EFFECT OF ATRAZINE ON TESTES IN *XENOPUS LAEVIS* TADPOLES AND JUVENILES

A Report of a Senior Study

by

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ABSTRACT

Environmental contaminants in recent years have been researched because of a speculated correlation with the decline in amphibian populations (Boyer & Grue, 1994; Carey & Bryant, 1995). Atrazine is the world's most commonly used herbicide (Leu, Singer, Stamm, Muller, & Schwarzenbach, 2004). Recent studies have found that atrazine inhibits the aromatase enzyme that triggers the conversion of androgens to estrogens (e.g., Reeder et al., 1998; Tavera-Mendoza et al., 2002; Hayes et al., 2003). This study exposed tadpole and one-year-old *Xenopus laevis*, the African clawed frog, to 2 ppb atrazine to see whether or not there were any adverse side effects on gonad organization and activation. Histology slides were made of the gonads. Experimental results demonstrated that atrazine-exposed frogs had slightly larger testes and more seminiferous tubules than did the control frogs. Although slight differences were found, none of the results were significant. A future study that exposes tadpoles at developmental stages below stage 52, which is when gonad differentiation is presumed to occur, would be beneficial in determining whether or not atrazine affects development of the gonads in *X. laevis*.

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CHAPTER I

INTRODUCTION

Environmental Contaminants

The prevalent use of environmental contaminants has become a global issue because of the current decline in amphibians (Boyer & Grue, 1994; Carey & Bryant, 1995). Through advancements in biological engineering and chemistry, agricultural industries have produced many herbicides and pesticides that solve almost any weed, bug, parasite, or fungal problem. However, many of these helpful chemicals have damaging effects on aquatic and terrestrial habitats (Atrazine, 2002). In the United States alone, more than 100 types of "new generation" pesticides are applied at approximately 200 million acre treatments annually (Hill, 1995). As usage of these pesticides and herbicides increases, environmental contamination is becoming more of a concern. One animal group of particular concern is amphibians (Carey & Bryant, 1995).

Numerous amphibian populations have declined as a result of the excessive usage of these contaminants. Some pesticides, for example DDT, have been banned as a result of harmful side effects that were unknown when the chemical was released to the market. Many of these chemicals not only influence amphibians but also have adverse side effects on other organisms,

including humans (Schreinemachers, 2003). It is important to study high-use chemicals to ensure safety for amphibians and other organisms. Of the many herbicides used, atrazine is the most common because of its low price and positive results.

Atrazine Usage

The U.S. Department of Agriculture (USDA) approximates that 68 % of all corn crops in America were treated with atrazine at a rate of 1.04 lbs per acre (United States Department of Agriculture, 2004). Atrazine is the world's most commonly used herbicide, accounting for 40% of all herbicides used in the United States (Leu et al., 2004). More than 70 million pounds are being used annually in the United States (Withgott, 2002). Figure 1 provides a map of the United States showing atrazine use based on sales.

Atrazine has been banned in numerous European countries, including Switzerland, Sweden, Germany, Italy, Norway, France, and, most recently, Belgium, because it has been recognized as an endocrine disruptor. Syngenta Crop Protection, through Ecorisk, Inc, is a leading agribusiness and manufacturer of atrazine in the United States (Leu et al., 2004). 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 the Syngenta Crop Protection, Inc., have released information providing evidence that atrazine is relatively harmless (e.g., Carr et al., 2003; Hecker et al., 2004; Smith, 2003). However, studies conducted by Hayes and coworkers and some other private foundations have found evidence that atrazine

has a feminizing effect on amphibians as a result of activation of the aromatase enzyme that is essential for the conversion of testosterone to estradiol (e.g., Reeder et al., 1998; Tavera-Mendoza et al., 2002; Hayes 2004; Hayes et al., 2003). An understanding of testis development and spermatogenesis is necessary to better understand how feminizing effects from environmental contaminants may occur.

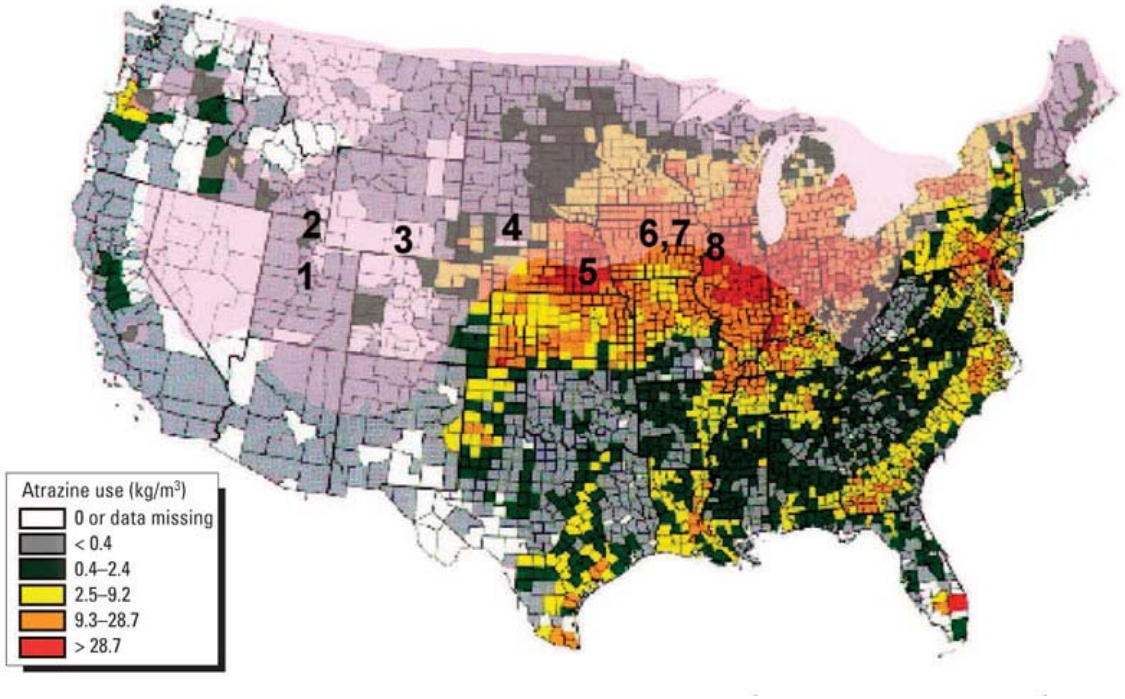

Figure 1. Map of the United States showing atrazine usage. Numbers indicate sites where water and frogs were collected (Withgott, 2002, p447).

Xenopus Normal Testes Development

Nieuwkoop and Faber (1994) describe normal testes development in the model amphibian *Xenopus laevis*. Development begins at stage 40 in the future genital tract, with the primordial germ cells being located in the dorsal ectoderm. After segregation along the dorsal median line at stage 41, they migrate toward the dorsal base of the mesentery where an unpaired genital ridge begins to form. Migration towards the coelomic wall along the sides of the dorsal mesentery continues until there are two separate genital ridges at stage 45. By stage 47, the migration is complete and the genital ridges are becoming denser and commence to protrude into the coelomic cavity. Medullary tissue begins forming from the somatic cells that are descending along the lateral walls of the vena cava, and the cells begin penetrating into the genital ridges. At this point, the germ cells are in the wall of the genital ridge. During stage 50, the germ cells begin to lose their yolk material while the amount of medullary tissue increases.

Sexual differentiation of the gonads begins at stage 52. In males, the germinal cells migrate into the medullary tissue where they will eventually form spermatogonia. During stages 53 and 54, spermatogonia increase in number until small tubular cavities begin to appear at stage 55. These cavities that are considered primordia of the seminiferous tubules further develop until stage 66. From this point, development of the spermatocytes continues until the frog's testes are fully developed. The *Xenopus* developmental process in frogs from juvenile to adult is controlled by the sex steroid testosterone.

Amphibian Spermatogenesis

After formation of the amphibian testes, androgens are produced under hypothalamic-hypophyseal control (Feder & Burggren, 1992). Testicular binding sites for the two types of gonadotropins, luteinizing hormone and follicle-stimulating hormone, are present. Androgen concentrations increase when physiological conditions are good for spermatid formation in the adult amphibian (Feder & Burggren). Often, adult amphibians follow circannual rhythms for their reproduction cycles. Sex steroid concentrations surge during these reproductive periods, heightening the process of spermatogenesis in adult males.

Spermatogenesis commences with the mitotic divisions of germinal epithelial cells called spermatogonia, located on the inner surface of the tubule. This process continues through the meiotic spermatocyte stages until the maturation stage is completed as the spermatogenic wave, ending with the insertion of spermatozoan bundles in the Sertoli cells (Feder & Burggren). The length of the spermatogenic wave from the nests of secondary spermatogonia to the maturation of spermatozoans is between five to six weeks (Feder & Burggren). This wave begins simultaneously after the first generation of seminiferous tubules has differentiated in the testes. As the amphibian grows and more tubules differentiate, spermatogenesis becomes increasingly asynchronous; thus, spermatogenesis in the seminiferous tubules occurs at different stages (Feder & Burggren).

During the frog's metamorphosis, the sex organs are susceptible to deviation coming from environmental contaminants like herbicides and

pesticides. According to Feder and Burggren, both drastic temperature change and the administration of estrogen or testosterone cause the frog's sex to change according to the sex steroid administered. Occasions on which male frogs are administered estrogen during early larval stages cause a sex change in the frogs (Feder & Burggren, 1992; Deuchar, 1975). Thus, a herbicide that causes an increase in estradiol in frogs is expected to lead to physiological feminization, if not a complete sex change, depending on the concentration of the steroid.

Amphibian Exposure to Atrazine

Amphibians are highly susceptible to adverse side effects resulting from environmental contaminants because of their skin permeability. For example, frogs respire both through lung respiration and tiny pores in their epidermis. Not only can oxygen be passed through these pores but also water that is essential for maintaining the frog's hydration. A frog's skin permeability greatly increases the absorption of contaminants such as atrazine into the frog's body. Most frogs lay their eggs in aquatic areas such as temporary water holes, ponds, and streams. Once the eggs germinate into tadpoles, survival for the tadpole is dependant on the aquatic habitat in which they are hatched. Because of the tadpoles' dependence on their habitat, it is necessary to examine whether or not the traces of pesticides and herbicides such as atrazine, a result of bioaccumulation, influence frog development and reproduction. The demasculinization (failure to induce spermatogenesis) and feminization in frogs happens as a result of prohibiting the release of putative spermatogenesis-inducing factor that is necessary for denaturing oocytes in the testes (Hayes et

al., 2003). Therefore, it is not uncommon to find oocytes in the testes of male amphibians that were exposed to atrazine. Figure 2 shows a photograph taken by Tyrone Hayes of hermaphroditic gonads of a male *Xenopus* (Hayes et al., 2002).

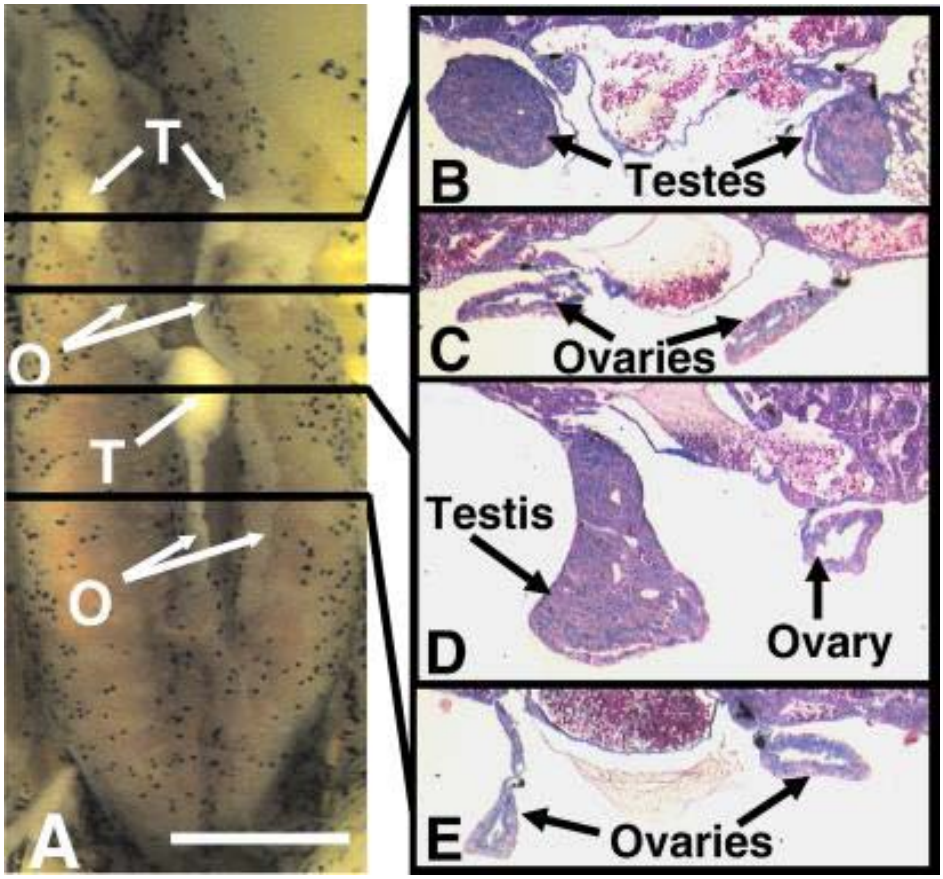

Figure 2. Hermaphroditic gonads in model of amphibian *Xenopus laevis* (Hayes et al. 2002a, p. 5478).

Endocrine Disruption

Atrazine is hypothesized to increase the production of endogenous estrogen by inducing aromatase, which is the enzyme necessary for the conversion of androgens to estrogens in the *Xenopus laevis*. Tyrone Hayes and coworkers of the University of California, Berkely, first began studying the effects of atrazine on amphibians in 2002. He discovered exposure to atrazine caused the testes to produce enough estrogen to feminize them, but it did not completely stop testosterone production that would cause a complete sex change (Hayes et al., 2003). Hayes, along with organizations such as the Midwest Society of Toxicology, National Science Foundation, and W. Alton Jones Foundation, has found, in both laboratory and field studies, feminizing effects on frogs exposed to atrazine at varying concentrations (Hayes, 2004). Although some evidence suggests that atrazine feminizes frogs, there is also research sponsored by Syngenta Crop Protection that found no such effects on frogs.

The herbicide atrazine affects the endocrine system in amphibians, which, in turn, causes alterations in the hormones released from the anterior pituitary of the nervous system as well as the sex organs. The pituitary and the infundibulum of the ventral posterior hypothalamus, both in the nervous system, maintain gonadotropin secretion during testicular cycles (Feder & Burggren, 1992). Since atrazine causes the endocrine system to affect the central nervous system, further experimentation is needed to study altered steroid secretion of the gonadal axis as a direct result of endocrine disruption. Future testing will provide more evidence determining whether or not atrazine's effect on the

endocrine and nervous systems results in the demasculinization of *Xenopus* frogs.

Research has provided examples of differentiation in which feminization occurred to the extent of causing hermaphroditism in frogs. However, other studies show no gross effect on "gonadal morphology or histopathology of the gonads in post-metamorphic frogs" (Coady et al., 2004). One study conducted on frogs showed that the males treated with 0.1 and 0.25ppb, which is far under the EPA drinking standard of 3ppb, were sexually differentiated and suffered from gonadal dysgenesis (Hayes et al., 2003). As mentioned earlier, there is evidence for both the feminizing effects of atrazine on amphibians and also some studies that show no adverse side effects.

Atrazine Controversy

Several experiments in which no atrazine-related feminizing effects on *Xenopus* frogs were observed were conducted by James Carr of the Department of Biological Sciences, Texas Tech University, and sponsored by Syngenta Crop Protection (Coady et al., 2004). However, an article written by Tyrone Hayes in *BioScience* reviewed Carr's experiments and found evidence of improper experimental procedure or misinterpreted results (Hayes, 2004). A Syngenta press release quoted James Carr as saying, "We have been unable to reproduce the low concentration effects in the larynx and gonads of the *Xenopus laevis* tadpole that have been reported elsewhere in the scientific literature" (Kendall et al., 2002). Oddly enough, once the data were published, Carr's results actually supported evidence pointing towards an adverse effect.

Syngenta Crop Protection funded nine experiments on atrazine exposure to frogs; eight of these were deemed inappropriate by Hayes for a few reasons (Hayes, 2004). First, in most of the holding tanks, the atrazine levels were not maintained and therefore could not be correctly monitored. The holding tanks were found to contain varying amounts of atrazine with traces of atrazine in the control tank as well. Second, the mortality rates in Carr's experiments were very high. The mortality rate averaged 76.5% across all treatments. If the majority of the frogs in the research experiment die, data collection for a valid conclusion is difficult. Furthermore, should a conclusion be drawn, it would most likely be a poor deduction from the small population size collected. Statistically, the law of large numbers is applicable to experimental research; the more specimens mean a better representation of the actual population. Next, Hayes lists five potential factors that may contribute to the experimental results, whether positive or negative: (1) species, (2) study type, (3) study design, (4) principal authors, and (5) financial sponsorship. It is interesting to note that all of the studies sponsored by Syngenta Crop Protection found that atrazine had no effect on gonads; however, five of the six other studies found conclusive results showing that atrazine exposure led to adverse side effects on frog specimens.

Experimental Proposal

The Environmental Protection Agency (EPA) only recently passed legislation that allows atrazine to be used in America as long as it is maintained less than 3 ppb in drinking water. In agriculture, atrazine is successful in reducing weed growth, but it has been found to exceed the EPA standard of 3

ppb in some runoff and aquatic areas. Regardless of the EPA standard, previous studies have found feminizing effects of amphibians exposed to atrazine at numerous concentrations, including some as low as 0.01 ppb (Hayes, 2002). For this experiment, it is hypothesized that male *Xenopus* frogs treated with atrazine at a concentration of 2 ppb will be demasculinized as a result of inducing the production of aromatase enzyme in the enzymatic conversion of testosterone to estradiol.

CHAPTER II

MATERIALS AND METHODS

Juvenile *Xenopus*

Atrazine exposure to *Xenopus laevis* was conducted in two different parts. The chronic study exposed one-year juvenile frogs to 2 ppb atrazine in a 95% ethanol solution from April 19, 2005, until September 1, 2005. Measuring pipettes were used to administer the appropriate concentrations of atrazine and ethanol. A group of frogs was also exposed to 95% ethanol solution to control for the use of ethanol in the atrazine solution used with the exposed frogs. Plastic Rubbermaid containers were used to hold both frog groups. The frogs were kept in approximately 6 L of aged distilled water that was changed every 3 days. A dilute bleach and water solution was used to clean the containers when algae and slime mold were evident. This was done approximately once a week. A slime mold contaminated the frog containers mid-May. At this point, Mela-Fix was added to the water to maintain and reduce the harmful effects of the slime mold. This was continued through July 26, 2005. On this date, a 10 % Holtfreter's solution composed of 35 g NaCl, 2 g $NaHCO_3$, 0.5 g KCl, 6.66 mL $MgSO_4$, 6.66 ml $CaCl_2$, was added to distilled water in a five-gallon bucket (1.2 L / 4.6 L respectively), and the Mela-Fix was stopped. Treatments of

Holtfreter's solution were administered until September 1, 2005, when the frogs were anesthetized in Chlorotone.

Tadpole *Xenopus*

The acute study was conducted on tadpoles of two different stages: stage 48 tadpoles and stage 56 tadpoles. These tadpoles were ordered from Nasco Fort Atkuson, WI, and received on August 9, 2005. The tadpoles were allowed 2 days to acclimate to their Rubbermaid containers containing 6 L of aged distilled water mixed with 10 % Holtfreter's solution (contents listed above for chronic study). The tadpoles at stage 56 were divided into two groups of 12. Treatments began on August 11, 2005. Atrazine exposure was conducted in the same manner as in the chronic study. A complete atrazine or 95% ethanol change was completed every 3 days to reduce algae and slime mold growth and ensure precise concentrations of the contaminant, atrazine. A dilute bleach and water solution was used to clean the tanks when algae and slime mold were evident. Another group of tadpoles at stage 48 was divided into an atrazine group of 15 and a control group of 16. The same procedures were followed in exposing these tadpoles as was followed in the stage 56 tadpoles.

Dissection of *Xenopus*

On September 1, 2005, the juvenile frogs and stage 56 tadpoles (which had now completely metamorphosed) were placed in Chlorotone for a time of 4-7 minutes depending on frog body mass. The frogs were then weighed and dissected. The stomach and intestines were removed first, followed by the adipose tissue found in the stomach cavity. A drop of Bouin's fixative was placed

in the stomach cavity, which made the transparent gonads appear on top of the kidneys. The kidney-gonad combination was removed by using forceps and dissection scissors and placed in Bouin's fixative. After the samples were retrieved, the tissue was stored in Bouin's fixative until the tissue was ready to be prepared for the paraffin wax.

Slide Preparation

The tissue was dehydrated using 70%, 80%, 95%, and 100% concentrations of ethanol, and then the dehydrated tissue was mounted in wax blocks. The wax samples were placed in a paraffin block for sectioning and mounting. A Shandon Finesse 325 microtone was used to section the tissue into ribbons, and the ribbons were placed in water to remove wrinkles and then placed on microscope slides. Typically, 8-12 tissue sections were on each microscope slide. The slides were then incubated overnight, stained with hematoxylin, and covered with a cover slip and Permount. The procedures are outlined in detail in *Humason's Animal Tissue Techniques,* 5th Edition (Presnell & Schreibman, 1997).

Slide Analysis

The slides were analyzed, and the best tissue samples were circled for measurements. The largest diameter of testis cross section was measured by using a dissecting microscope with an ocular micrometer. Next, the total number of seminiferous tubules was counted in a single testis. It is important to note that most of the tadpoles had not developed enough to distinguish individual seminiferous tubules. A sperm scale measuring the approximate amount of

mature sperm seen in each testis was conducted. The sperm scale measure was 1-5 with 5 signifying that more than 90 % of the seminiferous tubules had mature sperm. A four on the sperm scale was representative of approximately 75 % of the seminiferous tubules having mature sperm. If there were no evident mature sperm, the specimen was given a one on the sperm scale.

The data from the control and the atrazine-exposed males were then compared. To determine whether or not frog weight had an influence on testis diameter, a diameter per body weight was calculated and the data were compared among the frogs. Statistical analyses were conducted showing whether or not the testis size difference between the two samples was significant. Digital photographs of the testis were later taken using a digital camera with a Zeiss triocular phototube attached to a Zeiss microscope.

CHAPTER III

RESULTS

A 2 ppb atrazine exposure to juvenile and tadpole *Xenopus laevis* was found to have no gross observable effect on testes development. A clear distinction between testis and ovaries was seen in both the tadpoles and juveniles upon removal (Figure 3). Ovaries were much longer and had a granular appearance, while testes were shorter in length and had a smooth appearance.

A comparison of the diameters of the greatest testis cross section showed that atrazine juvenile frogs had slightly larger testes than did control frogs (Figure 4). The results did not have a *p*-value less than 0.05, meaning they were not significant. Figure 5 provides an image of the testes of a control juvenile. However, it was also important to compare testis diameter per individual body weight, so that any size differences among frogs could be eliminated. Data showed that, on average, the atrazine-exposed frogs had slightly larger testis diameter per body weight (Figure 6). The results were not significant.

In addition to larger testis diameters, the number of seminiferous tubules in each testis was greater in atrazine-exposed juveniles than in control juveniles (Figure 7). However, the difference in size was not significant. Tadpoles showed

very little variation in the total seminiferous tubule count because their testes had not fully developed, and thus, no significant results were found. Furthermore, all juvenile male frogs had mature sperm in their testes, yet none of the tadpoles had developed mature sperm. Figure 8 is an image at 125x magnification showing seminiferous tubules and sperm formation at various stages. A sperm scale showed that control juvenile frogs had more mature sperm in their testes than did those juveniles exposed to atrazine, but the results were not significant (Figure 9).

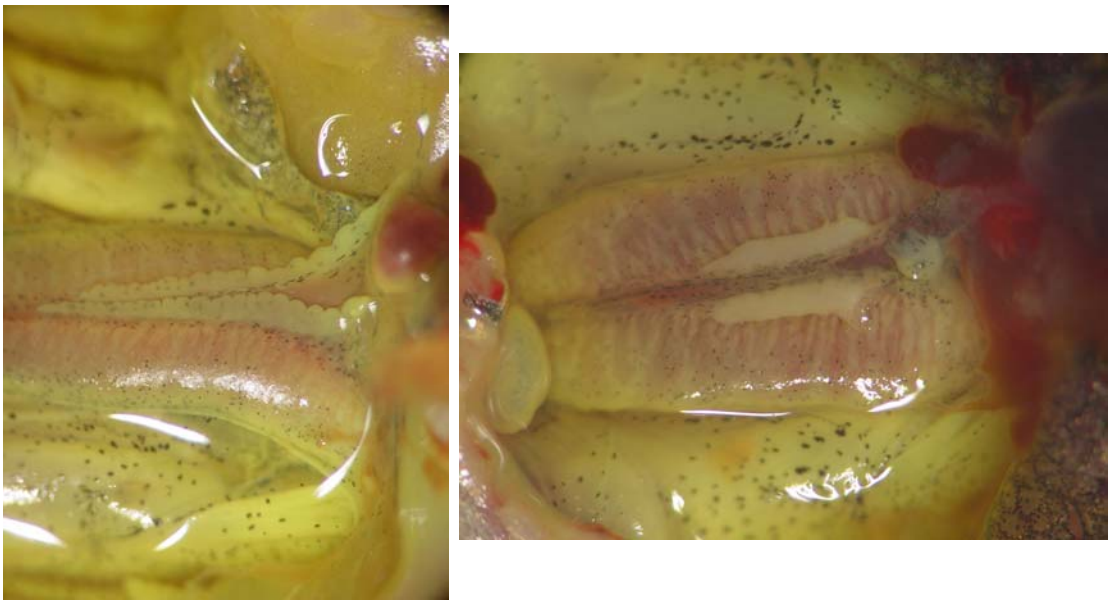

Figure 3. Clear distinction between ovaries (left) and testes (right) in tadpole *Xenopus laevis*, both at 30x magnification.

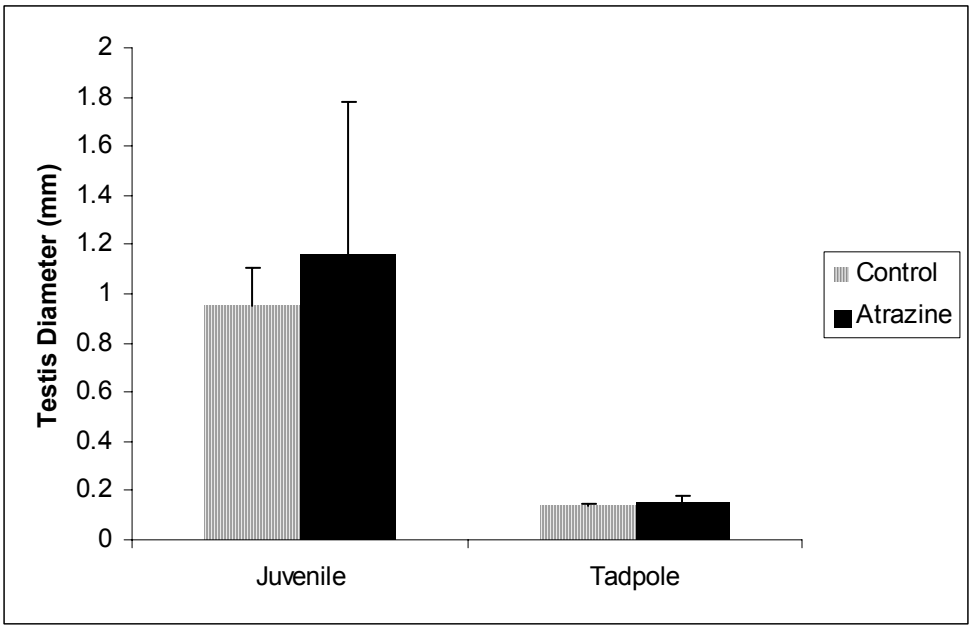

Figure 4. Comparison of average testis diameter (+1 SE) among atrazine-exposed and control *Xenopus laevis*. Tadpole comparison (p=0.4247) and juvenile comparison (p=0.7058) were not significant.

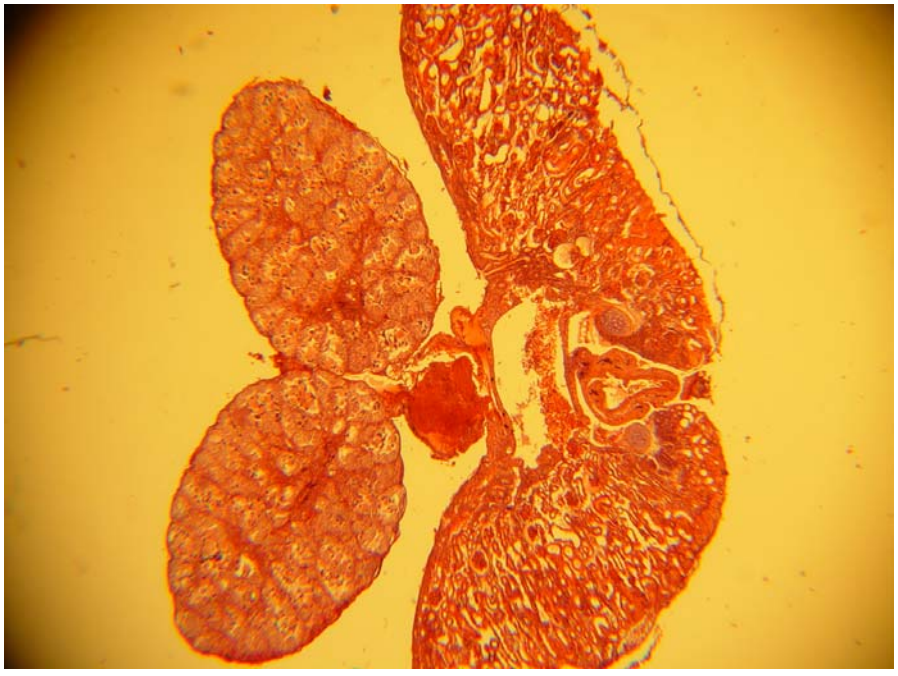

Figure 5. Testes and kidneys at 125x magnification from a control juvenile.

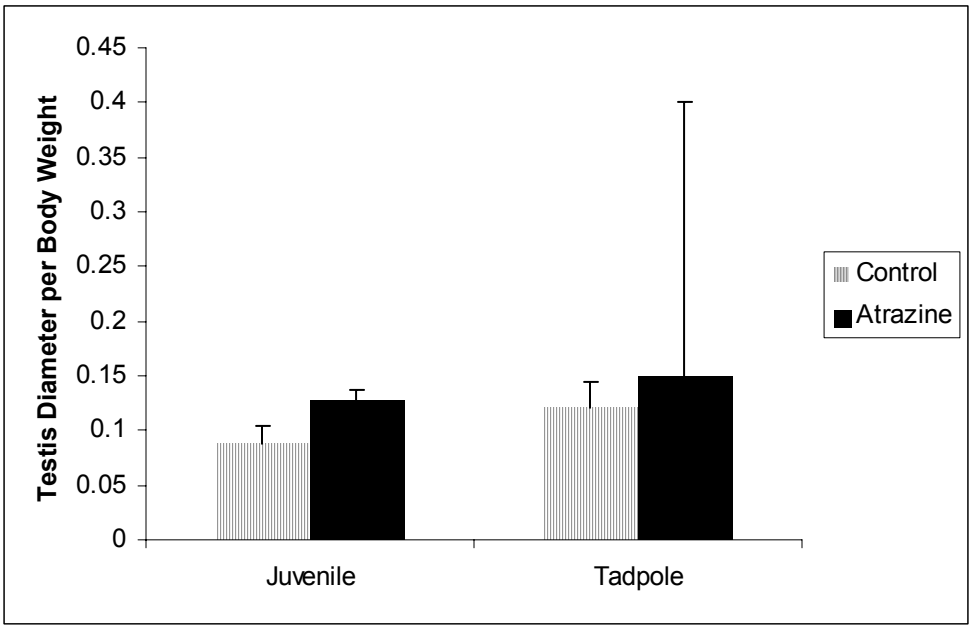

Figure 6. Comparison of average testis diameter per body weight (+1 SE) among atrazine-exposed and control *Xenopus laevis.* Tadpole comparison (p=0.4838) and juvenile comparison (p=0.1964) were not significant.

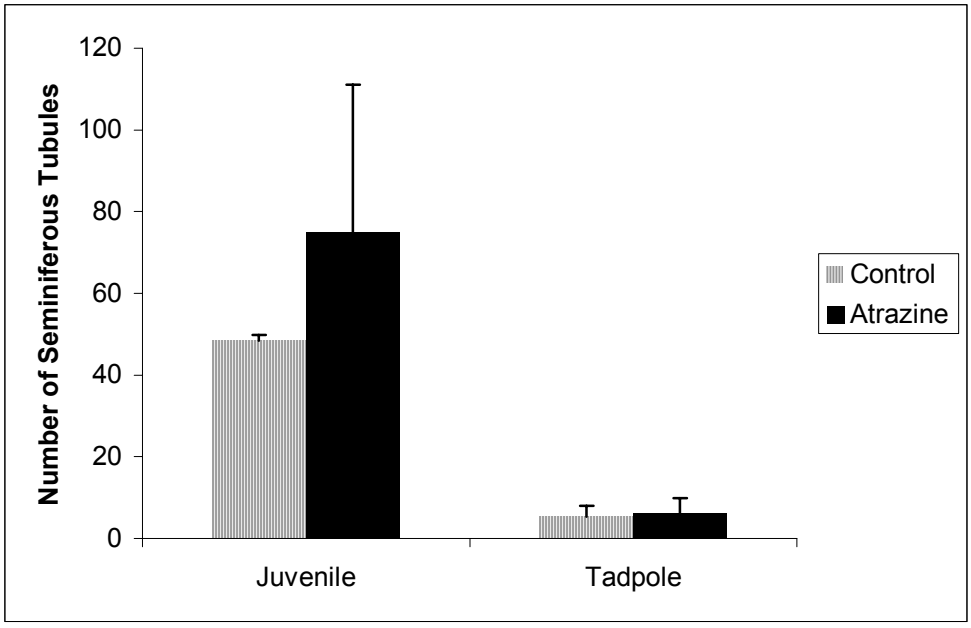

Figure 7. Comparison of the total number of seminiferous tubules in each testis (+1 SE) among atrazine-exposed and control *Xenopus laevis*. Tadpole comparison (p=0.8593) and juvenile comparison (p=0.3946) were not significant.

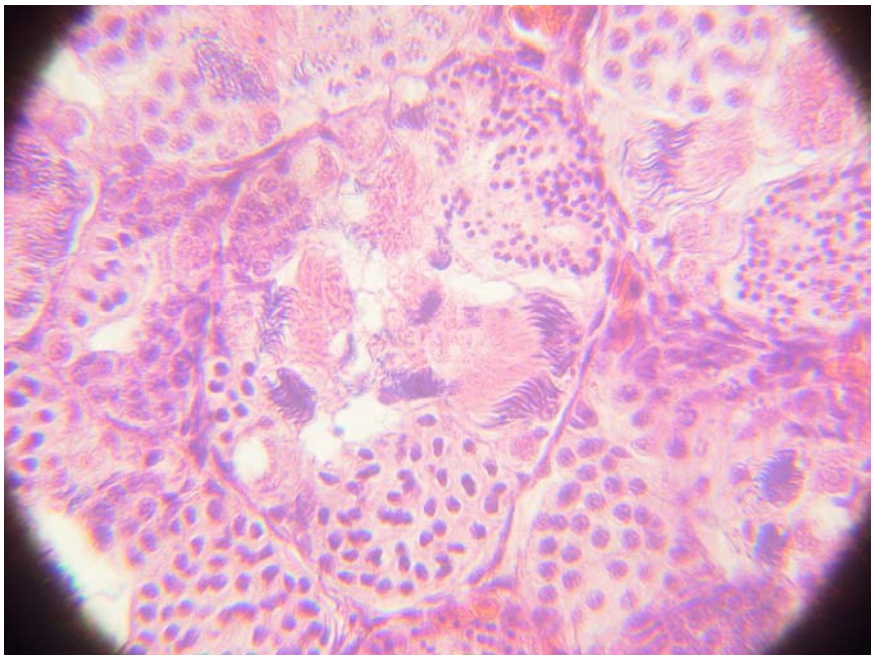

Figure 8. Seminiferous tubules in a control testis at 500x magnification.

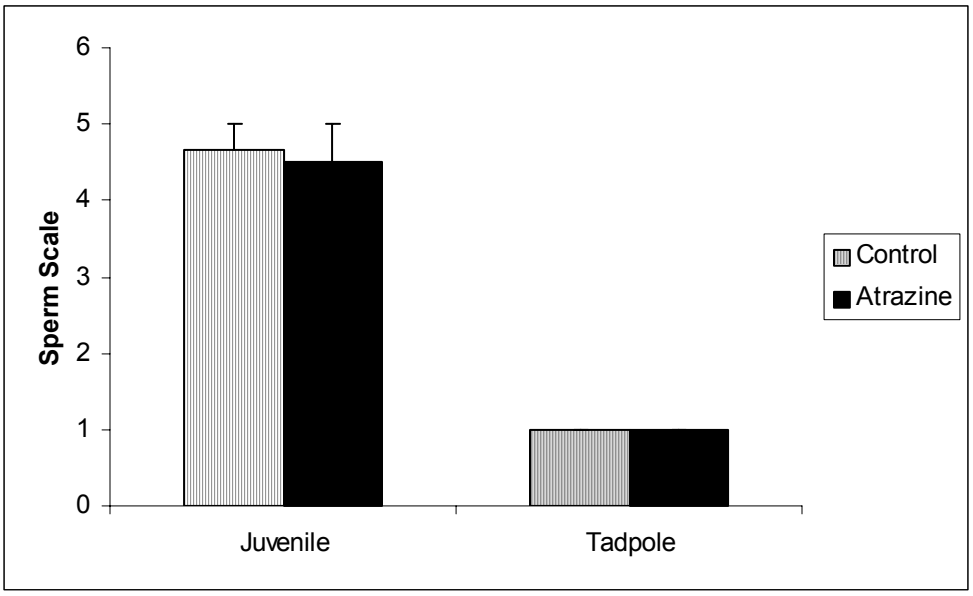

Figure 9. Comparison of the percentage of mature sperm seen in each testis (+1 SE) among atrazine-exposed and control *Xenopus laevis.* Juvenile comparison (p=0.7888) was not significant.

CHAPTER IV

DISCUSSION

The results found no significant difference in any of the comparisons made between the atrazine-exposed frogs and the control frogs. There was a high percentage of mortality among the juvenile frogs due to a foreign slime mold contamination that was brought into the laboratory by an adult pair of *Xenopus* purchased from Nasco. The slime mold reduced the sample size of the juvenile frogs by approximately 50 %. It is unclear what other effects the slime mold contamination had on the frogs. However, the reduction in sample size made it hard to gain sufficient data for comparison between the atrazine-exposed frogs and the control frogs.

It was interesting to note that the frogs exposed to atrazine were seen to have slightly larger testes (although not significantly larger) and more seminiferous tubules. This evidence would argue that atrazine does not have a demasculinizing effect on amphibians when exposed at 2 ppb. It has been theorized that exposure to greater concentrations of atrazine may trigger an immune response that would reduce the demasculinizing effects of atrazine on males. However, from the experiment conducted it is impossible to determine whether or not an immune response occurred. In future studies using atrazine, a

blood sample would be beneficial to determine exact atrazine concentrations and any immune response occurring in the frogs.

This experiment showed that atrazine had no effect on juvenile frogs exposed for approximately 3 months at 2 ppb. However, these frogs were exposed post-differentiation of the testis. A future study in which frogs were exposed from an earlier stage for approximately 1 year would provide more evidence as to whether or not a chronic exposure to atrazine at a low concentration had any effect on sex differentiation. A chronic study commencing after sex differentiation would ultimately show if atrazine had any effect on sperm production in mature testis.

Previous experiments conducted by Tyrone Hayes and coworkers found that frogs exposed to atrazine at 0.1, 0.4, 0.8, 1.0, 10.0, 25.0, and 200.0 ppb had gonadal abnormalities (Hayes et al., 2002). They found significant evidence showing demasculination in both male *Rana pipiens* and *Xenopus laevis* exposed to atrazine. Adult frogs exposed to 25 ppb atrazine showed a significant decrease in testosterone (Hayes et al., 2002). In fact, testosterone levels were found to be lower in atrazine-exposed males than in control females. Control males had significantly higher testosterone concentrations. It is probable that the high concentration of atrazine (25 ppb) caused a significant decrease in testosterone concentration by activating the aromatase enzyme. In this study frogs exposed to concentrations of 2 ppb atrazine had no gonadal abnormalities or anatomical distinctions compared to the control frogs. Concentrations of 2

ppb were likely not high enough to significantly decrease testosterone in atrazine-exposed males, as was seen in the results.

In their experiments, Hayes and coworkers began exposing frogs in the larval state, prior to any gonadal differentiation. However, comparative to Hayes' experiments, this study began exposure at stage 56, which is after gonadal differentiation has begun. It has been proposed that atrazine activates the aromatase enzyme prior to differentiation, leading to gonadal abnormalities, but after differentiation, gonadal development may be effected less by atrazine. Follow up research should be conducted to see whether or not atrazine at 2 ppb has an effect on testis development prior to stage 56.

Short-term studies would be beneficial in determining if atrazine at high concentrations affected frogs during development. This would mimic a habitat of rainwater accumulation and agricultural runoff in which up to 40 ppb atrazine have been currently detected (Hayes et al., 2002). It is important for both acute and chronic atrazine exposure studies to be conducted for a comparison to all types of environmental conditions.

The data in this study show that atrazine did not have an effect on testes in the *Xenopus laevis*; however, future research would be beneficial to further investigate whether or not atrazine has any effects on different developmental stages of frogs or other amphibians. Because results found by Tyrone Hayes showed that atrazine does effect differentiation and activation of testes in frogs, further research on atrazine is warranted.

APPENDIX

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