The Combinatory Effects of 17β-estradiol and Atrazine on *Xenopus laevis* Development

Abstract

The presence of chemical toxicants such as pesticides and estrogens in local watersheds has become an issue of concern in recent years. Environmental reports have shown that amphibian populations have declined, along with the health of the overall ecosystems (Gardner, 2001). Organisms exposed to endocrine disrupting chemicals (EDCs) experience interruption of the natural organogenesis that takes place during development (Lenkowski et al 2008). Amphibians, fish, and other aquatic organisms are the most at-risk because of constant EDC exposure due to water-dependent survival. The model organism *Xenopus laevis* is a perfect sentinel for studying EDCs because of its short life cycle that is spent entirely in water, hormone-dependent development, and thin epidermis (Cong et al 2005). It is also being tested in multiple laboratories, providing for better optimized techniques and protocols. In the laboratory, EDC experiments are often single-chemical experiments, which are not representative of the natural environment. The purpose of this research is to propose an experimental setup that evaluates two ecologically relevant chemicals in combination. Firstly, I will develop an atlas of gonad malformations to standardize the evaluation process and reproducibly gauge the morphological defects associated with endocrine disruption. Secondly, I aim to assess the internal effects of EDCs on *X. laevis* using histology, directly observing the intersex and feminization proposed due to EDC exposure. Furthermore, the methods will be extrapolated to a local species of frog, *Rana pipiens* to test for similar effects found in *X. laevis*.

Background and Significance

Despite conscious and continuous efforts to save the natural environments, the human race continues to burden the earth’s ecosystems with
exogenous chemicals. Compounds such as pesticides, estrogens, and organic compounds enter natural water sources via agricultural run-off and human waste products. Environmental health reports show that some amphibian populations have experienced a 50% decline over recent years, contributing to a decrease in the biomass necessary to maintain a healthy ecosystem (Gardner, 2001). With the health of our ecosystems at risk, the observed decrease in amphibian populations should be examined.

Exposure to such run-off chemicals can lead to an interruption of the natural organogenesis that takes place as an organism develops into a mature adult (Lenkowski et al 2008). Compounds that interrupt the normal organogenesis of exposed organisms are endocrine disrupting chemicals (EDCs). According to Kortenkamp, these chemicals may be classified as estrogenic, antiandrogenic, or thyroid-disrupting agents (2007). How the chemicals affect the organism, by increasing estrogens, increasing androgens, or disrupting the thyroid, and to which receptor the chemical binds determine the class of the endocrine disruptor (Kortenkamp, 2007). Many chemical toxicants are estrogenic and work by binding to estrogen receptors in the body, disrupting the homeostatic hormone levels, and leading to an interruption of normal growth and development (Nishimura et al 1997). Several mechanisms of action are possible when an organism is exposed to EDCs. The exogenous chemicals may affect the sex steroid levels present in the body via second-messenger systems or altering the activity of steroidogenic enzymes (Crews et al 2000). Other mechanisms of action include steroid receptor competition and binding (Crews et al 2000). This competition for receptors is the means by which estrogenic EDCs disrupt the endocrine system. Many estrogenic compounds bind to the estrogen receptors (ER) in the organism exposed and thus compete with endogenous estrogens, resulting in an overabundance of estrogen in the body (Crews et al 2000). Androgenic EDCs likewise compete and bind to the androgen receptors (AR) in the exposed organism (Crews et al 2000).
EDCs pose a specific threat to aquatic animals, such as amphibians, because they mimic or interfere (Crews et al 2000; Reeder et al 1998) with endogenous hormones, thus interrupting an organism’s natural development. A lack of maturation due to organogenesis disruption can lead to mortality in organisms exposed to anthropogenic chemicals and pollutants in the watershed (Bevan et al 2003).

Exposure to such chemical toxicants can also affect an organism’s overall health. Health effects to organisms exposed to EDCs can include inefficient nutrient metabolism and/or secretion processes, suppression of an organism’s immune system, and DNA damage, which can also lead to gross developmental abnormalities (Filby et al 2006). Even at very low concentrations (0.1 ppb to 1 ppb), endocrine disrupting chemicals can lead to disruption or dysfunction in an organism’s natural life processes and hormonal regulations (Filby et al 2006). Developmental perturbations, depending on the severity, can lead to fatal deformities or the inability to procreate. With that, organisms exposed to such endocrine disrupting compounds are at risk for population decline, further affecting the health of the surrounding ecosystems.

**17β-estradiol**

Interruption of normal endocrine function can lead to malformations that may significantly hinder an organism’s ability to develop normally and reproduce. Water-dwelling organisms are particularly at risk because they are in constant exposure to chemicals that endocrine disruption. A study done by Gross-Sorokin and colleagues aimed to assess the effects of toxic chemicals released into the environment (2006). The group referenced prior field studies that had documented significant changes to the reproductive physiology, namely feminization of males, and the well-being of aquatic organisms living in rivers around the world (Gross-Sorokin et al 2006). The Environment Agency of England and Wales verified the findings of reproductive disruption and further
stated that a risk management strategy to control the chemicals being discharged into aquatic ecosystem should be put into place. First however, a system of determining such reproductive disruption needs to be developed in order to assess the severity of reproductive disruption seen in organisms that are classified as feminized or intersex. My research will aim to address the quantification of these intersex or feminization incidences found in prior studies of endocrine disruption in aquatic species.

A prevalent chemical responsible for endocrine disruption is 17β-estradiol (E₂), a natural estrogen produced by females and excreted into waste effluents (Bevan et al 2003). It is present in many water sources and has the most significant estrogenic properties of all EDCs quantified (Filby et al 2006). The presence of this chemical in watersheds is concerning because of the effects exogenous estrogens have on organisms exposed. When exposed to estrogens in the external environment, competition with the estrogen produced by the organism results in an overabundance of estrogen in the body system (Crews et al 2000). Figure 1 shows the chemical structure of 17β-estradiol.

![Chemical structure of 17β-estradiol](image)

**Figure 1.** The chemical structure of the natural estrogen 17β-estradiol, produced in female ovaries, adapted from Hayes 2006a.

Estrogens released into the environment via anthropogenic or human-derived waste often contaminate watersheds and other effluents (Filby et al 2006). In a study done by Filby and colleagues, the researchers questioned how combinations of EDCs would affect the reproductive integrity of organisms in
contaminated watersheds (2006). This study examined the effects of environmental estrogens in wastewater treatment effluents and the reproductive effects on the wild fish *Pimephales promelas* or fathead minnow. Chemicals from anthropogenic sources often act as EDCs, including pesticides and chemical pollutants (Filby et al 2006). The fathead minnow was used in two experiments by this group to demonstrate the effects of estradiol in isolation (Filby et al 2006) in experiment 1 and the effects of estrogen combinations in experiment 2. To test the combinatory effects of multiple estrogens, experiment 1 exposed fathead minnows for 21 days to a wastewater runoff with what was designated as high estrogenic content. Experiment 2 consisted of fathead minnows being exposed for 21 days to a wastewater effluent that was defined to have a weak estrogenic content. These experiments studied the effects of multiple estrogens in water sources; the groups were compared with a group exposed to a single estrogen (ethinyl estradiol) to examine the combination effects (Filby et al 2006). The study reported that health effects linked with estrogen overexposure include immunosuppression, “genotoxic damage,” toxicity due to lack of excretion functions, and stunted growth (Filby et al 2006). The study also reported a decrease in the presence of secondary sex characteristics, (dorsal fat pad and tubercle development) in males in experiment 1 with a high estrogenic content, whereas there was no significant decrease found in experiment 2 with a weak estrogenic content (Filby et al 2006). The fish exposed to ethinyl estradiol alone showed a significant suppression of secondary sex characteristics (Filby et al 2006).

While Filby and colleagues found a reduction in the expression of secondary sex characteristics in the high estrogen treatment and no significant decrease in secondary sex characteristics in the low estrogen treatment, the morphological effects on the gonads were not explicitly stated. Further experimentation should be done to assess the effects of such estrogenic compounds on the gonad morphology of exposed fish. This is because exposure
to estrogenic compounds, leading to feminization can result in the presence of female tissue in male organisms. The direct observation of gonadal tissue provides more information on the actual feminization taking place in the organisms exposed. Such direct examination can be done using histological techniques, dissecting and fixing the gonadal tissue from organisms exposed in their natural habitat.

In the environment, certain species are at an elevated risk for harm by endocrine disruption. Fish exposed to such EDCs with estrogenic qualities are at risk for malformations, including intersex (Gross-Sorokin et al. 2006). A study done by Gross-Sorokin and colleagues aimed to address the increase in reproductive disruption in various fish species exposed to waste water effluents (2006). The researchers noted that field studies done in the United Kingdom, the United States, Europe, and Japan yielded data showing a positive correlation to the between the degree of intersex an organism exhibits and the amount of estrogenic chemicals in the water (Gross-Sorokin et al 2006). Intersex organisms are those with male and female gonadal tissue present in the same organism (Gross-Sorokin et. al. 2006). The intersex phenomenon has been documented at E₂ concentrations of 10 ppb and shown to elicit effects such as inconsistent mating behavior, irregular female to male ratios, inhibited testicle development, and a decrease in reproductive capacity (Gross-Sorokin et al 2006). The group studied the expression of vitellogenin, a female-specific protein and found that many male organisms also expressed the gene (Gross-Sorokin et al 2006). This improper induction of vitellogenin is a result of estrogen exposure, and according to Gross-Sorokin and colleagues, may result in outward effects such as disrupted lipid and protein production and a decrease in calcium present in the scales (2006). The induction of this female-specific protein is one indicator of feminization, as is the decrease in sperm quality of exposed male fish (Gross-Sorokin et al 2006). Further and more direct indications of feminization can be found by using histology techniques. By dissecting the gonads of exposed
organisms, we could observe actual feminized tissue, further affirming the instances of intersex. Intersex, hermaphroditism, and other developmental malformations are observable in fish, as well as aquatic species that are perhaps more conducive to laboratory experimentation.

A model organism for studying the impacts of EDCs is *Xenopus laevis*, the South African clawed frog. This organism is ideal for study because complete maturation is observable in a laboratory setting (Cong et al. 2005). Nieuwkoop and Faber (1994) developed a staging protocol to describe *X. laevis* development from fertilization to the development of its four limbs and tale reabsorption (referred to as NF stages). This table is used to gauge the growth of organisms and predict when certain critical points in development occur (Nieuwkoop and Faber, 1994). Lenkowski and colleagues note that gonadal development occurs at NF stage 66, after tale reabsorption, in *X. laevis* (2008).

Laboratory experimentation on the organism is also facile because of its thin skin, water-dependent fertilization and hormone-regulated development (Hayes et al. 2006b). In addition to these qualities, the aquatic life stage of *Xenopus laevis* make the organism exceptionally vulnerable to EDCs. Cong and colleagues also showed this organism to have a high degree of gonad sensitivity to estrogens in particular (2005). Thus, *Xenopus laevis* has become a sentinel for laboratory examination of environmental stressors and reproductive effects of EDCs because of its short maturation cycle, thin epidermis and dual life phases (Cong et al. 2005).

*X. laevis*’s hormone-sensitive processes, as described by Bevan et al (2003) have been exploited by many scientific studies in order to test the impact of pesticides and estrogens on the organism’s natural development. The group aimed to look at how such chemicals, including the synthetic nonylphenol, octylphenol, methoxychlor and the natural E₂ affected *X. laevis* embryos when exposed at a very early stage in development (Bevan et al 2003). Development and feminization in *X. laevis* was analyzed when the organisms were exposed to
concentrations of 1 ppb \( E_2 \). Bevan and colleagues state that exposure to estrogens at or before the critical stage of development (defined as before stage 27 of development in the NF system). Bevan and colleagues also report that treating \( X. laevis \) starting at NF stage 3 with \( E_2 \) at 1 ppb results in increased incidence of organism death and increased accounts of malformations (Bevan et al 2003). Such deformities includes crooked spines, increase in abdomen size, smaller heads and eyes, retarded digestive organ growth, and slowed development of the nervous system (Bevan et al. 2003). The organisms exposed to environmental estrogens at concentrations of 1 ppb at or before the critical period experienced a consistent increase in malformations, resulting in death before NF stage 42 (Bevan et al. 2003). Bevan and colleagues also suggest that due to the aquatic nature and extreme sensitivity to hormones, amphibians like \( X. laevis \) as well as other water-dwelling organisms are prime examples to study the effects of environmental estrogens on wildlife development (Bevan et al 2003).

Exposure to estrogenic EDCs cause abnormal thyroid function in fish and birds, a lower fertility rate in birds and mammals and potential feminization in birds, fish, and mammals (Nishimura et al 1997). In a study done by Nishimura and colleagues, \( X. laevis \) was treated with 10 ppb \( E_2 \) and exhibited a shorter distance between the eyes and retarded digestive organ development when compared to the control group (Nishimura et al 1997). Figure 2a shows an image of a tadpole raised in a negative control solution while 2b shows a tadpole reared in 10 ppb \( E_2 \).
Figure 2. X. laevis tadpole raised in negative control solution (a) and tadpole raised in 10 ppb E$_2$ solution (b). The difference in spinal curvature and enlarged abdomen from (a) to (b) is evident. Adapted from Nishimura et al 1997. Magnification x 11.

Other studies have shown that complete feminization of X. laevis is attained when the organisms are exposed to E$_2$ 100µg/L for 7 days at NF stages 50-53, 14 days at NF stages 50-55, or 49 days NF stages 50-66 (Hayes et al. 2006a). X. laevis’s water-dependent existence, in addition to its sensitive hormonal processes and sensitivity to EDCs, it is logical to note that the outward malformations are only a small fraction of the damage being done. Internal analysis of the malformations, specifically the gonads, will provide a more complete picture of the feminization taking place in organisms exposed to EDCs such as the natural estrogen E$_2$.

Atrazine

In addition to estrogens, herbicides such as atrazine have also been evaluated for potential effects on the sexual development of water-dwelling organisms. Atrazine is an herbicide that targets broadleaf weeds and interferes with photosynthetic pathways and aerobic respiration (Hecker et al 2005). It is widely used in the United States to control the presence of broadleaf weeds in
corn crops (Fan et al 2007). Its prevalent use for agricultural benefit makes atrazine the number one pesticide toxicant found in groundwater and surface water (Fan et al 2007; Lenkowski et al 2008). With a water solubility of 30 mg/L and crop application concentrations of 213 g/L, atrazine is often found in greatest concentrations (12.7 mg/L to 4.9 mg/L) in areas with shallow water collection. Agricultural runoff often contains atrazine at concentrations of many parts per million (ppm) and 40 parts per billion (ppb) measured in rainfall collections (Hayes et al 2002).

Multiple studies have shown the endocrine disrupting potential of atrazine in organisms including laboratory mice and X. laevis (Fan et al. 2007). Atrazine interferes with both androgenic and estrogenic pathways; however it has very little affinity for the estrogen receptors of gonadal cells (Hayes et al 2006a). Despite its non-estrogenic properties, studies have shown that atrazine in water systems disrupts androgen synthesis and increases estrogen levels in various species (Hayes et al 2006a). Hayes and colleagues showed that atrazine works via the aromatase mechanism in amphibians, fish, turtles, human cells, and human tissues (2006a). The aromatase mechanism works to increase estrogen levels, with atrazine binding to and inhibiting the enzyme phosphodiesterase (Fan et al 2007). Phosphodiesterase inhibition leads to an increase in aromatase levels within the organism system. Aromatase uses testosterone as a substrate and subsequently converts testosterone to estrogen (Fan et al 2007; Hayes et al 2006a), resulting in an increase of estrogen in the body system. This overabundance of estrogen leads to feminization and intersex in the exposed organism (Crews et al 2000). Figure 3 shows the aromatase mechanism as presented by Hayes and colleagues.
Figure 3. The proposed aromatase mechanism, explaining the effect of atrazine on X. laevis. According to Hayes, the induction of aromatase by atrazine results in a decrease of testosterone via conversion to estradiol. Adapted from Hayes et al 2006a.

Atrazine exposure increases estrogen levels within the animal and chemically induces female characteristics in genetically male frogs (Hayes et al 2006). Atrazine is known to cause a decrease in testicular volume and a decrease in the number of nursing cells present in amphibians (Tavera-Mendoza et al 2002). In addition, it is documented that atrazine exposure significantly decreases spermatogonial nests in amphibians and fish (Tavera-Mendoza et al 2002). Parshley and colleagues (2000) also demonstrated demasculinization in male X. laevis male tadpoles, encompassing a significant decrease in larynx size, resulting from androgen depletion in the organism’s body system (Parshley, 2000).

However, there is a case of controversy in the use of atrazine and its reported effects from previous studies. In multiple experiments performed by laboratories opposing atrazine use, investigators have shown hermaphroditism (testes and ovaries present) and demasculinization occurs at atrazine levels of ≥ 1 ppb (Hayes et al 2002). At lower, ecologically relevant doses (≥ 0.1 ppb), atrazine has been shown to produce gonad abnormalities, including multiple male gonads or hermaphroditism (Hayes et al 2002). Both aberrations were never seen in control organisms, according to Hayes and colleagues (2002).
Hayes and colleagues also asserted that atrazine itself is a detrimental pesticide that has been shown to feminize aquatic organisms (Hayes et al. 2006a). Atrazine, through the Aromatase mechanism, has been shown to affect the growth of the larynx in *X. laevis*, which is directly connected to the androgen concentration in male organisms (Hayes et al. 2006a). In addition, the study delved into the effects of atrazine on gonadal development. According to Hayes and colleagues’ histological procedures, the gonads of *X. laevis* are differentiated between NF stage 52 and 54 (2006a). Atrazine exposure, even at very low concentrations (0.1 ppb), was shown to cause animals with single sex polygonadism (SSP) and hermaphroditic animals with both ovaries and testes (Hayes et al. 2006a). The group hypothesized that exposure to atrazine at concentrations of 0.1, 0.4, 0.8, 1 and 25 ppb would either result in gonad deformities due to a loss of androgens in the system or in feminization due to the overproduction of estrogens via the aromatase mechanism (Hayes et al. 2006a). To test the first hypothesis, *X. laevis* was exposed to 5 mg/mL of the antiandrogen Cyproterone Acetate (CPA) (Hayes et al. 2006a). According to Hayes, CPA exhibited effects similar to the pesticide atrazine on *X. laevis* as both were shown to elicit the occurrence of non-pigmented ovaries. SSP was also seen in atrazine-exposed larvae, but absent from frogs exposed to CPA.

Although atrazine has been reported to disrupt sexual development and gonad differentiation in *X. laevis*, other studies have presented controversial results that are inconsistent with the aforementioned sexual malformations. In other studies similar to those of Hayes and colleagues (2002), no accounts of multiple gonads, single sex polygonadism (SSP) (Hayes et al. 2006a) or hermaphroditism were documented (Lenkowski et al. 2008).

Hecker and colleagues (2005) set out to replicate the work of Hayes and colleagues indicated that while exposing three groups of adult *X. laevis* to concentrations of 1, 25, or 250 µg/L atrazine, levels of aromatase activity were at minimal levels, and no increase in estrogen was seen. Furthermore, the study
indicated that atrazine did not disrupt natural hormone levels by means of aromatase activation suggested by Fan et al (2007) and Hayes et al 2006a; (Hecker et al. 2005). The contradicting results from these studies present a question of the true effects of atrazine.

Another study done by Kloas and colleagues (2008) further investigated the effects of atrazine on the development and differentiation of gonads in X. laevis (2008). The group exposed X. laevis tadpoles to atrazine concentrations of 0.01, 0.1, 1, 25, or 100 ppb from day 8 after fertilization until full tail resorption (Kloas et al 2008). The group found that all treatment groups showed similar survival and development to negative control groups (Kloas et al 2008). The positive control in this study was E2, in which the authors observed gonadal feminization (2008). Despite the findings presented by this research team, controversy is still present. The Hayes findings come from an environmentally conscious lab, that is working to discontinue the use of atrazine, whereas the Kloas group’s research is funded by Syngenta, the company that produces atrazine (2008). By doing this research in an undergraduate laboratory, without the pressures felt in corporate or government-funded labs, I aim to resolve this controversy of atrazine’s true effect as an EDC when it is present in the environment. Furthermore, my research aims to accurately represent concentrations and combinations of EDCs in the natural environment.

**Combinatory Effects**

Chemicals in the environment are present in water sources often in various combinations and concentrations. Rarely is a watershed contaminated with only one pollutant or endocrine disruptor. However, most laboratory studies examine the effects of only one EDC on exposed organisms (Hayes et al. 2006b). Combinations of chemicals such as pesticides may produce an increased effect on an exposed organism than any of the chemicals singly (Hayes et al 2006b). This outcome is possible because a chemical that does not cause harm to
an organism may alter the effects of another EDC also in the watershed (Hayes et al. 2006b).

In a study done by Hayes, Case and colleagues (2006b) the effects of pesticide mixtures were analyzed in watersheds with a decreasing amphibian population. The group aimed to study the effects of such endocrine disrupting compounds at concentrations much lower than in previous experiments (Hayes et al. 2006b). By addressing the effect of such pesticides at concentrations used by corn farmers in Your County, Nebraska, the group aimed to show the impact of multiple pesticides on water-dwelling species. These anthropogenic chemicals pose a constant threat because they are present in the environment year-round (Hayes et al 2006b). Hayes and colleagues assert that pesticides, including the common herbicide atrazine, are usually applied in combination as a comprehensive agricultural treatment (2006b). The local species of Northern Leopard Frog (*Rana pipiens*) was used in this study and exposed to the pesticides singly and in combination experiments (Hayes et al 2006b). Nine pesticides, including atrazine were analyzed in isolation at 0.1ppb and in combination at either 0.1ppb or 10 ppb the lab showed that while some of the pesticides individually retarded the growth of larvae, exposure to chemical mixtures at concentrations of 0.1ppb resulted in a significant delay in time to metamorphosis based on ANOVA (p < 0.0001), with the delay being near 20 days (Hayes et al 2006b). The organisms exposed to the pesticide mixtures were also smaller than those in control groups (p < 0.05) (Hayes et al 2006b). Histological analysis described males as having undifferentiated gonadal tissue, with a cortex and medulla still intact (Hayes et al 2006b). Analysis of females showed an incomplete extension of the gonadal medulla and very few developing oocytes in the gonads (Hayes et al 2006b). Other adverse effects of these chemical combinations on *Rana pipiens* include spinal integrity, lacking the ability to sit erect, meningitis, and septicemia from bacterial growth in the water (Hayes et al 2006b). However, Hayes and colleagues did not confirm which pesticides in the
mixtures were responsible for the detrimental effects in exposed organisms (Hayes et al 2006b). The group proposed that some pesticides disrupt endocrine function and normal gonadal development to an extreme degree while others may have little or no effect on the normal maturation of the organism (Hayes et al 2006b). From this study, it can be gathered that using single chemicals at environmentally unrealistic concentrations to estimate the effects of EDCs on amphibians and other water-dwelling organisms is under-representative of a true aquatic environment (Hayes et al 2006b).

Chemicals in combination can work together in an animal system, either synergistically or additively. According to Crews and colleagues (2000), when two chemicals elicit a disruptive effect in a small number of organisms exposed to either chemical in isolation, but elicit an effect in more than 95% of the organisms exposed to the chemicals in combination, the effect is synergistic. On the other hand, when chemicals elicit effects in half of the organisms exposed to either chemical in isolation, and elicit an effect in more than 95% of the organisms exposed to the chemicals in combination, the effect is additive (Crews et al 2000). It has been shown in previous studies that the natural estrogen E2 causes feminization in a majority of the organisms exposed to ecologically relevant doses (Bevan et al 2003; Nishimura et al 1997). In addition, Hayes and colleagues have shown in multiple experiments that atrazine also elicits intersex and multiple gonad malformations in X. laevis. Therefore, I propose that the effects of the estrogen E2 and the pesticide atrazine at ecologically relevant doses (10 ppb and 1 ppb, respectively) will elicit additive effects in X. laevis tadpoles (Crews et al 2000).

The additive combinatory effects of such EDCs on X. laevis should result in an increase of gonad malformations when compared to single-chemical experiments. I expect to see an increase of intersex in X. laevis when it is exposed to a combination of chemicals that work together in an additive fashion.
Alternatively, if the effects of the proposed chemical combinations are not seen to be additive, the effects may be synergistic or antagonistic. This would result in either a significant increase in gonad malformations in the case of chemical synergy, or a decrease in malformations (fewer organisms malformed) in the case of chemical antagonism (Crews et al 2000). However, if one of these alternative hypotheses is supported, the development of a malformation standard will allow researchers to examine the severity of malformation in the synergistic or antagonistic sense. It will also present an alternative to how the chemicals affect the hormone balance and endocrine system in an organism.

With an antagonistic effect, the EDCs an organism is exposed to may not work through the same pathway as previously described by Nishimura et al (1997). Antagonistic effects on the organism may be due to the suppression of one chemical by the other, or as one chemical disrupts chemical processes in the organism, the other chemical restores the homeostatic balance via an alternative mechanism (Fan et al 2007).

**Specific Aims**

The goal of this study is to examine the combinatory effects of two endocrine disrupting chemicals (EDCs) 17β-estradiol and atrazine, found at ecologically relevant concentrations (10 ppb and 1 ppb, respectively) in Midwestern agricultural watersheds on the model organism *Xenopus laevis*. I propose that the chemicals when present in combination, as they are in the natural environment, will have an additive effect on the endocrine disruption and subsequent gonad malformations in the organisms exposed. I expect to see an increase in intersex organisms, not only qualitatively, but quantitatively as a result of combinatory chemical exposure.

1. To establish an atlas of intersex gonad malformations in *X.laevis*.

Although numerous investigators report sexual differentiation defects in
amphibians exposed to endocrine disruptors, a comprehensive scale of severity does not exist. My research will create a set of standards to reproducibly gauge the morphological defects associated with endocrine disruption (for future work in our laboratory and others). With this gauging system, I further propose a method of quantifying the degree to which organisms are affected in a population exposed to a combination of chemicals.

2. To perform histology on *X. laevis* tadpoles exposed to the endocrine disrupting chemicals 17β-estradiol, atrazine, and 17β-estradiol + atrazine treatments. Because no outward malformations are manifested, I will examine the internal effects of these chemicals on *X. laevis* using histology. Specifically, I will look for the presence of both male and female gonadal tissue in a single organism, and characterize the degree of intersex based on the atlas developed in the previous section of my research aims.

**Research Design and Methods**

**Materials**

Three females and two male *Xenopus laevis* will be obtained from Nasco (Ft. Atkinson, WI). The chemicals used in this experiment include atrazine (2-chloro-4-ethylamino-6-isopropylamine-1,3,5-triazine), to be obtained from Chemservice Inc. (Chester, PA), 17β-estradiol (1,3,5-estratrien-3,17-β-diol), human choriogonadotropin, 95% Ethanol, Bouin’s fixative and benzocaine obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Food will be obtained from Carolina Co.

**Methods**
Specific Aim #1: Developing a set of standards used to assess the severity of intersex in *X. laevis* tadpoles in 3 different dose treatments of the EDC 17β-estradiol.

I will characterize the degree of malformation qualitatively based on other organisms in the treatment group and then graphing the accounts of malformations relative to others in the same treatment group.

This aim focuses on developing a scale for gonad malformations of *X. laevis* when exposed to endocrine disrupting chemicals. It has been shown in many previous studies that exposure to such chemicals can result in gonad malformations, including feminization and intersex (Nishimura et al 1997; Hayes et al 2002; Gross-Sorokin et al 2006). However, since the degree of feminization and intersex is a qualitative measure, it should be evaluated based on a continuum of malformation levels. A standard set of characterizations has yet to be developed, and there are very few scales for evaluating the effects of endocrine disruption. Hayes and colleagues have developed an atlas of gonad malformations, yet they neglect to account for the varying degrees of intersex and feminization seen in exposed organisms (2002). Gross-Sorokin and colleagues, along with Filby and colleagues lacked an actual method of characterizing the feminization and intersex they were studying (2006; 2006). A consistent method for assessing the degree of severity is therefore needed to more accurately quantify the amount of intersex and feminization seen in organisms exposed to endocrine disrupting chemicals.

In this experiment, the 3 adult female frogs are each injected with 1,000 IU of human choriogonadotropin 18 hours before eggs are harvested. Once injected, the females are returned to a tank and placed in an incubator set for 16°C overnight, consistent with the method used by Dr. Heathcoat’s lab at the University of Milwaukee (2009). After incubating overnight, the females are removed and squeezed for eggs three times; squeezing takes place in two-hour intervals (Heathcoat, unpublished protocol, 2009). Once squeezed from the
female, the eggs are placed in 0.2% L-cysteine for one hour to remove the jelly coat (May and Escobar, unpublished protocol, 2008). Next, one adult male frog is sacrificed, first placed in a solution of 1% benzocaine until movement ceases (Hayes et al, 2002). The process is completed by slicing through the upper jaw and probing into the spinal cord. The organism is sliced open and the ventral organs are moved aside to expose the testes. The testes are excised and placed in a Petri dish of Ringer’s Solution (Hayes et al 2002). After one hour, the dejellied eggs are also placed in the Ringer’s Solution with the dissected testes. Fertilization is allowed to occur.

Eggs hatch 4 days after fertilization. Approximately 200 eggs will be obtained from the squeezings. The tadpoles will be split into 4 groups; 3 groups will receive a separate estrogen treatment, with the fourth group as the FETAX control. The Frog Embryo Teratogenesis Assay for Xenopus is an accepted negative control that causes no harm to X. laevis when the organisms are grown in this solution. Each tank will be rotated on the shelving unit every 3 days to eliminate position bias (Hayes et al 2002).

The estrogen treatments assigned to the experimental groups are low estrogen content 1 ppb, the ecologically relevant does of 10 ppb, and the high dose of 25 ppb. Tadpoles will be exposed to the estrogen from fertilization to NF stage 66, with water changes and chemical replenishments every 3 days (Hayes et al 2002).

The area where the tanks are kept will be kept at a constant temperature (22°C according to Hayes, 2002) and will be equipped with a 12h light/12h dark schedule to reduce confounds and simulate a natural environment. Adult frogs will be fed rabbit food obtained from Carolina Co. once daily while the tadpoles are fed sera micron twice daily (Hayes et al 2002).

The organisms in each treatment are exposed to the respective chemicals from hatching (NF stage 48) until the tail is fully reabsorbed (NF stage 66). Once the organism have reached stage 66, organisms are removed from the tanks and
given identification codes based on the color tank they were taken from, the date
they were removed, and a number corresponding to order of organism removal
(J. Bickle, unpublished lab protocol). Stage 66 is the point in development when
the gonads are completely formed; therefore histology can be performed on the
mature gonads (Nieuwkoop and Faber, 1994). They will then be anesthetized
with a 1% Benzocaine solution (Hayes et al 2002). Organisms are then placed in
100% Bouin’s fixative solution for 24 hours, then the solution is diluted with
ethanol to a 70% ethanol/Bouin’s mixture. The organisms can be stored
indefinitely in 70% ethanol/Bouin’s or after 24 hours can undergo dissection.
Dissections are performed under an Olympus Dissecting Scope; the kidney-
interrenal-gonadal complex is excised and placed in 100% Bouin’s fixative
solution. Sex is determined based on gross morphological analysis of the kidney-
interrenal-gonadal complex (Hayes et al 2002). All organisms are dissected, but
histological procedures are carried out for 10 organisms per tank and on those
organisms whose sex is undeterminable based on gross morphological analysis
(Hayes et al 2002).

Histology is performed based on a fixation-dehydration-embedding
protocol developed in our lab (JB, unpublished protocol). The tissue is fixed in
70% ethanol/Bouin’s and then diluted into 80%, 95%, and finally 100% ethanol.
After that, the tissue solution is diluted in 25% increments into 100% Histo-clear.
After that, the solution is slowly changed into 100% paraffin wax. The tissue is
then embedded into plates and cooled. The wax is trimmed around each gonad
and mounted on wood blocks for sectioning.

Sectioning is done with a microtome; the gonadal tissue is sliced into 8 µm
sections and floated on 4% formalin, consistent with the Hayes laboratory
protocol (2002). The formalin allows the tissue to adhere to the slide for further
processing. Once the tissue has been adhered to the slide, the slides are carried
through a hematoxylin and eosin staining series whose steps have been
optimized based on conditions and equipment used at Carthage College (J. Bickle, unpublished protocol, 2009).

To establish the atlas of gonad malformations, I will analyze the sections of gonad tissue for each of the estrogen treatments, as well as the negative controls. I will assign the gonads of negative control organisms with the degree of normal development. For the low estrogen treatment, the gonads will be assigned a degree of intersex ranging from normal, to describe organisms with few or no malformations, to mild, describing organisms with malformations notably greater than negative control organisms. The gonadal structures in organisms classified as normal will look similar to those in negative controls. Next, I will take photos of each degree documented, establishing the qualitative atlas for future reference. I will evaluate the ecologically relevant dose treatment and the high estrogen treatment in a similar fashion.

For the ecologically relevant treatment, the gonads will be assigned a degree of intersex ranging from mild to intermediate. Organisms classified as mild present similar malformations as seen in the previous treatment, while organisms classified as intermediate will present more prevalent malformations. Again, photos of each degree will be taken in order to establish the atlas. The same procedure will be employed for the high estrogen treatment, with the intersex ranges being intermediate to severe. Intermediate organisms will present gonad malformations similar to those seen in the intermediate organisms of the previous estrogen treatment, while organisms classified as severe will present malformations that are far more prevalent than any of the organisms documented. Photos again will be taken to document the degrees and complete the atlas.

This collection of intersex accounts, ranging from mild to severe will be used to qualitatively assign the gonads of organisms exposed to EDCs a degree of severity related to the ensuing malformations. My second research aim,
evaluating the combinatory effects of two ecologically relevant chemicals, will use this atlas to analyze and present these effects in a quantitative fashion.

**Specific Aim #2:** Performing histology on X. laevis tadpoles exposed to 4 experimental groups, FETAX, 10 ppb 17β-estradiol, 1 ppb atrazine, and 10 ppb 17β-estradiol + 1 ppb atrazine.

The organisms used to carry out specific aim 1 will not be involved in the pursuit of specific aim 2. The standard of gonad malformations will be established first and then employed for evaluating gonad malformations in this experiment. The procedure for inducing egg production, male sacrifice, and squeezing the female frogs will be the same as specific aim 1.

Eggs hatch approximately 4 days after fertilization according to Hayes and colleagues (2002). The larvae are then split up randomly and put into 4 groups of 30 organisms (Hayes, 2002). Four experimental groups are established as follows: FETAX negative control, 10 ppb 17β-estradiol positive control, 1 ppb atrazine group, and 10 ppb 17β-estradiol + 1 ppb atrazine combinatory experimental group. In addition each treatment group, including FETAX contains up to 0.004% ethanol as a vehicle for each chemical to be distributed in the water (Hayes et al 2002). This experiment is done in triplicate; three tanks and 90 organisms are subject to each treatment (Hayes, 2002). The tanks are color-coded by treatment and the experiment itself is conducted blindly to reduce bias (Hayes et al 2002). Tanks will be rotated around the shelving apparatus every 3 days to minimize positional advantages in growth or temperature differences (Hayes et al, 2002). Frog rearing and histology techniques are consistent with the techniques used in specific aim 1.

The quantitative numbers of intersex will be taken from the gonadal malformations standard developed in specific aim 1. By evaluating the degree of intersex in specific aim 1, I will be able to assess the intersex data from specific aim 2 using statistical techniques. Each organism evaluated in experiment 2 will
be assigned a degree of intersex based on the standard I will develop. After this, I will document the number of organisms for each gonad malformation standard category. The numerical data obtained will be statistically evaluated using an ANOVA, comparing the negative FETAX control with the single-chemical treatments and the combinatorial treatment, testing for significant differences in the degree of gonadal malformations seen in each of the treatment groups.

**Preliminary Results**

To date, my research lab has optimized protocols for fixation, dehydration, sectioning and hematoxylin and eosin staining. Dissection techniques have also been developed and lab assistants have been trained in excising the kidney-interrenal-gonadal complex that is described by Hayes and colleagues (2002). We have currently examined the gross morphology of both male and female organisms and carried tissue through the entire histological process. The gonadal tissue from organisms exposed to the FETAX treatment has been analyzed and documented using hematoxylin and eosin staining and microscopy, consistent with previous studies (Hayes et al 2002, Hayes et al 2006a)

Relatively speaking, testicular tissue is shorter and more compact than ovarian tissue in *X. laevis* (Hayes et al 2006a). Testes from negative control organisms are non-pigmented and are generally one-third the size of the *X. laevis* kidney (Hayes et al 2006a). Figure 2 shows a 8 µm section of *X. laevis* testis at 200x magnification.
Figure 4. Anterior-to-posterior negative control testis tissue section (8 µm) from NF stage 66 *X.laevis* male in hematoxylin and eosin stain, 200x magnification. (Photo by J.Bickle). The blue arrowhead shows a primary spermatocyte, while the red arrowhead shows a collection of sertoli cells.

Ovarian tissue is identified on a gross scale by the presence of lobes in the tissue and the presence of melanin pigment (Hayes et al 2006a). The ovary itself is the length of the kidney in *X. laevis* and is distinguishable from testes microscopically by the development of a central cavity that is encapsulated by connective tissue (Hayes et al 2006a). Figure 3 is an 8 µm section of ovarian tissue at 40x.
Figure 5. Anterior-to-posterior negative control ovarian tissue section (8 µm) from NF stage 66 *Xenopus laevis* female in hematoxylin and eosin stain, 40x magnification. (Photo by J.Bickle). The blue arrowhead shows the primary oocytes, dyed purple. The large canal in the center is the ovarian canal, characteristic of ovarian tissue.

Currently, *X.laevis* organisms from each of the 4 treatments are being dissected and carried through the fixation process. The tissue will be sectioned and analyzed via microscopy. We propose to test for an increase in hermaphroditism (ovarian and testicular tissue within one organism) and/or single-sex polygonadism, in particular multiple testes as seen by Hayes and colleagues (2006a).
Summary and Conclusions

Endocrine disrupting chemicals (EDCs) are present in our local watersheds and have the potential to affect the organisms in the natural ecosystems through steroid receptor binding and inducing Aromatase activity. Many of these chemical toxicants are due to agricultural run-off (atrazine) and human excretion (E_2). Studies have shown the effects of estrogens as EDCs, and are consistent in findings of feminization of organisms exposed. Atrazine as an EDC has faced controversy from groups supporting its use, such as the company Syngenta and groups such as the Hayes lab advocating against it.

My research goal of discovering the nature of the effects of these ecologically relevant chemicals in combination is attainable through fulfilling my aims of developing an atlas of standard gonad malformations for reference and quantification. Once the atlas is developed, the next step is analyzing the internal gonad malformations using histology. By quantifying the degree of gonad malformations, my study will show the effects of atrazine and E_2 in combination on *X. laevis*.

Future Directions

At this point, further analysis of gonadal tissue from organisms exposed to 10 ppb 17β-estradiol, 1 ppb atrazine, and the combinatory 10 ppb 17β-estradiol + 1 ppb atrazine experimental group is needed. Dissection and fixation as well as sectioning, staining and microscopic analysis will be performed, with malformations as well as gender numbers recorded and documented visually. Statistical analysis will aid in determining the effect of endocrine disruptor combinations and the nature of the combinatory effects, being additive, synergistic, or antagonistic.

Since the effects of atrazine on *X. laevis* and other water-dwelling organisms has been disputed by researchers in the field, another future direction will be to come to a sound conclusion on the effects of atrazine as an EDC and
quell the controversy between the Hayes lab and Syngenta-funded projects. The main goal is to determine if atrazine causes similar gonad malformations at concentrations used by Hayes and colleagues or if the findings of groups like Kloas and colleagues is more representative of atrazine’s impact.

A final direction for this research will be to extend the analysis of the combinatorial effects of these endocrine disrupting chemicals on local amphibians such as the Northern Leopard Frog *Rana pipiens*, as well as fish native to our local watersheds.

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