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AN INVESTIGATION INTO THE MECHANISM OF ACTION OF ATRAZINE AND ITS EFFECTS ON DEVELOPING *RANA CLAMITANS* AND*XENOPUS LAEVIS*

presented by

Katherine Kemler Coady

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AN INVESTIGATION INTO THE MECHANISM OF ACTION OF ATRAZINE AND ITS EFFECTS ON DEVELOPING *RANA CLAMITANS* AND *XENOPUS LAEVIS*

By

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ABSTRACT

AN INVESTIGATION INTO THE MECHANISM OF ACTION AND EFFECTS OF ATRAZINE ON DEVELOPING AMPHIBIANS

By

Katherine Kemler Coady

Concern for the amphibian class has increased in the last two decades. A high incidence of deformed frogs among certain populations and a growing awareness of amphibian population declines across the globe have contributed to the fear that amphibians are facing environmental stressors that may be deleterious to their future survival. Pesticide exposure, whether from agricultural runoff or atmospheric deposition, is a suspected environmental stressor thought to be adversely affecting amphibian communities. Atrazine, one of the most widely used herbicides in the United States and Canada, has been reported to cause abnormal reproductive development in amphibian populations via disruption of the endocrine system. This laboratory investigation was undertaken to elucidate the mechanism of action of atrazine in amphibians and to examine the effects of atrazine on developing frogs. Two frog species, the native green frog (*Rana clamitans)* and the non-native African-clawed frog (*Xenopus laevis*), were chronically exposed to waterborne test solutions, including 0, 0.1, 1.0, 10, or 25 μg/L atrazine, 0.005% ethanol (EtOH), or 0.1 mg/L estradiol or dihydrotestosterone carried in 0.005% EtOH. Test solutions were renewed by 50% every 72 hrs. Survival, metamorphosis, growth, sex ratios, as well as gonad and larynx development were examined as endpoints in this study. In addition, sex steroid concentrations, and P450 aromatase activity were

examined in *X. laevis* frogs. Aromatase, an important enzyme in reproductive development, is responsible for converting androgens into estrogens. Atrazine exposure at environmentally relevant concentrations did not significantly affect frog mortality in either species. Time to metamorphosis as well as length and weight of frogs at metamorphic completion did vary between treatments in some cases, but these parameters were not consistently affected across atrazine concentrations. Sex ratios were not altered in the green frog or the African-clawed frog in response to atrazine exposure. However, when green frog tadpoles were exposed to DHT, over 97% of the metamorphosing frogs were classified as males. Neither laryngeal nor gonadal development appeared to be affected by atrazine in the exposed frogs. As expected DHT-exposed frogs had significantly larger larynx muscle areas compared to frogs in other treatments. Sex steroid levels, including 17β-estradiol and testosterone, were not affected in response to atrazine exposure. Likewise, aromatase activity in the brains and gonads of atrazine-exposed frogs was not different from controls. However, aromatase activity was significantly altered in E2 exposed frogs. In conclusion, chronic atrazine exposure at environmentally relevant concentrations did not affect amphibian survival, growth, or reproductive development. Based on these findings, it appears that low-level atrazine exposure does not disrupt the amphibian endocrine system via interference with the steroidogenic enzyme, aromatase. Future research using a greater range of atrazine concentrations and focusing on the possible interactions of atrazine with the amphibian endocrine system, including the hypothalamus-pituitary axis, is necessary to discount atrazine as an endocrine disruptor in amphibians.

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TABLE OF CONTENTS

LIST OF TABLES

CHAPTER ONE

CHAPTER TWO

CHAPTER THREE

LIST OF FIGURES

CHAPTER ONE

CHAPTER TWO

CHAPTER THREE

INTRODUCTION

In recent decades, there has been a growing concern among scientists and conservationists for the status of amphibian populations worldwide. Diverse members of the amphibian class, including Costa Rica's golden toad (*Bufo periglenes*) and Australia's unique gastric brooding frog (*Rheobatrachus silus*), have been completely eliminated in recent years, with scientists left trying to discover the cause of their disappearance (Pounds and Crump, 1994; Laurance et al., 1996). In addition to amphibian extinctions, a greater number of limb deformities among frog populations have also been noted in the United States and Canada (Ouellet et al., 1997; Sower et al., 2000). By some accounts, the increasing reports of amphibian declines and deformities indicate that amphibian populations are now expiring at an unprecedented rate. This has fueled increased research into the causes of amphibian declines.

The mechanisms underlying amphibian declines are complex, and several causal factors are probably involved depending on region and habitat (Blaustein and Kiesecker, 2002). These causal factors include climate changes (Pounds and Crump, 1994), ultraviolet radiation (Ankley et al., 1998; Blaustein et al., 2003), acid rain (Rowe et al., 1992), introduction of exotic species (Kats and Ferrer, 2003), parasitism (Lefcort and Blaustein, 1995), infectious diseases (Daszak et al., 2003), habitat destruction, and environmental contaminants (Blaustein et al., 2003).

Environmental contaminants, especially pesticides and industrial byproducts, are ubiquitous in the environment due to atmospheric transport. Amphibians, due to their permeable skins and life history (incorporating both aquatic and terrestrial habitats), are considered especially at risk of exposure to environmental contamination. Pesticide use,

in particular, has been a suspected contributor to amphibian declines. This is especially true in cases where there is a close association between wetland habitat and agricultural areas. Low-lying wetlands can act as a sink for various agricultural toxicants, and amphibians, due to their dependence on aquatic systems, can be exposed to various toxicants via agricultural runoff, drift, or aerial deposition. Most pesticides are applied in the spring when frogs and other amphibians are congregating near water bodies in preparation for reproductive activities. Pesticide exposure may affect larval amphibians to a greater extent than adults, since, in most cases, the larvae are restricted to water bodies that may be contaminated. In addition, early life stages are often more sensitive to pollutants, due to the complex and sensitive developmental processes taking place (McKim, 1977).

Pesticide exposure has been shown to alter growth and metamorphosis in developing anurans. Juvenile ranids exposed to triphenyltin fungicides showed decreased growth rates and increased time to metamorphosis (Fioramonti et al., 1997). Atrazine exposure at concentrations greater than 40 μg/L caused a decrease in the length and weight of *Xenopus laevis* and *Hyla versicolor* metamorphs as compared to controls (Diana et al., 2000; Sullivan and Spence, 2003).

Pesticide exposure has also been linked to immunosuppresion in certain frog populations. A reduction in antibody production was observed in northern leopard frogs (*Rana pipiens*), when the frogs were injected with sub-lethal doses of various pesticides (Gilbertson et al., 2003). Studies have suggested that pesticide exposure alters various immune responses in amphibians, which consequently affects their ability to combat parasitic infections (Kiesecker, 2002; Christin et al., 2003).

Some pesticides target the nervous system in frogs, causing an array of adverse effects. For example, exposure to the insecticide endosulfan affected the neuromuscular development in ranid and bufonid tadpoles, causing paralysis (Berrill et al., 1998). Organophosphate insecticides, used in California's Sierra Nevada, may be contributing to amphibian declines in that area by interfering with cholinesterase activity in the nervous system. Depression of cholinesterase activities has been shown to reduce tadpole activity, increase uncoordinated swimming behavior, and lead to greater tadpole mortality (Sparling et al., 2001). Triphenyltin exposure also appears to affect the nervous system of developing frogs. Triphenyltin-exposed tadpoles exhibited decreased swimming and feeding activity compared to tadpoles in the control treatment (Semlitsch et al., 1995).

Some pesticides may have the ability to interact with and consequently disrupt the amphibian endocrine system, thereby altering hormone-dependent processes, such as development and reproduction. For example, methoxychlor has been shown to inhibit progesterone-induced maturation of *X. laevis* oocytes (Pickford and Morris, 1999). Dichlorodiphenyl trichloroethane (DDT), a now banned insecticide, is suspected of disrupting corticosterone activities in exposed amphibians (Hayes et al., 1997). During a single year of a multi-year study, the prevalence of intersex individuals among cricket frog populations (*Acris crepitans*) was increased in those areas with greater pesticide exposure (Reeder et al., 1998). In addition, deformed frogs collected in New Hampshire were found to contain lower levels of androgens and gonadotropin releasing hormones. Both the deformities and the disrupted hormone levels in these frogs were attributed to pesticide exposure (Sower et al., 2000).

Atrazine (6-chloro-4-ethylamino-6-isopropyl-amino-*s*-triazine) is a suspected endocrine-disrupting chemical (Cooper et al., 2000) and is also the most widely used herbicide in the Midwestern United States and Canada (Solomon et al., 1996). The herbicide was first registered for commercial use in the United States in 1959 and has been applied mainly on corn, sorghum, and sugarcane crops to control annual broadleaf and grass weeds. The water solubility of atrazine is 33 mg/L at 22°C, thus the herbicide can partition into the water and reach aquatic systems via runoff from agricultural fields (Solomon et al., 1996). The half-life of atrazine can be as little as 2 d to as long as 800 d, depending on pH and other environmental factors (Solomon et al., 1996). Environmental concentrations of atrazine rarely exceed 20 μg/L in North American water bodies; however, runoff events periodically contaminate nearby aquatic systems with greater atrazine concentrations (Solomon et al., 1996).

While atrazine is not acutely toxic at environmentally relevant concentrations (Morgan et al., 1996), chronic exposure has been linked to endocrine disruption in amphibians (Hayes et al., 2002; Hayes et al., 2003). Field surveys of frog populations have suggested a trend between atrazine-exposure and altered reproductive development (Reeder et al., 1998; Hayes et al., 2003; Hecker et al., 2003). However, a causal relationship between atrazine-exposure and reproductive responses has not been established in these cases. There have been several studies investigating the effects of atrazine exposure on laboratory populations of African clawed frogs (*Xenopus laevis*) (Hayes et al., 2002; Carr et al., 2003). One series of laboratory exposures reported that atrazine increased the incidence of gonadal anomalies, decreased laryngeal muscle size, and altered sex steroid concentrations in male *X. laevis* (Hayes et al., 2002). However, in

a similar exposure study, there were no observable changes in laryngeal muscle size and fewer observed incidences of gonadal anomalies in frogs exposed to atrazine (Carr et al., 2003). Other studies reported that exposure to 21μg/L atrazine for 48 hrs during development resulted in decreased testicular volume in male *X. laevis* tadpoles and decreased numbers of primary germ cells in females (Tavera-Mendoza et al., 2002a; Tavera-Mendoza et al., 2002b). Thus, some authors have suggested that atrazine may be affecting gonadal development and consequently reproduction in some sensitive amphibian populations via endocrine disruption.

The proposed mechanism of action by which atrazine disrupts the amphibian endocrine system is via induction of P450 aromatase (aromatase) (Hayes et al., 2002). The aromatase enzyme is a product of the CYP 19 gene family, so named because it converts androgens to estrogens by removing the methyl group at carbon 19 (C19) and aromatizes the steroid A ring (Melo and Ramsdell, 2001). Atrazine, at relatively high concentrations, was reported to induce aromatase activity in a mammalian cell line (Sanderson et al., 2000). Thus, it has been proposed that atrazine increases the activity of aromatase not only *in vitro,* but also *in vivo*, reducing plasma concentrations of testosterone (T) and increasing plasma concentrations of estradiol (E2), resulting in demasculinization or feminization of male frogs (Hayes et al., 2002; Hayes et al., 2003). Aromatase activity and plasma sex steroid concentrations were investigated in *Xenopus laevis* collected from reference areas and areas of atrazine use in South Africa (Hecker et al., 2003). In this study, there were no significant differences in gonadal aromatase activity between frogs collected from reference areas and areas of atrazine use. However, female *X. laevis* collected from areas of atrazine use had significantly lower plasma

concentrations of both T and E2 in comparison to female frogs collected from reference locations (Hecker et al., 2003).

While the effects of atrazine on frogs are equivocal, they suggest that atrazine may be affecting steroidogenesis and/or gonad development in amphibian populations via endocrine disruption. Thus, the following research was conducted to elucidate the mechanism of action of atrazine in amphibians and to investigate the effects of environmentally relevant concentrations of atrazine on developing frogs.

Two anuran species, one North American native and one non-native, were selected as test animals for laboratory exposures involving atrazine. The green frog (*Rana clamitans*) was selected as a representative amphibian of North American wetlands. Green frogs commonly occur in agro-ecosystems of the Midwestern United States, where atrazine is frequently used in corn crop production (Gillilland et al., 2001). These frogs deposit eggs in quiet waters and their larval stage is adapted to life in a pond. Green frog tadpoles may not undergo metamorphosis for a year or two depending on environmental conditions. Following metamorphosis, the adult green frog is terrestrial, but remains closely associated with ponds and wetlands (Conant and Collins, 1998).

African clawed frogs (*Xenopus laevis*) are native to South Africa (Duellman and Trueb, 1994). Unlike native North American frogs, *X. laevis* will breed year round under laboratory conditions. *X. laevis* is purely aquatic throughout its lifecycle, and is an easy amphibian to care for in the laboratory. Thus, these frogs are commonly used in various scientific investigations. Since there is widespread use of African-clawed frogs in toxicology research, the selection of this test species allows for more precise comparisons between this and other toxicology studies.

CHAPTER ONE

EFFECTS OF ATRAZINE ON METAMORPHOSIS, GROWTH, AND GONADAL DEVELOPMENT IN THE GREEN FROG (*RANA CLAMITANS*)

Abstract

Embryos of the green frog (*Rana clamitans*) were collected from the field and exposed to one of six waterborne treatments for 273 d (mid July 2001 to mid-April 2002). The treatments were 0, 10, or 25 μg/L atrazine, 0.005% ethanol (EtOH), or 0.1 mg/L estradiol or dihydrotestosterone carried in 0.005% EtOH. The exposures began approximately 5 d post-hatch. Treatments were applied in a static renewal system with a 50% test solution replacement approximately every 3 d. Following the exposure period, tadpoles were reared in freshwater until metamorphosis or until study termination (at day 506). Time to initiate and complete metamorphosis, stage-specific mortality, length and weight at metamorphosis, and gross morphology and histology of the gonads were examined. At environmentally relevant concentrations, atrazine did not consistently affect growth or metamorphosis. Compared to controls, the length of the larval period was greater in tadpoles exposed to 10 μg/L atrazine. However, the length of the larval period was not different between tadpoles in the control and 25 μg/L atrazine treatments. Neither gross gonadal morphology nor histopathology of the gonads was altered in response to atrazine exposure in post-metamorphic frogs. This study provides evidence that environmentally relevant concentrations of atrazine do not adversely affect the growth or reproductive development of *Rana clamitans*.

Study Objectives

The overall hypothesis investigated in this study was that waterborne concentrations of atrazine could affect gonadal development of the green frog (*Rana clamitans*) when exposed during critical phases of development. The objectives of this

study were: (1) to develop and validate methods of husbandry and exposure for conducting laboratory studies with *R. clamitans* and (2) to determine the response of larval *R. clamitans* to atrazine by assessing metamorphosis and reproductive indices when animals are exposed during larval development. Indices evaluated included the number of frogs initiating and completing metamorphosis, time to metamorphosis, fresh post-mortem body weight and snout-vent length at metamorphic completion, and the incidence of gross and histological gonadal anomalies.

Test Materials

Atrazine (CAS number 1912-24-9; purity 97.1%) was obtained from Syngenta Crop Protection Inc. (Greensboro, NC, USA). Estradiol (E2) (CAS number 50-28-2, purity 98%) and dihydrotestosterone (DHT) (CAS number 521-18-6) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ethanol (EtOH) (CAS number 64-17-5, purity 200 proof, 100% USP grade) was purchased from AAPER alcohol (Shelbyville, KY). All chemicals were dissolved in UV-treated laboratory freshwater. EtOH was used as a carrier solvent to deliver DHT and E2 treatments in the freshwater, thus the 0.005% EtOH treatment group served as a solvent control.

Methods

Exposure methods

R. clamitans eggs were collected as a single mass of fertilized eggs from a rural pond (42°41'20''N, 084°16'59''W) in Michigan on July 10, 2001. This pond has no history of pesticide contamination and no predatory fish populations, but does have a

stable population of *R. clamitans*. Eggs were brought back to the laboratory and hatched in 600-mL beakers containing 500 mL of aerated pond water. Pond water was gradually replaced with laboratory freshwater. Hatching began on July 13, 2001. On July 18, 2001 tadpoles had reached Gosner stage 25 (Gosner, 1960) and the exposure period began. Approximately 30 free-swimming tadpoles were randomly assigned to 10-L exposure tanks containing 4 L of test solution. Each exposure treatment was replicated in 9 tanks for a total of 54 exposure tanks in the study. Tadpoles were housed in a windowless environmental chamber with a light exposure regime of 12 hrs of light followed by 12 hrs of darkness.

The test solutions used in the exposure were untreated control (UV-treated freshwater only), 0.005% EtOH, 0.1 mg/L E2, 0.1 mg/L DHT, as well as 10 or 25 μg/L atrazine. Test materials were applied in a static renewal exposure regime and were renewed by 50% every 72 hours with minor exceptions. During test solution renewals, 2 L of medium were removed by siphoning from each tank. Each treatment had its own siphon tube assigned by color code to avoid cross-contamination. New batches of the various test solutions were made up in large glass tanks and delivered by siphon to 20-L polypropylene carboys. The carboys were then used to deliver the new test solutions to the exposure tanks.

On September 22, 2001, after 67 d of exposure, tadpoles were transferred from 10-L glass tanks into 20-L tanks containing 16 L of test solution. The transfer was necessary due to tadpole growth and increasing levels of nitrite and ammonia nitrogen in the smaller exposure tanks. After a total of 273 d of static renewal exposure, tadpoles were transferred to a continuous flow-through system of clean freshwater. Flow-through

tanks were made by boring two 4 cm diameter holes on opposing sides of 10-L glass tanks, and covering these holes with 1 mm mesh material to allow water flow in and out of the tanks. Flow-through tanks were housed in large acrylic tanks containing 80 L of continuously renewing freshwater. From that time, tadpoles were reared in laboratory freshwater until metamorphosis occurred. At the initiation of metamorphosis, marked by forelimb emergence (Gosner stage 42), tadpoles were removed from the flow-through system and were housed as individuals or in small groups in 10-L glass tanks containing approximately 500 mL of freshwater. Tanks holding metamorphosing frogs were tilted at an angle such that both a wet and dry surface was available for the developing frogs. In total, the study lasted for 506 d. Tadpoles were exposed for 273 days and then reared in clean freshwater for the remaining 233 d of the study. After 506 d, tadpoles that had not reached initiation of metamorphosis were sacrificed in tricaine methanesulfonate (MS-222) and stored at -20°C.

Water quality and exposure verification

Prior to test solution renewals, water quality parameters such as pH, total ammonia nitrogen, nitrite nitrogen, hardness, and dissolved oxygen were measured in select replicate tanks. Water quality parameters were measured using Lamotte test kits for ammonia nitrogen, nitrite nitrogen, and hardness (Aquatic Ecosystems, Apopka, FL, USA). Dissolved oxygen was measured by an YSI model 57 oxygen meter (YSI Inc. Marion, MA, USA), and pH was measured using an Orion research pH meter, model 710A (Thermo Orion Inc., Beverly, MA, USA).

Water samples were collected from all treatments following each test solution renewal in order to measure atrazine concentrations in the exposure water. Concentrations of atrazine were measured by enzyme-linked immunosorbent assay (ELISA) at Michigan State University (MSU) and also by ELISA at Syngenta Crop Protection Inc. (Greensboro, NC, USA). At MSU, concentrations of atrazine in dilute stock solutions and replicate tanks were measured using the Envirogard® Triazine 96-well plate kit (Strategic Diagnostics Newark, DE, USA; product # 7211000). The method detection limit of the Envirogard® Triazine 96-well plate kit was 0.025 μg/L atrazine. A subset of the water samples analyzed at MSU was also analyzed at Syngenta Crop Protection Inc. At Syngenta, concentrations of atrazine were measured with the Beacon Analytical triazine plate kit (Beacon Analytical Systems, Portland, ME, USA). The method detection limit of the Beacon kit was 0.05 μg/L atrazine. When the concentrations of atrazine, as measured by ELISA, deviated from expected concentrations, water samples were compiled and analyzed by gas chromatography/mass selective detection (GC-MS) at Syngenta Crop Protection Inc. to verify the results of the ELISA. For GC-MS analyses, water samples were buffered to a pH of 10, and analytes were extracted by partitioning into ethyl acetate. Samples were then evaporated to dryness and reconstituted in acetone. GC-MS analyses were performed in the selected ion monitoring mode (Yokley and Cheung, 2000).

Methods at exposure termination

Throughout the study, mortalities and number of frogs initiating and completing metamorphosis were recorded daily. At metamorphic completion, marked by complete

tail resorption (Gosner stage 46), frogs were euthanized by immersion in MS-222, weighed, and measured for snout-vent length. Then the frogs were preserved in Bouin's fixative for 48 hrs followed by 70% ethanol for long-term storage. When frogs were transferred into ethanol, code numbers were assigned so that the frog's treatment origin would be unknown in subsequent analyses. All frogs completing metamorphosis and some frogs near metamorphic completion (Gosner stages 44 - 46) were examined for gross gonadal morphology. Frogs were dissected under an Olympus SZ40 stereomicroscope and gonads were examined for sex classification and gross gonadal anomalies. Digital photographs were taken of each specimen. The incidence of gross gonadal anomalies was enumerated and the nature of the anomaly described.

A random subset of the frogs examined for gross morphology of the gonads was also examined for histology of the gonads. Gonads were removed from the fixed frogs, embedded in paraffin and serially cross-sectioned at 5 μm intervals. Every fourth cut section of the gonad was mounted onto a slide, such that every 20 μm was represented in the analysis. Slides were stained with eosin and hematoxylin. All slides of sectioned ovaries and testes were observed under a compound microscope without knowledge of sample origin. When they occurred, histological anomalies were described and enumerated.

Historically, a number of terms have been used to describe both normal and abnormal gonadal development in vertebrates, including frogs (Dumont, 1972). More recently, other terms have been used to describe the phenomenon in fish (Sumpter et al., 1996; Gimeno, 1998) and frogs (Carr et al., 2003). In this study, juvenile frogs containing both testicular and ovarian tissue are referred to as rudimentary

hermaphrodites. Rudimentary hermaphrodites function as one sex, but also contain gonadal tissue of the opposite sex (Van Tienhoven, 1983). Rudimentary hermaphroditism can be characterized by two different morphologies. The intersex morphology occurs when testicular and ovarian tissues are separated rostral/caudal or left/right in the same individual. The mixed sex morphology occurs when both testicular and ovarian tissue are mixed together in the same gonad. When observed at the histological level, the mixed sex morphology is characterized by the occurrence of testicular oocytes. Testicular oocytes were characterized as such if the oocytes within the testes contain an intact nucleus, nucleoli within the nucleus, and a surrounding squamus epithelial layer.

Statistical methods

Fresh batches of treatment solutions (before being added to exposure tanks) and replicate exposure tanks were sampled and analyzed for concentrations of atrazine. These concentrations were incorporated into the total average atrazine concentration calculated for that treatment. Measured concentrations of atrazine from replicate tank samples were averaged across tanks for a single date. This average concentration was then used as a single data point when calculating treatment averages. For any given treatment, concentrations in the fresh batches of treatment solutions were indicative of atrazine exposure for the date that they were used. Non-parametric 95% confidence intervals were calculated for mean measured concentrations of atrazine by bootstrapping the data array.

Kolmogorov-Smirnov's One Sample test with Lillifor's transformation was used to assess whether observations were normally distributed. When data were normally distributed, analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) was used to detect significant differences between treatment groups. When observations for a parameter were not normally distributed, the non-parametric Kruskal-Wallis test was used to detect differences in the treatment groups. The non-parametric Mann-Whitney U test was used to test for significant differences between treatment groups. The Chi-square test was used to detect significant deviations in expected sex ratios in each replicate tank, and Pearson's Chi-square was used to test for significant differences in the incidences of gross and histological gonadal anomalies. The criteria for significance that was used in all statistical tests was $p<0.05$. The probability of a type II error ($1-\beta$), or power, in statistical tests was set at 0.8, and the detectable differences among treatment groups were calculated based on sample size and the average variation of the parameter under investigation.

Data sets were analyzed in two separate groups such that the treatment groups were compared to the appropriate control treatment in the experimental design. The atrazine data set included all atrazine treatments and the untreated control. The positive control data set included the DHT, E2 and EtOH solvent control treatments.

Results

Atrazine exposure verification

When measured by ELISA at MSU, concentrations of atrazine were similar to the nominal concentrations. Similar results were found when water samples were analyzed

by immunoassay at Syngenta Crop Protection Inc. (Table 1). Small concentrations of atrazine were detected in control exposure water (mean ± standard deviation = 0.10 ± 0.16 μg/L atrazine). To verify the presence of atrazine in control treatment tanks and to cross check the results of the ELISA methods, select water samples from each treatment (having the greatest concentrations of atrazine as measured by ELISA) were compiled and measured by GC-MS at Syngenta Crop Protection Inc. The ranges of GC-MS-measured atrazine concentrations in the control, 10 and 25 μg/L treatment samples were $4.0x10^{-1}$- $4.3\ x10^{-1}$, 18-29, and 33-50 μg/L atrazine, respectively. These values represent the greatest concentrations of atrazine present during the exposure period for each treatment.

Table 1: Nominal and measured concentrations of atrazine (μg/L) in exposure water as measured by ELISA at Michigan State University (MSU) and Syngenta Crop Protection Inc. (95% confidence intervals in parentheses)

Treatment	Nominal concentrations	(Syngenta) Average measured concentration	(MSU) Average measured concentration
[a] Control	0	0.14 (0.07-0.23)	0.10 (0.06 - 0.17)
10 μg/L atrazine	10	15.91 (12.03 –19.90)	11.76 (10.21 -13.65)
25 μg/L atrazine	25	27.95 (24.92-31.24)	28.23 (25.14 - 31.60)

[a] For calculations, a proxy value of either 0.0125 or 0.025 μg/L atrazine was assigned to values below the ELISA method detection limits at MSU or Syngenta, respectively.

Water quality

Water quality was measured in both the static exposure tanks and the flow-through freshwater tanks. The average temperature (± standard deviation) in the static exposure tanks was 21.3 ± 1.4 °C. The average nitrite nitrogen and total ammonia nitrogen concentrations (± standard deviation) in static exposure tanks were 0.22 ± 0.44 and 0.04 ± 0.05 mg/L, respectively. Static tanks had an average pH of 7.87, an average hardness concentration (± standard deviation) of 426 ± 45 mg/L $CaCO_3$, and an average dissolved oxygen content (± standard deviation) of 8.0 ± 0.7 mg/L. The average temperature (± standard deviation) in the flow-through tanks was 24.8 ± 0.5 °C. Both nitrites and total ammonia nitrogen had average concentrations of 0.02 mg/L in the flow-through tanks. The average dissolved oxygen concentration (± standard deviation) in the flow- through tanks was 6.1 ± 1.7 mg/L, and the average hardness concentration (± standard deviation) was 424 ± 26 mg/L $CaCO_3$.

Mortality

Mortality was greatest during the first month of the exposure period, and tended to decrease as the tadpoles aged. Mass mortality events occurred early in the study within a time span of 24 hrs. Due to tank-specific mortality, several experimental replicates were lost. Mortality decreased after transferring the tadpoles from 4 L to 16 L in the static exposure regime. Mortality also decreased after transferring tadpoles from the 16 L static tanks into the flow-through water system after 273 d (Figure 1). Average total mortality over 506 d was 76.5%. There were no significant differences in mortality among atrazine treatments and the untreated controls (ANOVA, $p=0.34$). Given the

sample sizes and variation in mortality in this experiment, a distinction of 19% mortality was differentiated between treatments with 80% power (1-β). Mortality did differ among the positive control treatments and the EtOH solvent control (ANOVA, p=0.01). The DHT treatment had fewer deaths than the E2 treatment (LSD, p=0.003), but mortality in this group was not different from the EtOH control treatment. There were also no significant differences in mortality between the E2 treatment and the EtOH controls. Average mortality is listed for each treatment group (Table 2).

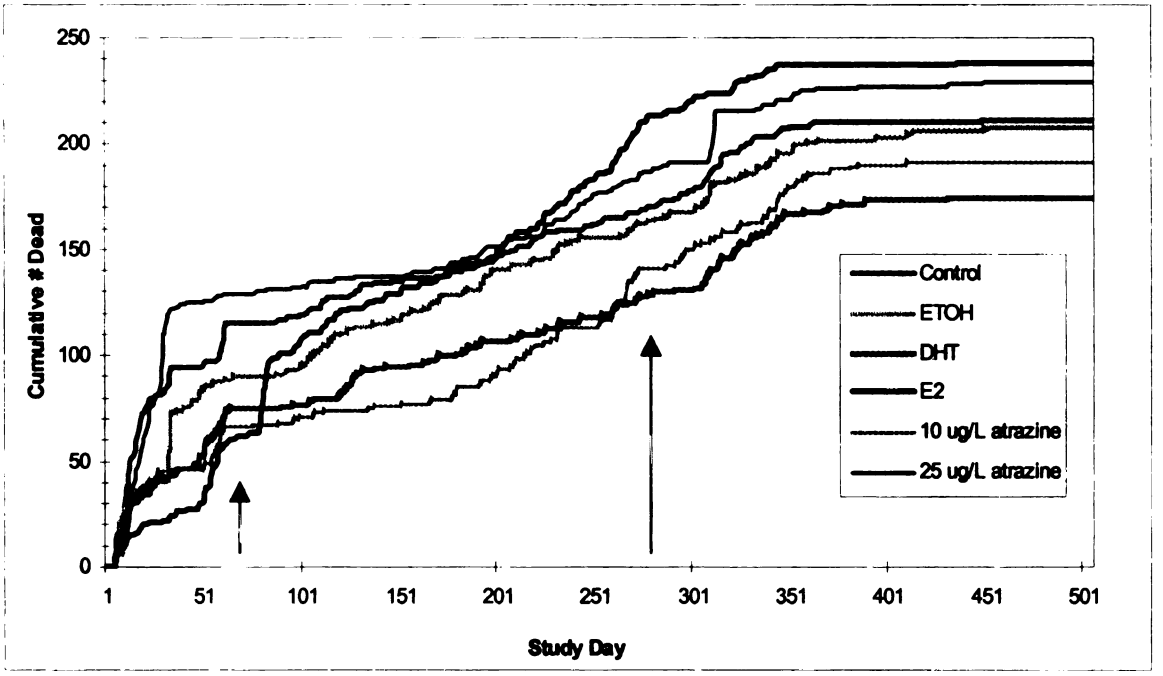

Figure 1: Cumulative mortality of *R. clamitans* for each treatment group

Arrows indicate the days in which tadpoles were transferred to new exposure vessels.

Table 2: Average percent (%) mortality for *R. clamitans* in different treatment groups over the 506 d study period

Treatment	Initial N	Average % Mortality
Untreated controls	285	79.2
EtOH	280	74.8
DHT	291	62.7
E2	282	* 85.7
10 µg/L atrazine	292	73.1
25 µg/L atrazine	292	83.3

EtOH = 0.005% ethanol, DHT = 0.1 mg/L dihydrotestosterone, E2 = 0.1 mg/L estradiol

* indicates a significant difference from the DHT treatment at $p < 0.05$

Metamorphosis

The first incidence of initiation of metamorphosis was observed on exposure day 99. The first incidence of completion of metamorphosis was observed on exposure day 112. As of exposure day 143, 10 tadpoles had completed metamorphosis. Between exposure day 143 (December 7, 2001) and 285 (April 28, 2002), no tadpoles initiated metamorphosis. An increased rate in development occurred after exposure day 285 (post April 28, 2002) (Figure 2). Total numbers of green frogs surviving to initiation and completion of metamorphosis are listed (Table 3).

Table 3: Number of *R. clamitans* exposed to different treatment groups over 506 d and surviving to initiation and completion of metamorphosis

Treatment	Initial N	# Frogs Initiating Metamorphosis	# Frogs Completing Metamorphosis
Untreated controls	285	58 (20.4%)	44 (15.4%)
EtOH	280	69 (24.6%)	47 (16.8%)
DHT	291	104 (35.7%)	75 (25.8%)
E2	282	40 (14.2%)	33 (11.7%)
10 µg/L atrazine	292	77 (26.4%)	64 (21.9%)
25 µg/L atrazine	292	48 (16.4%)	37 (12.7%)

EtOH = 0.005% ethanol, DHT = 0.1 mg/L dihydrotestosterone, E2 = 0.1 mg/L estradiol

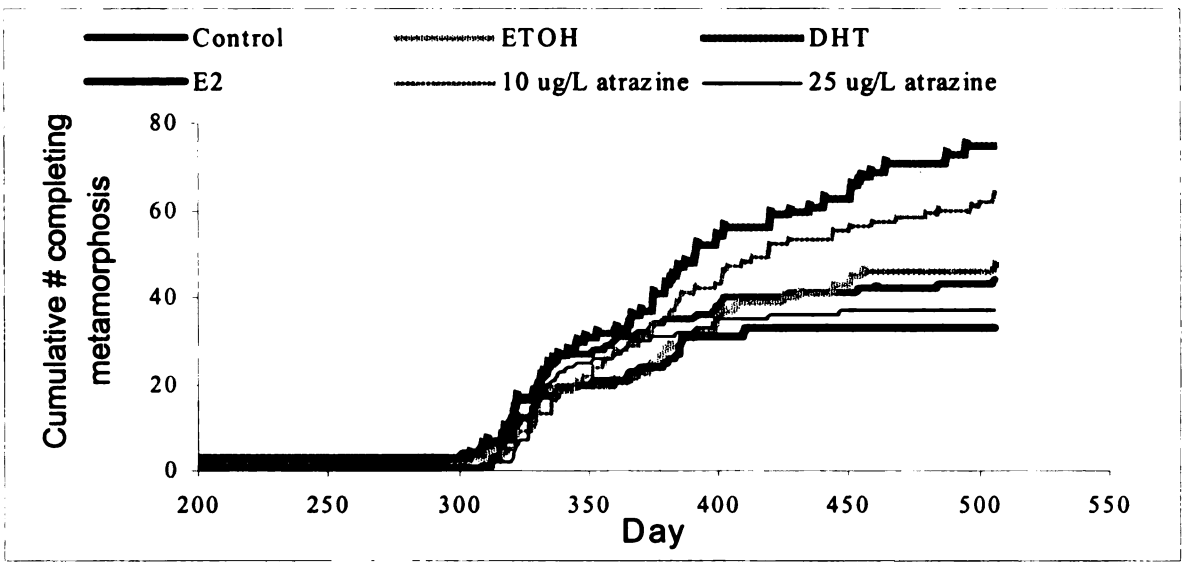

Figure 2: Cumulative number of *R. clamitans* frogs completing metamorphosis for each treatment group

Age at initiation and completion of metamorphosis was different among the atrazine treatment groups and the untreated controls (Kruskal-Wallis, p=0.021 [initiation] p = 0.010 [completion]). Pair-wise comparisons revealed that frogs in the 10 µg/L

atrazine treatment initiated and completed metamorphosis at a significantly older age as compared to both untreated control frogs and frogs exposed to 25 μg/L atrazine (Figure 3).

Age at initiation of metamorphosis was also different among the positive control treatment groups and the EtOH controls (Kruskal-Wallis, p=0.029). Pair-wise comparisons revealed that frogs in the E2 treatment initiated metamorphosis at a younger age as compared to both EtOH control frogs and frogs exposed to DHT (Mann-Whitney U, p=0.049 and 0.013, respectively; Figure 3).

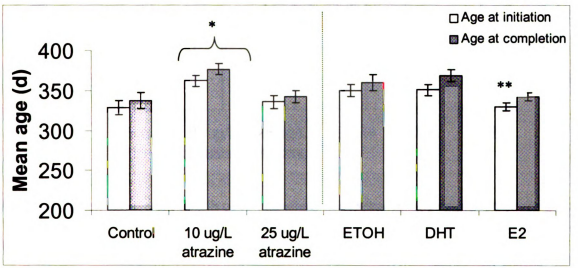

Figure 3: Mean ages of *R. clamitans* initiating and completing metamorphosis (error bars represent the standard error of the mean)

* Signifies a significant differences from untreated control and 25 μg/L atrazine treatments at $p < 0.05$

** Signifies significant differences from the EtOH and DHT treatments at $p<0.05$

Snout-vent length at metamorphic completion was different among the atrazine treatment groups and the untreated controls (ANOVA, p=0.03). Fisher's LSD test

revealed that frogs in the 10 μg/L atrazine treatment were shorter than frogs in the 25 μg/L atrazine treatment (p=0.009) but were not shorter than the controls (p=0.15). There were no differences in snout-vent length among frogs in the positive control treatments and the EtOH controls (ANOVA, p= 0.15) (Figure 4). At 80% power, a difference in length of 0.22 cm was detectable among these treatments.

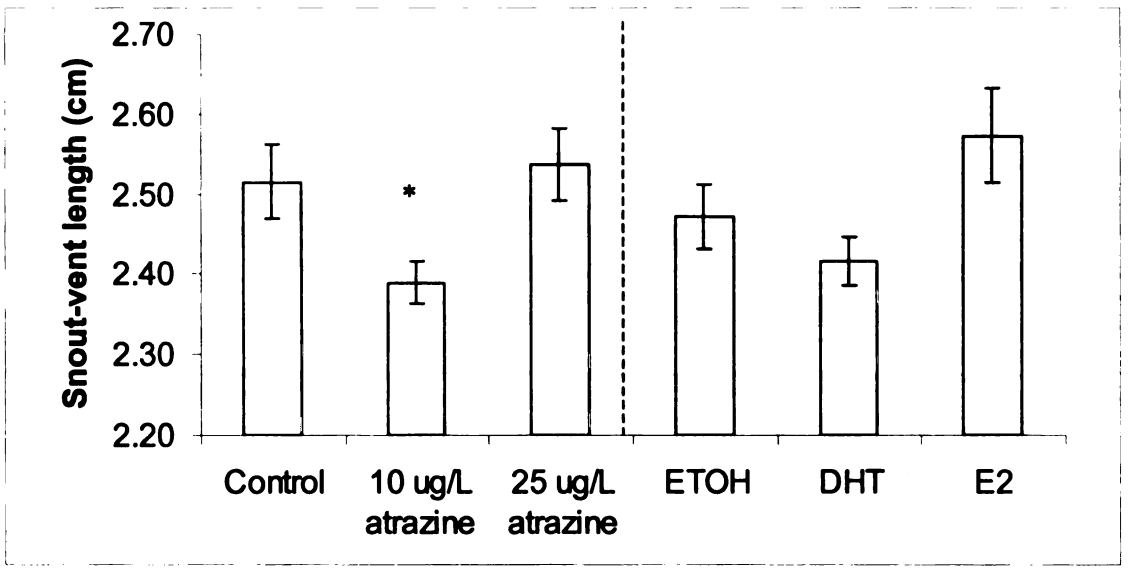

Figure 4: Mean snout-vent length of *R. clamitans* at metamorphic completion (error bars represent the standard error of the mean)

* Signifies a significant differences from the 25 μg/L atrazine treatment at p< 0.05

There were no significant differences in body weight at metamorphic completion among the atrazine treated frogs and the untreated controls (ANOVA, p=0.07). Likewise, there were no differences in weight at metamorphic completion among frogs in the positive control treatments and the EtOH controls (ANOVA, p=0.47). The detectable weight difference among all treatment groups in this experiment was found to be 0.36 g

at 80% power. Average ages, lengths, and weights at metamorphosis are listed for each treatment (Table 4).

Table 4: Mean ages, lengths, and weights at metamorphosis for *R. clamitans* exposed to positive controls and various concentrations of atrazine (Std. error in parentheses)

Treatment	Mean age at initiation (d) [a]	Mean age at completion (d) [b]	Mean weight (g)	Mean snout-vent length (cm)
Untreated Control	328 (8.5)	337 (10.0)	1.76 (0.05)	2.52 (0.08)
EtOH	350 (7.4)	360 (9.5)	1.56 (0.04)	2.47 (0.06)
DHT	350 (7.1)	369 (7.9)	1.50 (0.03)	2.42 (0.05)
E2	** 330 (4.8)	342 (4.6)	1.64 (0.09)	2.57 (0.06)
10 µg/L atrazine	* 362 (6.5)	* 377 (7.3)	1.48 (0.03)	* 2.39 (0.04)
25 µg/L atrazine	335 (7.7)	342 (7.9)	1.64 (0.05)	2.54 (0.07)

EtOH = 0.005% ethanol, DHT = 0.1 mg/L dihydrotestosterone, E2 = 0.1 mg/L estradiol

[a] Initiation of metamorphosis was marked by the first emergence of forelimbs.

[b] Completion of metamorphosis was defined as complete tail resorption.

* Indicates a significant difference from the untreated control at $p < 0.05$.

** Indicates a significant difference from the EtOH control at $p < 0.05$.

Sex ratios

Sex was determined during gross examination for gonadal anomalies. The sex ratios of frogs from untreated control and atrazine treatments were not significantly different from an expected 50:50 male to female ratio (Chi-square). Likewise, sex ratios in the EtOH and E2-exposed tanks were not significantly different from an expected 50:50 male to female ratio (Chi-square). However, all tanks in the DHT treatment had sex ratios skewed in favor of more male frogs (Chi-square). A total of 97.7% of the

examined frogs in the DHT treatment were males. Sex ratios for each treatment are listed (Table 5).

Table 5: Percent male and female *R. clamitans* exposed to positive controls and various concentrations of atrazine [a]

Treatment	N	Males	Females
Untreated Control	51	43.1	56.9
EtOH [b]	57	47.4	50.9
DHT	86	97.7	2.30
E2	38	36.8	63.2
10 µg/L atrazine	72	40.3	59.7
25 µg/L atrazine	44	40.9	59.1

EtOH = 0.005% ethanol, DHT = 0.1 mg/L dihydrotestosterone, E2 = 0.1 mg/L estradiol

[a] All frogs surviving to Gosner stage 44 were examined for sex determination

[b] As the gonads from one frog in the EtOH treatment were not located, the percentages do not total 100%

Gonadal anomalies

Several types of gross gonadal anomalies were observed: discontinuous gonad, multiple testes, and size irregularity. Discontinuous gonad was characterized by abnormal segmentation of the gonadal tissue. Frogs with multiple testes were characterized by multiple testicular-like gonads on one side of the frog. Size irregularity was characterized either by large size discrepancies between gonad pairs or unusually large or small gonads (Figure 5).

The incidence of gross gonadal anomalies ranged from 0.0 – 5.9% across all treatment groups (Table 6). A large size incongruity between gonad pairs was the most

commonly observed gross gonadal anomaly (Table 6). No hermaphroditic gonads (testicular and ovarian tissue in one individual) were identified during gross gonadal investigations. A discontinuous gonad was detected only in the 25 μg/L atrazine treatment (Table 6). Gonad or gonad pairs could not be located in five frogs. It is unclear whether this was due to dissection errors or if these constituted an anomaly. Two frogs in both the E2 and DHT treatments were missing one gonad upon inspection. In addition, the gonads in one frog in the EtOH control treatment could not be located. Using Pearson's Chi-square test, no differences in the incidence of gross gonadal anomalies were found among all treatment groups (Chi-square, p= 0.404 [discontinuous gonads], p=0.527 [multiple testes], p=0.456 [size incongruity]).

When gonads were examined at the histological level, gonadal anomalies, such as rudimentary hermaphroditism, were observed in the green frogs. Two different morphologies of rudimentary hermaphroditism were observed: intersex gonads and testicular oocytes (Figure 6).

When examined histologically, the incidence of gonadal anomalies, across all treatment groups, ranged from 0.0 to 12.9%. Most of the gonadal anomalies occurred in male frogs (Table 7). However, one frog in the E2 treatment group, identified as a female during the gross gonadal inspections, had testicular oocytes. Testicular oocytes were the most common histological anomaly. This anomaly occurred in untreated control, EtOH, and E2-exposed frogs, but not in DHT, 10, or 25 μg/L atrazine-exposed frogs (Table 7). Using Pearson's Chi-square test, no differences in the incidence of histological gonadal anomalies were found among all treatment groups (Chi-square, p= 0.299 [testicular oocytes], p=0.673 [intersex]).

Table 6: Percent gross gonadal anomalies in *R. clamitans* exposed to positive controls and various concentrations of atrazine [a]

Treatment	N	Size incongruity between gonads	Discontinuous gonad	Multiple testes	Missing gonad
Control	51	5.9	0.0	2.0	0.0
EtOH	57	0.0	0.0	0.0	1.8
DHT	86	3.5	0.0	0.0	2.3
E2	38	5.3	0.0	0.0	5.3
10 μg/L atrazine	73	1.4	0.0	1.4	0.0
25 μg/L atrazine	44	2.3	2.3	0.0	0.0

EtOH = 0.005% ethanol, DHT = 0.1 mg/L dihydrotestosterone, E2 = 0.1 mg/L estradiol

[a] All frogs surviving to Gosner stage 44 were examined for gonadal anomalies

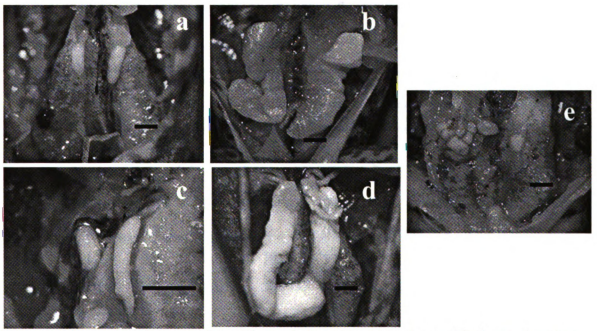

Figure 5: Normal and abnormal gonad morphology in post-metamorphic *Rana clamitans* a) Normal testes, b) Normal ovaries, c) Gonad with size irregularity from the dihydrotestosterone treatment, d) Discontinuous ovary from the 25 μg/L atrazine treatment, e) Multiple testes from the 10 μg/L atrazine treatment (Bar represents 1 mm)

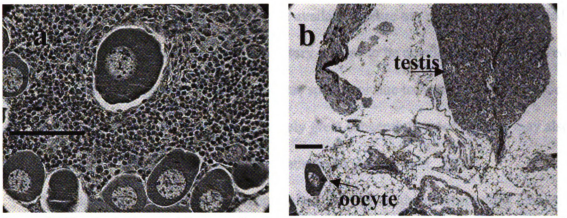

Figure 6: Histological gonadal anomalies in post-metamorphic *Rana clamitans* a) Gonad with testicular oocytes from the untreated control treatment b) Intersex gonad from the 25 μg/L atrazine treatment (Bar represents 200 μm)

Table 7: Percent histological gonadal anomalies in *R. clamitans* exposed to positive controls and various concentrations of atrazine

Treatment Group	N males	Rudimentary hermaphroditism		N females	Rudimentary hermaphroditism		N total	Rudimentary hermaphroditism	
		Testicular oocytes	Intersex		Testicular oocytes	Intersex		Testicular oocytes	Intersex
Control	17	12	5.9	25	0.0	0.0	42	4.8	2.4
EtOH	20	5.0	0.0	24	0.0	0.0	44	2.3	0.0
DHT	22	0.0	4.5	2	0.0	0.0	24	0.0	4.2
E2	12	25	0.0	19	5.3	0.0	31	13	0.0
10 μg/L atrazine	20	0.0	0.0	25	0.0	0.0	45	0.0	0.0
25 μg/L atrazine	16	0.0	6.3	20	0.0	0.0	36	0.0	2.8

EtOH = 0.005% ethanol, DHT = 0.1 mg/L dihydrotestosterone, E2 = 0.1 mg/L estradiol

Discussion

Since there were no standard protocols available before our research was initiated, methods for rearing *R. clamitans* in the laboratory needed to be developed. The observed level of mortality may have been natural for this species or due to overcrowding in static tanks. At times, water quality, such as relatively great concentrations of nitrites, may also have contributed to tadpole mortality in the static tanks (Marco et al., 1999).

In addition to its possible effects on mortality in this study, overcrowding also is known to decrease rates of growth and development in amphibians (Gillespie, 2002; Tejedo and Reques, 1992). In this study, when frogs were transferred to larger aquaria or removed from static tanks and placed in a flow-through system, mortality decreased and the rate of development increased (Figure 1). The increased rate of development for tadpoles in the continuous flow-through system was most likely due to the combined influences of an increase in temperature, better water quality, and the onset of spring. However, seasonal cues appeared to have the most important role in the regulation of metamorphic development. Even though green frog tadpoles were kept under constant temperature and lighting conditions in the laboratory, they still responded to seasonal cues. This was evident in that no tadpole initiated metamorphosis in the winter during the natural hibernation period for *R. clamitans*. However, metamorphosis greatly increased in the spring when the natural hibernation period was over.

In this study, the use of *Rana clamitans,* a species with a long larval period, simulates a worst-case scenario of atrazine exposure in an aquatic ecosystem. However, the long spans of time from hatching to completion of metamorphosis makes *R. clamitans* a difficult species for routine developmental toxicology tests. Age at metamorphosis was variable. A small set of tadpoles underwent metamorphosis the fall of the year they were

collected (2001), however the majority of tadpoles underwent metamorphosis spanning the period from late April until November of the following year (2002). This result is consistent with what has been observed in wild populations of *R. clamitans*. In the field, most *R. clamitans* eggs laid near the end of the summer (after July 10th) did not hatch until the following year in the spring. Egg masses that were laid before June 25th tended to metamorphose by early or mid fall of the same year (Martof, 1956). The *R. clamitans* eggs used in this study were collected near the end of the spawning period. Individuals that initiated metamorphosis during the first season after their collection were generally from tanks that had experienced high mortality rates during the first month of the exposure. Therefore, the few frogs that underwent metamorphosis in the first year experienced lesser tadpole densities during the pre-metamorphic phase of development. It is possible that the reduced crowding early in development allowed for increased growth, which enabled an earlier onset of metamorphosis (Tejedo and Reques, 1992).

To limit mortality and maximize development in future laboratory exposures with *R. clamitans*, it is recommended that tadpoles be reared at lower densities in static tanks (<1 tadpole/ L) or in a continuous flow-through system. In addition, although they are not as closely associated with aquatic systems throughout their life span, other species, such as the wood frog (*Rana sylvatica*) or the northern leopard frog (*Rana pipiens*) have shorter developmental periods and are possible alternative ranids that could also be used in developmental toxicology research in aquatic systems. They were not selected as experimental animals for this study because their populations are not as strongly established in Michigan.

The results are discussed in terms of nominal exposure concentrations. However,

the actual average concentrations of atrazine were slightly different. The nominal concentrations of 10 and 25 μg atrazine/L were actually approximately 12 and 28 μg atrazine/L, respectively. The average concentration of atrazine measured in the control exposure was 0.1 μg atrazine/L. Thus, the actual measured concentrations of atrazine were slightly greater than the nominal concentrations. Average concentrations of atrazine measured by Syngenta were greater than the concentrations measured at MSU. This was due in part to the sub-selection of water samples that deviated from the expected nominal atrazine concentrations for further analysis by Syngenta. It is unknown why small concentrations of atrazine were measured in the control tanks. It is possible that other compounds were able to cross-react with the triazine ELISA resulting in false positive measures of atrazine in the control water. However, it seems most likely that small amounts of atrazine were periodically present in the laboratory water supply. The small concentrations of atrazine in the control water did not affect the conclusions of the study, since there were no statistically significant, dose-dependent effects of atrazine observed on any of the parameters investigated.

Concentrations of atrazine in the aquatic environment rarely exceed 20 μg/L (Solomon et al., 1996). Atrazine is generally applied in the spring and reaches aquatic systems in pulses due to agricultural runoff events. The continuous exposure of 10 and 20 μg/L atrazine to green frog tadpoles for 273 days overestimates the expected duration of atrazine exposure in the environment. Thus, the exposure regime in this laboratory study models a worst-case scenario of atrazine exposure in natural frog populations.

Continuous exposure to 10 or 25 μg/L atrazine did not affect mortality of *R. clamitans*. This result is consistent with other amphibian toxicology research involving

atrazine (Morgan et al., 1996; Battaglin and Fairchild, 2002). Atrazine causes amphibian mortality at much greater concentrations (LC50 = 410 μg/L atrazine) than those used in this study (Battaglin and Fairchild, 2002).

Length and age at metamorphic completion were significantly different among treatment groups. However, atrazine exposure did not affect these parameters in a concentration-dependent manner. Tadpoles exposed to 10 μg/L atrazine had lower mortality (73.1%) in comparison to the control (79.2%) and 25 μg/L atrazine (83.3%) treatments (Table 2). Thus, there were more tadpoles per tank in the 10 μg/L atrazine treatment, and these frogs were older and smaller when they underwent metamorphosis as compared to frogs in the control and 25μg/L atrazine treatments. Tadpoles exposed to 25 μg/L atrazine had similar mortality as the controls. Consequently, frogs exposed to 25 μg/L atrazine did not differ from controls in age, weight, or length at completion of metamorphosis. Therefore, the difference in time to complete metamorphosis between treatment groups appeared to be a result of density-dependent tank effects on relative growth rates rather than atrazine treatment. Frogs exposed to E2 had greater mortality and metamorphosed sooner than frogs in the EtOH controls. Since frogs exposed to 10 μg/L atrazine metamorphosed at an older age as compared to controls, and frogs in the E2 treatment metamorphosed at a younger age as compared to controls, this suggests that any effects on the maturation process caused by atrazine are not the result of an E2-mediated mechanism. In an exposure study with larval gray tree frogs (*Hyla versicolor*), atrazine did not affect size and age at metamorphosis of tadpoles exposed to atrazine concentrations < 200 μg/L (Diana et al., 2000). In our study, chronic exposure to 10 and 25 μg/L atrazine did not consistently affect age, length, or weight of *R. clamitans* at

completion of metamorphosis. However, an exposure to more than two concentrations of atrazine would be necessary to fully elucidate a possible dose-response relationship between atrazine exposure and tadpole development.

Even though tadpoles were not exposed to atrazine for their entire developmental history, sex ratio data indicated that the exposure period of 273 d was a critical time for green frog gonad development. Since *R. clamitans* exposed to DHT were 97.7% male, it can be assumed that the exposure period and duration were sufficient to allow effects of exogenous substances on gonadal development. Unlike African clawed frogs (*X. laevis)*, *R. clamitans* are not feminized when exposed to exogenous estradiol, but instead are masculinized when exposed to exogenous androgens, such as DHT (Foote and Witschi, 1939). This difference in responses to hormones demonstrates that endocrine regulation of maturation and sexual development is different among frog species (Wallace et al., 1999). Therefore, hormones and hormone mimics could induce different responses in different frog species.

The fact that exposure to atrazine did not affect sex ratios in *R. clamitans* is similar to the results reported for *X. laevis* exposed to atrazine, where the sex ratio was not altered from an expected 50:50 ratio (Carr et al., 2003). While it has been hypothesized that atrazine can feminize male frogs through up-regulation of aromatase, the enzyme that converts testosterone to estradiol (Hayes et al., 2002; Hayes et al., 2003), sex reversal was not observed in *R. clamitans.*

It has been reported that atrazine exposure causes hermaphroditism in some species of frogs (Hayes et. al, 2003). The fact that the incidence of gross and histological gonadal anomalies was not different in control and atrazine-treated *R. clamitans* suggests

that this is not the case for all species exposed to similar concentrations of atrazine. It is possible that different species or strains of amphibians have different types and magnitudes of response to atrazine exposure. In addition, some amphibians are known to have hermaphroditic characteristics during phases of their natural reproductive development. In particular, testicular oocytes have been reported to be a natural developmental occurrence in some strains of ranid species, including, but not limited to, *Rana temporaria* (Witschi, 1929), *Rana catesbeiana* (Hsu and Liang, 1970), and *Rana curtipes* (Gramapurohit et al., 2000). In addition, bufonid toads, whether genetic males or genetic females, develop ovaries, which become compressed into the Bidder's organs in front of the definitive gonad at the time of metamorphosis (Wallace et al., 1999). Therefore, based on the results of this study and the reported incidences of natural background levels of hermaphroditism in the scientific literature, gonadal anomalies in *R. clamitans* do not appear to be related specifically to the presence or absence of atrazine.

Conclusion

In summary, chronic exposure to environmental concentrations of atrazine did not affect mortality, growth, sex ratios, or incidence of gonadal anomalies in developing *R. clamitans*. While another study has linked atrazine exposure to the occurrence of hermaphroditism in leopard frogs (*Rana pipiens*) (Hayes et al., 2003), we did not observe a similar phenomenon in laboratory exposed *Rana clamitans*. In our study, hermaphroditism in *Rana clamitans* occurred irrespective of exposure to atrazine. The difference in the results of the two studies could be attributed to the difference in frog species examined. Gonadal development may differ between *Rana pipiens* and *Rana*

clamitans. In addition, part of the Hayes et al. 2003 study relied on data from field collected frogs. An array of factors, apart from atrazine, could influence the development of hermaphroditism in field-collected frogs. In a controlled laboratory exposure, we observed that exposure to atrazine at environmentally relevant concentrations did not affect mortality, growth, or reproductive development in *R. clamitans*.

CHAPTER TWO

EFFECTS OF ATRAZINE ON METAMORPHOSIS, GROWTH, AND LARYNGEAL AND GONADAL DEVELOPMENT IN THE AFRICAN-CLAWED FROG (*XENOPUS LAEVIS*)

Abstract

Eight replicates of 30 African clawed frogs (*Xenopus laevis*) were exposed to one of eight waterborne treatments including: 0, 0.1, 1.0, 10, or 25 μg/L atrazine, 0.005% ethanol (EtOH), or 0.1 mg/L estradiol (E2) or dihydrotestosterone (DHT) containing 0.005% EtOH. Frogs were exposed from 72 h post-hatch until 2 to 3 mo post-metamorphosis via a 3-d static renewal exposure regimen. Multiple endpoints were measured in the developing frogs, including time to complete metamorphosis, mortality, length and weight at metamorphosis, gross morphology of the gonads, and histology of the larynx and gonads. Male frogs had larger laryngeal dilator muscles than females, whereas male and female frogs exposed to DHT had larger laryngeal dilator muscle areas as compared to frogs in all other treatments. Frogs exposed to E2 had greater incidences of testicular oocytes. Atrazine at concentrations between 0.1 and 25 μg/L did not significantly affect any of the parameters measured in this study. These results suggest that environmentally relevant concentrations of atrazine do not affect growth, metamorphosis, or gonadal or laryngeal development of *X. laevis*.

Study Objectives

The present study was undertaken to evaluate the effects of chronic atrazine exposure on growth, development, and reproductive morphology of developing *X. laevis*. The objectives of this study were to determine the effects of atrazine on growth, metamorphosis, and reproductive indices of larval *X. laevis* exposed from 72 hr after hatching until completion of metamorphosis. Indices evaluated at the completion of metamorphosis included: number of frogs initiating and completing metamorphosis, time

to metamorphosis, body weight, snout-vent length, gonad development, and laryngeal dilator muscle size. Gonads were examined at the gross and histological levels for structural anomalies, such as rudimentary hermaphroditism (defined as the presence of both ovarian and testicular tissue in the same individual).

Test Materials

Atrazine (CAS number 1912-24-9; purity 97.1%) was obtained from Syngenta Crop Protection Inc. (Greensboro, NC, USA). Estradiol (E2) (CAS number 50-28-2, purity 98%) and dihydrotestosterone (DHT) (CAS number 521-18-6) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ethanol (EtOH) (CAS number 64-17-5, purity 200 proof, 100% USP grade) was purchased from AAPER alcohol (Shelbyville, KY). All chemicals were dissolved in the Frog Embryo Teratogenesis Assay- *Xenopus* (FETAX) test medium comprised of laboratory reverse osmosis water containing salts in the following concentrations: 0.625 g/L NaCl; 0.030 g/L KCl; 0.015 g/L $CaCl_2$; 0.096 g/L $NaHCO_3$; 0.060 g/L $CaSO_4*2H_20$; and 0.075 g/L $MgSO_4$ (ASTM, 1991). EtOH was used as a carrier solvent to deliver DHT and E2 treatments in FETAX; thus, the 0.005% EtOH treatment group served as a solvent control for these hormone exposure groups.

Methods

Exposure methods

Sexually mature *X. laevis* obtained from Xenopus Express® (Plant City, FL) were induced to breed in the laboratory by injecting human chorionic gonadotropin into the dorsal lymph sac. Twelve hours later, fertilized eggs were collected and de-jellied in a

2% solution of L-cysteine in FETAX medium (ASTM, 1991). Viable embryos were sorted into lots of 100 and left in FETAX medium overnight at 22° C. On the next day, larvae were randomly assigned with the use of random number tables to glass Petri dishes (100 mm diameter) containing 20 mL of FETAX (35 embryos per dish). The exposure period began after the larvae were 72 hr old, at which time all viable larvae were transferred from glass petri dishes containing FETAX solution and randomly assigned to 10L glass tanks containing 4 L of the appropriate test solution. At the start of the exposure, approximately 30 *X. laevis* larvae were placed in each replicate tank. Each treatment group was replicated eight times, for a total of 64 replicate tanks. Tadpoles were housed in a windowless environmental chamber with a light exposure regime of 12 hrs of light followed by 12 hrs of darkness.

Test materials were applied in a static renewal exposure regime. Test solutions were renewed by 50% replacement every 72 hr. The test solution renewal process began with the removal of 2L of the test solution by siphoning followed by replacement with 2L of the appropriate fresh test solutions. Each treatment had its own siphon tube designated by color code to avoid cross-contamination. At the time of test solution renewals, 15 mL water samples were taken from the stock solutions and the atrazine-treated tanks to test for actual atrazine concentrations.

Throughout the study, mortalities were monitored and recorded on a daily basis for each replicate tank. In addition, the number of tadpoles initiating and completing metamorphosis was recorded daily for each replicate tank. The Nieuwkoop-Faber (NF) staging method was used to determine when tadpoles initiated and completed metamorphosis (Nieuwkoop and Faber, 1967). At the initiation of forelimb emergence

(NF stage 58), glass dividers surrounded by a 1 mm mesh material were constructed and placed into the 10L aquariums, such that tadpoles at different stages of metamorphosis could be kept physically separate and be easily enumerated. At the time of metamorphic completion (NF stage 66), time to initiate and complete metamorphosis, snout-vent length, and weight data were recorded for each metamorphosing frog. A subset of the frogs was euthanized at NF stage 66 for assessment of gross morphology of the gonads and for histological analyses of gonad and larynx tissue. Frogs were euthanized by immersion in MS-222.

The remaining NF stage 66 *X. laevis* were reared in 4L of test solution until approximately 1 mo post-metamorphosis, at which time they were transferred to 40L aquaria containing 10 L of the test solution to increase space and maximize growth. These frogs were exposed to the various test solutions until 2-3 mo post-metamorphosis. In total, the exposure period was 185 d (from Dec 21, 2001 – June 24, 2002).

Analytical methods

Concentrations of atrazine in the exposure solution were measured by Enzyme-Linked Immunosorbent Assay (ELISA) at Michigan State University (MSU) and Syngenta Crop Protection Inc. (Greensboro, NC, USA). At MSU concentrations of atrazine in fresh renewal batches and in replicate tanks were measured using the Envirogard® Triazine 96-well plate kit (Strategic Diagnostics Newark, DE; product # 7211000). The method detection limit of the Envirogard® Triazine 96-well plate kit was 0.025 μg/L atrazine. A subset of the treatment solution samples analyzed at MSU was also analyzed at Syngenta Crop Protection Inc. At Syngenta, concentrations of atrazine

were measured with the Beacon Analytical triazine plate kit (Beacon Analytical Systems, Portland, ME, USA). The method detection limit of the Beacon kit was 0.05 μg/L atrazine. When the concentrations of atrazine, as measured by ELISA, deviated from expected concentrations, treatment solution samples were analyzed by gas chromatography/mass selective detection (GC-MS) at Syngenta Crop Protection Inc. to verify the results of the ELISA. For GC-MS analyses, treatment solution samples were buffered to a pH of 10, and analytes were extracted by partitioning into ethyl acetate. Samples were then evaporated to dryness and reconstituted in acetone. GC-MS analyses were performed in the selected ion monitoring mode (Yokley and Cheung, 2000).

Aquarium water quality parameters were measured weekly using Lamotte test kits for total ammonia nitrogen, nitrite nitrogen, and hardness (Aquatic Ecosystems, Apopka, FL, USA). Dissolved oxygen was measured by a YSI model 57 oxygen meter (YSI Inc. Marion, MA, USA), and pH was measured using an Orion research pH meter -model 710A (Thermo Orion Inc., Beverly, MA, USA).

Gross morphology of the gonads was examined in a subset of NF stage 66 *X. laevis* and in a subset of frogs that were allowed to develop to 2-3 mo post metamorphosis. Frogs examined for gross gonadal morphology were euthanized in MS-222 and preserved in Bouin's fixative for 48 hr and then transferred to a solution of 70% ethanol for storage. Frogs were dissected under an Olympus SZ40 stereomicroscope and gonads were evaluated for sex classification and gross gonadal anomalies. Digital photographs were taken of each specimen. The incidence of gross gonadal anomalies was enumerated and types of anomalies described.

A subset of the frogs examined for gross morphology of the gonads was also processed for gonad histology. A total of 25 NF stage 66 frogs found to have anomalous gonadal morphology were sectioned and stained at Texas Tech University (Lubbock, TX, USA). Gonads were embedded in paraffin wax and serially cross-sectioned at 7 μm intervals. All sections were mounted and stained with eosin and hematoxylin by standard methods. In addition, a total of 50 frogs per treatment (25 males and 25 females) were examined for gonad histology 2 –3 mo post-metamorphosis. These frogs were sectioned and stained at Michigan State University (East Lansing, MI, USA). Gonads were removed from the frogs, embedded in paraffin and serially sectioned at 5 μm intervals. Every fourth 5 μm section of the gonad was mounted onto a slide, such that every 20 μm was represented in the analysis. Slides were stained with eosin and hematoxylin. All slides of sectioned ovaries and testes were evaluated under a compound microscope with the analyst having no knowledge of sample treatment group. When they occurred, histological anomalies were noted.

NF stage 66 frogs were processed for laryngeal histology at Texas Tech University as described by Carr et al. (2003). The laryngeal regions were removed from preserved *X. laevis* and imbedded in paraffin. Specimens were coronally sectioned at 8 μm and stained with hematoxylin and eosin. Every twentieth section of the laryngeal dilator muscle was digitally photographed and the cross-sectional areas of the laryngeal dilator muscle was measured using "Simple PCI" software (Compix Inc. Imaging Systems, Cranberry Township, PA, USA) (Carr et al., 2003). After measuring a series of laryngeal muscle cross-sectional areas for each frog, the largest measured area for the right and left dilator muscle was selected and used in statistical analyses.

Statistical methods

Fresh batches of treatment solutions (before being added to exposure tanks) and replicate exposure tanks were sampled and analyzed for concentrations of atrazine. These concentrations were incorporated into the total average atrazine concentration calculated for that treatment. Measured concentrations of atrazine from replicate tank samples were averaged across tanks for a single date. This average concentration was then used as a single data point when calculating treatment averages. For any given treatment, concentrations in the fresh batches of treatment solutions were indicative of atrazine exposure for the date that they were used. Non-parametric 95% confidence intervals were calculated for mean measured concentrations of atrazine by bootstrapping the data array.

Mortality was calculated based only on the observed number of tadpoles/frogs that died in each replicate tank. Percent mortality for each treatment tank was calculated using the Kaplan-Meier method, since frogs were removed from the study at different times during the exposure (Lee, 1992). This method estimates mortality rates based on changing numbers of individuals at risk for mortality throughout the study period.

Kolmogorov-Smirnov's One Sample test with Lillifor's transformation was used to assess whether or not data sets were normally distributed. When data were normally distributed, analysis of variance (ANOVA) and Fisher's least significant difference (LSD) post hoc test were used to detect significant differences between treatment and control groups. When data were not normally distributed, the non-parametric Kruskal-Wallis test was used to detect differences among treatment groups. If the Kruskal-Wallis test showed significant differences among the treatment groups, the Mann-Whitney U test

was used to evaluate differences between treatment groups. The Chi-square test was used to detect significant deviations in expected sex ratios in each replicate tank. Pearson's chi-square was used to detect differences in incidences of gross and histological gonadal anomalies among treatment groups. The criteria used for significance in all statistical tests was $p<0.05$. The probability of a type II error ($1-\beta$), or power, in all statistical tests was set at 0.8, and detectable differences among treatment groups were calculated based on sample size and the average variation of the parameter under investigation.

Data sets were analyzed such that treatment groups were compared to the appropriate control treatment in the experimental design. Atrazine treatments were compared to the untreated controls. E2 and DHT treatments were compared to the EtOH solvent controls.

Results

Atrazine exposure verification

Atrazine concentrations measured by ELISA in the control, 0.1, 1.0, 10, and 25 µg/L atrazine treatments were greater than expected (Table 1). Similar results were found when a subset of the treatment solution samples was analyzed by immunoassay at Syngenta Crop Protection Inc. (Table 1). To verify the concentrations of atrazine as measured in the immunoassays, several treatment solution samples were compiled and atrazine concentrations were measured by GC-MS at Syngenta Crop Protection Inc. Concentrations of atrazine ranging from <0.05 to 0.27 µg/L were detected in the untreated control solution when measured by GC-MS. Two composite treatment solution

samples from the 25 µg/L atrazine treatment were analyzed by GC-MS. The measured concentrations of atrazine for these composite samples were 33 and 35 µg/L.

Table 1: Nominal and measured concentrations of atrazine in test solutions as measured by Enzyme-Linked Immunosorbent Assay at Michigan State University (MSU) and Syngenta Crop Protection Inc. [a]

Treatment	Nominal concentration of atrazine (µg/L)	MSU Average measured concentrations of atrazine (µg/L)[b]	Syngenta Average measured concentrations of atrazine (µg/L)[b]
Control	0	0.16 (0.11 – 0.20)	0.11(0.07-0.15)
EtOH	0	0.01 (0.01-0.01)	Not measured
DHT	0	0.02 (0.01-0.02)	Not measured
E2	0	0.04 (0.03-0.04)	Not measured
0.1 µg/L atrazine	0.1	0.22 (0.17-0.26)	0.23 (0.16 – 0.31)
1.0 µg/L atrazine	1.0	1.0 ($9.3x10^{-1}$ –1.1)	1.4 (1.2-1.7)
10 µg/L atrazine	10	16.4 (14.1-18.9)	11.4 (9.72-13.3)
25 µg/L atrazine	25	28.9 (24.2-35.1)	25.1 (22.3-28.1)

EtOH = 0.005 % ethanol; DHT = 0.1 mg/L dihydrotestosterone; E2 = 0.1 mg/L estradiol

[a] For these calculations, a proxy value of either 0.0125 or 0.025 µg/L atrazine was assigned to values less than the ELISA method detection limits of either 0.025 or 0.05 µg/L atrazine, respectively.

[b] 95% confidence intervals in parentheses

Water quality

Water temperatures over the 185 d exposure ranged from 17 to 23 °C. The mean water temperature (± standard deviation) during the 185 d exposure was 20 ± 1.1 °C. The mean nitrite nitrogen and total ammonia nitrogen concentrations (± standard deviation) in exposure water were 0.47 ± 0.78 and 0.23 ± 0.41 mg/L, respectively. The mean dissolved oxygen concentration (± standard deviation) was 7.4 ± 0.8 mg/L. The mean hardness of the exposure water (± standard deviation) was 138 ± 15 mg/L $CaCO_3$, and the mean pH (± standard deviation) was 7.6 ± 0.3.

Mortality

The number of surviving frogs at risk for mortality changed during the exposure period due to either euthanization of some tadpoles failing to initiate metamorphosis or scheduled subsampling of NF stage 66 frogs. In addition, five frogs escaped from the tanks. Therefore, the number of surviving frogs was taken into account when calculating percent mortality for each replicate tank. Mortality in each replicate tank was averaged to calculate overall percent mortality for each treatment group. Average percent mortality in the control treatment was 11.3%. Average percent mortality in the 0.1, 1.0, 10, and 25 µg/L atrazine treatments was 20.4, 11.5, 18.7 and 20.3%, respectively. Average percent mortality in the EtOH, DHT, and E2 treatments was 14.4, 21.3 and 8.3%, respectively. The total average observed mortality across all treatment groups was 16.1%. There were no statistical differences in observed mortality among treatments (n=8 tanks/ treatment) (Kruskal-Wallis, p= 0.298). Given the sample sizes and variation

in mortality in this experiment, a difference of 21.7 % mortality was detected between treatments with 80% power (1-β).

Metamorphosis

At completion of metamorphosis (NF stage 66) age, snout-vent length, and weight were determined for each frog. Due to tank effects, the experimental units in these analyses were the mean tank values for each measured parameter (n=8/treatment). In the entire study, a total of 17 surviving tadpoles did not initiate metamorphosis. Therefore, 98.9% of surviving frogs initiated metamorphosis during the study period. There were no statistically significant differences in age at completion of metamorphosis among treatment groups when treatments were compared to the appropriate controls (ANOVA atrazine and control treatments, p= 0.986; ANOVA E2, DHT and EtOH treatments, p= 0.703). Given the sample sizes and variation in age at metamorphosis in this experiment, an age difference of 5.25 d was discernable between treatments with 80% power (1-β). The average age at metamorphic completion across all treatments was 72.8 d with a standard deviation (SD) of 8.19 d.

There were no statistically significant differences in snout-vent length at completion of metamorphosis when treatment groups were compared to the appropriate control treatment (ANOVA atrazine and control treatments, p= 0.066; ANOVA E2, DHT and EtOH treatments, p=0.512). In this experiment, a 0.13 cm difference in length was discernable between treatments at 80% power. A pair-wise comparison of snout-vent lengths between EtOH and untreated control frogs showed that EtOH frogs were longer (mean ± SD = 1.89 ± 0.15 cm) than untreated control frogs (mean ± SD = 1.75 ± 0.20

cm; t test, p= 0.032). The mean snout-vent length at completion of metamorphosis across all treatment groups was 1.85 cm (SD = 0.17 cm).

There were no significant differences in weight at completion of metamorphosis when treatment groups were compared to the appropriate control treatment (ANOVA atrazine and control treatments, p=0.220; ANOVA E2, DHT and EtOH treatments, p= 0.311). Given the sample sizes and variation in weight in this experiment, a difference of 0.19 g was detected between treatments with 80% power. A pair-wise comparison found that EtOH -treated frogs were significantly heavier (mean ± SD = 0.85 ± 0.23 g) than the untreated controls (mean ± SD = 0.70 ± 0.24 g; t test, p= 0.046). The total average weight at metamorphic completion across all treatment groups was 0.78 g (SD =0.22 g).

Gross gonadal anomalies

Three types of gross gonadal anomalies were observed: discontinuous gonad, rudimentary hermaphroditism, and size irregularity. Discontinuous gonad was characterized by abnormal segmentation of the gonadal tissue. Rudimentary hermaphroditism was characterized by the appearance of immature testicular and ovarian tissue in a single individual (Van Tienhoven, 1983). Two types of rudimentary hermaphroditism were observed in juvenile *X. laevis*. The gonads of some individuals contained masses of ovarian and testicular tissue separated left/right or rostral/caudal. This type of rudimentary hermaproditism was termed intersex. In other hermaphroditic individuals, there was co-occurrence of both ovarian and testicular tissue in a single gonad, and this was termed mixed sex. Size irregularity was characterized either by large

size discrepancies between gonad pairs or unusually large or small gonads (Figure 1). Other gonadal anomalies, regarding shape and pigmentation, were noted as well.

The most commonly occurring gross gonadal anomaly in the stage 66 frogs was discontinuous gonad (Table 2). There were no statistically significant differences in the incidence of gross gonadal anomalies among treatments for NF stage 66 frogs (Chi-square). Gross gonadal anomalies observed in individuals 2-3 mo post-metamorphosis were similar to those observed in stage 66 frogs, with the most commonly observed gross gonadal anomaly being discontinuous gonad (Table 3). No statistically significant differences in the incidence of gross gonadal anomalies were observed among treatments when frogs were 2-3 mo post-metamorphosis (Chi-square).

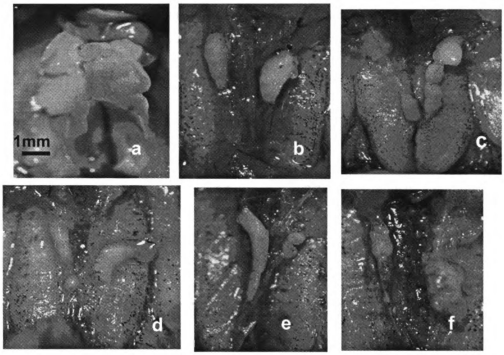

Figure 1: Normal and abnormal gonadal morphology of juvenile male and female *X. laevis* 2-3-months post metamorphosis. a) Normal ovaries b) Normal testes c) Mixed sex gonad from a 10 μg/L atrazine-exposed frog d) Discontinuous testes from a 0.1 μg/L atrazine-exposed frog e) Intersex gonad in a frog from an estradiol-exposed frog f) Gonad size irregularity in a frog from the 10 μg/L atrazine treatment

Table 2: Percent gross gonadal anomalies in Nieuwkoop-Faber stage 66 *X. laevis* exposed to EtOH, DHT, E2, or various concentrations of atrazine.

Treatment Group	N	% Discontinuous Gonads	% Rudimentary Hermaphrodites		% Size Irregularities	% Other Anomalies
			% Mixed Sex	% Intersex		
Control	45	2.2	0.0	0.0	2.2	2.2
EtOH	45	0.0	0.0	0.0	0.0	2.2
DHT	42	4.8	2.4	0.0	2.4	4.8
E2	46	6.5	4.4	2.2	0.0	2.2
0.1 µg/L atrazine	40	5.0	0.0	0.0	0.0	7.5
1.0 µg/L atrazine	46	2.2	0.0	0.0	2.2	0.0
10 µg/L atrazine	43	7.0	0.0	0.0	0.0	4.7
25 µg/L atrazine	39	5.1	2.6	0.0	0.0	0.0

EtOH = 0.005 % ethanol; DHT = 0.1 mg/L dihydrotestosterone; E2 = 0.1 mg/L estradiol

Table 3: Percent gross gonadal anomalies in *X. laevis* exposed to EtOH, DHT, E2, or various concentrations of atrazine until 2-3 mo post-metamorphosis.

Treatment Group	N	% Discontinuous Gonads	% Rudimentary Hermaphrodites (mixed sex)	% Size Irregularities
Control	75	1.4	0.0	2.7
EtOH	75	2.7	0.0	1.3
DHT	72	1.4	0.0	0.0
E2	77	2.6	0.0	0.0
0.1 µg/L atrazine	71	4.2	0.0	0.0
1.0 µg/L atrazine	79	1.3	0.0	2.5
10 µg/L atrazine	73	4.1	2.7	0.0
25 µg/L atrazine	67	3.0	0.0	0.0

EtOH = 0.005 % ethanol; DHT = 0.1 mg/L dihydrotestosterone; E2 = 0.1 mg/L estradiol

Histological gonadal anomalies

The most common gonadal anomaly among NF stage 66 frogs at the histological level of observation was rudimentary hermaphroditism (Van Tienhoven, 1983). At the histological level, rudimentary hermaphroditism was characterized either by rostral/caudal or left/right separation of testicular and ovarian tissue (intersex) or by the occurrence of testicular oocytes. Testicular oocytes were coded as such if the oocytes had an intact nucleus, nucleoli within the nucleus, and a surrounding squamus epithelial layer. Rudimentary hermaphroditism was observed in four NF stage 66 individuals, all of them in the E2 treatment group. In addition, one control frog appeared to have unidentified tissue surrounding the testes that appeared abnormal.

When the gonads from frogs exposed to the treatments for 2-3 mo post-metamorphosis were examined at the histological level, a greater percentage of rudimentary hermaphrodites, including intersex gonads (separated ovarian and testicular tissue) and testicular oocytes, were observed (Table 4). Testicular oocytes occurred in male frogs from all treatments, while intersex gonads were observed in male frogs from the control and EtOH treatments, as well as the 0.1 and 1.0 μg/L atrazine treatments (Table 4). In general, when testicular oocytes were observed, only one or two oocytes were present in the entire testis (Figure 2). However, several gonads in the E2 treatment and one gonad in the10 μg/L atrazine treatment had greater numbers of oocytes mixed in with testicular tissue (Figure 2). Few histological anomalies were noted during gonadal examinations from frogs classified as females during gross gonadal inspections. Most of the anomalies noted in female frogs were small or underdeveloped ovaries containing relatively few or no eggs. Two frogs (8.0%) in the E2 treatment classified as females

from gross gonadal inspections had testicular tissue containing oocytes. These were most likely genetic males developing female gonads in response to E2 exposure (Witchi, 1967).

There were no significant differences in the occurrence of any histological gonadal anomaly observed among control and atrazine treated frogs (Chi-square). However, during histological examinations, a significant difference in the occurrence of testicular oocytes was observed between DHT and EtOH treated frogs (Chi-square, p=0.039). DHT-treated frogs had significantly fewer gonads with testicular oocytes than did frogs exposed to EtOH (Table 4).

Table 4: Percent gonadal anomalies at the histological level in male *X. laevis* exposed to EtOH, DHT, E2, or various concentrations of atrazine until 2-3 mo post-metamorphosis.

Treatment Group	N	% Rudimentary Hermaphrodites		% Other Anomalies
		% Testicular Oocytes	% Intersex gonads	
Control	25	8.0	16.0	0.0
EtOH	25	20.0	4.0	0.0
DHT	25	* 4.0	0.0	0.0
E2	25	32.0	0.0	0.0
0.1 µg/L atrazine	25	12.0	4.0	0.0
1.0 µg/L atrazine	25	8.0	4.0	0.0
10 µg/L atrazine	25	12.0	0.0	0.0
25 µg/L atrazine	25	8.0	0.0	0.0

EtOH = 0.005 % ethanol; DHT = 0.1 mg/L dihydrotestosterone; E2 = 0.1 mg/L estradiol

* Indicates a significant difference from the EtOH control at $p < 0.05$.

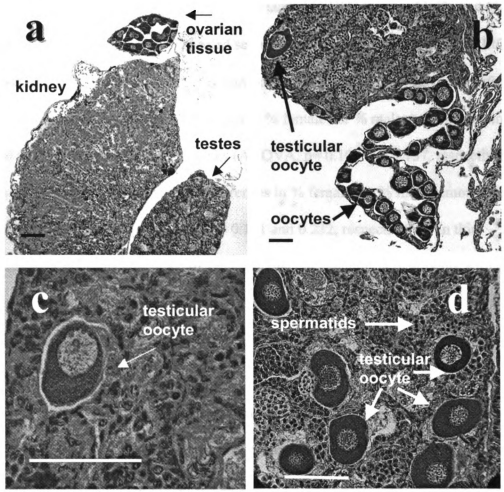

Figure 2: Cross section of rudimentary hermaphroditic gonads of 2-3 mo post-metamorphic *X. laevis*. a) Intersex gonad from an ethanol-exposed frog b) Testicular oocytes from an estradiol-exposed frog c) Testicular oocytes from a control frog d) Testicular oocytes from a 10 μg/L atrazine exposed frog (Bar in each picture represents 200 μm)

Sex ratios

There were no consistent deviations from the expected 50:50 sex ratio (Chi-square). Several tanks did have a significantly different sex composition from the expected 50:50 ratio, but the ratio was not consistently skewed in favor of one sex over the other, and

varied from tank to tank. One EtOH-exposed tank, one DHT-exposed tank, and two 0.1 μg/L atrazine-exposed tanks had skewed sex ratios in favor of male frogs. One EtOH - exposed tank and one E2-exposed tank had skewed sex ratios in favor of female frogs.

There were no significant differences in % females or % males among atrazine treated tanks and untreated control tanks (ANOVA, p= 0.108 and 0.137, respectively). Likewise, there were no significant differences in % females or % males among the DHT, E2, and EtOH treatments (ANOVA, p= 0.111 and 0.232, respectively). In this experiment, a sex ratio difference of 18% was discernable among treatments with 80% power.

Laryngeal dilator muscle area

Overall, male frogs had laryngeal muscle dilator cross-sectional areas that were significantly greater than those of females (Mann-Whitney U, p= 0.0001). In most treatment groups, laryngeal dilator muscles in males were larger than females (Figure 3). Due to the statistically significant differences in muscle area between the two sexes, the data was stratified by sex for subsequent statistical analyses.

There were no statistically significant differences in laryngeal muscle area among male frogs in the atrazine treatments and the untreated controls (Kruskal-Wallis, p= 0.476; Table 5). Given the sample sizes and variation in male laryngeal dilator muscle area in this experiment, a difference of 0.08 mm^2 was detected between treatments with 80% power. A significant difference in male laryngeal muscle area was detected among the E2, DHT, and EtOH treatments (Kruskal-Wallis, p=0.008). Pair-wise comparisons

revealed that male frogs exposed to DHT had greater laryngeal muscle area as compared to males in all other treatment groups (Figure 3).

There were no significant differences in laryngeal muscle area among female frogs in the atrazine treatments and the untreated controls (Kruskal-Wallis, p= 0.181; Table 5). Given the sample sizes and variation in female laryngeal dilator muscle area in this experiment, a difference of 0.12 mm^2 was detected between treatments with 80% power. Female frogs exposed to DHT had significantly greater laryngeal muscle area (Kruskal-Wallis, p=0.0001) than did females in all other treatment groups (Figure 3). The laryngeal muscle area of female frogs exposed to DHT was not significantly different from the muscle area of male frogs exposed to DHT (Mann-Whitney U, p= 0.815).

Table 5: Laryngeal dilator muscle cross-sectional areas for male and female *X. laevis* exposed to EtOH, DHT, EtOH, or various concentrations of atrazine

Treatment	N Males	Mean Left Laryngeal Muscle Area in Males (mm^2)	Standard error	N Females	Mean Left Laryngeal Muscle Area in Females (mm^2)	Standard error
Control	13	0.214	0.009	10	0.153	0.01
EtOH	14	0.192	0.020	5	0.185	0.028
DHT	14	* 0.304	0.022	10	* 0.346	0.068
E2	7	0.223	0.023	10	0.147	0.008
0.1 µg/L atrazine	14	0.212	0.024	9	0.211	0.025
1.0 µg/L atrazine	14	0.228	0.011	9	0.166	0.012
10 µg/L atrazine	14	0.217	0.013	8	0.147	0.013
25 µg/L atrazine	13	0.204	0.013	9	0.152	0.007

EtOH = 0.005 % ethanol; DHT = 0.1 mg/L dihydrotestosterone; E2 = 0.1 mg/L estradiol

* Indicates a significant difference from all other treatment groups at $p < 0.05$.

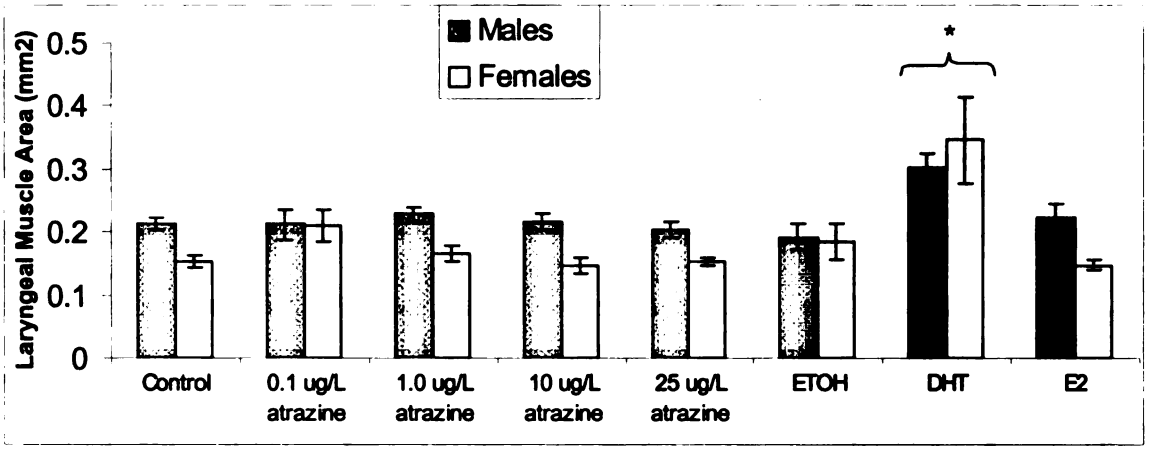

Figure 3: Laryngeal dilator muscle areas in Nieuwkoop-Faber stage 66 *X. laevis* exposed to EtOH, DHT, E2, and various concentrations of atrazine (Bars represent mean ± standard error of the mean)

EtOH = 0.005% ethanol; DHT = 0.1 mg/L dihydrotestosterone; E2 = 0.1 µg/mL estradiol

* indicates a significant difference from all other treatments, $p<0.05$

Discussion

Results are discussed in terms of the nominal exposure concentrations. However, the actual concentrations of atrazine measured by immunoassay at Michigan State University were slightly greater than the nominal concentrations. Concentrations of atrazine measured by immunoassay at Syngenta Crop Protection Inc. were similar to concentrations measured by ELISA at Michigan State University. On occasion, the ELISAs indicated that there was a small amount of atrazine in the control tanks. Atrazine was detected in control treatment solution at an average value ranging from 0.11 to 0.16 µg/L atrazine. When a subset of control treatment solution samples was analyzed by GC-MS, atrazine was detected at similar concentrations. The presence of atrazine in control tanks may be explained by aerial transport and deposition of droplets of treatment

solutions produced by bubbling air stones from nearby tanks. It is also possible that small amounts of atrazine were periodically present in laboratory RO water used in the FETAX solution. Even though small concentrations of atrazine were detected in untreated control tanks, this does not affect the conclusions of the study, since there were no statistically significant, dose-dependent effects of atrazine observed on any of the parameters investigated. The fact that there were no measurable concentrations of atrazine in the ethanol control tanks allows it to be used as a no-atrazine control treatment. When this was done, there was still no dose-response relationship for any of the measurement endpoints. This observation argues against any atrazine related effects.

The observation that atrazine, under the conditions and at the doses used in this experiment, did not have significant effects on mortality of *X. laevis* is consistent with other amphibian toxicity assays (Morgan et al., 1996; Battaglin and Fairchild, 2002). Atrazine causes mortality in amphibians at much greater concentrations ($LC_{50} \geq 410$ µg/L atrazine) than those used in this study (Battaglin and Fairchild, 2002).

The observation that chronic exposures to atrazine at concentrations between 0.1 and 25 µg/L did not affect age, length, nor weight at completion of metamorphosis is consistent with the results of other studies that have also observed no effect of atrazine on these parameters at similar concentrations (Allran and Karasov, 2000; Diana et al., 2000). In an exposure study with larval gray tree frogs (*Hyla versicolor*), atrazine did not affect tadpole growth at environmentally relevant concentrations (Diana et al., 2000).

Frogs exposed to EtOH were longer and heavier than those in untreated control tanks. This may have resulted from increased availability of food in ethanol containing tanks. Ethanol in exposure tanks appeared to aggregate food particles in the water

column. This was observed by the noticeably greater murkiness in EtOH -containing tanks.

In this study, exposure to environmentally relevant concentrations of atrazine did not affect the incidence of either gross or histological gonadal anomalies in post-metamorphic *X. laevis*. However, other researchers have linked atrazine exposure with the demasculinization of gonads in male *X. laevis* (Hayes et al., 2002; Hayes et al., 2003). The results from this study indicate that gonadal anomalies in *X. laevis* exposed to atrazine until they were 2-3 mo post-metamorphosis are not related to atrazine concentration. The lack of a significant concentration-response relationship and the presence of testicular oocytes and intersex gonads in EtOH controls (containing no measurable concentrations of atrazine) suggest that these types of deformities may not be related to atrazine exposure, but rather are a component of normal ontological development. Hermaphroditism occurs normally in other anurans (Witchi, 1929; Hsu and Liang, 1970; Gramapurohit et al., 2000) and therefore may also be a normal occurrence in some strains of developing *X. laevis*. Further research into the histological morphology of *X. laevis* gonads is necessary to determine the background levels of rudimentary hermaphroditism in juvenile frogs under both laboratory and field conditions.

The results of this study are in contrast to the studies of Hayes et al. (2002), who also studied the effects of atrazine on growth, metamorphosis, and gonadal and laryngeal development in *X. laevis*. It is not possible to directly compare the results obtained in our study with those of Hayes et al. (2002) due to differences in the age and strain of the examined frogs. In the study by Hayes et al. (2002), *X. laevis* were exposed to atrazine

until metamorphic completion (NF stage 66), but no *X. laevis* were exposed to atrazine 2-3 mo post-metamorphosis and then examined for gonadal anomalies. In addition, different strains of *X. laevis* were used in the two studies, and gonadal development and metamorphosis are not necessarily synchronized in different frog strains (Gramapurohit et al., 2000). As a result, the differences between our study and that of Hayes et al. (2002) could be explained by differences in the degree of gonadal development in the examined frogs.

The observation that exposure to atrazine did not affect sex ratios is consistent with the results of other studies with *X. laevis* (Carr et al., 2003). While it was expected that E2 exposed *X. laevis* would have skewed sex ratios in favor of increased numbers of females, this phenomenon was not observed in this study. Some researchers have reported 90-100% females when *X. laevis* were exposed to similar concentrations of E2 (Miyata et al., 1999; Miyata and Kubo, 2000; Hayes et al., 2002). However, exposure to E2 under a similar regime as used in the present study produced a proportion of female frogs that was similar to the present study (Carr et al., 2003). The balanced sex ratio in most E2-exposed tanks in this study may have resulted from rapid metabolism and breakdown of the E2. Since E2 was only applied every third day during the exposures, it is possible that the hormone was metabolized too rapidly by the frogs and therefore was below the threshold concentration required to cause altered sexual development. If E2 was administered daily or in a flow-through system, the results may have been different. E2 exposure was used as a positive control for possible estrogen receptor mediated effects, but was not used to model the fate of waterborne atrazine treatments.

Laryngeal development in frogs is a sexually dimorphic process, and the formation of a larynx capable of male calling behavior is androgen-dependent. Under normal conditions the laryngeal dilator muscle of male *X. laevis* is larger than that of females (Fischer et al., 1995; Tobias et al., 1991; Tobias et al., 1993). It has been hypothesized that atrazine could decrease plasma concentrations of testosterone (T) in *X. laevis* by up-regulating the expression of aromatase, the enzyme that converts T to E2 (Hayes et al., 2002). This decrease in available androgen for conversion into DHT could then result in a decrease in laryngeal dilator muscle volume (Hayes et al., 2002). Thus, dilator muscle size was selected as an indicator of disruption of plasma hormone balance, especially of androgens during development. Theoretically, this measurement endpoint can serve as an integrating measure of androgen-dependent processes that would respond to subtle changes in androgen status during critical periods of development when it is not possible to measure plasma hormone concentrations. Such a measure should be able to detect changes that occur in small windows during development as well as changes in androgen status in tissues that might not be observed in the plasma.

The observation that exposure to atrazine had no significant effect on the size of the laryngeal dilator muscle size in either males or females is consistent with a similar study that observed no effect on laryngeal dilator muscle size (Carr et al., 2003). Unlike the findings of this study, a decreased larynx muscle size in atrazine exposed *X. laevis* has been previously reported (Hayes et al., 2002). However, in that study, there was no concentration-dependent response and the relationship was only elucidated when the proportions of individuals greater than the controls was investigated, instead of investigating the central tendencies or variances in more standard statistical assessments.

As expected, male *X. laevis* in this study had greater larynx muscle size than females. Because the DHT treatment resulted in statistically greater laryngeal muscle sizes for both male and female *X. laevis*, it can be concluded that the frogs were responsive to changes in androgen status. Thus, the response to DHT was as would be expected. Therefore, if atrazine was acting as an androgen agonist or was increasing androgen levels, we would have expected to detect a change in the laryngeal muscle size.

Conclusion

In summary, chronic exposure to environmental concentrations of atrazine did not affect mortality, growth, sex ratios, laryngeal dilator muscle area, or incidence of gonadal anomalies in developing *X. laevis*. Alternative findings resulting from other studies could be due, in part, to differences in the exposure regime, *X. laevis* age or strain, larynx muscle area measurement techniques, diet, or exposure conditions. However, based on the findings in these studies, it appears that exposure to atrazine at environmentally relevant concentrations does not disrupt development in juvenile *X. laevis*.

CHAPTER THREE

EFFECTS OF ATRAZINE ON AROMATASE ACTIVITY AND PLASMA SEX STEROID LEVELS IN AFRICAN-CLAWED FROGS (*XENOPUS LAEVIS*)

Abstract

Eight replicates of thirty African clawed frogs (*Xenopus laevis*) per replicate were exposed to one of eight waterborne treatments including: 0, 0.1, 1.0, 10, or 25 μg/L atrazine, 0.005% ethanol (EtOH), or 0.1 mg/L 17β-estradiol (E2) or dihydrotestosterone (DHT) carried in 0.005% EtOH. Frogs were exposed from 72 hr post-hatch until 2 to 3 mo post-metamorphosis. Exposures were via a 3-d static renewal exposure regimen. Aromatase activity in brain and gonadal tissue and circulating concentrations of both E2 and testosterone (T) were examined in juvenile frogs at the end of the exposure. Gonadal aromatase activity was statistically greater in female frogs as compared to male frogs. There were no atrazine-related effects on aromatase activity in the brains or gonads in either male or female *X. laevis*. However, exposure to E2 decreased gonadal aromatase activity in female frogs and increased brain aromatase activity in male frogs. Female *X. laevis* had greater plasma E2 concentrations as compared to male frogs. Male frogs exposed to 1.0 μg/L atrazine had statistically lower concentrations of E2 in their plasma as compared to controls; however, this effect was not consistent across atrazine doses. In addition, male frogs exposed to E2 had statistically greater concentrations of E2 in their plasma as compared to controls. There were neither sex nor treatment-related effects on plasma T concentrations due to atrazine exposure. We conclude that at environmentally relevant concentrations, atrazine does not affect aromatase activity or plasma concentrations of E2 or T in juvenile *X. laevis*.

Study Objectives

The objectives of this study were to determine the effects of atrazine on 1) brain and gonadal aromatase activity and 2) concentrations of circulating T and E2 in control and atrazine-exposed *X. laevis* reared under laboratory conditions.

Methods

Test materials and exposure methods

X. laevis were exposed to one of eight treatment solutions from 72 hrs post-hatch until 2-3 mo post-metamorphosis. Treatment solutions were dissolved in the Frog Embryo Tertatogenesis Assay-*Xenopus* (FETAX) medium (ASTM, 1991). The treatment groups were 0 (Control), 0.1, 1.0, 10, and 25 μg/L atrazine, 0.005% ethanol (EtOH), 0.1 mg/L dihydrotestosterone (DHT), or 0.1 mg/L 17β-estradiol (E2). DHT and E2 were delivered to FETAX medium in EtOH, so the EtOH treatment group functioned as a solvent control for the DHT and E2 treatments. Each treatment was replicated in eight tanks, for a total of 64 tanks in the entire study. Test materials were applied in a static renewal exposure regime. Test solutions were renewed by 50% replacement every 72 hr. The light cycle during the exposure was 12 hrs of light followed by 12 hrs of darkness. Throughout the study, treatment solution samples were collected to test for actual atrazine concentrations. In total, the exposure period was 185 d (from Dec 21, 2001 – June 24, 2002).

Sample collection methods

At the termination of the exposure period, a subset of the frogs was selected for blood and tissue collection, with the use of random number tables. Sixty-four individuals

(one from each replicate tank) were randomly selected, and necropsies were conducted between June 11 and June 24, 2002. To control for circadian cycles in circulating hormone levels, individual frogs were sampled within a 3 hr time window from 8:30 to 11:30 am. Blood, as well as gonad and brain tissue, were collected to test for treatment effects on sex steroid hormone concentrations and aromatase activity, respectively.

Blood collection and sex steroid measurements

Frogs were anesthetized by immersion in tricaine methanesulfonate (MS-222). As soon as frogs were anesthetized, blood was collected by cardiac puncture. Heparin coated syringes equipped with 25G 5/8 needles were used in the blood collection. Blood samples were kept on ice for several hours and then centrifuged at 11,000g to separate the plasma. Plasma samples were stored at –80 °C until extraction for sex steroids. Plasma samples (50 μL) were extracted twice with 2.5 mL diethyl ether. Ether fractions were nitrogen evaporated to dryness and the hormone extract was re-constituted in 250 μL phosgel buffer (1.4375 g Na_2HPO_4, 0.32 g NaH_2PO_4, 0.25 g gelatin, and 0.025 g thimersol in 250 mL nanopure water) for use in the enzyme-linked immunosorbent assay (ELISA). Ten μL of 0.0002 μCi/μL ^{3}H-labeled testosterone [1,2,6,7-^{3}H(N)] (Perkin Elmer Life Sciences, Boston, MA, USA) was dosed into each plasma sample before extraction. Following the extraction procedure, the radioactivity in a fraction of the final extract was quantified in a liquid scintillation counter to test for recoveries. Concentrations of T and E2 in plasma extracts were measured by competitive ELISA as described by Cuisset et al. (1994) with modifications (Hecker, 2002). In the competitive ELISA, steroids of the sample extract compete with acetylchloinesterase-labeled steroid

for the binding site on the polyclonal rabbit anti-steroid antibody. Antiserum to T was obtained from Dr. D.E. Kime (Sheffield, UK). Cross reactivities of the T antiserum are described in Nash et al. (2000). The antiserum to E2 (Cayman Chemical product # 482250, Ann Arbor, MI) was reported to cross-react with estradiol-3-glucoronide (17%), estrone (4%), estriol (0.57%), T (0.1%) and 5α-dihydrotestosterone (0.1%). For all other steroids, cross-reactivities were reported to be less than 0.1%. The steroid ELISAs were performed using 96-well COSTAR high binding plates (Corning Inc, product # 9018).

Tissue collection and aromatase activity measurements

Following blood collection and euthanasia, both gonads were removed and flash frozen in liquid nitrogen. Frog carcasses were then stored at -80°C until frozen solid. At that point, the entire brain mass was removed from the frog carcass by slicing open the cranium of the frozen frogs using a razor blade. Brain tissue was then flash frozen in liquid nitrogen.

The tritium labeled water release assay was used to measure aromatase activity in brain and gonad tissue (Lephart and Simpson, 1991). Gonad tissue was removed from liquid nitrogen storage, and homogenized in 600-900 μL of buffer containing 50 mM KPO_4, 1 mM EDTA, and 10mM glucose 6-phosphate. Brain tissue was removed from liquid nitrogen storage and homogenized in a similar buffer containing 10 mM KPO_4, 100 mM KCl, 10mM dithiothreitol, 1 mM EDTA, and 10 mM glucose 6-phosphate. A 500 μL aliquot of the tissue homogenate was incubated with 395 μL 1β-^{3}H-androstenedione (Perkin Elmer Life Sciences, Boston, MA, USA), 100 μL of 10 mM NADP, and 5 μL glucose 6-phosphate dehydrogenase (100 IU/ml) for 2 hrs at 37 °C. After incubation,

samples were centrifuged for 2 min at 11,000g, and the supernatant was split into two 200 μL fractions. These fractions were extracted with 500 μL chloroform by vortex mixing for 1 min. Samples were then centrifuged for 2 min at 11,000g. Following centrifugation, 100 μL of the supernatant was vortexed for 1 min with 100 μL of a dextran-coated charcoal suspension (5% charcoal and 0.5% dextran) to remove remaining ^{3}H-labeled androstenedione substrate. Samples were then centrifuged for 15 min at 11,000g. A 125 μL-aliquot of the supernatant was added to 4 mL of scintillation cocktail and quantified in a liquid scintillation counter. Protein content was quantified for each tissue homogenate (Bradford, 1976). Aromatase activity for gonad tissue was reported as fmol/h/mg protein, and aromatase activity for brain tissue was reported as pmol/h/mg protein.

Statistical methods

Kolmogorov-Smirnov's One Sample test with Lillifor's transformation was used to assess whether or not data sets were normally distributed. When data were normally distributed, analysis of variance (ANOVA) and Fisher's least significant difference (LSD) post hoc test were used to detect significant differences between treatment and control groups. When data were not normally distributed, the non-parametric Kruskal-Wallis test was used to detect differences among treatment groups. If the Kruskal-Wallis test showed significant differences among the treatment groups, the Mann-Whitney U test was used to decipher differences between treatment groups. The criteria used for significance in all statistical tests was $p<0.05$. The probability of a type II error ($1-\beta$), or power, in statistical tests was set at 0.8, and detectable differences among treatment

groups were calculated based on sample size and the average variation of the parameter under investigation.

Data sets were analyzed as two separate groups such that treatment groups were compared to the appropriate control treatment. The atrazine treatments were compared against the untreated control treatment. The DHT and E2 treatments were compared against the EtOH solvent control.

Results

Atrazine concentrations

The concentrations of atrazine in the exposure tanks were measured by ELISA at Michigan State University and Syngenta Crop Protection Inc. In short, measured concentrations of atrazine were close to nominal concentrations, however, small amounts of atrazine were detected in the control treatment solution from time to time. At Michigan State University an average measured concentration of 0.16 ± 0.17 μg/L atrazine was detected in the control solution. When measured by GC-MS, atrazine was detected in control solutions at concentrations ranging from <0.05 to 0.27 μg/L. Average measured concentrations of atrazine in the nominal 0.1, 1.0, 10, and 25 μg/L atrazine treatments were 0.22 ± 0.13, 1.0 ± 0.30, 16.4 ± 7.23, and 28.9 ± 16.7 μg/L atrazine, respectively. Average measured concentrations of atrazine in the EtOH, DHT, and E2 treatments were 0.01 ± 0.00, 0.02 ± 0.01, and 0.04 ± 0.03, respectively. At Syngenta Crop Protection Inc. average measured concentrations of atrazine in the nominal 0, 0.1, 1.0, 10, and 25 μg/L atrazine treatments were 0.11 ± 0.08, 0.23 ± 0.13, 1.44 ± 0.42, 11.4 ± 2.91, and 25.1 ± 4.77 μg/L atrazine, respectively.

Aromatase activity

Aromatase activities in the gonads of juvenile female *X. laevis* were significantly greater than in males (Mann-Whitney U, p = 0.0001; Figure 1). The mean (± SD) ovarian aromatase activity was 420 ± 486 fmol/h/mg protein, while the mean (± SD) testicular aromatase activity was 10.8 ± 52.8 fmol/h/mg protein. There was no detectable aromatase activity in 77% of the male frogs examined.

There were no statistically significant differences in testicular aromatase activity among male frogs exposed to atrazine and untreated control frogs (Kruskal-Wallis, p = 0.075). Neither were there statistically significant differences in testicular aromatase activity among DHT, E2 and EtOH -exposed frogs (Kruskal-Wallis, p = 0.382; Figure 1; Table 1). Given the sample size and variability in testicular aromatase activity, a difference of 95.5 fmol/h/mg protein was discernable among treatments with 80% power.

There were no statistically significant differences in ovarian aromatase activity among atrazine treatments and untreated controls (Kruskal-Wallis, p = 0.821). Given the sample size and variability in ovarian aromatase activity, a difference of 766 fmol/h/mg protein was discernable among treatments with 80% power. Females exposed to E2 had significantly less ovarian aromatase activity than did females exposed to EtOH (Mann-Whitney U, p = 0.0003; Figure 1; Table 1).

Table 1: Gonadal aromatase activity (fmol/h/mg protein) in male and female juvenile *X. laevis* exposed to positive controls and various concentrations of atrazine [a]

Treatment	N	Males		Standard Error	N	Females		Standard Error
		Mean	Median			Mean	Median	
Control	19	8.92	0.0	4.19	16	532	272	162
EtOH	21	5.55	0.0	2.67	17	614	403	127
DHT	21	7.22	0.0	3.21	15	202	111	52.3
E2	25	13.1	0.0	4.19	15	* 106	* 37.9	44.7
0.1 ug/L atrazine	24	8.66	0.0	4.67	10	331	232	117
1.0 ug/L atrazine	23	35.8	0.0	28.8	15	490	209	156
10 ug/L atrazine	18	1.92	0.0	1.92	18	507	332	116
25 ug/L atrazine	21	0.94	0.0	0.94	16	496	354	113

[a] All frogs with aromatase activity less than the method detection limit (MDL) were assigned a value of 0.0 for statistical analyses and summaries.

* Indicates a significant difference from the EtOH control at $p < 0.05$.

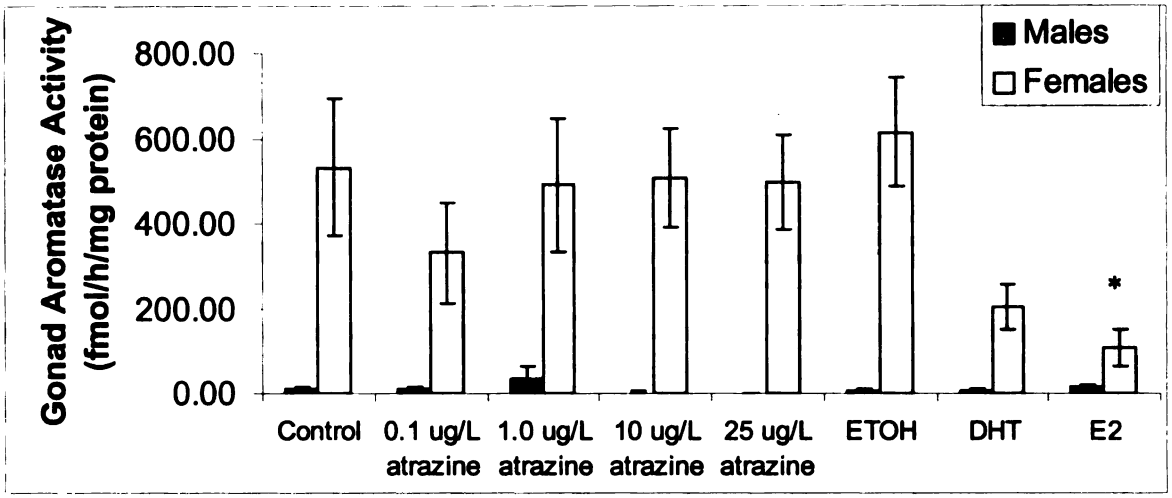

Figure 1: Gonadal aromatase activity in male and female *X. laevis* exposed to positive controls and various concentrations of atrazine. Bars represent mean ± standard error of the mean. * indicates a significant difference from ethanol control, $p<0.05$.

EtOH = 0.005% ethanol; DHT = 0.1 mg/L dihydrotestosterone; E2 = 0.1 mg/L estradiol

Brain aromatase activity also was statistically different between male and female *X. laevis* (Mann-Whitney U, p = 0.024; Figure 2; Table 2). Overall, there was greater aromatase activity in the brains of female frogs as compared to male frogs. However, this was not a consistent trend in all treatment groups. Male frogs in the EtOH and E2 treatment group had greater aromatase activity than the female frogs (Table 2). The mean aromatase activity in the brains of male and female frogs was 0.73 ± 0.64 and 0.90 ± 0.69 pmol/h/mg, respectively.

There were no statistically significant differences in brain aromatase activity among male atrazine-exposed *X. laevis* and untreated controls (Kruskal-Wallis, p = 0.410). Given the sample size and variability in male brain aromatase activity, a difference of 0.83 pmol/h/mg protein was discernable among treatments with 80% power.

Significant differences in brain aromatase activities of male *X. laevis* were observed among the DHT, E2, EtOH treatments (Kruskal-Wallis, $p = 0.024$). The E2-exposed *X. laevis* had significantly greater brain aromatase activity as compared to the DHT-exposed *X. laevis* (Mann-Whitney U, $p = 0.012$; Figure 2; Table 2).

Among the female *X. laevis*, there were no significant differences in brain aromatase activity among atrazine-treated *X. laevis* and the untreated controls (Kruskal Wallis, $p = 0.885$). Likewise, in female *X. laevis* there were no significant differences in brain aromatase activities among the DHT, E2, and EtOH treatments (Kruskal-Wallis, $p = 0.597$; Figure 2; Table 2). Given the sample size and variability in female brain aromatase activity, a difference of 1.1 pmol/h/mg protein was discernable among treatments with 80% power.

Table 2: Brain aromatase activity (pmol/h/mg protein) in male and female juvenile *X. laevis* exposed to positive controls and various concentrations of atrazine [a]

Treatment	N	Males		Standard Error	N	Females		Standard Error
		Mean	Median			Mean	Median	
Control	18	0.71	0.54	0.12	15	1.05	0.81	0.22
EtOH	21	0.93	0.89	0.17	15	0.81	0.70	0.12
DHT	19	0.54	0.47	0.09	15	0.63	0.46	0.11
E2	24	* 1.07	* 0.91	0.16	15	0.86	0.48	0.20
0.1 µg/L atrazine	24	0.58	0.55	0.09	10	0.93	0.85	0.22
1.0 µg/L atrazine	22	0.71	0.47	0.15	16	0.95	0.86	0.17
10 µg/L atrazine	18	0.43	0.27	0.10	18	0.98	0.90	0.16
25 µg/L atrazine	21	0.80	0.81	0.16	16	0.94	0.59	0.23

[a] All frogs with aromatase activity less than the MDL were assigned a value of 0.0 for statistical analyses and summaries.

* Indicates a significant difference from the DHT treatment at $p < 0.05$.

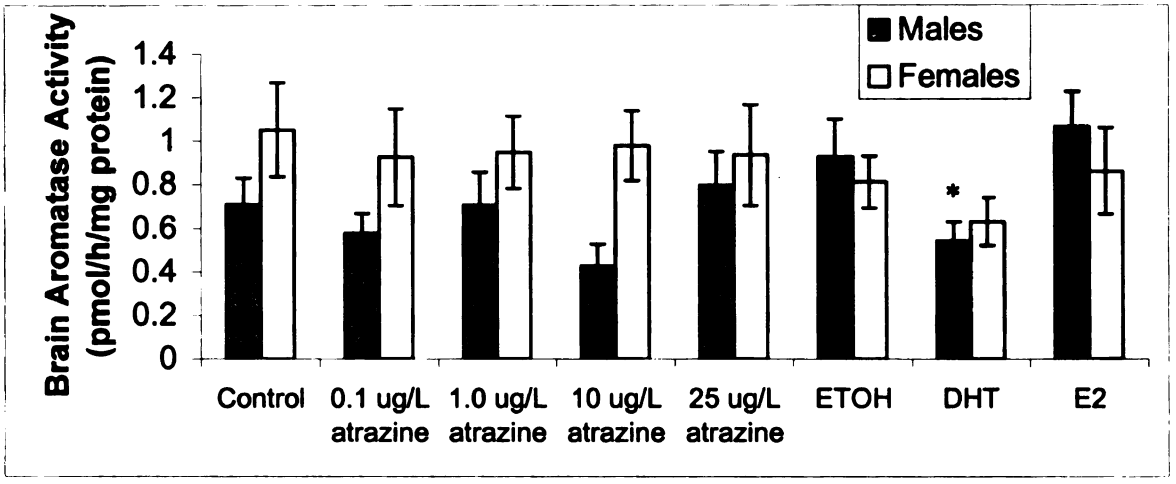

Figure 2: Brain aromatase activity in male and female *X. laevis* exposed to positive controls and various concentrations of atrazine. Bars represent mean ± standard error of the mean. * indicates a significant difference from estradiol treatment, $p<0.05$.

EtOH = 0.005% ethanol; DHT = 0.1mg/L dihydrotestosterone; E2 = 0.1mg/L estradiol

Hormones

Concentrations of T and E2 were measurable in the plasma of both male and female *X. laevis* by ELISA. However, E2 concentrations in both male and female juvenile frogs were sometimes less than the method limit of detection (7.8 pg/mL). Juvenile female *X. laevis* had greater plasma E2 concentrations than juvenile male frogs (Kruskal-Wallis, $p = 0.020$). The mean concentrations of E2 were 27.0 ± 126 and 40.8 ± 227 ng/mL for males and females, respectively.

There were statistically significant differences in plasma E2 concentrations among male frogs exposed to control and atrazine treatments (Kruskal-Wallis, $p = 0.003$; Table 3). Male frogs exposed to 1.0 μg/L atrazine had significantly lower E2 concentrations than untreated control, 0.1 μg/L and 25 μg/L atrazine exposed male frogs (Mann-Whitney U, $p = 0.001$, 0.015, and 0.0001, respectively; Figure 3). However,

concentrations of E2 in *X. laevis* exposed to 0.1, 10 and 25 μg/L atrazine were not different from untreated controls. Significant differences in plasma E2 concentrations were also observed among males exposed to the DHT, E2, and EtOH treatments (Kruskal-Wallis, $p = 0.025$). Plasma concentrations of E2 in male *X. laevis* exposed to E2 were significantly greater than concentrations of E2 in the EtOH -exposed frogs (Mann-Whitney U, $p = 0.008$; Figure 3).

Among the female *X. laevis*, there were no significant differences in E2 concentrations among any of the atrazine treatments and the untreated controls (Kruskal-Wallis, $p = 0.079$). Likewise, there were no significant differences in E2 concentrations among the DHT, E2, and EtOH treatments (Kruskal-Wallis, $p = 0.086$). Given the sample size and large variability in female estradiol levels, a difference of 495 ng/mL was discernable among treatments with 80% power.

Table 3: Plasma estradiol concentrations (ng/mL) in juvenile *X. laevis* exposed to positive controls and various concentrations of atrazine [a]

Treatment	N	Males		Standard Error	N	Females		Standard Error
		Mean	Median			Mean	Median	
Control	20	35.2	3.47	15.4	15	97.5	1.88	60.4
EtOH	21	6.03	0.30	5.08	18	0.66	0.23	0.22
DHT	21	18.7	1.47	5.96	16	149	0.35	147
E2	25	** 68.6	** 3.92	57.8	15	22.2	2.44	8.61
0.1 µg/L atrazine	26	12.9	0.80	4.64	12	42.5	0.17	26.3
1.0 µg/L atrazine	24	* 0.29	* 0.24	0.03	16	5.14	0.27	2.74
10 µg/L atrazine	20	58.0	0.53	39.6	17	8.98	0.18	8.52
25 µg/L atrazine	23	18.2	3.52	5.32	17	12.4	0.32	6.12

[a] All estradiol concentrations less than the method detection limit (0.0078 ng/mL) were set to a value of 0.005 ng/mL for statistical analyses and summaries. * Indicates a significant difference from the control treatment at $p < 0.05$. ** Indicates a significant difference from the EtOH control at $p < 0.05$.

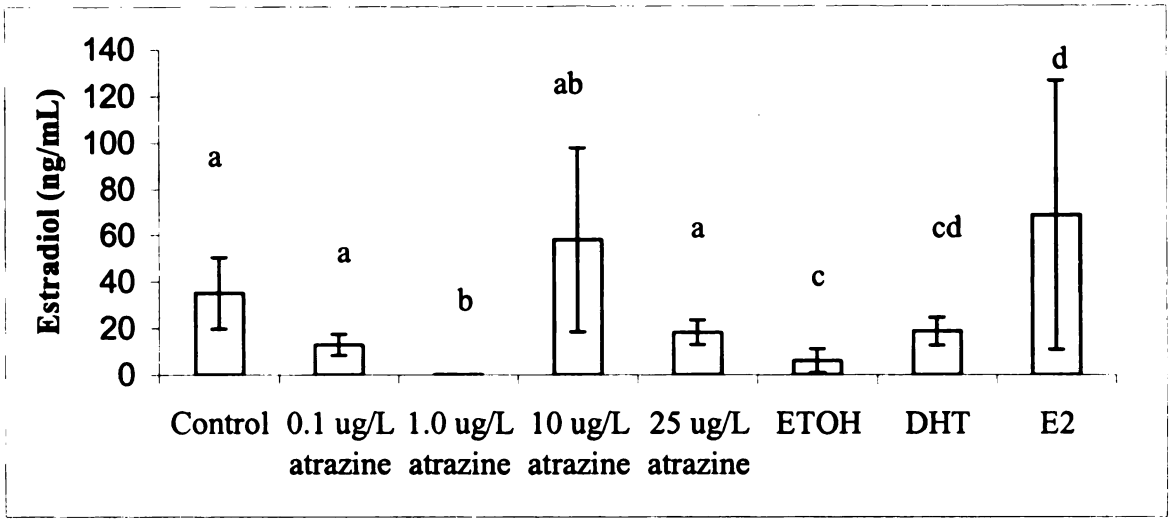

Figure 3: Estradiol concentrations in juvenile male *X. laevis* exposed to positive controls and various concentrations of atrazine. Bars represent mean ± standard error of the mean.

a, b signify tests for significant differences at $p < 0.05$ among the atrazine data set; c, d signify tests for significant differences at $p<0.05$ among the positive controls; EtOH = 0.005% ethanol; DHT = 0.1 mg/L dihydrotestosterone; E2 = 0.1 mg/L estradiol

There were no significant differences in plasma T concentrations between juvenile male and female *X. laevis* (Kruskal-Wallis, $p = 0.170$). There were no statistically significant differences in T concentrations among male frogs in the control and atrazine treatments (Kruskal-Wallis, $p = 0.270$). There also were no statistically significant differences in concentrations of T among the E2 and DHT treatments and the EtOH controls (Kruskal-Wallis, $p = 0.187$; Table 4). Given the sample size and variability in male testosterone levels, a difference of 37 ng/mL was discernable among treatments with 80% power.

There were no statistically significant differences in concentrations of T among atrazine treatments and the untreated controls for female *X. laevis* (Kruskal-Wallis, p =

0.179). Likewise, there were no statistically significant differences in plasma concentrations of T among females in the DHT, E2, and EtOH treatments (Kruskal-Wallis, $p = 0.363$; Table 4). Given the sample size and variability in female testosterone levels, a difference of 83 ng/mL was discernable among treatments with 80% power.

Concentrations of E2 and T were positively correlated ($R^2 = 0.81$). Individual frogs with greater concentrations of T also tended to have greater concentrations of E2. However, neither concentrations of T nor E2 were correlated with either gonad or brain aromatase activity ($R^2 < 0.005$ in all cases).

Table 4: Plasma testosterone concentrations (ng/mL) in juvenile *X. laevis* exposed to positive controls and various concentrations of atrazine [a]

Treatment	N	Male		Standard Error	N	Female		Standard Error
		Mean	Median			Mean	Median	
Control	20	17.2	1.39	8.17	15	24.6	1.39	12.9
EtOH	21	5.60	0.74	3.57	18	1.11	0.61	0.30
DHT	21	3.30	1.46	0.99	16	25.7	1.04	24.3
E2	25	11.7	1.56	7.74	15	6.61	0.70	4.16
0.1 µg/L atrazine	26	3.57	0.77	1.15	12	24.2	0.94	17.3
1.0 µg/L atrazine	24	1.10	0.67	0.18	16	1.00	0.75	0.23
10 µg/L atrazine	20	16.3	1.48	11.0	17	2.79	0.82	1.82
25 µg/L atrazine	23	2.49	1.24	0.57	17	1.69	0.71	0.58

[a] All testosterone concentrations less than the method detection limit (0.0078 ng/mL) were set to a value of 0.005 ng/mL for statistical analyses and summaries.

Discussion

During the 185 d exposure, small concentrations of atrazine were detected in untreated control tanks. This may have resulted from volatilization and deposition of atrazine from exposure tanks into control tanks, or atrazine may have been periodically present at low levels in the laboratory water supply. However, this does not affect the conclusions of the study, since there were no statistically significant, concentration-dependent effects of atrazine observed on any of the parameters investigated.

Aromatase is a critical enzyme for ovarian differentiation in fish and amphibians (Miyata et al., 1999; Baroiller and D'Cotta, 2001). Therefore, as expected, female *X. laevis* had greater aromatase activity in their gonads compared to male frogs. As in teleost fishes, there was lower aromatase activity (per unit protein) in the gonadal tissue of *X. laevis* as compared to the brain tissue (Melo and Ramsdell, 2001; Pasmanik and Callard, 1988).

Overall, female *X. laevis* had greater brain aromatase activity than male frogs, but this result was not consistent in all treatment groups. In fish, brain aromatase activity differs spatially in the male and female brain (Melo and Ramsdell, 2001). In addition, brain aromatase activity was reported to vary seasonally in both frogs and fish (Pasmanik and Callard, 1988; Guerriero et al., 2000) and was quantitatively different in male and female goldfish (*Carassius auratus*) only during a single month of the year (Guerriero et al., 2000). In the present study, it is possible that the sex difference in brain aromatase activity among *X. laevis* is also a transient observation.

Aromatase activity was measurably different in frog gonads and brains exposed to E2 and DHT. Exposure to E2 resulted in lesser gonadal aromatase activity in female *X. laevis* relative to controls. This phenomenon was not apparent in the male frogs. Most

male individuals had gonadal aromatase activity that was less than the method detection limit, resulting in poor resolution for detecting significant decreases in aromatase activity among treatment groups. In the case of female *X. laevis*, exogenous application of E2 may have been functioning in a negative feedback system with the aromatase enzyme, thus lowering the enzyme's activity (Yue et al., 2001). In male frogs, brain aromatase activity was elevated in E2-exposed frogs as compared to DHT-exposed frogs. This result is similar to other studies that report a positive feedback mechanism between E2 administration and male brain aromatase activity (Melo and Ramsdell, 2001).

There were no atrazine related effects on either gonad or brain aromatase activity in either male or female *X. laevis*. These results are consistent with another study in which aromatase activity did not differ between *X. laevis* collected from reference and atrazine use areas (Hecker et al., 2003). Aromatase activity was not correlated with circulating concentrations of either T or E2 in *X. laevis*. This result does not support the hypothesis that atrazine feminizes male frogs by increasing estrogen or demasculinizes frogs by decreasing testosterone levels via upregulation of aromatase, the enzyme that transforms T into E2 (Hayes et al., 2002; Hayes et al., 2003).

Atrazine exposure did not affect circulating levels of T in either male or female *X. laevis*. In addition, there were no differences in circulating concentrations of E2 in female frogs exposed to atrazine. In males, however, a significant difference in plasma concentrations of E2 was detected among frogs in the 1.0 μg/L atrazine treatment as compared to controls. Yet, this result was not consistent across atrazine concentrations, leading to the conclusion that atrazine was not responsible for depressed levels of E2 in this case. Since circulating concentrations of both E2 and T levels were variable in

juvenile *X. laevis*, and because sex steroids are not at their maximum level in juvenile frogs (Tobias et al., 1998; Kang et al., 1995), alterations in T and E2 concentrations may be more difficult to detect in juvenile frogs. In adult *X. laevis,* the plasma concentrations of both E2 and T were depressed in female frogs collected from atrazine use areas as compared to reference areas (Hecker et al., 2003). However, this decrease in hormone levels could not be causally linked with atrazine exposure, since multiple pesticides and other unknown environmental factors also could have resulted in lowered hormone levels in these frogs (Hecker et al., 2003). Thus, it cannot be concluded that atrazine alone is capable of causing depressed sex steroid concentrations in *X. laevis*. For a clearer picture of how exogenous compounds may disrupt sex steroid levels in amphibians, increased research into the normal background titers of circulating E2 and T and an increased understanding of normal sex steroid cycling is necessary in juvenile and adult frogs.

Conclusion

At measured concentrations ranging from 0.01 to 28.9 μg/L, atrazine did not affect aromatase activity or circulating concentrations of T or E2 in developing *X. laevis*. This conclusion does not support the hypothesis that environmentally relevant concentrations of atrazine disrupt hormone levels in frogs via upregulation of the enzyme aromatase (Hayes et al., 2002; Hayes et al., 2003). While atrazine may cause an effect on aromatase activity *in vitro* in mammalian cell lines (Sanderson et al., 2000), the same may not be true *in vivo* in frogs. Therefore, it appears that atrazine, at environmentally relevant concentrations, does not affect reproductive development in *X. laevis* frogs via upregulation of the aromatase enzyme.

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