THE EFFECT OF ATRAZINE ON THE LARYNGEAL TISSUE IN *XENOPUS LAEVIS*

A Report of a Senior Study

by

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ABSTRACT

Over the past two decades, there has been a noted decline in amphibian populations. One of the major factors contributing to this decline are environmental contaminants. Atrazine is one of these contaminants and is a ubiquitous herbicide found in almost all water supplies at varying concentrations. This experiment explored the potential ramifications of 2 ppb atrazine, a concentration below the EPA standards for drinking water, on the development and activation of the M. dilator laryngis muscles and the elastic cartilage in the larynx of *Xenopus laevis*. The experiment was spilt into two age groups with exposure beginning at stage 56 (tadpoles) and 1 year into development (juveniles). It was hypothesized that atrazine would cause demasculinization of the laryngeal tissue of male *Xenopus laevis,* resulting in a decrease in the diameter of the laryngeal muscle and elastic cartilage due to the increase in aromatase activity which converts androgens into estrogen. No significant differences were found in the juveniles with respect to elastic cartilage diameter or laryngeal muscle diameter. There was a significant reduction in the diameter of the laryngeal muscle found between the tadpole control males and the atrazine exposed males (p=0.027), supporting the original hypothesis. However, no difference was found in the diameter of elastic cartilage in the tadpole group. Similar results have been found in other studies in which atrazine has been shown to significantly reduce the M. dilator laryngis muscle.

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CHAPTER I

INTRODUCTION

Amphibian Decline

Over the past two decades, there have been a significant number of studies focusing on the decline in amphibian populations (Alford, Dixon, & Pechmann, 2001). A recent analysis of amphibians in 37 countries and 8 regions of the world showed global decline in their populations (Semlitsch, 2003). In the United States, 8 new amphibian species were added to the endangered species in 2001 alone (see Table 1). Most scientists believe that habitat destruction is the major cause for the reduction in population size (Carey & Bryant, 1995). However, many other factors, such as chemical contamination, disease, global climate changes, and invasive species are becoming increasing threats to amphibian populations (Semlitsch, 2003).

Table 1. Amphibians protected under U.S. Endangered Species Act 2001 (based on Semlitsch, 2003).

Year Listed	Scientific Name	Common Name
2001	*Necturus alabamensis*	Black warrior waterdog
2001	*Bufo boreas boreas*	Boreal toad
2001	*Ambystoma californiese*	California tiger salamander
2001	*Rana pretiosa*	Oregon spotted frog
2001	*Rana luteiventris*	Columbia spotted frog
2001	*Rana chiricahuensis*	Chiricahua leopard frog
2001	*Rana muscosa*	Mountain yellow-legged
2001	*Rana capito sevosa*	Mississippi gopher frog
2000	*Ambystoma californiense*	California tiger salamander

Loss of both terrestrial and wetland habitats has greatly influenced the size of many amphibian populations. Most amphibians have a dual life cycle that depends on both a terrestrial habitat and a wetland habitat for survival (Semlitsch, 2003). In the past decade many of the natural wetland areas have been filled in order to accommodate development. The removal of these wetlands limits the breeding sites that these animals can exploit. According to Dahl in 2000, approximately 40.6 million hectares of freshwater wetlands remain in the continental United States, with an average net loss of 23,700 hectares per year from 1986 to 1997. The removal of trees and other vegetations around these wetlands is destroying the terrestrial shelter and food used by the adults of the species.

Many concerns have been voiced over the introduction of new invasive species. The stocking of predatory game fish and the introduction of the American bullfrog and other exotic species have caused concern primarily because of competition between the native amphibians and the invasive species (Semlitsch, 2003). With respect to predatory fish, the threat to amphibian population is very direct. More than 160 species of predatory fish have been released into 120 different countries (Semlitsch). A severe

decline in the population of *Rana muscosa* was correlated with the introduction of non-native salmonid fish into the habitat of the *Rana muscosa* negatively influencing their distribution (Semlitsch). Where the introduced fish were present, the frog populations were severely reduced as a result of predation by the introduced fish (Semlitsch).

Chemical contamination can be found in both terrestrial and aquatic habitats. Herbicides, pesticides, fertilizers, pharmaceuticals, heavy metals, and other chemicals can easily contaminate aquatic systems due to runoff from agriculture and output from water treatment facilities. Recently, the endocrine disrupting ability of many chemical contaminants has become a major area of interest (Boone & Bridges, 2003). Information about environmental endocrine disruptors in amphibians is scarce and this information is of particular concern in view of the worldwide decline of amphibians (Kloas, 2002). Endocrine disruptors could contribute to changes of amphibian populations via adverse effects on reproduction and the thyroid system (Kloas).

Endocrine Disruption

Many chemical contaminants affect the endocrine system, the physiological system consisting of glands that secrete hormones for use inside the body. The endocrine system of amphibians is very sensitive to environmental changes (Herman, 1992). It consists of the hypothalamus, pituitary, pineal, thyroid, parathyroid, pancreas, adrenal, and the gonads (Herman). In amphibians, the endocrine system controls the response of the integument to changes in the environment, maintenance of calcium homeostasis, metabolism and physiological adjustments to stress, cardiovascular regulation, reproduction and sexual behavior, growth, development, and sexual differentiation (Herman). The hypothalamo-hypophyseal-gonadal axis is fundamental to the endocrine

system and is involved in the control of amphibian reproduction as it is in many other vertebrates (Herman). The production of gametes and gonadal hormones is controlled by this system. Amphibian reproduction is controlled by GnRH (gondatropin releasing hormone), which is made and released by the hypothalamus and controlled by negative feedback. GnRH stimulates the release of LH (luetinizing hormone) and FSH (follicle-stimulating hormone) from the anterior pituitary. Unlike humans, these hormones stimulate physiological responses equally; however they are not required for ovulation, androgen secretion, or ovarian progesterone secretion (Herman). Amphibian testes produce androgens under the hypothalamo-hypophyseal control in addition to receptor-mediated control of LH and FSH (Herman).

Endocrine disruption is defined as the interference with endogenous hormone synthesis, secretion, receptor binding, activity, or degradation (Hayes, 2004). Although most environmental chemical contamination is below the lethal limit for most organisms, it still has the capacity to alter food web dynamics and disrupt endocrine systems (Smelitcsh, 2003). As a result, the EPA created the Endocrine Disruption Screening and Testing Advisory Committee to investigate and study various compounds (Hayes, 2004). Endocrine disruptors (EDs) have the potential to effect reproduction by abnormal sexual differentiation and the thyroid causing acceleration or retardation of metamorphosis (Kloas, 2002).

In an experiment studying the endocrine-disrupting potential of pesticides, rainbow trout cells were exposed to four different pesticides (Bisson & Hontela, 2002). With respect to the EC_{50} (concentration that inhibits cortisol secretion by 50%) the pesticides ranked endosulfan > diazinon > mancozeb > atrazine. The LC_{50} (concentration

that kills 50%) ranked the pesticides as diazinon > endosulfan > mancozeb <>atrazine, with atrazine being the least cytotoxic (Bisson & Hontela, 2002). Although shown to be the least cytotoxic, atrazine is considered an endocrine disrupter due to its ability to cause changes in the gonads of many animals.

Atrazine

Atrazine (2-chloro-4-ethylamino-6-isopropylamine –1,3,5-triazine) is one of the most widespread pesticides and is used in many countries around the world, including the United States (Withgott, 2002). For this reason, atrazine is a very common contaminant found in the ground and surface water. Although 8 countries have banned the use of atrazine, atrazine is still commonly used in the United States and many other countries (Withgott). Over 70 million pounds is applied in the United States every year to golf courses, lawns, and primarily to agricultural crops such as corn, accounting for 40% of all the herbicides in the United States (Withgott). In experimental trails, larval amphibians demonstrated greater survival at higher concentrations (100 ppb) of atrazine contamination than at lower concentrations (3 ppb) (Storrs & Kieseckar, 2004). This pattern was evident in a three different species (*Pseudacris crucifer, Rana clamitans,* and *Rana sylvatica)* exposed to atrazine (Storrs & Kieseckar).

Atrazine elevates estrogen levels and decreases androgens in amphibians (Hayes, 2004). It has been shown that the estrogenic effects associated with the triazine herbicides in vivo are not estrogen receptor-mediated, but may be explained partly by their ability to induce aromatase in vitro (Sanderson et al., 2001). Once activated aromatase is an enzyme that converts testosterone into estrogen (see Figure 1), thus assaulting male development due to the imbalance of sex hormones (Hayes et al., 2004).

Atrazine has demonstrated aromatase induction in mammals (Sanderson et al., 2001), reptiles (Crain et al., 1997), and amphibians (Hayes et al., 2002).

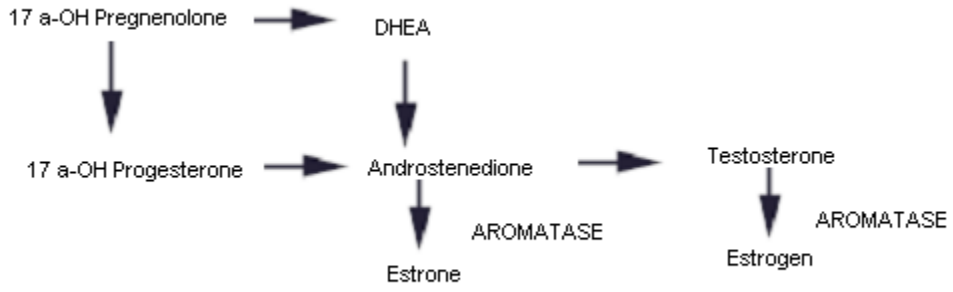

Figure 1. Flow diagram of the conversion of steroid hormones by aromatase.

Since the aromatase disrupts the sex hormone balance, the two most effected organs are the gonads and the larynx due to the integral role of testosterone in the development in these organs. Many studies have been preformed to test the effect of atrazine on gonadal development. One study by Tyrone Hayes et al. in 2003 examined wild American leopard frogs (*Rana pipiens)* and took water samples from the frog's habitat and performed laboratory exposures. The concentration of atrazine was determined from the wild water samples and compared with the histology of the gonads from the frogs living in that concentration (Hayes et al., 2003). They found that the atrazine-treated males in the laboratory had underdeveloped testis at 0.1 and 25 ppb. Some of the animals at 25ppb showed sex-reversal (testis with oocytes) (Hayes et al., 2003). Similar effects were shown in the specimens taken from the wild. In an earlier study by Hayes et al. (2002), *Xenopus laevis* were exposed to atrazine concentrations of 0.01, 0.1, 0.4, 0.8,1.0,10.0, 25.0, and 200 ppb. In this experiment, atrazine had no effect on mortality; however at all

concentrations (except 0.01), atrazine produced gonadal abnormalities (Hayes et al., 2002). Some had multiple gonads (up to 6 in one frog) and others were hermaphrodites (with both testis and ovaries in one animal) (Hayes, 2002).

Gonadal disruption has not only been shown in amphibians. A study on goldfish (*Carassius auratus)* exposed both sexes to 100 and 1000 ppb atrazine concentration for 21 days. Suppression of plasma testosterone, an increase in plasma estrogen, and structural disruption in the testis of males at 1000 ppb was found (Spano et al., 2004). Not all studies are in agreement that atrazine causes gonadal deformities and intersex individuals. One such study performed by Carr et al. (2003) reported no difference between the experimental and the control groups. No significant difference was found in the forelimb emergence, tail reabsorption, larval growth, larynx size, or delay in metamorphosis (Carr et al., 2003). Although intersex individuals were found in the atrazine exposure group, intersex individuals were also present in the control group (Carr et al., 2003). Therefore, it was concluded that the interconversion of testis and ovaries must be a naturally occurring abnormality (Carr et al., 2003).

Larynx

The larynx is a box-like structure consisting of muscle and cartilage, and is connected to the lungs and the buccal cavity. The skeletal construction of the larynx consists of several types of cartilage, predominantly hyaline cartilage. Male *X. leavis* are known for their elastic cartilage which is absent in the females (Kelley, Tobias, & Horng, 2001). The type of cartilage is not the only difference between the male and female larynx; the males also tend to have a different size and shaped hyaline cartilage that results in a more elaborate lumen in the male larynx (Kelley et al., 2001). The major

muscle associated with the larynx is the *M. dilator laryngis* (Niewkwoop & Faber, 1994). Developmentally, the *M. dilator laryngis* originates from the mesenchyme that surrounds the larynx and is faintly delimited at Stage 40 (Niewkwoop & Faber). At Stage 65, *M. dilator laryngis* gains a new origin so that it originates from the thyroid process and inserts on the arytenoids (Niewkwoop & Faber).

In all anurans, the vocal apparatus is the larynx, which is sexually dimorphic (see Figure 2) in size and form (Holmes, 1954). The dimorphism occurs in response to circulating androgens during postmetamorphric maturation (Kelley et al., 2001). One difference between the female and male larynx is that the male laryngeal tissue consists entirely of fast twitch muscle, which is dependent on gonadal secretions (Kelley et al.). By the end of metamorphosis, males and females have the same number of laryngeal muscle fibers that consists mainly slow twitch muscles (Kelley et al.). After metamorphosis males continue to add muscle fibers and convert their slow twitch into fast twitch muscles. Experimental data has also showed that there is a distinct difference between the size of the male and female larynges, with the male's larynx being larger than the female (Hayes et al., 2002). The conversion from slow to fast twitch muscle is directly dependant on androgen production (Kelley et al.). In an experiment in which males were castrated after metamorphosis, the conversion from slow to fast twitch muscles was halted (Tobias, Martin, & Kelley, 1991). Testis transplants and exogenous androgens have been shown to reverse the effects of early castration and laryngeal tissue develops normally after the androgens begin circulating in the tissue (Tobias et al.). This evidence showing the dependence of laryngeal masculinization on androgens has given rise to studies exploring the effects of chemical castration on laryngeal development.

Atrazine has been one such chemical castrator investigated, due to studies concluding that it has the ability to disrupted gonadal development. In a study performed by Tyrone Hayes et al. (2002), it was found that, when male *Xenopus laevis* are exposed to an atrazine concentration greater than 1 ppb during development, the *M. dilator laryngis* muscle was significantly reduced when compared to the control. However, in a counter study no significant difference was found in the diameter of the laryngeal muscles of *X. laevis* exposed to 1, 10, or 25 ppb of atrazine in comparison with the control (Carr et al., 2003).

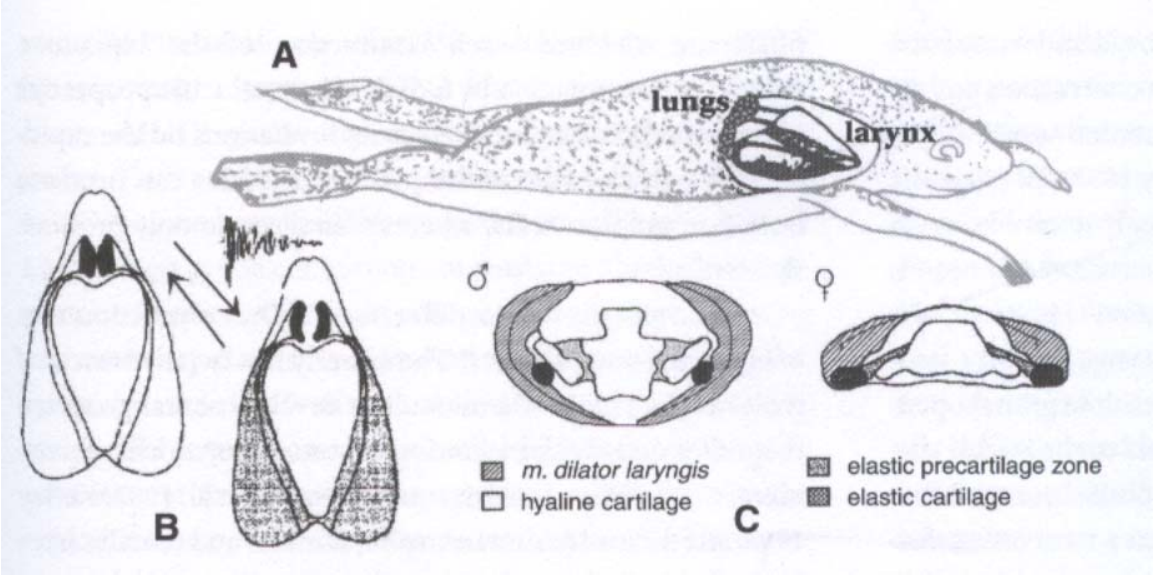

Figure 2. The larynx of *Xenopus laevis.* (A) The larynx is just inside the body cavity, dorsal to the heart. It is located posteriorly to the buccal cavity and anterior to the lung. (B) Contracted laryngeal muscles. (C) Transverse section of an adult male and female larynx. (Drawing by D. Kelley and L. Fischer)

Xenopus laevis

Amphibians are an excellent model for the study of endocrine disruption (Kloas, 2002). *Xenopus laevis* is an entirely aquatic species and only rarely cross land in the wild (Deuchar, 1975). They are flattened dorsoventrally and are mottled greenish-grey dorsally and yellowish-white ventrally (Duechar). The hind legs are massive and muscular in comparision with the small forelimbs (Deuchar). Females are significantly larger than the male (Deuchar). *Xenopus laevis* (the African clawed frog) tadpoles are a good model for the disruption of gonadal development in response to endocrine disrupting contamination. This is true because of their quick developmental and sexual differentiation period, easy laboratory maintenance, and high survival rate. The developmental stages of *Xenopus laevis* are defined by the Normal Table of *Xenopus laevis* by Niewkwoop and Faber (1994), in which stage 1 is a one-celled egg through total tale reabsorption in stage 66.

Hypothesis

The controversy contrived by the two differing results of atrazine on the male *Xenopus laevis* justifies further investigation. This study will investigate the effects of atrazine (2 ppb) on the larynx of both developing and fully developed *Xenopus laevis*, in order to determine whether atrazine in low concentrations has the potential to cause demasculinization of laryngeal tissue. The hypothesis for this experiment is that atrazine will cause demasculinization of the laryngeal tissue of male *Xenopus laevis*.

CHAPTER II

MATERIALS AND METHODS

Animal Husbandry

The frogs were exposed to a 12:12 light/ dark cycle. A 100% water change was completed in the juveniles and tadpoles every three days. Animals were netted from their containers and placed in a designated holding tank while the containers were cleansed with a weak bleach solution. The *X. laevis* were fed crushed frog brittle daily.

Experimental Design

Tadpoles: Three stages (stage 46, 48-50, and 56-58) of *Xenopus laevis* were obtained from Nasco (Wisconsin) on 9 August 2005. The tadpoles were randomly separated into control and experimental groups and placed in their corresponding plastic tubs. The frogs were acclimated for 48 hours prior to the beginning of the experiment. The stage 56-58 and stage 48-50 were placed in 6L plastic containers. The water for the control groups in these two stages contained 1.2L of Holfreter's Solution (Holfreter's Solution consists of 35g of NaCl, 2g of $NaHCO_3$, 0.5g of KCl, 6.66mL of $MgSO_4$, and 6.66mL of $CaCl_2$ mixed in 5 gallons of water), 4.6L of aged/dechlorinated water, and 120µL of 95% ethanol. The water for the experimental group contained 1.2L of Holfreter's Solution, 4.6L of aged/dechlorinated water, and 120µL of Stock 2 Atrazine (Stock 2 Atrazine consists of 100µL of Stock 1 Atrazine in 9.9mL of 95% ethanol. Stock

1 Atrazine contained 10mg of solid dry atrazine in 1mL of 95% ethanol). The control stage 46 tadpoles were placed in 3L containers and their water consisted of 0.6L of Holfreter's Solution, 2.4L of aged water, and 60 µL of 95% ethanol. The water for the experimental stage 46 tadpoles contained 0.6L of Holfreter's Solution, 2.4L of aged water, and 60 µL Stock 2 Atrazine. These three stages were exposed continually for 25 days.

Juveniles: A male and female *X. laevis* were injected with hCG, which stimulated them to copulate. The eggs were collected, placed in dechlorinated water and allowed to develop into completely metamorphosed juveniles, approximately one year prior to exposure. The wet weights of the juveniles were taken and they were divided into control and experimental groups maintaining a close average weight between the two groups (p=0.877). They were acclimated to their new plastic 6L plastic containers and covered with lids containing air holes. The juveniles were placed in 6L plastic tanks under the same water conditions as the stage 56-58 tadpoles for 135 days.

Tissue Processing and Histology

The frogs were anesthetized by emersion in chlorotone and the kidneys, gonads, liver and larynx were removed. The laryngeal tissue was removed by unhinging the lower jaw and removing all of the muscular tissue beginning with the lower palate cutting posteriorly towards its attachment on the anterior of the pericardial sac. Once the tissues were removed, they were fixed in Bouins fixative. Following fixation, the tissues were clear, dehydrated, infiltrated with wax, and embedded in a wax block following the histotechnique outlined in *Humason's Animal Tissue Techniques* (1998). In brief the tissues were exposed to 80% ethanol for 2 hours, 95% ethanol for 1.5 hours, 100%

ethanol for hour twice, and finally Citrasolv for 1 hour twice. They were infiltrated with wax in a vacuum oven, in which the tissues were placed in wax I for 1 hour at 12 psi, wax II for 1 hour at 15 psi, wax III for 1 hour at 21 psi and wax IV for 1 hour at 25 psi. The tissues were removed from wax IV and placed into the wax pot and embedded in a wax block. The blocks mounted onto the microtome and sectioned at12 μm. The tissue ribbons were then mounted on glass slide and stained using hematoxylin and eosin. The coverslips were adhered using Permount.

Tissue Analysis

The larynx consists of the M. dilator larynges muscles, which are bipennate muscles that surround the elastic and hyaline cartilage of the larynx. The sections were scanned to find the sections with the largest muscle area and the diameter of the M. dilator larynges muscles was measured. The cartilage was measured from the same section as the largest muscle diameter.

CHAPTER III

RESULTS

Analysis of Musculature

The laryngeal muscle and elastic cartilage diameter was determined in order to ascertain the effect of atrazine on laryngeal development and post metamorphic activation. In the female tadpoles, there was no significant difference in the muscle diameter when the control group ($p = 0.283$) was compared to the atrazine treatment group ($p = 0.257$) (See Figure 4). However, there was a significant reduction ($p = 0.0268$) in the diameter of the M. dilator muscle found between the control and the *X. laevis* tadpoles exposed to 2 ppb atrazine (see Figure 5). Since the diameter of the atrazine treated males laryngeal muscles were greatly reduced, they closely resembled the larynges of control females (see Figure 7).

There was an apparent decrease in M. dilator laryngis muscle diameter in atrazine-exposed juvenile males (see Figure 5), however this was not significant ($p = 0.1464$). Similarly, there was no significant difference ($p = 0.4055$) seen in the females with respect to the diameter of the laryngeal muscle (see Figure 6).

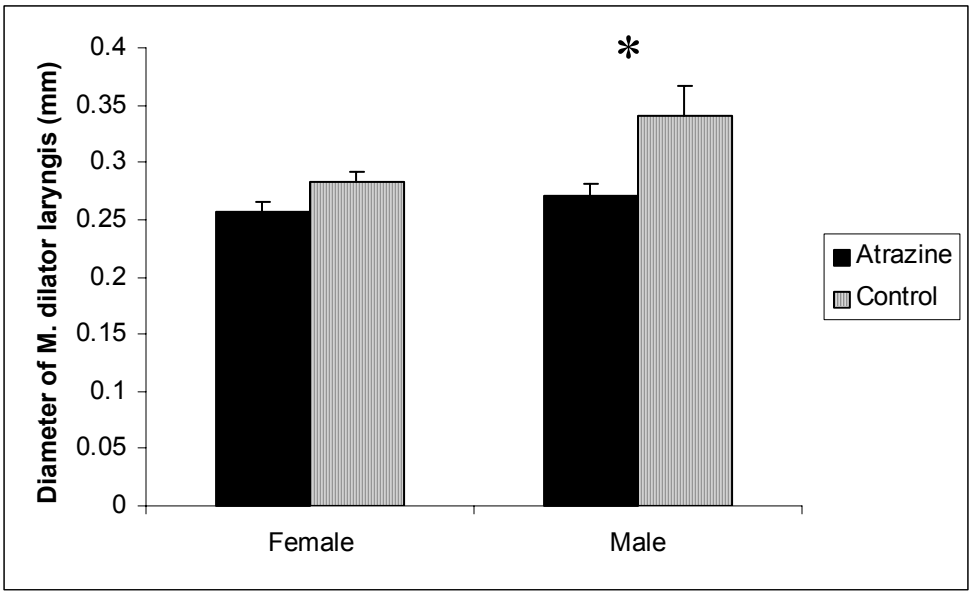

Figure 3. Mean diameter of M. dilator laryngis in tadpole control and Atrazine treated groups (+/- 1SE). Asterisks indicate values that were significantly different (p=0.0268).

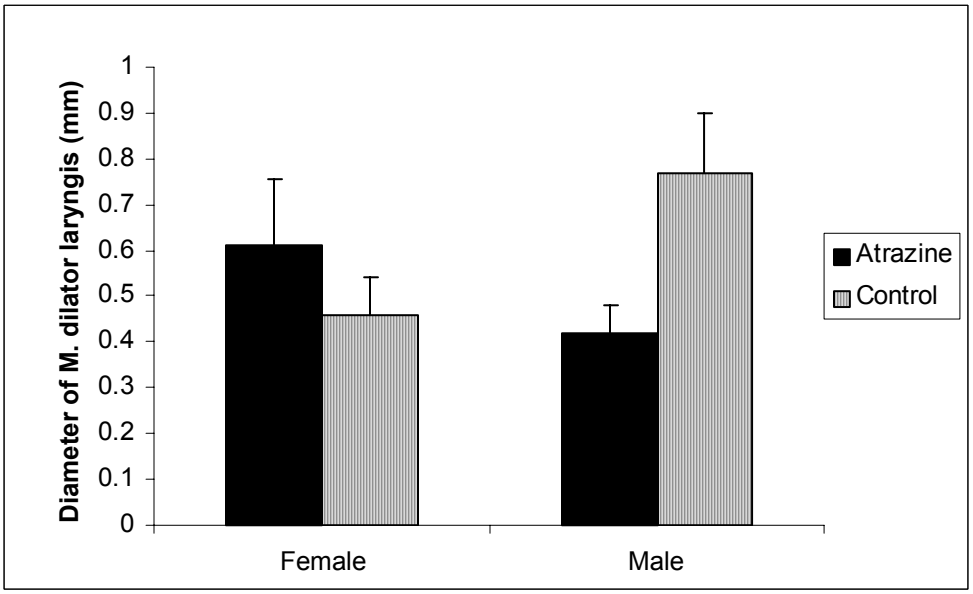

Figure 4. Mean diameter of M. dilator laryngis in juvenile control and Atrazine treated groups (+/- 1SE). No significant differences were seen.

Analysis of Cartilage

The diameter of the laryngeal elastic cartilage was very conserved between gender as well as experimental groups. No significant differences were seen in the diameters of the elastic cartilage between the control and atrazine treated group for male (p = 6647) or female tadpoles (p = 0.7822) (see figure 5). There was also no significant difference found between the control and the experimental groups for both the male (p = 0.7132) and female juveniles (p = 0.5141) (see Figure 6).

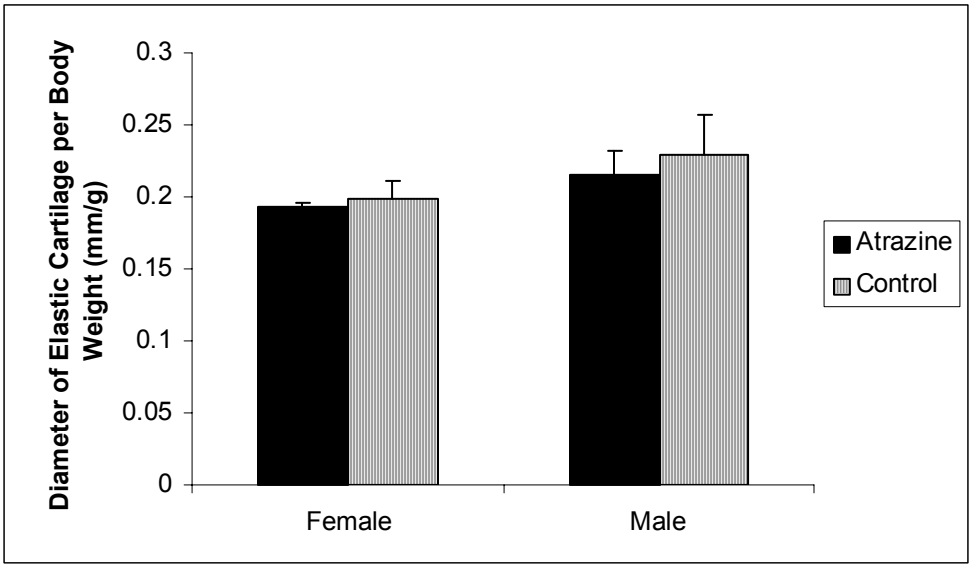

Figure 5. Mean diameter of elastic cartilage in tadpoles standardized for body weight for control and Atrazine treated groups (+/- 1SE). No significant differences were seen.

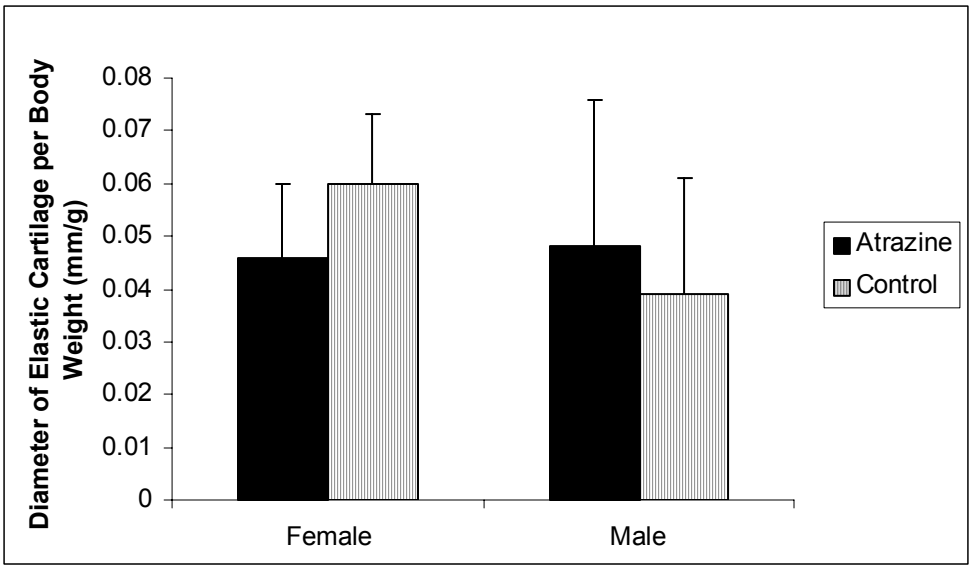

Figure 6. Mean diameter of elastic cartilage in juveniles standardized for body weight for control and Atrazine treated groups (+/- 1SE). No significant differences were seen.

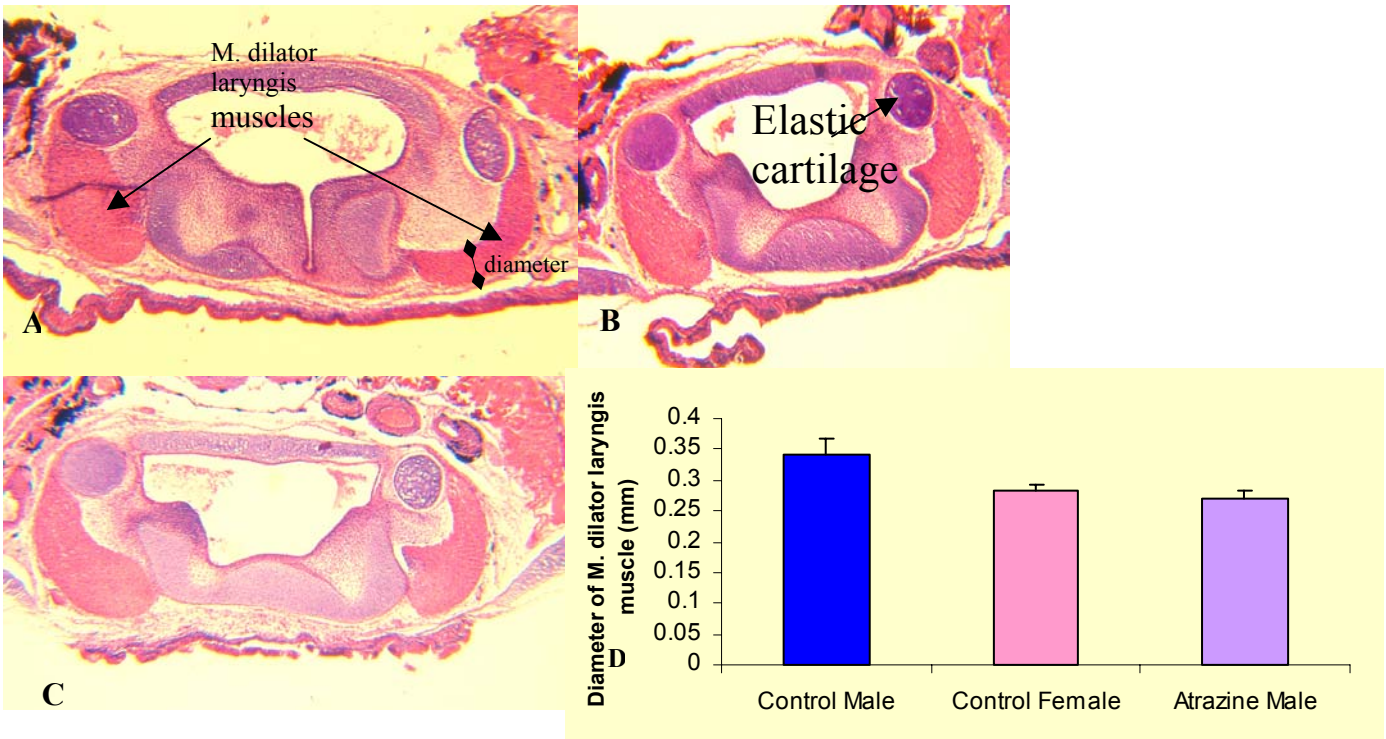

Figure 7. Histology of the larynx (40x) for (A) control female tadpole larynx, (B) control male tadpole larynx, (C) atrazine-exposed male tadpole larynx. (D) Comparison of diameter of M. dilator laryngis (+/- 1SE) in control male, control female, and atrazine male.

CHAPTER IV

DISCUSSION

Atrazine was shown to have an extreme effect on the atrazine-exposed male tadpoles (treatment began at stage 56). There was a significant decrease in the muscle diameter in those tadpoles exposed to atrazine when compared with the control males. However, no difference was found in the juvenile males when exposure began at the age of 1-year. The diameter of the elastic cartilage was highly conserved between the females and males as well as the control and atrazine-exposed groups and no significant results were found.

Similar studies have supported the results found in this experiment. In a study performed by Tyrone Hayes et al. (2002), it was found that when male *Xenopus laevis* were exposed to an atrazine concentration greater than 1 ppb during development, there was a significant reduction in the *M. dilator laryngis* muscle when compared to the control. However, studies funded by the manufacturers of atrazine have shown no significant difference in the diameter of the laryngeal muscles of *X. laevis* when exposed to 1, 10, or 25 ppb of atrazine (Carr et al., 2003).

Male and female *Xenopus laevis* begin post-metamorphic development with the same number of muscles fiber in their respective larynges (Martin, Tobias, & Kelley, 1990). During this developmental period, males increase the number of muscle fibers by

adding more fibers more quickly than the females (Martin, 1990). This sexual dimorphic development arises due to androgen concentration; increased androgen results in the increased deposition of muscle fiber and thus increased muscle diameter. Therefore, exposure to atrazine has the potential (as seen in this experiment) to prevent increased muscle deposition in males and cause the retention of the smaller, "female" larynx, due to the increase aromatase activity.

It has also been shown that muscle fiber addition in the larynx has a sensitive period in which exposure to androgens has a permanent masculinizing effect (Martin, Tobias, & Kelley, 1990). Since the maculinization is completed fairly quickly after metamorphosis (PM 2), this supports the null results found in the exposed juveniles since exposure began after this sensitive temporal window had closed.

Studies have also linked the thyroid in the androgen sensitive laryngeal development and the sexually dimorphic laryngeal innervation (Robertson & Kelley, 1996). In this experiment, tadpoles were exposed to thyroxine synthesis inhibitor propylthiouracil (PTU). PTU was found to block the morphological development of the larynx. Tadpoles treated with PTU for 50 or 100 days had larynges, which structurally resembled less developed control stages and not exhibit the extensive development in the laryngeal cartilage seen in control animals. Unlike the untreated animals, the tadpoles exposed to PTU did not show marked expansion of the dilator muscles. Laryngeal growth initiated by exposure to exogenous androgen was completely prevented when exposed to PTU. No difference was found in the number of axons between in the control and treatment group or between male and females.

Implications of reduced laryngeal muscles could have a direct effect on an individual's ability to call in the wild. Calling is extremely important to the males in amphibian populations. Although vocalization is essential for warning calls and evasion of predators, most calls are directed to the attraction of females. In many species, calls are not only an advertisement of where the males are in the habitat, but some females use calling as a tool for sexual selection. Males that have a larger call and more extensive repertoire and have the ability to sustain calling for long periods of time increase the number of females they have the chance to mate with, thus increasing their reproductive success. The reduction of the laryngeal muscle could affect the pitch of the call (making the males sound like females) and in extreme reduction could inhibit calling completely. Therefore, exposure to atrazine can have a drastic effect on the reproductive success of amphibian populations.

Because amphibians are a very sensitive groups and are commonly used as biological indicators of the health/illness of the environment, the drastic effects that are being seen in amphibians could be a indication that health of other vertebrate classes are in being influenced by atrazine contamination. The enzyme aromatase is not specific to amphibians; reptiles and mammals also posses this essential enzyme can therefore be negatively effected by atrazine (Crain et al., 1997).

Future studies should examine the effect of atrazine at earlier developmental stages in order to explore whether exposure prior to the "sensitive temporal window" could elicit an effect on the development of the cartilage, muscles, and organization of the larynx. Other studies should also explore the ramifications of atrazine on mammals I think there also needs to be further research on atrazine's effect of other organs in the

body, like the thyroid. Since the thyroid has a role in the development of the laryngeal tissue, the effect of atrazine this organ could provide a more complete explanation for the reduction in laryngeal muscle diameter found in atrazine treated males.

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