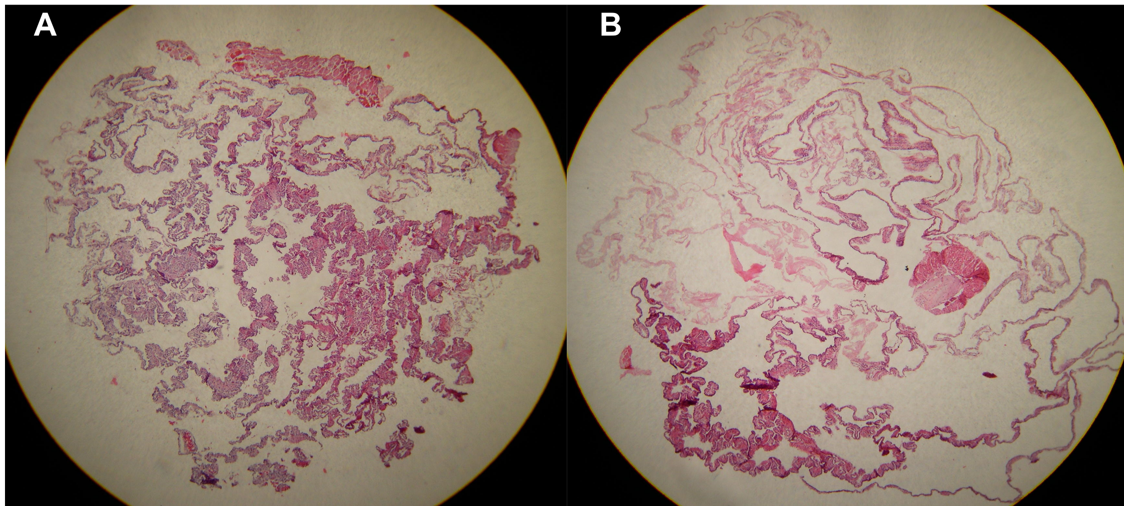


Figure 2. Bladder histology of A) normally structured bladders stained darkly indicating the presence of epithelial cells and smooth muscle, and B) abnormally structured bladders with portions of normal staining and very light staining (upper half) indicating a lack of epithelial cells and smooth muscle.



CHAPTER 5

BEHAVIORAL RESPONSE AND KINETICS OF TERRESTRIAL ATRAZINE EXPOSURE IN AMERICAN TOADS (*BUFO AMERICANUS*)

ABSTRACT

Amphibians in terrestrial environments obtain water through a highly vascularized pelvic patch of skin. Chemicals can also be exchanged across this patch. Atrazine (ATZ), a widespread herbicide, continues to be a concern among amphibian ecologists based on potential exposure and toxicity. Very few studies have examined its impact on the terrestrial juvenile or adult stages of toads. In the current study, we asked: 1) will juvenile American toads (*Bufo americanus*) avoid soils contaminated with atrazine, 2) can they absorb ATZ across their pelvic patch, and 3) if so, how is it distributed among the organs and eventually eliminated? We conducted a behavioral choice test between control soil and soil dosed with ecologically relevant concentrations of ATZ. In addition, we examined the uptake, distribution and elimination of water dosed with ^{14}C-labeled ATZ. Our data demonstrate that toads do not avoid ATZ-laden soils. ATZ crossed the pelvic patch rapidly and reached an apparent equilibrium within five hours. The majority of the radiolabeled ATZ ended up in the intestines, while the greatest concentrations were observed in the gall bladder. Thus, exposure of adult life stages of amphibians through direct uptake of ATZ from soils and runoff water should be considered in risk evaluations.

INTRODUCTION

Amphibians move across the landscape including agricultural fields to reach suitable habitats for foraging and reproduction (Guerry and Hunter 2002, Semlitsch 2008). These movements occur for anurans at multiple life stages either as metamorphs dispersing from the pond or as adults migrating to and from breeding sites (Semlitsch 2008). Migration of adults in particular is more likely to occur during or soon after a rain event; they utilize this wet environment to reduce the likelihood of desiccation (Hurlbert 1969, Semlitsch and Pechmann 1985, Pechmann and Semlitsch 1986, Todd and Winne 2006). Amphibians in terrestrial habitats obtain most of their water dermally, predominantly through a highly vascularized pelvic patch of skin (McClanahan and Baldwin 1969, Marrerro and Hillard 1985, Viborg and Hillard 2005). Toads usually hydrate on moist surfaces, such as soil, rather than in open water (Sullivan et al. 2000). Along with water, chemicals are also exchanged across the pelvic patch. These include salts and ions necessary for organ function and osmotic balance (Hillard 1999, Sullivan et al. 2000, Viborg and Hillard 2005) as well as metals (James et al. 2004) and pesticides (Willens et al. 2006). Thus, amphibians moving across agricultural fields may be at risk for chemical exposure when they come in contact with moist soils. Agricultural chemicals present in these soils can leach or diffuse into the small pockets of water and subsequently could accumulate in amphibians.

Atrazine (ATZ, 2-chloro-4-ethylamino-6-isopropylamino-s-triazine), a widespread herbicide commonly applied to corn and sorghum, continues to be a concern among amphibian ecologists based on potential exposure and toxicity. Residues from the

previous year's use can be detected in surface runoff prior to application (Ghidey et al. 1997). Post-application levels in surface runoff can range from 0.1 -6.7 µg/L in amphibian breeding ponds in mid to late July (Hayes et al. 2003). Much of the research to date has examined the effects of ATZ on reproductive development of amphibian larvae exposed in a fully aquatic stage of life with contradicting results as to the ability of atrazine to induce aromatase, the enzyme that converts testosterone to estrogen (Hayes et al. 2002, Hayes et al. 2003, Carr et al. 2003, Hecker et al. 2004, Coady et al. 2005, Hecker et al. 2005, Murphy et al. 2006, Storrs and Semlitsch 2008).

Very few studies have examined the impacts of ATZ on the terrestrial juvenile or adult stages of amphibians. Although atrazine is applied as a pre-emergent herbicide, it can be detected in agricultural soils in late May (1608 µg ATZ/kg of soil; Ghidey et al. 1997), when many toad metamorphs are emerging and dispersing from ponds (*pers obs*), and remain at detectable levels in November (16 µg ATZ/kg of soil; Ghidey et al. 1997). Behavioral studies have demonstrated that adult northern leopard frogs (*Rana pipiens*) exposed to ATZ exhibit a dose-dependent increase in buccal ventilation as well as increased thoracic ventilation and decreased feeding at some doses of atrazine (Allran and Karasov 2001). Stream-side salamanders (*Ambystoma barbouri*) exposed to ATZ as larvae exhibit higher activity levels, fewer water conservation behaviors, and a higher desiccation risk as terrestrial juveniles (Rohr and Palmer 2005). *In vitro* studies of amphibian skin have demonstrated that ATZ can cross the pelvic patch and create an increase in sodium uptake (Cassano et al. 2006). In addition, *in vitro* studies of mammalian skin have illustrated that one commercial formulation of ATZ (Aatrex 4L) is

absorbed more than the pure form of ATZ (Brand and Mueller 2002). *In vivo* studies have illustrated that ATZ can be absorbed by fully-aquatic African-clawed frog juveniles (*Xenopus laevis*; Edgington and Rouleau 2005). However, to our knowledge, no studies have examined behavioral avoidance or *in vivo* uptake of ATZ by terrestrial amphibians. In the current study, we asked: 1) Do juvenile American toads (*Bufo americanus*) avoid soils contaminated with ATZ?, 2) Can toads absorb ATZ across their pelvic patch *in vivo*?, and 3) If so, how is ATZ distributed and eventually eliminated in these toads?

MATERIALS AND METHODS

Animal Collection and Maintenance

Toad egg masses were collected from Thomas S. Baskett Wildlife Area in Boone County, MO. Three free-swimming tadpoles (Gosner 25; Gosner 1960) were placed in cups and randomly assigned to rearing containers at a density of one tadpole per liter on10 May 2007. Water was completely changed every third day. When the first metamorph was found, containers were checked every day. Individuals with at least one forelimb (Gosner stage 42 – 46) were removed and allowed to fully metamorphose (Gosner stage 46, complete tail resorption) in a 29.2 x 11.4 x 16.5 cm container with about 1 cm of water and tilted to allow both wet and dry areas. Metamorphs were then placed in individual terraria for rearing. Toads were fed mealworms and calcium-dusted crickets until experiments were conducted.

Behavioral Choice Test Design

The choice test was conducted at a research facility of the University of Missouri on 18 and 19 July 2007 at 22° C. Test chambers (N = 27) contained a plexiglass partition (16.5 x 2.5 cm) down the center to separate the two soil types and a fiberglass screen release platform (diameter = 9.8 cm, mesh = 0.16 cm) in the center that allowed toads to receive cues present in the soil on both sides of the partition. Test chambers were oriented north-south or east-west to control for direction biases in movements. Sides of the chamber were randomly assigned low ATZ soil (80 μg/kg) vs. control or high ATZ soil (1430 μg/kg) vs. control.

American toads (*Bufo americanus*) were randomly assigned to one of 18 chambers on night one and nine chambers on night two. Both nights included all treatments and were conducted in the same room. Trials began at 1930 hours and each toad was only used once. After the initial mass of each toad was recorded to the nearest 0.0001 g, individuals were placed on the release platform and under a plastic cup for six minutes. During this acclimation period, the lights were turned off and no humans were present. Following acclimation, we reentered the room, removed the cups, and observed toads using a headlamp with red light to minimize any distractions of our presence. We recorded the initial choice by noting the side each toad left the release platform. The location of each toad on either side of the chamber or on the release platform was noted at three-minute intervals thereafter for one hour and then every twelve hours for 2.5 days.

Choice Test Dosing

Stock solutions of analytical grade ATZ (99.2% ATZ; Sigma-Aldrich, Allentown PA) were created with an acetone solvent. High (1430 µg/kg) and low (80 µg/kg) terrestrial ATZ concentrations were chosen based on those measured in agricultural fields in Missouri where ATZ was applied immediately after application (high) and approximately fifty days later (low; Ghidey et al. 1997). Five milliliters of the appropriate stock solution was mixed with 800 ml UV-sterilized, carbon-filtered water before dosing soil.

Experimental soil (mixture of 3 kg peat moss, 10 kg top soil, 7 kg sand) was weighed with a Pesola scale (5 kg capacity) and mixed with a stainless steel drill bit (length 29cm, diameter 7cm). This soil was then dosed with the appropriate treatment solution. In addition 4.45 liters of reverse osmosis water was added to soil and mixed with the same drill bit. Three soil samples from each treatment (n = 9) were collected after the experiment was completed and stored in glass jars under foil at 4°C. Soil analysis was conducted five months after the choice test was conducted (Midwest Laboratories, Inc., Omaha, NE).

Uptake and Elimination Design

Seventeen toads from the choice-test experiment were randomly assigned to treatment groups in the examination of uptake and elimination of ^{14}C-ATZ (Sigma, St. Louis, MO, USA) at a research facility of the Columbia Environmental Research Center (United States Geological Survey) on 1-3 November 2007. Test chambers consisted of 1L glass jars and were housed inside a vented hood. Temperature, humidity, and wind speed were

recorded several times throughout the experiment (n=6). All data were measured in the center of the experimental setup at ground level. Each jar was tilted such that when dosed water was added, toads had space available for both wet and dry areas. Location of the pelvic patch (in or out of water) was recorded throughout the experiment. Jars were covered with fiberglass mesh screen to allow for evaporation.

Animals did not receive food for 72 hours prior to the start of the experiment, according to standard protocol (Preest and Pough 2003). On day one, animals were weighed and then their bladders were emptied by applying gentle pressure to obtain a standard mass (Rubial 1962). However 10 of the 17 animals did not empty their bladders after four minutes of pressure. All animal masses were recorded to the nearest 0.001 g. To dehydrate toads each individual was placed in an empty test chamber (n = 17) overnight to allow dehydration.

Each toad was removed from the test chamber and weighed 13 hours later to determine the percentage mass lost overnight. Each toad [except time zero (t_0) toads; t_0 = initiation of uptake experiment] was then placed back in its jar and both toad and jar were weighed together to the nearest 0.05 g. All remaining weights were obtained by weighing toads in their jars to prevent disturbing the toad. Further, this mass would be compared to the mass of toad + jar before dissection of the toads so as to assess any loss of mass due to evaporation.

Two toads were removed from the experimental chambers, weighed, euthanized with a lethal dose of MS-222, and dissected at t_0 to measure baseline radioactivity. Each remaining jar (n = 15) was dosed with 20 ml of reserve solution (460 μg/L*; see ^{14}C Atrazine Stock Solution*) using serological pipettes. Water was selected for dosing, as opposed to soil, to avoid any confounding factors and to ensure only uptake and elimination of atrazine was examined. Three toads each were euthanized with MS-222 and dissected at 2 (t_2), 4 (t_4), 8 (t_8), 12 (t_{12}), and 24 (t_{24}) hours after dosing. At t_8 and t_{12}, any remaining water was removed, the jar and toad rinsed with tap water, and 20 ml of fresh tap water was added to each experimental chamber to examine elimination. Toads removed from the experiment were euthanized using MS-222. Each toad was weighed after euthanasia and selected organs were dissected. The gall bladder, liver, stomach, intestines, kidneys and gonads were dissected (Edgington and Rouleau 2005) and weighed (to the nearest 0.001 g). Fat bodies were also dissected and weighed at this time but were not examined radiometrically for ATZ content. One gall bladder at t_0 and one at t_2 burst during dissection. After dissection, organs were placed in tubes with sterile phosphate-buffered saline (PBS) and bodies were frozen for proper disposal. The uptake phase of the experiment occurred from t_0 until t_8, while the elimination phase of the experiment occurred after any dosing solution was removed (t_8 and also at t_{12}).

Organs were homogenized and 500 μl of each homogenate added to 5 ml scintillation cocktail (Ecolume, Solon, OH) in a scintillation vial. If volumes of 500 μl of the organ homogenate were not obtained, the actual volume used was recorded for subsequent calculations. Duplicate water samples (500 μl) from the jars were placed in empty

scintillation vials at the time toads were removed ($t_0 - t_{24}$) to account for any radioactivity remaining in the water. Scintillation cocktail (5 ml) was added to the sample water and vortexed before counting. Limited water samples were obtained for t_8 likely due to uptake. All samples were counted (Beckman-Coulter, Inc., Model 6500 LS) for twenty minutes for determination of disintegrations per minute (dpm) in each sample. Triplicate samples were read for quality control at approximately every fifteenth organ sampled (excluding gall bladder, testes, and kidneys due to sizes of the samples).

^{14}C ATZ Stock Solution

ATZ labeled with ^{14}C (Sigma, St. Louis, MO, USA) had a specific activity of 9.5mCi/mmol of ATZ. A syringe (1cc tuberculine syringe with an 18 gauge needle) was used to add 0.5 ml of RNAse-Free HPLC ethanol (Sigma, St. Louis, MO, USA) to the ^{14}C-ATZ vial; this stock solution was vortexed for several seconds. Then, 34 µl of this solution were added to 340 ml of aged tap water to create the reserve solution (460 µg/L) from which each jar was dosed with 20 ml of ATZ-treated water. This concentration was chosen to ensure that radioactivity would be high enough in the smallest organs for the scintillation counter to detect with accuracy. Radioactivity (dpm; disintegrations per minute) is reported here as opposed to concentration in µg/L because radioactivity is the measure of radiolabel detected by the scintillation counter.

Statistical analysis

Choice test data were analyzed using a two-tailed exact binomial test with an expected proportion of 1:1 (alpha = 0.05) for each time period. Data for the first hour represent the soil location on which the toad was found most often.

Scintillation values were standardized with corrections for background dpm (t_0), PBS volume, and volume (μl) added to scintillation cocktail. Uptake and elimination were analyzed on a dpm/organ and dpm/mg basis. To examine differences over time, an analysis of variance was conducted for each organ with a general linear model. Whole organ values for the gallbladder were $\log_{10}$ transformed for normality. All other organs were normally distributed for both the mass balance and the concentration. Pairwise comparisons were made using a Tukey test. Alpha was set at 0.05 for all tests.

RESULTS

No statistically significant choice preference for soils was apparent during our test of juvenile American toads (Figure 1). Although more toads were found on the atrazine soil in general, there were only three significant departures from the expected (1:1) and no pattern of significance. The exact binomial test demonstrated significant departures from the expected in the control v. low experiment [36 hours ($P = 0.003$) and 60 hours ($P = 0.022$)] and in the control v. high experiment [during the first hour ($P = 0.013$)]. Measured concentrations of ATZ in soils (control = nondetectable, low = 33 ± 1.3 μg/kg, high = 582 ± 44.4 μg/kg) were 59% lower than nominal concentrations (control = 0

μg/kg, low = 80 μg/kg, high = 1430 μg/kg). The average mass of toads in the choice test was 0.833 ± 0.03 g (average ± standard error mean).

The uptake experiment was conducted at 20.6 ± 0.5 degrees C (average ± standard error mean), 25.4 ± 1.5 percent humidity, and 1.3 ± 0.1 mph wind speed. Radiolabeled ATZ was taken up rapidly across the pelvic patch of American toads as they rehydrated. At t_0, 93% of toads placed their pelvis immediately in the water. Radioactivity was removed from the water (Figure 2a) in parallel to an increase in body mass (Figure 2b). Radioactivity increased in the water between t_4 and t_8 (Figure 2b) and then decreased after the t_8 water change. Body mass of the toads remained relatively constant after the water changes at t_8 and t_{12}.

Organ-specific uptake of radioactivity was monitored over time (Figure 4). The greatest radioactivity per organ was found in the intestines which surpassed the gall bladder only after eight hours. Radioactivity in the other organs dissected (liver, stomach, kidneys) was much less. Values from these organs peaked at two hours and gradually decreased. Radioactivity values of the liver, stomach and kidneys were not significantly different from the control (t_0) at t_{12} and t_{24} (Table 1). The water changes appear to have had no effect on radioactivity values for the mass balance. However the whole intestine values were significantly higher after the water change at t_{12} (Table 1).

The concentration of radioactivity (radioactivity per milligram of organ) was also examined (Figure 5). The greatest concentration was found in the gall bladder, the least

contributor to body mass (Figure 3). Concentrations of ATZ in both the gall bladder and intestines appear to have peaked at 8 hours after the first water change, while concentrations peaked in all other organs (liver, stomach, kidneys) at two hours. Radioactivity in the kidneys was not significantly different from the control (t_0) after t_4, in the liver after t_8, and in the stomach after t_{12}. The water changes appear not to have significantly affected the concentration of radioactivity (Table 2).

DISCUSSION

Our study demonstrates that toads absorbed ATZ across their pelvic patch. When exposed to radiolabeled ATZ in water, dehydrated toads absorbed both water and ATZ rapidly. After being taken up across the highly vascularized pelvic patch, radiolabeled ATZ was thought to have entered the blood via diffusion and absorbed by the liver as a part of the normal filtration process of the blood. The absorbed ATZ in the liver was likely metabolized; however, we did not attempt to measure metabolites of ATZ. The liver of toads removes many contaminants from the blood and some are then emptied into the gall bladder, as is the general process in other vertebrates (Diamond 1962). ATZ kinetics and disposition in the American toads was consistent with this pattern of uptake and distribution. Bile from the gall bladder is highly concentrated and empties into the intestines for excretion. The pattern and time course of ATZ in the toads is consistent with this generalized model of toxicokinetics. The temporal pattern of ATZ observed in toads from our study demonstrates peak concentrations of ATZ in liver, gall bladder, and then intestine, respectively, following the expected toxicokinetic pattern of uptake and elimination. The smaller amounts of radioactivity observed in whole stomach and

kidneys in our study suggest that neither uptake nor elimination of ATZ occurred to a major extent through these organs. Measurements from water support these findings. A decrease in water radioactivity from t_0 to t_4 was assumed to reflect toad uptake, while an increase in water radioactivity from t_4 to t_8 was assumed to reflect toad.

As expected, the greatest concentrations of ATZ were found in the gall bladder followed by the intestines, kidneys, liver and stomach. The liver ranks low in this list presumably due to the sheer size of this organ (Figure 3) and its efficiency in removal of ATZ from the liver into the gall bladder. While the data of whole organs suggests that neither the stomach nor kidneys were major routes of uptake or elimination, the data of organ concentrations suggests that some radiolabeled ATZ passes through these organs. The order of these concentrations suggests that some filtration of ATZ occurs in the kidneys, likely residual ATZ not removed from the blood by the liver. The concentration of ATZ in the stomach was the least due to the route of uptake (pelvic patch directly into the vascular system) of the ATZ.

Edginton and Rouleau (2005) exposed African-clawed frog metamorphs (*Xenopus laevis*) to radiolabeled ATZ and followed the toxicokinetics over a period of 8 hours. They observed the greatest concentrations in the gall bladder followed by the intestines, yet the levels found in the stomach were higher than the liver in their study. Thus, the distribution of atrazine in the body in these two studies was consistent with one another. The difference between the results of these two studies is most likely due to the differences in the routes of uptake, a consequence of the life-histories of each species. *X.*

laevis are fully aquatic frogs, while *B. americanus* have a fully aquatic larval stage and terrestrial adult stage. The routes of uptake for the *X. laevis* metamorphs studied by Edginton and Rouleau would have been dermal and ingestion. The adults used in our study and the exposure design resulted in uptake only through the pelvic patch. No data were reported for the kidneys in the *X. laevis* metamorph ATZ exposure study (Edgington and Rouleau 2005). Although metabolism was not examined in our study, Edginton and Rouleau (2005) conducted a metabolism study and found the majority of the total body radioactive residues in *X. laevis* consisted of atrazine, deethyldeisopropylatrazine (DACT), deisopropylatrazine (DIA), and deethylatrazine (DEA), respectively, 24 hours after exposure to ATZ.

American toads had no statistically significant behavioral preference when presented with a choice between control and ATZ-contaminated soils. Other studies have demonstrated similar lack of response by amphibians exposed to soil contaminated with urea fertilizer (Hatch et al. 2001). The lack of choice suggests that toads could not detect the ATZ in either the soil or their body. Alternatively, the toads may have had no consistent behavioral or physiological response to the presence of ATZ in the soil or in their body. The lack of choice is important when considering the conservation of amphibians in terrestrial habitats in or around agricultural areas. Amphibians moving through agricultural landscapes may readily traverse ATZ-treated soils, making exposure to ATZ and potentially other chemicals present in terrestrial habitats very plausible. Amphibians in general are highly susceptible to contamination due to their semipermeable skin and biphasic life cycle (Hall and Henry 1992). There is overwhelming evidence of the

sensitivity of amphibians in the aquatic stage of their life cycle to the effects of chemical exposure; however few studies have demonstrated this same sensitivity in the terrestrial environment of an adult life stage. Our study demonstrates rapid and substantial uptake of a model pesticide, atrazine, by an amphibian in the terrestrial environment. Thus, examination of the sensitivity of amphibians towards pesticides or other chemicals found in a terrestrial environment is warranted.

The focus of our study was to examine the interactions between the terrestrial stage of amphibians and an ATZ-treated environment. We found that American toads do not avoid ATZ-treated soils. Moreover, ours is the first study to demonstrate the uptake of radiolabled ATZ in the terrestrial stage of amphibians and to examine its distribution and excretion *in vivo*. Our data indicate that when amphibians interact with agricultural soils they can accumulate pesticides, such as ATZ, across their skin (pelvic patch) and enter directly into their bloodstream. This interaction may occur in newly metamorphosed amphibians as they disperse from their natal pond as well as in adults migrating toward a breeding site. It is important to consider that amphibians traversing or inhabiting agricultural lands will likely encounter not only pesticides in the soils but also water contaminated through runoff. These exposures, if great enough, may act to affect amphibian populations by impacting both the aquatic and terrestrial stages of amphibians along an agricultural landscape.

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Table 1. Atrazine Mass Balance. ANOVA table for each organ. P-values for time are shown for each organ. Values for the gall bladder were $\log_{10}$ transformed for normality. Tukey's test was performed (alpha = 0.05). Overall P-value given in second column. P-values are displayed for each time compared to the control (t_0) as well as the P-values for comparisons between t_8 and t_{12} and between t_{12} and t_{24}. For all tests, df = 5, error = 11.

Organ	*F*	*P*	t_2	t_4	t_8	t_{12}	t_{24}	t_{8-12}	t_{12-24}
Gall bladder	19.21	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	1.000	0.664
Liver	19.47	<0.001	<0.001	0.001	0.007	0.094	0.365	0.492	0.890
Stomach	9.85	0.001	0.001	0.005	0.076	0.207	0.673	0.974	0.867
Intestines	13.08	<0.001	0.687	0.103	0.005	0.037	<0.001	0.734	0.037
Kidneys	24.08	<0.001	<0.001	<0.001	0.001	0.088	0.299	0.092	0.932

Table 2. Atrazine Concentration. ANOVA table for each organ. P-values for time are shown for each organ. Tukey's test was performed (alpha = 0.05). Overall P-value given in second column. P-values are displayed for each time compared to the control (t_0) as well as the P-values for comparisons between t_8 and t_{12} and between t_{12} and t_{24}. For all tests, df = 5, error = 11.

Organ	*F*	*P*	t_2	t_4	t_8	t_{12}	t_{24}	t_{8-12}	t_{12-24}
Gall bladder	1.06	0.433	--	--	--	--	--	--	--
Liver	15.77	<0.001	<0.001	0.002	0.038	0.108	0.379	0.973	0.913
Stomach	14.15	<0.001	<0.001	0.001	0.010	0.039	0.311	0.928	0.657
Intestines	2.91	0.065	--	--	--	--	--	--	--
Kidneys	9.98	0.001	0.001	0.031	0.251	0.133	0.764	0.996	0.598

Figure 1. Number of toads who chose (a) control vs. low atrazine soil (n = 13) and (b) control vs. high atrazine soil (n = 14). Control (white) = 0 μg/kg, low (gray) = 80 μg/kg, high (black) = 1430 μg/kg. a = significant difference between observed and expected proportion.

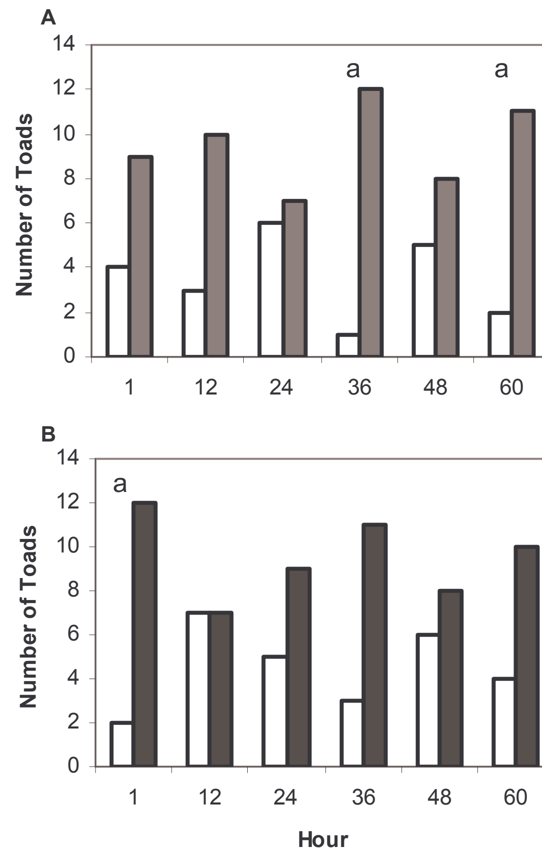


Figure 2. (a) Radioactivity in dosed water. Downslopes with arrows are assumed to represent uptake by the toad, while the upslope with an arrow is assumed to represent elimination from the toad and evaporation. Water changes occurred at t_8 and t_{12}. Bars represent standard error mean. (b) Change in percent standard body mass. Water changes occurred at t_8 and t_{12}. Bars represent standard error mean.

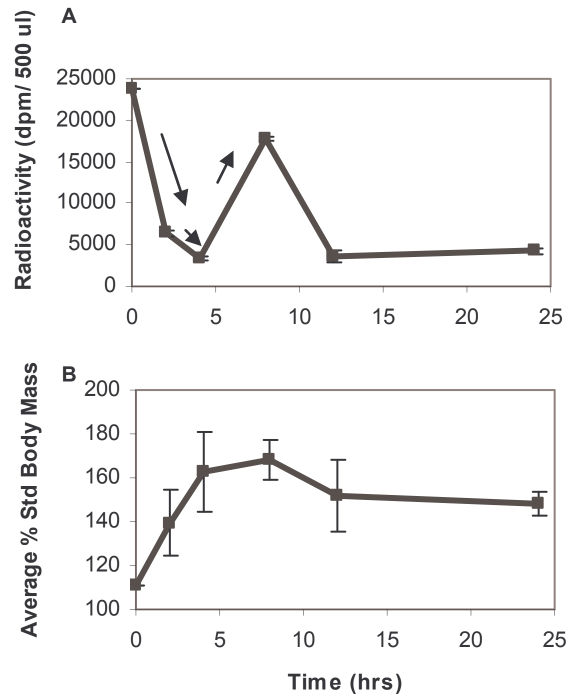


Figure 3. Proportion of body mass for organs dissected. Bars represent standard error mean.

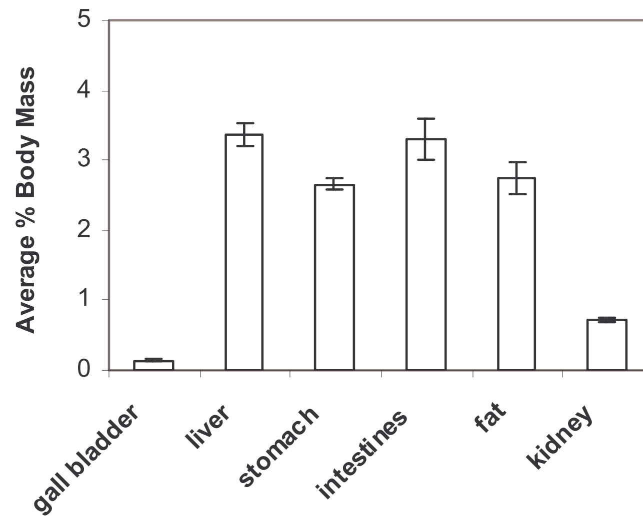


Figure 4. Radioactivity in each organ over time. Water changes occurred at t_8 and t_{12}. Bars represent standard error mean.

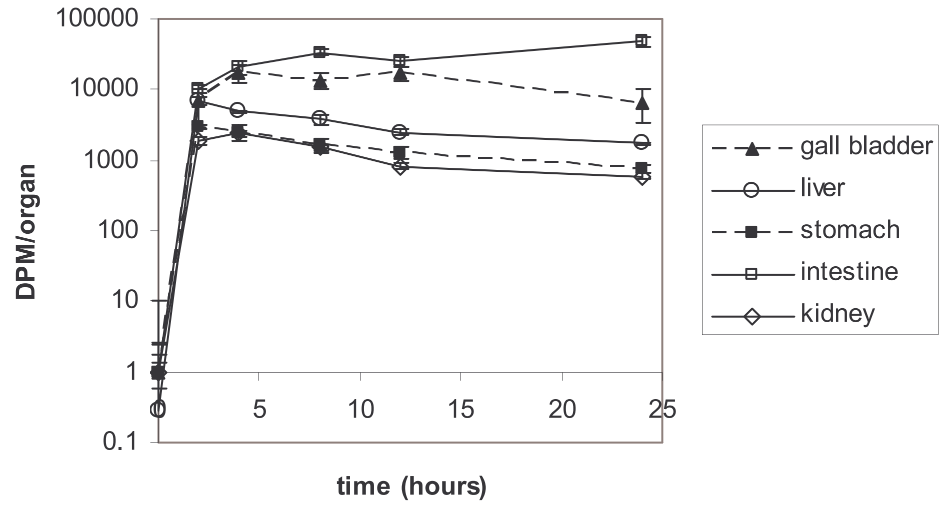
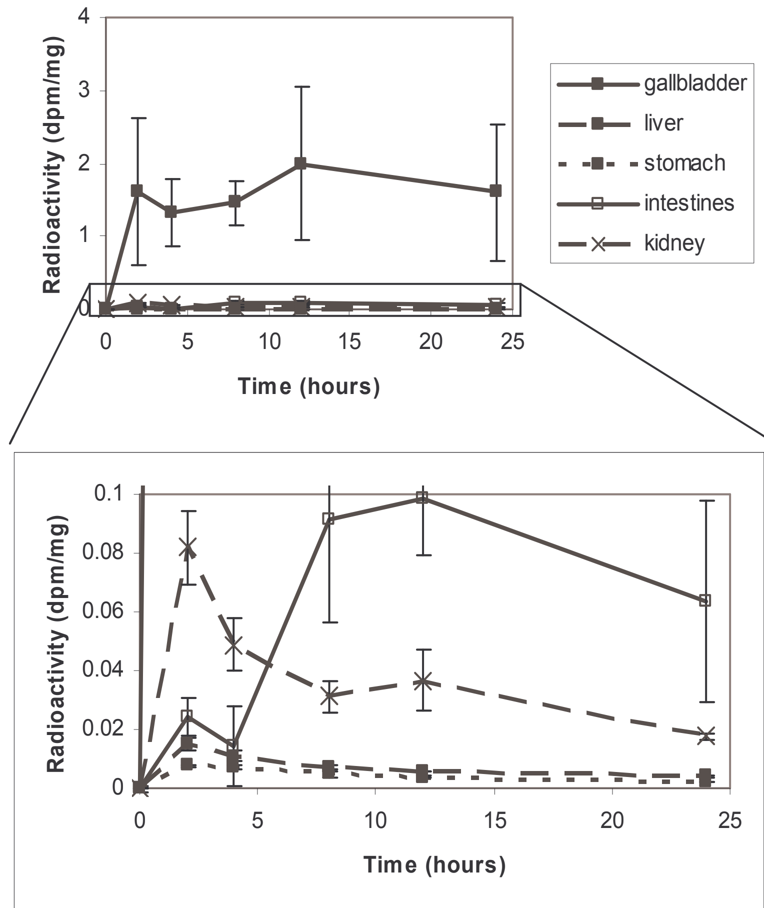


Figure 5. Radioactivity per milligram of each organ over time. Water changes occurred at t_8 and t_{12}. Bars represent standard error mean.



CHAPTER 6

CONCLUSIONS, IMPLICATIONS, AND FUTURE RESEARCH DIRECTIONS

The main research questions and resulting conclusions and implications are summarized below for each chapter:

I. Can larval period length aid in predicting ovarian differentiation rate? Do females from species with different larval periods and ovary differentiation rates respond differently to EDC exposures?

CONCLUSIONS: It appears that the length of the larval period can be an indicator for the type of ovary differentiation rate (e.g. retarded, basic, accelerated). The species with the fastest larval periods (fastest somatic rate) have the slowest ovary differentiation rates (retarded), while the species with the slowest larval period (slowest somatic rate) have the fastest ovary differentiation rate (accelerated). Those species with intermediate larval periods have the intermediate ovary differentiation rate (basic). It should be noted that the rates of ovary differentiation are measured in regard to the timing of metamorphosis and not measured by days from hatching.

The females examined here demonstrated that the faster of the two rates they displayed, either somatic or ovary differentiation, was the most susceptible to deleterious effects under exposure to EDCs. The species with the fastest and intermediate somatic rates

were more somatically susceptible to exposure in that those exposed took longer to develop limbs and metamorphose, while exposure of the species with the slowest somatic rate resulted in no significant slowing of limb development and metamorphosis. The species with the fastest ovary differentiation rate was more reproductively susceptible to exposure in that those exposed took longer to develop fully matured ovaries, while exposure of the species with the intermediate and slowest ovary differentiation rates displayed no significant slowing of ovary development.

IMPLICATIONS: These data illustrate that not all anurans are equally susceptible to harmful effects of EDCs. Moreover these data help to predict which species could be most affected, and therefore, those that should be species of concern under estrogenic EDC exposure conditions.

II. What is the normal developmental pattern of species following different rates of gonad development from the aquatic larval stage, through metamorphosis, to the terrestrial juvenile stage? Does EDC exposure significantly alter the normal pathway of each species?

CONCLUSIONS: Species representing all three gonad developmental rates displayed some intersex gonads under control conditions at some point in their development. Our data suggest that anurans go through a normal phase of intersex from which they recover *en route* to full sexual maturity as either males or females. However, it appears that

exposure to EDCs may increase the possibility of a persistent intersex condition, one that is prolonged past that of normal development.

IMPLICATIONS: It is possible that EDC exposure may be altering the reproductive success of anuran individuals and thus of populations. However, full recovery of EDC-exposed individuals from the persistent intersex condition or functionality is unclear and should be examined further. This study also highlights the importance of conducting longitudinal studies as opposed to single snapshots in time.

III. Can EDC exposure in the aquatic larval stage negatively impact the rate of dehydration in the terrestrial juvenile stage as well as the morphology of the urinary bladder, a critical organ in anuran water conservation?

CONCLUSIONS: EDC exposure appears not to significantly impact the dehydration rate in the species tested at the concentrations investigated. However, those exposed specifically to estradiol during their larval period displayed an altered bladder morphology, with an absence of smooth muscle and epithelial cells in some sections.

IMPLICATIONS: Although the rate of dehydration was not affected, it appears that EDC exposure can negatively affect the bladder. Further, our data demonstrate that exposure to estradiol can impact non-reproductive organs and may hinder the development of other systems. Studies including multiple species should be conducted.

Since the length of the larval period varies, and thus exposure time, differences in larval period between species may influence the severity of damage.

IV. Will anurans avoid soils contaminated with atrazine? Can anurans be exposed to atrazine by taking it up across their skin in the terrestrial juvenile stage? If so, how is it distributed among the organs and eventually eliminated?

CONCLUSIONS: Our data demonstrate that toads do not avoid atrazine-laden soils. Atrazine crossed the pelvic patch rapidly and reached an apparent equilibrium within five hours. The greatest concentrations were observed in the gall bladder, however the greatest amount ended up in the intestines. Material in the intestines is then excreted.

IMPLICATIONS: Amphibians moving through agricultural landscapes may readily cross atrazine-treated soils, making exposure to atrazine and potentially other chemicals present in terrestrial habitats very plausible. Exposure of adult life stages of amphibians through direct uptake of atrazine from soils and runoff water should be considered in risk evaluations.

FUTURE DIRECTIONS

The research conducted for this dissertation was conducted in the laboratory under highly controlled conditions; however future studies in mesocosms or in the field setting would offer more realism. In addition, these studies were conducted with exposure to one contaminant at a time. While these single-exposure studies aid in determining specific effects of a contaminant, it is more likely that amphibians are exposed to mixtures of contaminants as well as to both chemical and non-chemical stressors. Therefore, future studies should include multiple contaminants and/or stressors. Further, future studies should include higher sample sizes for each treatment group. The data presented in this dissertation can offer possible explanations of treatment effects based on small sample sizes. Future studies with higher sample sizes should offer greater predictive power in examining the effects of EDCs on amphibian populations. The following areas are in particular need of research:

- Contaminant mixtures
- Chemical and non-chemical stressors combined
- Aquatic and terrestrial exposure combined
- Longer-term effects of aquatic and/or terrestrial exposure
    - Survival to reproduction
    - Reproductive success
    - Maternal transfer of contaminants to offspring

Behavioral effects of amphibian EDC exposure (e.g. calling behavior)

VITA

Sara Inga Méndez (Storrs) was born May 10, 1979 in Solon, OH. After growing up all over Ohio, Maryland, and Pennsylvania, she graduated from Seneca Valley High School just outside of Pittsburgh, PA in 1997. She received a B.S. in Biology and an M.S. in Ecology from The Pennsylvania State University in 2001 and 2003, respectively. From there, she went on to receive her Ph.D. in Biology from the University of Missouri in 2009. She plans to continue doing stuff and things.