E F F E C T S O F A T R A Z I N E O N *X E N O P U S*

L A E V I S O V A R I A N D E V E L O P M E N T

A Report of a Senior Study

by

Austin Wes Mackens

Major: Biology

Maryville College

Fall, 2005

Date Approved _____, by _____

Faculty Supervisor

_____, by _____

Editor

ABSTRACT

Amphibian populations have been declining for over two decades. One hypothesis for this decline is that a commonly used herbicide, atrazine, is causing abnormalities in gonadal development. The Environmental Protection Agency has set the drinking water standard level of atrazine at three parts per billion (ppb). This experiment studied four groups: juveniles exposed to two ppb atrazine, juviles exposed to 95% ethanol (control), tadpoles exposed to two ppb atrazine, and tadpoles exposed to 95% ethanol. Juveniles were exposed for 107 days, while the tadpoles were exposed for 22 days. There were no gross morphological differences resulting from atrazine exposure. However, histological analysis of the tadpole groups showed that the cortex widths of atrazine-exposed females were significantly larger than control females ($p=0.0111$). Cortex width per body weight of the tadpoles showed no significant difference ($p=0.0776$), but previous research has shown that gonad size is not dependent on body weight. Further research is needed to determine if these findings are repeatable at this low dosage. If these findings are

further supported, the EPA standard level of atrazine of three ppb will need to be reevaluated to ensure safe environmental toxicant levels.

TABLE OF CONTENTS

LIST OF FIGURES

ACKNOWLEDGEMENT

I would like to sincerely thank all of those who have helped in any way during this project. I want to thank the other project members for their dedication and long hours put in to make the project a success. I also want to thank Dr. Jerilyn Swann for her help throughout the project and the expertise that she provided.

I owe my family much gratitude for all of their support over the years. I especially want to thank my mom for all of the sacrifices she has endured over the years so that my brother and I could have the opportunities that we have today. Her words of encouragement have been an incredible inspiration in my life and will guide me on the rest of my journey through life. I am eternally indebted to her and the rest of my family for their support and encouragement over the years.

Most of all, I would like to thank Dr. Drew Crain for his patience, trust, knowledge, support, and encouragement throughout this project. His knowledge of the subject provided invaluable information to the entire group and his

encouragement helped us stay dedicated when things looked grim. He also showed a great deal of patience with each member of the research group, especially when I would call him at home on a Sunday afternoon. My sincerest thanks go to Dr. Drew Crain for this project would have never occurred without him.

CHAPTER I

INTRODUCTION

Amphibian Decline

Recent studies have shown that some amphibian populations around the world are declining at an alarming rate while other populations have disappeared completely. In fact, the extinction rate of all known species is the highest ever recorded (Alford & Richards, 1999; Houlahan, Findlay, & Schmidt et al., 2002). Amphibians have been shown to be one of the groups with the highest risk of extinction with over 500 populations in decline or endangered. Several factors have been hypothesized to contribute to this decline, including ultraviolet light sensitivity, predation, habitat destruction, environmental contamination, disease, and climate changes (Alford & Richards, 1999). Amphibians have been shown to be particularly sensitive to environmental insults due to their highly permeable skin and their exposure to both terrestrial and aquatic habitats. This increased susceptibility to environmental insults has many adverse effects on amphibian populations. Many of these harmful

effects are directly related to environmental toxicants, which have been found to cause reproduction impairment, abnormal growth rates, abnormal development and reproduction, and immune system suppression.

Numerous environmental toxicants have been analyzed and the effects on amphibians have been strenuously studied. Atrazine is one of the toxicants that have been shown to have adverse effects on several amphibian populations. Atrazine is a widely used herbicide that was formulated to control weed growth in corn growing regions (Tavera-Mendoza, Ruby, Brousseau, Fournier, Cyr, & Marcogliese, 2002B). North America and Canada are the primary users of the herbicide and have been recorded to use up to 36,000 tons annually.

Atrazine's initial use was granted due to its ability to naturally degrade in as few as eight days, but other areas have shown degradation to take as long as 350 days (Tavera-Mendoza, Ruby, Brousseau, Fournier, Cyr, & Marcogliese, 2002B). Furthermore, it was believed that concentrations of atrazine rarely reach levels that cause harmful effects in lakes and rivers (i.e. approximately 21 μg/L). However, edge waters and runoff water from treated cornfields have been shown to reach levels as high as 740 μg/L. This increased atrazine concentration poses serious

threats to local amphibian populations as well as other environmentally sensitive organisms.

Xenopus laevis and Gonad Development

The African clawed frog, *Xenopus laevis,* is a native inhabitant of sub-Saharan Africa (Cannatella & De Sá, 1993). It is predominantly an aquatic amphibian that spends the majority of its life in lakes, rivers, wetlands, or other aquatic environments. In its native region, *X. laevis* has a mating season that spans from July to September (Deuchar, 1975). Originally, it was believed that temperature was the determining factor in mating periods, but further analysis showed that moisture was the key environmental factor regulating mating. Thus, reproduction is dependent on environmental moisture, which can be adequately controlled in the laboratory. This makes *X. laevis* an ideal model organism because it can reproduce year-round given the proper environment and necessary stimuli.

Both male and female *X. laevis* reproductive characteristics have rapid responses to gonadotropin injections. Gonadotropins induce secondary sexual characterization, including spermiogenesis in males and ovulation in females. A commonly used gonadotropin in laboratories is human chorionic gonadotropin (hCG). While

females require a significant dose of hCG to induce ovulation, males require minimal amounts to induce spermiogenesis. In fact, hCG can cause spermatozoa production in less than two hours after minimal exposure.

Another advantage of *X. laevis* as an experimental model is that its morphogenesis and development has been well studied for several decades (Feder & Burggre, 1992). Specifically, normal ovary development has been carefully studied and recorded for future references. In the embryo, the gonads exist merely as cortex and medulla in germinal ridges along the anterior mesonephros. Germ cells then migrate from the endoderm into the ridges once they have enlarged. Next, the medulla expands and differentiates testis development while ovary development is characterized by cortex expansion. However, ovary and testes distinction is not possible until approximately stage 52 of larval development. Once gonadal differentiation has occurred, the gonads continue development until approximately stage 66 when metamorphosis is complete.

Sex determination depends on many factors one of which being the external environment, specifically temperature (Feder & Burggre, 1992). A minor fluctuation in temperature can render 100% female or 100% male offspring. Studies have shown that temperatures exceeding 25°C cause

all larvae to develop testes, while temperatures below 15°C render all females in all tested amphibian species. Another factor that aids in sex determination is the administration of sex steroid hormones. Administration of testosterone in early larval development renders 100% male offspring, while estrogen administration renders 100% female offspring. Furthermore, low levels of estrogen can also cause differentiation of all male offspring.

Atrazine Effects on Gonadal Development

Studies have been done by numerous research groups for several decades to determine the possible causes of the ongoing decline in amphibian populations. Many mechanisms have been analyzed and environmental contaminants have been tested and retested. One of the most extensively studied contaminants is atrazine and its affect on gonadal development of *X. laevis.* The following studies are just a few of the projects that have shown the adverse effects of atrazine on gonadal development of *X. laevis*:

Demasculinized *X. laevis*

One of the effects found from atrazine exposure is demasculinized male *X. laevis.* Hayes et al. studied the effects on the testes of *X. laevis* from atrazine exposures above and below runoff water levels (Hayes, Collins, Lee, Mendoza, Noriega, Stuart, & Vonk, 2002). Hayes et al.

found that at 25 parts per billion (ppb) atrazine-exposured males had a 10-fold decrease in plasma testosterone levels. It was also shown that laryngeal size in males was significantly decreased at atrazine exposure greater than or equal to 0.1 ppb. It is believed that the decreased laryngeal size directly correlates to the decrease in plasma testosterone levels. Hayes et al. hypothesize that aromatase activation causes the lower testosterone levels by converting testosterone into estrogen. This study concludes that environmental levels of atrazine are high enough to be considered a major contributor in amphibian population declines.

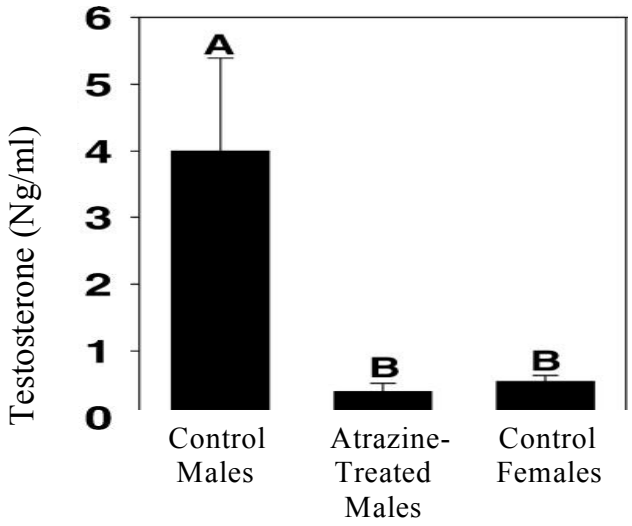

Figure 1. The above figure gives the plasma testosterone levels after a 46-day 25 ppb atrazine exposure (Hayes, Collins, Lee, Mendoza, Noriega, Stuart, & Vonk, 2002).

Tavera-Mendoza et al. (2002B) also found evidence for demasculanized effects of atrazine exposure on *X. laevis*

testis development. They found that 21 μg/L atrazine exposure for 48 hours just before gonadal differentiation caused testis volume to decreased 56% compared to the control group. Primary spermatogonial cell nests decreased in number by 70% and nursing cell number decreased by 74%. Furthermore, testicular reabsorption was observed in 70% of the treated tadpoles and aplasia, or failure to fully develop testes, was recorded in 10% of the treated frogs. Tavera-Mendoza et al. concluded that the adverse effects of atrazine exposure are correlated to a decrease in testosterone levels, due to increased aromatase activity and atrazine's ability to inhibit receptor binding of testosterone and dihydroxytestosterone. They conclude that atrazine levels in surface waters need to be reexamined in order to insure the safety of inhabiting organisms.

Tavera-Mendoza et al. (2002A) also found that ovaries had adverse effects after exposure to atrazine. *X. laevis* were exposed to atrazine at 21 μg/L for 48 hours. Histological analysis revealed a significant increase in the frequency of secondary oogonia with a p-value less than 0.05. Atresia was also found to have a significant increase in the atrazine-exposed models. The study concluded that the primary germ cells in atrazine-exposed

models were decreased by 20% as opposed to only 2% in control models.

Hermaphroditic *X. laevis*

Hayes et al., along with demasculinization, found that atrazine causes the development of hermaphroditic males (Hayes, Collins, Lee, Mendoza, Noriega, Stuart, & Vonk, 2002). Every exposure, except 0.01 ppb, showed induced gonadal abnormalities including up to 16-20 percent multiple gonad and hermaphroditic development. Hermaphroditic development is also believed to be due to a decrease in plasma testosterone levels in male *X. laevis* which has already been postulated to be caused by increased aromatase activity.

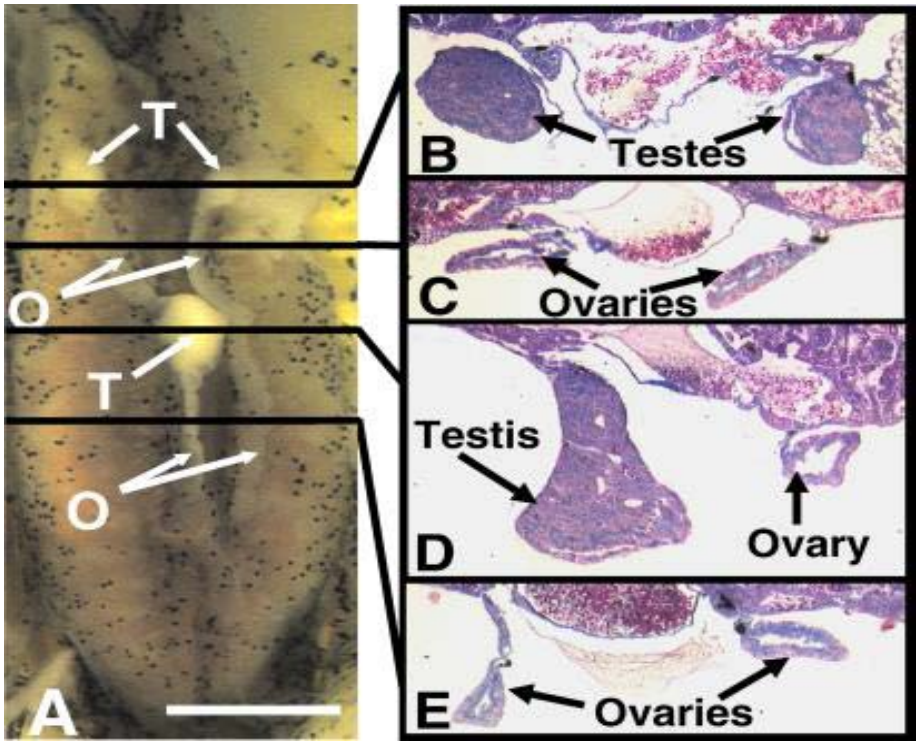

Figure 2. The above figure shows a one ppb atrazine-induced *X. laevis* (Hayes, Collins, Lee, Mendoza, Noriega, Stuart, & Vonk, 2002). Section A shows the kidney-gonad complex while B-E shows transverse cross-sections of the different gonads.

Compiled Findings of Atrazine Exposure

Recently, Tyrone Hayes compiled his findings on the effects of atrzine exposure in a response to Syngenta-funded research that predictably found different results since Syngenta is a manufacturer of atrazine (Hayes, 2004). Hayes first establishes that the adverse effects of atrazine exposure had been well studied before his group began researching. Previous groups concluded that atrazine exposure leads to increased levels of estrogen as well as mammary tumors in rodents. Coupled with the increased

estrogen levels is a decreased androgen level and inhibition of androgen activity.

Other groups confronted the issue of androgen and estrogen levels and found that atrazine stimulates aromatase production in human cells (Hayes, 2004). Similarly, Hayes also found effects of low androgen levels by observing impaired laryngeal growth at a mere 0.1 ppb exposure to atrazine. Later research by Hayes clearly showed that exposure to 0.1 ppb atrazine or higher also caused severe gonadal abnormalities in *X. laevis.* Furthermore, exposure to 0.01 ppb atrazine was shown by Hayes in 2002 to cause chemical castration of *X. laevis.* Hayes and another research group later confirmed that atrazine stimulated production and activation of aromatase.

Conflicting Studies

While the aforementioned research groups have repeatedly concluded that specific levels of atrazine exposure induce several types of abnormalities in amphibians, others claim that levels of atrazine in the wild have no affect on the local populations of organisms. One such study is that performed by James Carr and his team of researchers.

Carr exposed larval *X. laevis* to four different levels of atrazine (0, 1, 10, and 25 μg/L) (Carr et al., 2003).

The experimental results showed that atrazine exposure had no affect on posthatch mortality and larval growth. This was coupled with the results showing no posthatch success difference between any of the treated groups. There was also no significant effect of atrazine exposure on weight or snout-vent length (SVL). Body weight and SVL inversely affected time to metamorphosis completion in each experimental group. Atrazine was found to cause abnormal swimming, but only significantly in the group treated with 25 μg/L atrazine. Furthermore, atrazine-induced intersex was observed in many of the organisms, but again only those exposed to 25 μg/L atrazine. Laryngeal dilator muscle showed no significant difference in volume between each experimental group. Carr et al. concluded that environmental levels of atrazine have no significant adverse effects on developing frogs. Furthermore, they believe that the abnormalities induced by exposure to 25 μg/L atrazine would have no effect on survival rate of *X. laevis* in the wild.

Hayes addresses this study and the reasons for the conflicting findings. Hayes points out that previous studies done by Carr and his colleagues had similar results as his own studies involving atrazine and induced abnormalities (Hayes, 2004). However, later studies by

Carr, as in the one previously discussed, showed that atrazine had no significant adverse effects on amphibian populations at concentrations that can be found in the wild. Hayes has correlated the change in results with the fact that an atrazine manufacturer, Syngenta, funded Carr's later studies. Hayes also points out that Carr's earlier studies have shown that there is a 99% probability that the abnormalities in his studies are due to atrazine. Yet, Carr still concluded that the findings in Hayes' study that atrazine induced abnormalities were "weak trends" and could not be repeated.

Hayes addresses this accusation by pointing out the faults in Carr's experimental design (Hayes, 2004). He first points out that nearly 60% of the experimental organisms failed to metamorphose in some treatments while those surviving the treatment period showed a significant impediment of growth. Another problem with Carr's study is that dosing was incorrectly compared to Hayes' study. Hayes states that dosing in Carr's study at critical developmental stages was only 12.5% of that used in his own study due to overcrowding. Furthermore, the treatment tanks in Hayes' study were cleaned and atrazine was completely renewed every 72 hours. Carr's study involved only a 50% atrazine renewal every 72 hours.

Hayes also addresses a conflicting study done at Michigan State University that had results showing hermaphrodites in the control groups as well as the treated groups (Hayes, 2004). They concluded that since hermaphrodites were found in controls as well as treated organisms that it was not induced by atrazine exposure. Hayes points out that the control groups were contaminated with atrazine levels four times greater than threshold doses. Furthermore, this study had a mortality level as high as 85 percent. Hayes argues that in his studies, mortality greater than 15% call for cancellation of the entire study. Thus, Hayes concludes that due to poor experimental design, contamination, and negligence, the claim that atrazine has no effect on amphibian populations lacks credibility.

Experimental Justification and Hypothesis

Given the conflicting data stated previously, more studies are needed to support or reject the hypothesis that atrazine can induce gonadal abnormalities in developing *X. laevis.* This experiment will attempt to independently find results that are free of any outside influence. The affects of atrazine exposure on *X. laevis* ovary development will be closely analyzed and recorded and the results will be thoroughly analyzed and discussed. Furthermore, if any

abnormalities occur, a hypothesized mechanism will be proposed for the abnormality. It is hypothesized that female larval *X. laevis* exposed to two ppb atrazine will show significant adverse developmental effects. Such abnormalities are predicted to be increased follicular size and number, increased cortex width, and even intersex gonadal development.

CHAPTER II

MATERIALS AND METHODS

Juveniles

Stock one of atrazine was made using 10mg of atrazine and one ml of 95% ethanol. Stock two was made using 100µl of stock one and 9.9ml of 95% ethanol. Each stock was stored in a freezer. Eleven one-year-old juvenile *Xenopus laevis* were obtained from Jennifer Wilson's stock of experimental models and acclimated in approximately six liters of dechlorinated water at a temperature of approximately 23 degrees Celsius.

The juveniles were separated into two groups (Atrazine and Control) and put into separate containers containing six liters of 10% Holtfreter's solution with the following ingredients used for making the solution: 35g of NaCL, 2g of $NaHCO_3$, 0.5g of KCl, 6.66ml of $CaCl_2$, and 5 gallons of water. The atrazine tank was dosed with 120µl of atrazine to obtain a 2 ppb exposure. The control tank was dosed with 120µl of 95% ethanol to obtain a control exposure. A

100% water change was performed every three days for each group and the models were fed every day. Each group was exposed for 107 days until euthanasia and fixation was performed.

Tadpoles

Xenopus laevis tadpoles were ordered from Nasco at metamorphosis stage 56-58. The tadpoles were acclimated in dechlorinated water at 23 degrees Celsius and divided into Atrazine and Control groups.

The tadpoles were put into tanks with six liters of 10% Holtfreter's solution. The atrazine group was dosed with 120μl of atrazine to obtain a 2 ppb exposure. The Control group was dosed with 120μl of 95% ethanol to obtain a control exposure. A 100% water change was performed every three days and the groups were fed every day. The tadpoles were exposed for 22 days before euthanasia and fixation was performed.

Euthanasia, Fixation, and Mounting

Each experimental model was euthanized using chlorotone. After euthanized, the models were dissected and the kidney-gonad complex was removed using microscissors and a dissecting microscope. The kidney-gonad complexes were put in Bouin's fixative in a bullet

tube for three days and then washed with 70 percent ethanol three times with varying time intervals between washings.

Approximately seven days after the initial 70% ethanol wash the tissues were dehydrated by putting the tissues in 80% ethanol for two hours, 95% for one and a half hours, 100% for one hour, and 100% for an additional hour. The tissues were placed in Citrisolv (non-toxic clearing agent) for one hour and then placed in new Citrisolv immediately after the first hour and cleared for an additional hour.

The tissues were embedded in wax via the following procedure: tissues put in wax I for one hour at 12 pounds per square inch (psi); wax II for one hour at 15 psi; wax III for one hour at 21 psi; and wax IV for one hour at 25 psi. The tissues were then set in a paraffin wax block and the wax was cooled to set the tissue. The tissues were oriented so the kidneys were perpendicular to the surface. The paraffin block was then cut using a razor blade in order to decrease the amount of wax in the sections used for slide mounting. The trimmed block was mounted in the microtome and sectioned with a thickness of 12µm. The ribbons were floated in a warm water bath (with a pinch of gelatin) and mounted on slides. The slides were then dried on a slide warmer before staining.

Staining

The slides were stained in order to visualize various cell structures. Hematoxylin and Eosin staining was used via the following procedure: Citrosolv for 10 minutes; 100% ethanol for one minute; 95% ethanol for one minute; 70% ethanol for one minute; running water for four minutes; Hematoxylin for four minutes; running water for four minutes; Scott solution for two minutes; running water for four minutes; Eosin for three minutes; two dips in 70% ethanol; two dips in 95% ethanol; 100% ethanol for two minutes; 100% ethanol for two minutes; Citrosolv for four minutes; and Citrosolv for four minutes.

After staining was complete Permount was applied and a slide cover was set over the tissues. The tissues were then analyzed using a light microscope.

Analysis

After the tissue was stained and fixed on slides the ovaries were analyzed. The average follicle diameter, follicle diameter per body weight, maximum follicle diameter, and follicle number in a 100x magnification field of view were recorded for juvenile control and atrazine frogs. An unpaired *t*-test was performed for control and atrazine data and the p-value recorded.

Ovary cortex width, cortex width per body weight, medullary width, and medullary width per body weight was calculated and recorded for control and atrazine tadpoles. An unpaired *t*-test was used to compare control and atrazine data.

CHAPTER III

RESULTS

Analysis using a dissecting microscope showed no apparent ovotestes in either the control or atrazine groups. Each juvenile frog had apparent testes or ovaries under the dissecting microscope. Most of the tadpole frogs could be identified as having ovaries or testes using a dissecting microscope (see Figure 3), but some had to be determined during histological analysis.

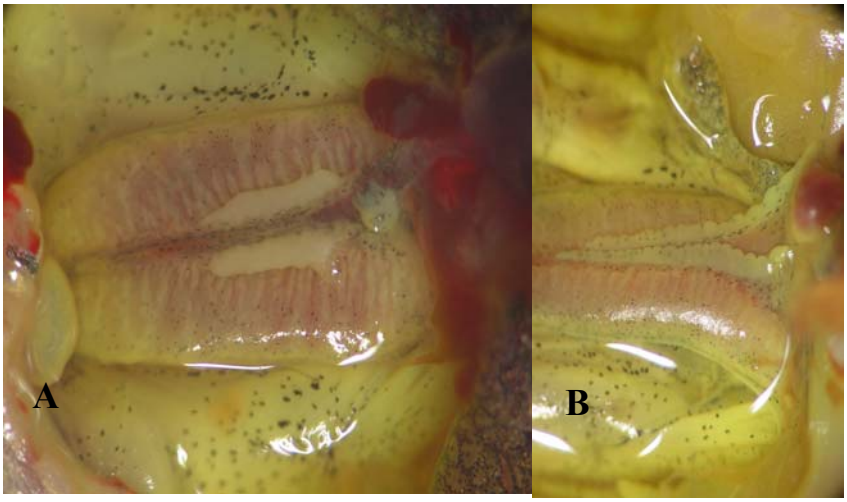

Figure 3. Gross gonadal anatomy (A) Testis (B) Ovaries. Magnification 30x.

The results from the juvenile data were inconclusive. The p-values showed no significance between each measured variable. The average follicle diameter for control and atrazine frogs had a p-value of 0.3998 (see Figures 4 and 5A). The average follicle diameter per body weight also showed no significant difference between control and atrazine frogs with p-value of 0.4895 (see Figure 6A). The number of follicles in a field of view at 100x magnification showed no significant difference with a p-value of 0.7247 (see Figure 6B).

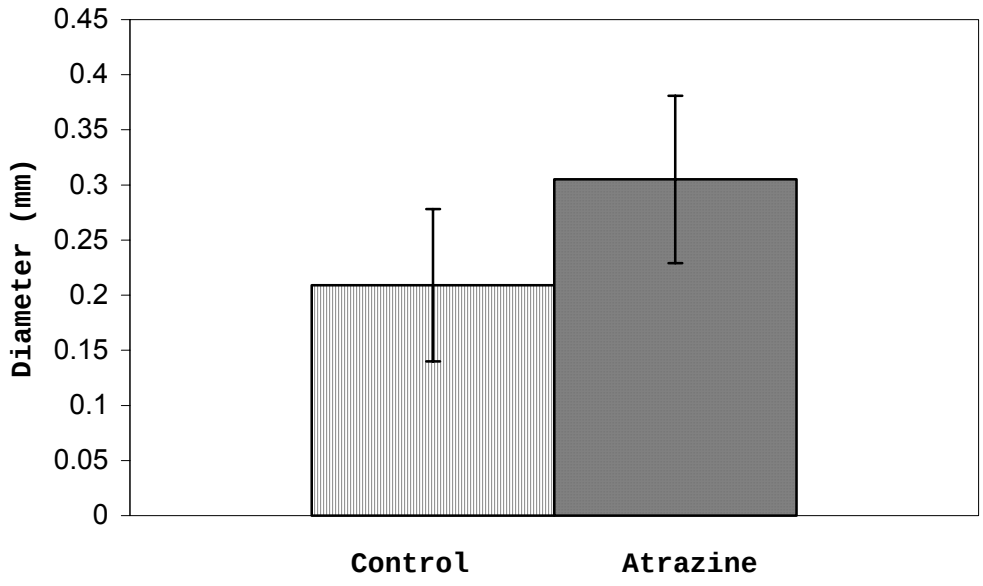

Figure 4. Control and atrazine average follicle diameter with standard error bars.

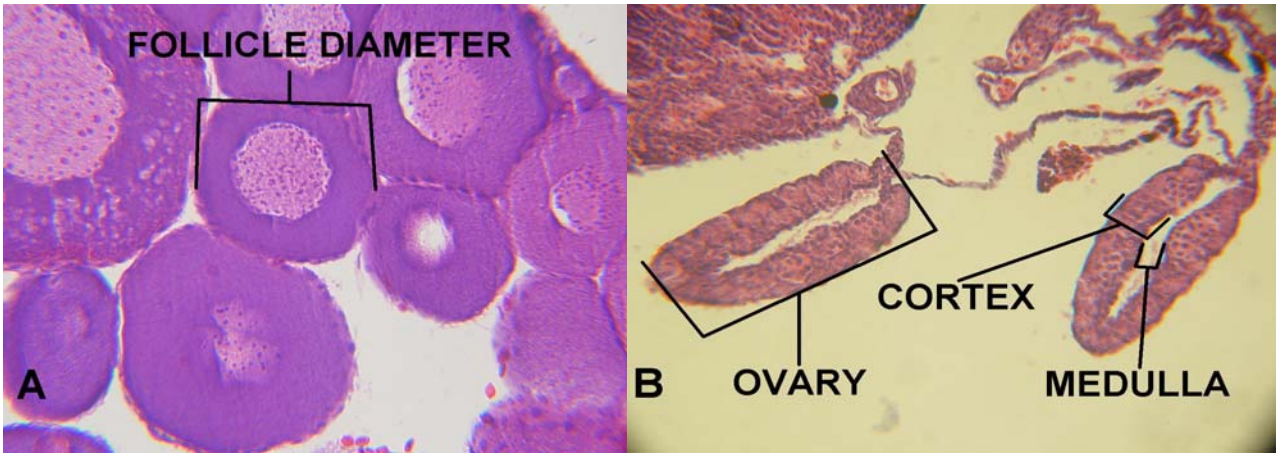

Figure 5. Histological view of (A) juvenile and (B)tadpole ovary including medulla and cortex at 200x magnification.

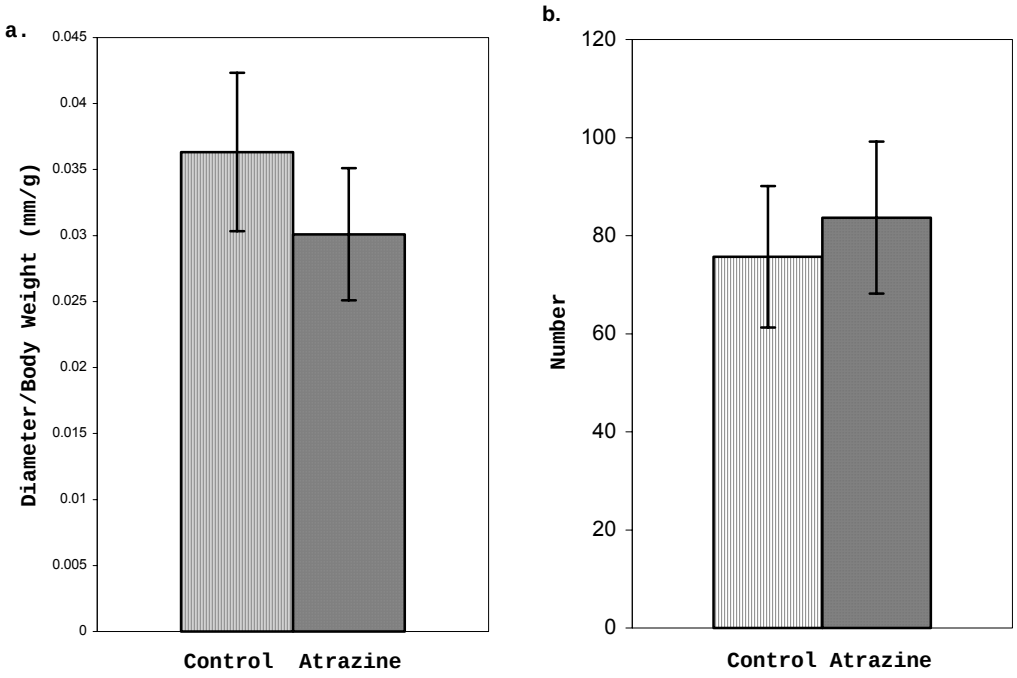

Figure 6. a. Juvenile control and atrazine follicle diameter per body weight with standard error bars. b. Juvenile control and atrazine number of follicles in 100x magnification field of view.

The ovarian cortex diameter from tadpoles exposed to atrazine was significantly larger compared to controls when body weight was not used as a correcting factor (p=0.0111, see Figures 5B and 7A). The p-value for cortex width per body weight was 0.0776 (see Figure 7B).

The results for analysis of tadpole control and atrazine medullary width were inconclusive (see Figure 8). The p-value for medullary width analysis was 0.5698, while the p-value for medullary width per body weight was 0.6735.

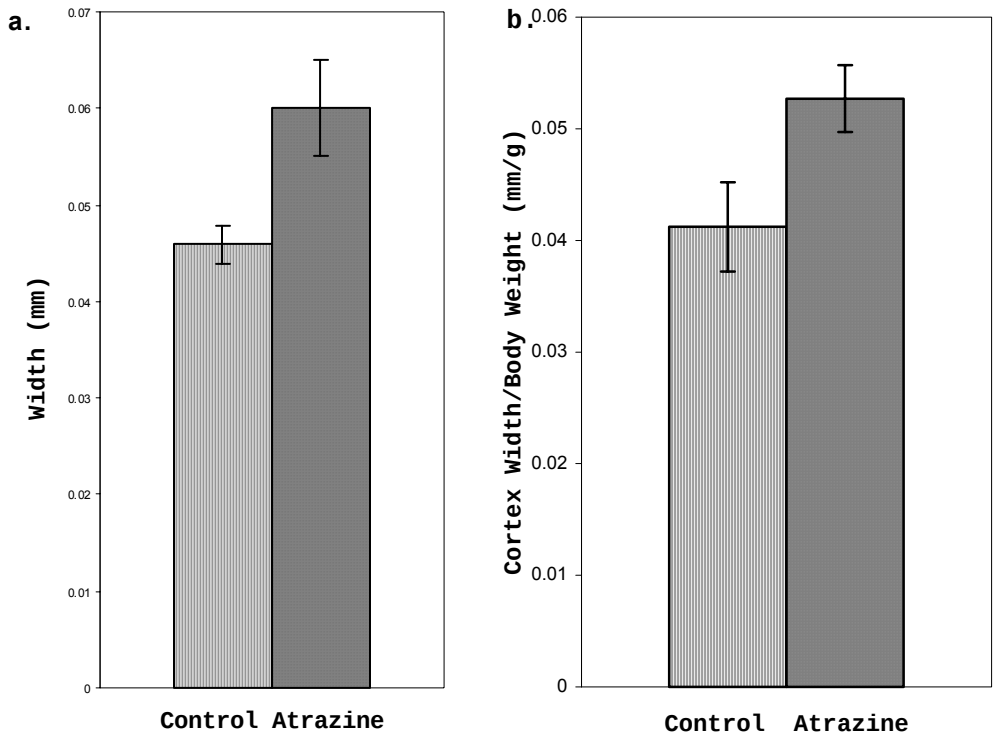

Figure 7. a. Tadpole control and atrazine cortex width with standard error bars. b. Tadpole control and atrazine cortex width per body weight with standard error bars.

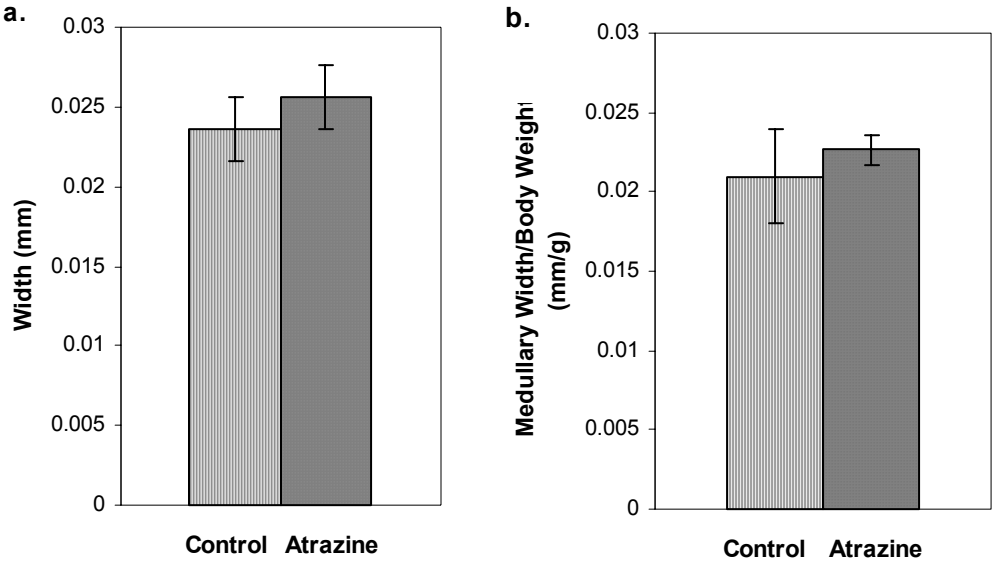

Figure 8. a. Control and atrazine tadpole medullary width and standard error bars. b. Control and atrazine tadpole medullary width per body weight with standard error bars.

CHAPTER IV

DISCUSSION AND CONCLUSION

The results for the statistical analysis of the one-year-old juveniles showed no significance in the separate measurements. The *t*-test results for average follicle diameter showed no significant difference between control and atrazine frogs. The *t*-test for average follicle diameter per body weight also showed no significant difference between control and atrazine frogs. The number of follicles in a field of view at 100x magnification showed no significance between control and atrazine frogs. It is hypothesized that because the frogs had completed gonadal formation before exposure to atrazine, the conversion of testosterone to estrogen via aromatase activation had little to no affect on gonadal development. The difference between juvenile control and atrazine frogs would be better studied by analysis of circulating testosterone and estrogen concentrations or gonadal aromatase activity. However, these were not measured due

to limited plasma volume and the need of each gonad for histological analysis.

The results for the statistical analysis of tadpoles beginning exposure at stage 56 showed no significance except for cortex width. The t-test for cortex width showed a significant difference between control and atrazine frogs. However, the t-test for cortex width per body weight did not show a significant difference between control and atrazine frogs. The t-test for medullary width and medullary width per body weight showed that there is no significant difference between control and atrazine frogs.

The difference between control and atrazine tadpole cortex width is most likely due to the timing of the exposure to atrazine, which was during the organization of the gonads. However, the cortex width per body weight ratio showed a lack of significant difference. It is undetermined at this time whether or not ovary cortex width or gonad size is dependent on body weight.

Previous studies have shown that atrazine can have several adverse affects on gonadal development. Tavera-Mendoza et al. (2002A) concluded that atrazine adversely affects ovary development by decreasing the number of germ cells. While this was not measured in the frog ovaries in this experiment, the tadpole cortex was found to be

significantly larger in atrazine frogs. Furthermore, Hayes et al. (2002) showed that a 25 ppb atrazine exposure significantly decreases plasma testosterone levels. The decrease in plasma testosterone coincides with the hypothesis that atrazine induces the conversion of testosterone to estrogen via aromatase. While Hayes' study showed a decrease testosterone level (i.e. an increase in estrogen) at a 25 ppb exposure, this experiment appeared to have the same findings at a 2 ppb exposure.

In summary, the ovaries of exposed frogs showed minimal differences between control and atrazine groups. The juveniles showed no significant difference between any of the measured variables, which was most likely due to the fact that the juveniles had completed gonadal formation prior to atrazine exposure. The tadpoles showed minimal differences in ovary morphology as well. However, the difference in cortex widths between control and atrazine frogs shows that gonadal development was in fact affected during atrazine exposure. Thus, it seems pertinent that more research be completed in order to either confirm these findings or reject them. Furthermore, if the findings are confirmed, the standard atrazine ground water level of 3 ppb set by the Environmental Protection Agency should be

reevaluated in order to maintain safe environmental toxicant levels.

APPENDIX

MARYVILLE COLLEGE HUMAN AND ANIMAL SUBJECTS
REVIEW COMMITTEE ANIMAL STUDY APPLICATION FORM

1. Student Name: Travis Groth, Heather Hedden, Ginger Lovingood, Austin Mackens, Lauren Ward
2. Date: March 15, 2005

3. Senior Thesis Advisor: Crain/Swann

4. Pain or Distress Category: B (See listing of Pain or Distress Categories below)

For categories C, D, or E, USDA regulations require that the investigator consider alternative procedures. Please provide a narrative (for instance the end of Chapter 1) describing the methods and sources used to determine that alternatives are not available. If a computer assisted literature search was conducted, provide the names of the database(s) and date(s) of the search.

PAIN OR DISTRESS CATEGORIES

A. ACUTE STUDIES
Studies performed under anesthesia from which the animals are not permitted to regain consciousness, or performed on excised animal tissues collected under anesthesia or following euthanasia.

B. PAIN OR DISTRESS - NONE OR MINOR
Chronic studies that DO NOT involve survival surgery, induction of painful or stressful disease conditions, or pain or distress in excess of that associated with routine injections or blood collection. Included are induction or transplantation of tumors in animals (so long as the tumors do not cause pain and the animals are terminated prior to becoming seriously ill), administration of mildly toxic substances or drugs that cause no significant disease or distress, and antibody production as long as significant disease does not result and antigen booster doses do not include Complete Freund's Adjuvant (CF A).

C. PAINFUL PROCEDURES WITH ANESTHESIN ANALGESIA
a. Survival surgical procedures.
b. Painful or potentially painful non-surgical procedures; e.g. bone marrow taps, injections into particularly sensitive areas such as foot pads, cardiac punctures, or traumatic procedures such as burns (burns may be category D, depending on severity).

D. MODERATE DISTRESS OR PAIN GENERALLY WITHOUT ANESTHESW ANALGESIN TRANQUILIZERS
Induction of moderately distressful or painful disease conditions (examples: arthritis, administration of toxic chemicals, infectious challenges, immunosuppression resulting in infectious disease, peritonitis, severe inflammation, especially of weight bearing surfaces or resulting in external sores), whole body irradiation, stress models, septic shock, hypotensive shock, moderate painful stimuli (examples: low level electrical shock or heat), survival surgical procedures that have the potential to result in long term distressful illness (organ transplants, for example), induction of cardiac ischemia, booster immunizations with CFA, tumor induction or animal cultures that cause significant distress or pain, sight deprivation, restraint for periods longer than 12 hours.

E. INTENSE SUSTAINED OR REPEATED PAIN WITHOUT ANESTHESIN ANALGESIA

Direct stimulation of CNS pain tracts, nociceptor stimulation by physical or chemical means that causes severe pain (e.g., corneal abrasions), or any category C (see above) procedure if performed without chemical relief of pain.

5. Species to be used *Xenopus laevis*

6. Age of animals egg-subadult

7. Number of animals in study 80 tadpoles, 22 subadults

8. Duration of study April 2005-October 2005

9. Location of animals during the study (building and room) 114 Sutton

10. List of personnel to call if problems with animals develop.

Name	Daytime Phone	Nighttime Phone	Emergency No.
Dr. Crain	981-8238	379-1706	
Dr. Swann	981-8068	981-5439	
Heather Hedden	680-4670		
Austin Mackens	865-254-3173		
Travis Groth	865-748-7809		
Lauren Ward	865-640-6202		
Ginger Lovingood	865-207-7794		

Investigator Assurance

The information provided in this protocol form accurately reflects the intended use of animals for this research activity. Significant changes in procedures will not be undertaken without prior notification and approval of the Human and Animal Subjects Review Committee.

All persons involved in the use of animals on this protocol have been informed of the experimental objectives and methods. Each has received training in the execution of animal-related procedures he/she will perform prior to participation in the protocol, and will participate in any educational or training programs deemed appropriate or necessary by the Human and Animal Subjects Review Committee.

I agree to follow the provisions of the Animal Welfare Act and the guidelines of the National Institutes of Health on the care and use of laboratory animals.

I agree to use anesthesia, analgesia and tranquilization to relieve pain or distress whenever use of these agents will not jeopardize the scientific validity of the data. I have specifically consulted with the Human and Animal Subjects Review Committee regarding any experiments that are classified in pain/distress categories C, 0, or E.

I will take appropriate steps to avoid exposure of persons working with these animals to any biohazardous agents used in the study.

State the reasons if you cannot attest to the accuracy of any of these statements:

11. Husbandry Requirements: Is anything other than routine care and equipment required? YES___ NO _x_ If yes, please list below.

12. It is likely that pain/discomfort will be experienced by animals in this protocol? YES ___ NO__x___ If "YES", describe:

13. What will happen to the animals at the end of the study? If euthanasia is required, state the methods.

    Animals will be euthanized immersion into a concentrated chlorotone solution.

14. Briefly describe your proposed research project (or attach a research proposal). Be sure to include a justification for the species number.

Atrazine is currently America's and probably the world's most commonly used pesticide. It is reported by the U.S. Department of Agriculture that approximately *800* million pounds of atrazine were used between the years 1980-1990 in America (USDA *2003).* It has been banned in numerous countries already because it has been recognized as an endocrine disruptor. Recent studies on the endocrine disrupting effects of atrazine have caused much controversy over whether or not this product should be banned in the United States. Numerous studies funded by Syngenta Crop Protection, Inc. have released information providing evidence primarily showing that atrazine is relatively harmless. However, other studies conducted by Hayes and other private foundations have found evidence showing that atrazine has a feminizing effect on amphibians as a result of inhibition of the aromatase enzyme that is essential for the conversion of testosterone to estradiol.

This study will examine the effect of 2 ppb atrazine-exposure on *Xenopus laevis* tadpoles and juveniles. Endpoints measured will be frog gonads and laryngeal tissue using both light microscopy and electron microscopy techniques. This experiment will provide evidence as to whether or not atrazine at concentrations of 2ppb have adverse side effects on the endocrine system of frogs.

Experimental design includes exposing 11 juvenile and 40 tadpole *Xenopus laevis* frogs to atrazine, while having equal numbers of each group serve as controls. An adult male and female *Xenopus* will be purchased from Nasco and injected with hCG. The frogs will then be allowed to mate and two groups of forty tadpoles taken at random and placed into separate groups.

Currently, twenty-three juvenile *Xenopus* are in the laboratory and they will be separated into two groups of eleven and twelve each. One group from the tadpoles and one from the juveniles will be exposed to an atrazine concentration of 2 ppb *for* approximately three months. Tanks will be cleaned and atrazine levels maintained every three days to assure chronic exposure. Atrazine exposure should begin at the beginning of April *2005* and conclude at the beginning of July *2005.* Laboratory analyses will be conducted during the summer and fall of 2005.

This project has been reviewed and approved by the Maryville College Human and Animal Use Committee.

se Committee.

APRIL 1, 2005

REFERENCES

Alford, R. A. , & Richards, S. J. (1999). Global amphibian declines: A problem in applied ecology. *Annu Rev Ecol Syst 30*: 133-65.

Cannatella, D. C. , & De Sá, R. O. (1993). *Xenopus laevis* as a model organism. *Systematic Biology 42*(4): 476-507.

Carr, J. A. , et al. (2003). Response of larval *Xenopus laevis* to atrazine: Assessment of growth, metamorphosis, and gonadal and laryngeal morphology. *Environmental Toxicology and Chemistry 22*: 396-405.

Deuchar, E. M. (1975). *Xenopus*: *The South African clawed frog*. London, England: Wiley-Interscience.

Feder, M. E. , & Burggre, W. W. (Eds.). (1992). *Environmental physiology of the amphibians*. Chicago: University of Chicago Press.

Hayes, T. B. (2004). There is no denying this: Defusing the confusion about atrazine. *BioScience 54*: 1138-1149.

Hayes, T. B. , Collins, A. , Lee, M. , Mendoza, M. ,

Noriega, N. , Stuart, A. A. , & Vonk, A. (2002). Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. *Proceedings of the National Academy of Sciences 99*: 5476-5480.

Houlahan, J. E. , Findlay, C. S. , & Schmidt, B. R. , et al. (2000). Quantitative evidence for global amphibian population declines. *Nature 404*: 752-55.

Tavera-Mendoza, L. , Ruby, S. , Brousseau, P. , Fournier, M. , Cyr, D. , & Marcogliese, D. (2002A). Response of the amphibian tadpole (*Xenopus laevis*) to atrazine during sexual differentiation of the ovary. *Environmental Toxicology and Chemistry 6*: 1264-7.

Tavera-Mendoza, L. , Ruby, S. , Brousseau, P. , Fournier, M. , Cyr, D. , & Marcogliese, D. (2002B). Response of the amphibian tadpole (*Xenopus laevis*) to atrazine during sexual differentiation of the testis. *Environmental Toxicology and Chemistry 3*: 527-31.