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Effects of Chronic Atrazine Exposure on Male Reproductive Function in Sprague Dawley Rats

Josh Quinn

A Thesis Submitted to the Graduate Faculty of

GRAND VALLEY STATE UNIVERSITY

In

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For the Degree of

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Abstract

Estrogens, in addition to testosterone, are much more physiologically relevant to normal sperm production in the testis and overall male fertility than previously thought. Current use herbicides, such as atrazine, are known endocrine disruptors and have been suggested to disrupt male reproductive health. The purpose of this study was to investigate the effect of chronic atrazine exposure on male reproductive function in Sprague Dawley rats. Three groups of rats were treated until 7 months of age. The first group was the control vehicle and given only water. The second group was given a low dose of atrazine (0.1 mg/kg) and the third group was given a high dose of atrazine (10 mg/kg) via oral gavage to mimic the most common exposure to atrazine in humans. At 7 months of age, animals were euthanized and reproductive tissues were collected for analysis. Concentrations of testosterone were not significantly different between groups, but levels of serum FSH and estradiol were significantly increased in the high dose group relative to the control. Sperm/gram of the testis was significantly lower in the low dose group and daily sperm production was significantly lower in the high dose group. Collectively, these results support the hypothesis that sperm production and overall male reproductive health is significantly disrupted by chronic exposure to atrazine, and the relationship between estradiol and male fertility is sensitive to environmental contaminants.

Table of Contents

List of Figures

Chapter 1. Introduction

A marked decline of semen quality (semen volume, sperm motility, sperm count and normal morphology) in normal men has been reported over the past few decades. Associations between current-use pesticides and reduced semen quality suggest that agricultural chemicals may contribute to the reduced semen quality seen in fertile men from agricultural regions of the United States (Swan, 2006). Results from a study comparing semen quality of men from different regions of the United States indicated that changes in semen quality were associated with exposure to several common pesticides currently being used (Swan et al., 2003) including the active ingredient atrazine.

It has been reported that in females, leuteninzing hormone (LH) release was suppressed (McMullin et al., 2004) while in males follicle stimulating hormone (FSH) was increased (Kniewald et al., 2000) after atrazine exposure. Leydig cells, responsible for synthesizing testosterone, were of irregular shape with unequal form. In Sertoli cell cytoplasm, the cells responsible for the maintenance of developing germ cells, atrazine provoked degenerative changes (Kniewald et al., 2000). This result in males has been hypothesized to be a possible mechanism by which the male reproductive tract and overall fertility have been affected by atrazine (Babić-Gojmerac et al., 1989). The mechanisms by which atrazine alters the male reproductive tract are poorly understood and have not been thoroughly analyzed, thus it is important to bridge the gap between infertility and atrazine exposure beyond serum hormone analysis.

Purpose

The purpose of this study was to determine the effects of chronic atrazine exposure on male reproductive function.

Scope

Male reproductive function includes spermatogenesis, hormone production, and maintenance of the testis and epididymis. Any alterations in these aspects of male reproductive endocrinology and sexual development can cause a significant drop in male fertility and overall male reproductive health. We will determine the extent that male reproductive function is altered in response to chronic atrazine exposure by focusing on sperm production and homeostasis of testosterone, estradiol, FSH, LH, and the enzyme CYP19, commonly known as aromatase.

Assumptions

1. Estradiol, within specific physiological ranges, is essential for maintaining proper male reproductive function.
2. Atrazine contributes to the adverse trends observed in male fertility and is currently attributed to disrupting endocrine function in developing and adult males.

Hypothesis

We hypothesize that atrazine disrupts proper endocrine function in developing and adult male rats and has negative effects on sperm production and fertility.

Significance

Because male sperm concentration has been reported to decline 52% and overall sperm count has dropped 59% over the past 40 years in North America, a better understanding of the effects of commonly known endocrine disruptors is essential. These effects on male fertility are relevant now more than ever as the United States population is waiting longer, on average, in attempting to conceive. Endocrine disrupting chemicals such as atrazine are commonly used in the United States and are highly concentrated in agricultural regions. More concrete knowledge on the developmental and life-long effects of chronic exposure to atrazine will prove useful in the battle for fertility. This report is, to our knowledge, the first to examine the direct effects of atrazine on hormone levels, sperm production, and expression of aromatase in the male mammalian testis in a single study.

Chapter 2. Review of Literature

Current State of Male Fertility

Male reproductive health has deteriorated in many countries (Toppari et al., 1996) and the latest meta-regression analysis from Levine et al. reports a significant decline in sperm counts between 1973 and 2011, driven by a 50-60% decline among men unselected by fertility from North America, Europe, Australia, and New Zeeland (Levine et al., 2017). Carlsen et al. (1992) published a provocative study which suggested that human semen quality had declined by roughly 50% from 1930 to 1990 (Fisher, 2004; Carlsen et al., 1992). Haimov-Kochman et al. conducted a 15-year longitudinal study from 1995 to 2009 by obtaining weekly sperm samples taken from 58 sperm donors in Israel and found that average sperm concentrations dropped from 106 x 10^6/ml with 79% motility to 68 x 10^6/ml with 66% motility (Haimov-Kochman et al., 2012). A decline in semen quality could constitute a decrease in semen volume, sperm motility, sperm count, and/or adverse deviations from normal sperm morphology. Many subsequent studies criticized or re-analyzed the original meta-analysis of Carlsen et al., but the trends and conclusions have been reaffirmed (Fisher, 2004; Swan et al., 2000). Initial results from prospective European studies have shown regional differences in semen quality between Denmark, France, Scotland, and Finland; the median sperm concentrations were 61 x 10^6/ml, 74 x 10^6/ml, 77 x 10^6/ml, and 82 x 10^6/ml, respectively (Jørgensen et al., 2001). Jørgensen et al. published a cross-sectional study in 2012 of 4,867 Danish men in Copenhagen, Denmark, that revealed only 23% of that sample population had optimal sperm concentration and sperm morphology (Jørgensen et al., 2012). Recent studies published in the United States, which used identical methodologies to the European-based studies, have also reported geographical

differences in sperm quality; the mean sperm count in Columbia, Missouri (58.7 x 10^6/ml) compared to New York, New York (102.9 x 10^6/ml) is striking (Fisher, 2004; Swan et al., 2003). Sperm concentrations below 40 x 10^6/ml are generally considered subfertile/infertile.

Sperm count is of considerable public health importance for several reasons. First, sperm count is closely linked to male fecundity and is a crucial component of semen analysis, the first step to identify male factor infertility (World Health Organization, 2010; Wang and Swerdloff, 2014). Second, the economic and societal burden of male infertility is high and increasing (Winters and Walsh, 2014; Hauser et al., 2015; Skakkebaek et al., 2015). Third, reduced sperm count predicts increased all-cause mortality and morbidity (Jensen et al., 2009; Eisenberg et al., 2014). Fourth, reduced sperm count is associated with cryptorchidism, hypospadias and testicular cancer, suggesting a shared prenatal etiology (Skakkebaek et al., 2015). Fifth, and most relevant to this study, sperm count and other semen parameters have been plausibly associated with multiple environmental influences, including endocrine disrupting chemicals (Bloom et al., 2015; Gore et al., 2015), pesticides (Chiu et al., 2016), and lifestyle factors (Afeiche et al., 2013; Jensen et al., 2013). Therefore, sperm count may sensitively reflect the impacts of the modern environment on male reproductive health and the male gonad throughout the course of life (Nordkap et al., 2012).

Testicular Anatomy and Function

The male gonad, the *testis and reproductive tract*, serves the dual function of sperm production and hormone synthesis. The testis and reproductive tract are composed of (in order): seminiferous tubules, rete testis, efferent ducts, and the epididymis (Figure 1). The two

principal functions of the testis, sperm production and hormone synthesis, are carried out in morphologically distinct compartments.

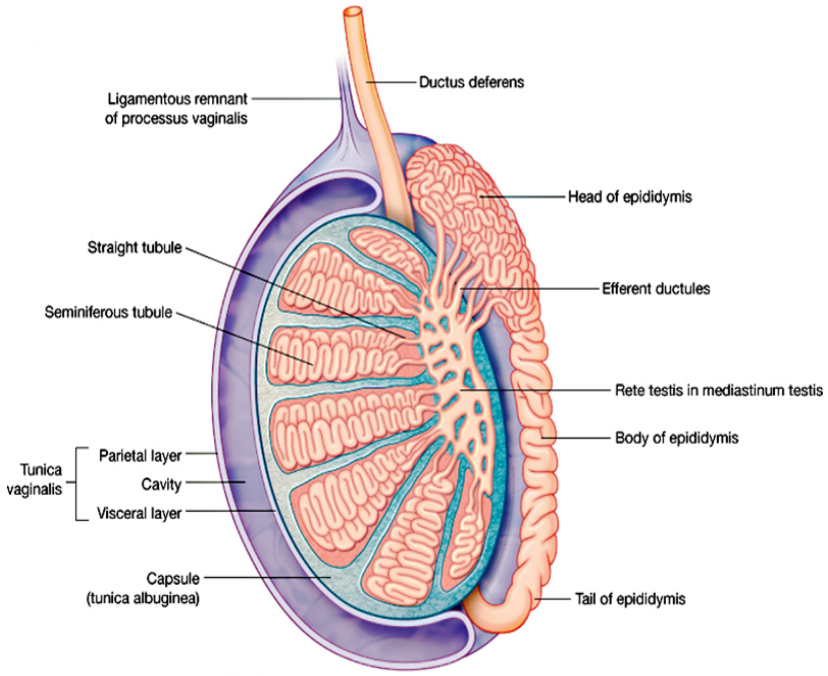

Figure 1. Anatomy of the Male Testis
Berne, Robert M.,, Koeppen, Bruce M.Stanton, Bruce, A., eds. Berne & Levy Physiology. Philadelphia : Mosby/Elsevier, 2008. Print.

The seminiferous tubules, in the first section of the male gonad comprising the bulk of testicular mass, house Sertoli cells and in the interstitial space are the Leydig cells. Sertoli cells are responsible for the maintenance and coordination of developing germ cells (immature sperm cells), in a process called spermatogenesis, and are located inside of the lumen of seminiferous tubules. Spermatogenesis can be divided into three discrete phases: 1. Mitotic divisions, which maintain a stem cell population of spermatogonia and provide the cells destined to become mature sperm; 2. Meiotic divisions, which reduce the chromosome number and produce a cluster of haploid spermatids; 3. Transformation of spermatids into mature

spermatozoa, a process involving the loss of the cytoplasm on their heads and the development of flagella. Sertoli cells also secrete the watery fluid that transports spermatozoa through the seminiferous tubules and into the epididymis, where 99% of the fluid is reabsorbed.

The primary role of Leydig cells is synthesizing and secreting the steroid hormone *testosterone* when stimulated by an upstream hormone. Although extensive at birth, Leydig cell function virtually disappears after the first six months of postnatal life in humans, only to reappear more than a decade later with the onset of puberty. In the adult, Leydig cells comprise 10-20% of testicular mass (Goodman, 2009). Testosterone released from Leydig cells can either diffuse into nearby capillaries for general circulation, or may diffuse into seminiferous tubules where it performs its essential role in spermatogenesis, which will be addressed later.

Developing sperm travel through the seminiferous tubules to the rete testis, through the efferent ducts, and then to the epididymis where they mature and gain motility. The primary function of the efferent ducts is fluid reabsorption in order to concentrate the sperm. Efferent duct-fluid reabsorption is under hormonal control of another steroid hormone, 17β-estradiol or simply estradiol, the primary form of estrogen in humans. Without proper estradiol signaling, fluid reabsorption will not occur and spermatogenesis will be disrupted (Hess, 2000).

Transit of spermatids through the epididymis is crucial for sperm maturation. The epididymis is divided into three major regions: caput, corpus, and cauda. The caput is the head of the epididymis, which receives immature sperm from the efferent ducts and begins the process of sperm maturation. The corpus is the body of the epididymis, and this is where sperm gain motility. The final section is the cauda, or the tail, where sperm are stored until ejaculation. Mature sperm then travel through the vas deferens from the cauda and are

expelled through the urethral orifice. The importance of testosterone and estradiol and their roles in the male reproductive tract and overall male health cannot be understated. They are the key messengers of reproductive and development information and are crucial for facilitating proper sperm production and proper male reproductive function.

In order for hormones to regulate physiologic processes, their secretion and subsequent actions must be turned on and off at precisely the right times. The organism must have some way of knowing when there is a need for a hormone to be secreted, how much is needed, and when that need has passed (Goodman, 2009). Secretion of most hormones is regulated by *negative feedback*. This means that some consequence of hormone secretion acts directly or indirectly on the endocrine cell secreting it in a negative way in order to inhibit further secretion. There is also secretion regulation by means of *positive feedback*. Positive feedback means some consequence of hormonal secretion acts on the endocrine cell secreting it to provide an augmented response resulting in further secretion of that hormone. Positive feedback systems are more rare and usually end in some sort of large and observable event, as is the case with oxytocin secretion and causing contraction of uterine muscle during childbirth.

The male reproductive tract, including the process of spermatogenesis, is under hormonal control of one branch of the endocrine system called the hypothalamic-pituitary-gonadal axis (HPG-Axis). The male HPG-Axis is a relatively simple negative feedback loop, in which it's designated effects (production and secretion of testosterone and inhibin) temporarily shut down the axis until hormone levels are reduced (Figure2).

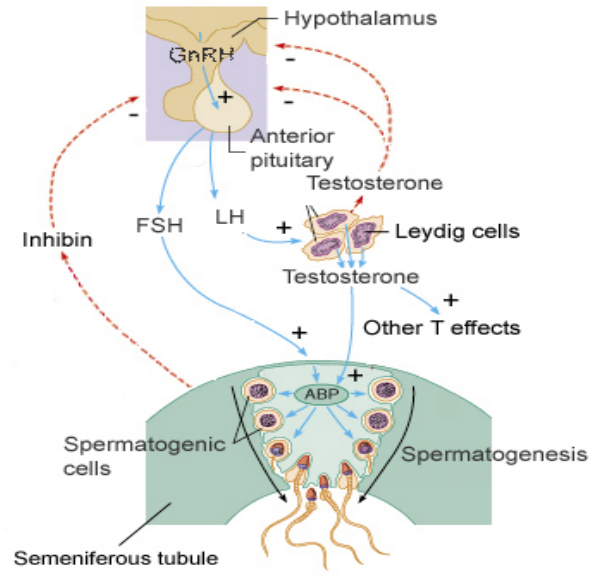

Figure 2. Hypothalamic-Pituitary-Gonadal Axis.
The HPG-Axis is the endocrine system that regulates male reproductive endocrinology. Campbell, Simon, Reese and Dicky Biology, Pearson/Benjamin Cummings 2010, Marieb; Human Anatomy & Physiology 5th edition, Benjamin Cummings, San Francisco 2001.

This HPG-Axis begins when Gonadotropin Releasing Hormone (GnRH) is secreted from the hypothalamus in response to low levels of testosterone in the blood. GnRH then travels to the pituitary gland via the blood and stimulates the anterior pituitary to secrete LH and FSH, which both subsequently travel to the testis via the blood. Inhibin is a protein hormone synthesized in Sertoli cells and stimulated by testosterone. It is designed to inhibit the further synthesis and secretion of FSH from the anterior pituitary (Skinner et al., 1989). Sertoli cells within the seminiferous tubules respond to FSH. Sertoli cells are the only cells known to express FSH receptors in human males and therefore the only targets of FSH. FSH stimulates Sertoli cells to proliferate and differentiate as well as produce growth and survival factors that prevent germ cell apoptosis.

Leydig cells within the interstitial space respond to LH and their principal role is stimulating the synthesis of testosterone. As previously stated, testosterone synthesized in Leydig cells is vital in supporting germ cell development within the seminiferous tubules. It also

has a number of physiological effects important for normal male development including: development of primary and secondary sex characteristics (testis formation/external genitals and pubic/facial hair, respectively), increases in bone and muscle mass, as well as a strong influence on masculine behavior. Sertoli cells lack receptors for LH, but are rich with androgen (testosterone) receptors, indicating the actions of LH on Sertoli cell function are indirect, and are mediated by testosterone, which reaches them in high concentrations via diffusion through the blood-testis barrier (Goodman, 2009). Developing human sperm cells lack androgen receptors, but do express receptors for estradiol, indicating that support of sperm cell development by testosterone is also exerted indirectly by that of Sertoli cells, yet directly by estradiol synthesis in the testis (Goodman, 2009). Interestingly, Leydig cells are also the primary source of testicular estradiol via the enzyme *aromatase* (CYP19), which converts testosterone into estradiol (Figure 3). Sertoli cells and Leydig cells, as well as seminiferous tubules, efferent ducts, and the epididymis express the aromatase enzyme, suggesting that estradiol may have other important responsibilities in normal sperm formation and maturation (Carreau and Hess, 2010).

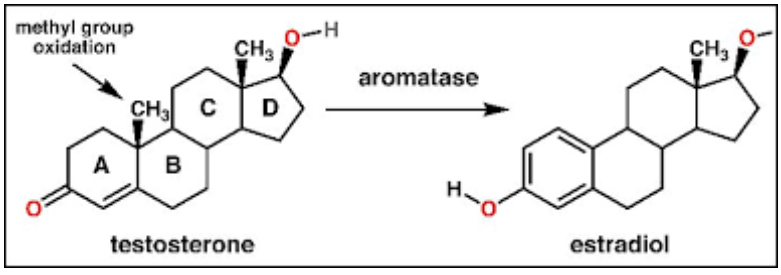

Figure 3. Aromatase Function.
The function of the enzyme aromatase is to convert, or aromatize, the A-ring of the steroid hormone testosterone, into estradiol. Leder BZ, Rohrer JL, Rubin SD, Gallo J, Longcope C (March 2004). "Effects of aromatase inhibition in elderly men with low or borderline-low serum testosterone levels". J. Clin. Endocrinol. Metab. 89 (3): 1174–80.

It has been well documented that testosterone is necessary for proper spermatogenesis and male development (Dohle et al., 2003; Kliesch, 2010; Wang et al., 2009; Collins et al., 2003). In recent years, the role of estradiol has been established as an integral component for spermatogenesis as well (Cooke et al., 2017). Previous studies have demonstrated that physiology of the male testis and epididymis are in part under the control of a balance of androgens (testosterone) and estrogens (estradiol), with the enzyme aromatase (CYP19) converting testosterone into estradiol (Carreau and Hess, 2010). Estrogen receptors α (ERα) and β (ERβ) are expressed within the testis and epididymis of multiple species, including humans and rats (Carreau and Hess, 2010), suggesting that the male reproductive tract is both a source and target for estrogen regulation (Clarke and Pearl, 2014). ERβ is present in the nuclei and cytoplasm of all epididymal regions in rats and other mammalian species, including humans (Zaya et al., 2012). ERα is present in epididymal epithelial cell nuclei and cytoplasm, and on the sperm in rats. Recently, a third form of the estrogen receptor, G protein-coupled estrogen receptor (GPER), has been identified (Martínez-Traverso and Pearl, 2015). These types of receptors are expressed on the cell surface within the membrane of cells, unlike ERα and ERβ, which are intracellular.

There are a number of important roles estradiol performs within the male testis and one such role is the regulation of fluid reabsorption within the efferent ducts, as stated previously. The loss of estrogen receptor function in males interferes with fluid reabsorption of the efferent ducts, a function essential for fertility (Hess, 2000). When efferent ducts fail to properly reabsorb fluid, a backpressure develops resulting in testicular atrophy and infertility (Hess et al., 1997). Also, in rodent models lacking complete estrogen production such as

aromatase knockout (ArKO) mice (Robertson et al. 1999), or when estrogen production is reduced by aromatase inhibitors (Kondarewicz et al., 2012), rats experience progressive infertility until spermatogenesis becomes severely impaired (Kondarewicz et al., 2012). A regional expression pattern of a G protein-coupled estrogen receptor (GPER) in the epididymis of multiple strains of rats has been demonstrated (Martínez-Traverso and Pearl, 2015) and the percentage of mature sperm greatly increases in the corpus where GPER expression is highest, which suggests GPER signaling may have a role in sperm maturation (Albanito et al., 2015).

Furthermore, estradiol appears to regulate apoptosis of rat Sertoli Cells (Royer et al., 2012) as well as the inhibition of apoptosis in human germ cells *in vitro* (Pentikäinen et al., 2000). Germ cell apoptosis appears to occur at a higher rate in the aged testis (Barnes et al., 1998), which is one mechanism daily sperm production could be reduced. Thus, it is possible that reduced estradiol within the testis during aging leads to increased germ cell apoptosis and lower overall daily sperm production (Clarke and Pearl, 2014).

The work previously described suggests too little estradiol adversely affects spermatogenesis and overall male fertility, and males lacking estradiol signaling in the reproductive tissues are infertile. However, it is equally important that serum and testicular estradiol levels and actions do not increase *above* the normal functional range in males. Estradiol is clearly involved in the negative feedback effects of testosterone on the brain to control pituitary gonadotropin secretion, and hence an absence of (or inappropriate exposure to) estradiol leads to disturbances in the delicate balance of the hypothalamic-pituitary-gonadal axis in both mice and men (O'Donnell et al., 2001). The fact that overexposure to estradiol during neonatal development can also produce similar defects in efferent duct function

highlights the need for a tightly coordinated series of estradiol-dependent events in this tissue (O'Donnell et al., 2001). Data have shown increases of estradiol in males alter the male reproductive tract as well as spermatogenesis and are consistent across vertebrae classes (Hayes et al., 2011).

Endocrine Disrupting Chemicals and Fertility

Recent patterns of infertility have been associated with chemicals that interfere with the body's *endocrine* system, and are commonly called endocrine disruptors. The endocrine system is comprised of many interconnecting and interacting axes involved in almost every process in the body including: development, behavior, neurologic activity, fertility, and maintenance of homeostasis and it is self-regulated by the synthesis, secretion, transport, binding, action, and clearance of natural hormones in the body. There are many hormones involved in these processes, for example: the commonly known hormone *insulin* is secreted from the liver in response to increases in blood sugar and performs important physiological actions to remove glucose out of the blood and into storage; a less commonly known hormone *thyroxine* is secreted from the thyroid gland located in the neck and is involved in physiological actions such as increasing metabolism and is therefore permissive for growth and development. Endocrine disruptors are chemicals (EDCs) that may interfere with the body's endocrine system at any level of these axes and produce adverse developmental, reproductive, neurological, and/or immune defects. EDCs may also mimic the actions associated with this system, thus amplifying its effects on the body.

The most widely known EDC to the general public currently is Bisphenol-A (BPA). When it was in use for cheap production of plastic bottles, it would leech into the bottle's fluid and was found to cause heart problems and was even associated with the failure of female egg implantation involved in In-Vitro Fertilization (Ehrlich et al., 2012). Common environmental contaminants can exhibit EDC activity and adversely affect male reproductive tracts and decrease overall fertility as well (Toppari et al., 1996). The most prevalently used herbicide applied to crops by the United States' agriculture industry is atrazine, a chlorotriazine (2-chloro-4-ethylamino-6-isopropylamino-s-trizine). Atrazine was introduced in the 1950s as a broad spectrum herbicide to control grasses and broadleaf weeds (Environmental Protection Agency, 2009). Currently, atrazine is most commonly applied to sweet corn, sorghum, and sugarcane, and atrazine works by binding to the QB protein of photosystem II in the chloroplast, halting photosynthesis (Stevens and Sumner, 1991). In agricultural regions of the United States, more than 32 pounds per square mile is applied to crops on average. For corn alone, an estimated 63,000,000 pounds of atrazine per year is used (Swan, 2006). As a result of this usage it is frequently detected in ground water, and atrazine seems to be relatively persistent compared to other common-use herbicides (J.R. Vogel and J. I. Linard, 2011). Coincidentally, a marked decline of semen quality (semen volume, sperm motility, sperm count and normal morphology) in normal men has been reported over the past few decades specifically in agricultural regions of the United States (Swan, 2006). These associations between current-use pesticides and reduced semen quality suggest that agricultural chemicals may contribute to the recently reduced semen quality observed in fertile men (Swan, 2006). Results from a study comparing semen quality of men from different regions of the United States indicated that changes in

semen quality were associated with exposure to several common pesticides currently being used (Swan et al., 2003), including atrazine.

While the reasons for these differences are currently unknown, both clinical and laboratory research suggest that the adverse changes may be inter-related and have a common origin in fetal life or childhood (Toppari et al., 1996; Swan, 2006). The growing number of reports demonstrating that common environmental contaminants and natural factors possess estrogenic activity presents the working hypothesis that the adverse trends in male reproductive health may be, at least in part, associated with exposure to estrogenic or other hormonally active environmental chemicals during fetal and childhood development in males (Jeng, 2014). A controlled study measured metabolites of eight non-persistent, current-use pesticides in urine samples the men had provided at the time of semen collection. Pesticide metabolite levels were elevated in cases compared with controls for the herbicides alachlor and atrazine (Swan, 2006).

It has been reported that in females LH release was suppressed and women who lived in communities where atrazine is used extensively experienced increased menstrual cycle irregularity, longer follicular phases, and decreased levels of menstrual cycle endocrine biomarkers of infertile ovulatory cycles (McMullin et al., 2004; Cragin et al., 2011). These results were reported with atrazine levels below the Environmental Protection Agency's MCL (Maximum Contaminant Level) for atrazine of 0.003 mg/L defined as: The level of a contaminant in drinking water below which there is no known or expected risk to health, which is alarming (Environmental Protection Agency, 2009). In a study performed with male rats, they experienced an increase in FSH synthesis after atrazine exposure, Leydig cells were of irregular

shape with unequal form, and atrazine provoked degenerative changes in Sertoli cell cytoplasm (Kniewald et al., 2000). This result in males has been hypothesized to be a possible mechanism by which the male reproductive tract and overall fertility have been affected by atrazine in agricultural regions of the United States (Babić-Gojmerac et al., 1989). The mechanisms by which atrazine alters the male reproductive tract are poorly understood and have not been thoroughly analyzed, thus it is important to bridge the gap between infertility and atrazine exposure beyond serum hormone analysis and epidemiological data.

The overall goal of this study is to investigate the hypothesis that changes in sperm production, maturation, and hormone levels of the male reproductive tract are consequences of chronic atrazine exposure.

Chapter 3. Methodology

Animals and Experimental Design

Sprague Dawley rats were chosen because they are a common and established model for the study of male reproductive tract function, including, but not limited to, the testes, epididymis and seminiferous tubules (Iannaccone and Jacob, 2009). Atrazine was administered daily via oral gavage to the mother while they were in gestation until rats were weaned off, as well as throughout their adult lives until seven months of age. Oral gavage was chosen because it mimics the most common mode of human exposure (drinking water), and it allows for the delivery of exact amounts of atrazine. Seven months of age was chosen because that is the age when rats are full adults, but before we begin to see any side effects of aging, which appears around twelve months of age. Three groups were included; the first group of ten rats was the vehicle control, the second group of nine rats was administered atrazine at a concentration of 0.1 mg/kg, and the third group of nine rats was administered atrazine at a concentration of 10 mg/kg. Serum of each rat was collected at time of euthanasia (via decapitation). The testis and epididymis of each subject were removed and frozen for later analysis, which were the sources of data for this study. Animals were housed and treated at Western Michigan University (WMU) and covered by a WMU animal use protocol. Dr. Chris Pearl, who has access to these tissues, brought the collected samples to GVSU.

Daily Sperm Production

Daily sperm production (DSP) was determined using procedures similar to those previously used (Clarke and Pearl, 2014). Frozen testis samples were thawed and homogenized in 0.9% NaCl/0.05% Triton X-100 at room temperature using an OmniTip tissue homogenizer. The homogenate was brought to a total volume of 30 ml, stored at 4°C for twenty-four hours, and the number of homogenization-detergent resistant spermatids was counted using a hemocytometer. The hemocytometer is comprised of two sides containing 25 squares each. Five squares on each side were counted and multiplied by five to account for the entire grid. This provided the number of elongated spermatids in the homogenate. The number of elongated spermatids counted, divided by the weight of testis homogenized provided sperm/gram of testis. Sperm/gram of testis provides a measure of efficiency for spermatogenesis. Sperm/gram multiplied by total testis weight equals the total number of elongated spermatids. Daily sperm production was calculated as the total number of elongated spermatids per testis divided by 6.1 days, which is the amount of time for elongated spermatids to be found in the rat seminiferous tubule.

Hormone Analysis

Blood and testicular concentrations of testosterone and estradiol were determined by competitive ELISAs (Enzo Life Sciences). For testicular concentrations, samples were thawed at room temperature and minced into small pieces. Pieces were homogenized in buffer, and subsequently centrifuged to remove any large portions of unsolubilized tissue. For testosterone, serum and tissue homogenates were mixed with a steroid displacement reagent

for 15 minutes and diluted in assay buffer to fall within the range of the standard curve. Testosterone samples were assayed in triplicate. The plates were analyzed using an Epoch Microplate Spectrophotometer and Gen5 statistical software. For estradiol, serum and testis samples were extracted in diethyl ether. After freezing the aqueous layer in liquid nitrogen, the ether was allowed to evaporate at room temperature overnight before the estradiol was reconstituted in assay buffer. Reconstituted samples were run in triplicate in the ELISA. LH and FSH serum samples were analyzed by following the Virginia Liquid Assay Core. Testicular steroid values were normalized to weight of tissue homogenized.

Androgen Receptor and Aromatase Immunohistochemistry

Tissues were paraffin embedded and sectioned at a thickness of 5 µm using a Leica RM 2125 rotary microtome, and placed on superfrost plus slides. The sections were deparaffinized in citrisolv and rehydrated in an alcohol series (100%, 95%, 70%, water). Antigen retrieval was performed by submerging slides in a citric acid based antigen unmasking solution in Coplin jars and steam heated at 93 degrees C for 5 minutes after which they were allowed to cool to room temperature. Aromatase immunohistochemistry (IHC) was done without antigen retrieval. After a blocking step, tissues were incubated overnight at 4 degrees C with antibodies to Aromatase (1:250; rabbit anti-rat) or Androgen Receptor (AR) (1:250; MC-20; rabbit anti-mouse AR; Santa Cruz Biotechnology). Following primary incubation, sections were incubated with goat anti-rabbit biotinylated secondary antibody followed by an avidin-biotin horseradish peroxidase complex (Vector Laboratories). Immunostaining was visualized using NovaRed chromagen

(Vector Laboratories). All slides were counterstained with Immunomaster Hematoxylin and evaluated by light microscopy at Grand Valley State University.

Statistical Analysis

Twenty-eight animals were used for analysis. Data were analyzed by two-way ANOVA using GraphPad Prism statistical software and values reported as means ± SEM. The data were tested for normality to ensure the assumptions of the ANOVA were satisfied. If the overall ANOVA was significant, differences between treatments were determined using Tukey's multiple comparison test. Differences were considered significant if $p \leq 0.05$. IHC was analyzed visually and staining intensity rated as strong positive, positive, weak positive, or negative.

Chapter 4. Results

To assess the overall health of the animals, we first looked at body weight. Body weights of the three atrazine-dose groups did not differ significantly. Rats raised in the control group had an average body weight of 463.6 ± 38.2 grams. Rats raised on the low dose of atrazine (0.1 mg/kg) had an average body weight of 497.2 ± 59.9 grams and rats raised on the high dose of atrazine (10 mg/kg) had an average body weight of 444.9 ± 59.4 grams (Figure 4).

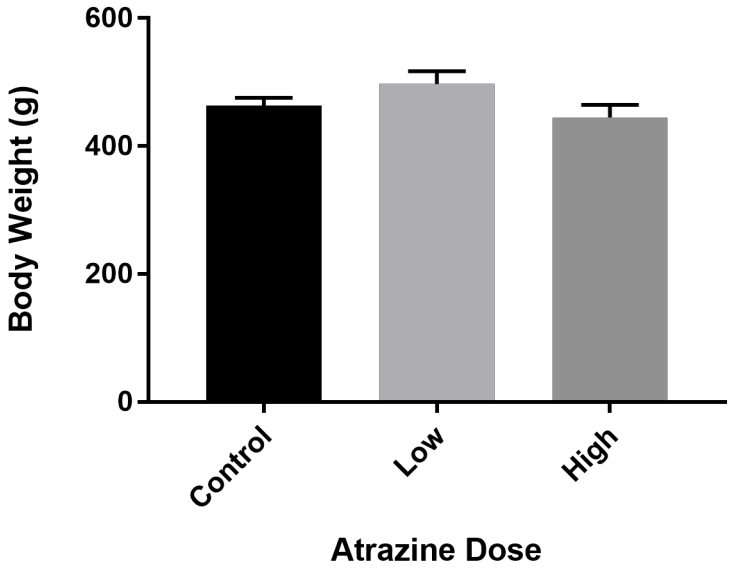

Figure 4. Body Weight
Body weights were similar between control, low dose, and high dose treated rats.

We next looked at paired testis weight to see if reproductive organs were affected. Atrazine exposure did alter testis weight depending on dose (Figure 5). Rats in the control group had an average paired testis weight of 4.17 ± 0.10 grams which did not differ significantly from rats in the high dose group that had an average testis weight of 3.95 ± 0.15 grams. Rats in the low dose group had significantly larger paired testis weights than both the control and the high dose groups with an average paired testis weight of 4.58 ± 0.11 grams.

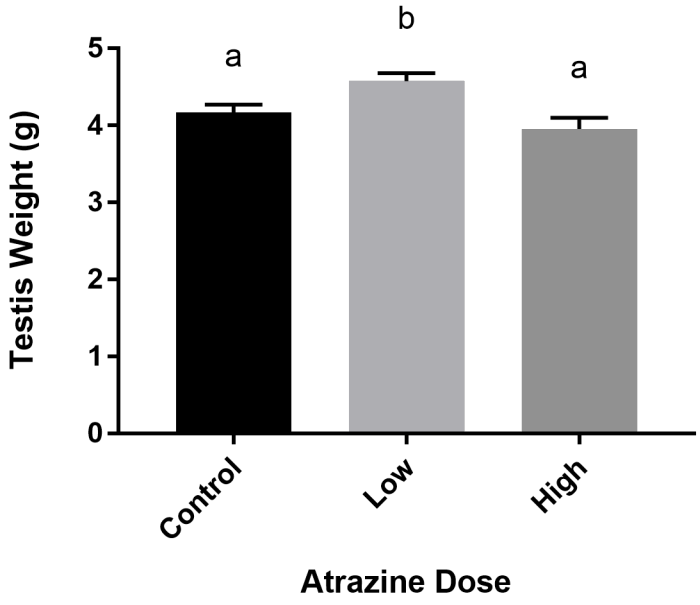

Figure 5. Paired Testis Weight
Paired testis weights were significantly higher in the low dose group compared to either control or high dose exposure. There was no significant difference in paired testis weights between the control and high dose groups. Columns labeled with different letters (a vs. b) indicate a significant difference at $p \leq 0.05$.

We next looked at sperm per gram of testis. Sperm per gram of testis was significantly lower in rats exposed to the low dose of atrazine (Figure 6). Control rats had an average sperm per gram of 94.88 ± 1.32 million which was similar to rats in the high dose with 90.48 ± 2.51 million sperm per gram. Rats exposed to the low dose of atrazine had a significantly lower sperm per gram of testis with an average of 86.07 ± 2.71 million sperm per gram.

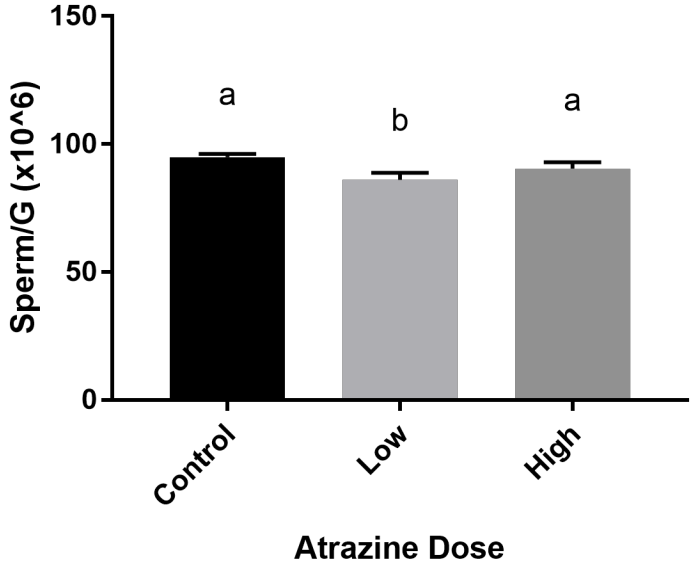

Figure 6. Sperm Per Gram of Testis
Rats exposed to the low dose of atrazine had significantly lower sperm per gram when compared to either the control or high dose animals. There was no significant difference between the control and high dose groups. Columns labeled with different letters (a vs. b) indicate a significant difference based on $p \leq 0.05$.

Daily sperm production is a critical measure of fertility potential and both testis weight and sperm per gram of testis contribute to this value. Daily sperm production (DSP) of the three groups differed significantly between doses (Figure 7). Rats in the control group had a DSP of 64.86 ± 1.87 million sperm, which did not differ significantly from rats in the low dose group with an average DSP of 64.24 ± 1.0 million sperm. Rats in the high dose group had a significantly lower DSP than both the control and low dose groups with an average DSP of 58.36

± 1.78 million sperm. Even though testis weight and sperm per gram of testis were not different from controls in the high dose group, it is our interpretation that the interplay of small changes in each of these variables was sufficient to cause a significant decline in DSP. Interestingly, DSP in the low dose group was not different from the control even though both testis weight and sperm per gram of testis were significantly different. However, these changes were in opposite directions with testis weight being larger and sperm per gram of testis reduced. When these variables were combined DSP appears similar to the control. Collectively, these data suggest that both low and high doses of atrazine may disrupt testicular function and potential fertility.

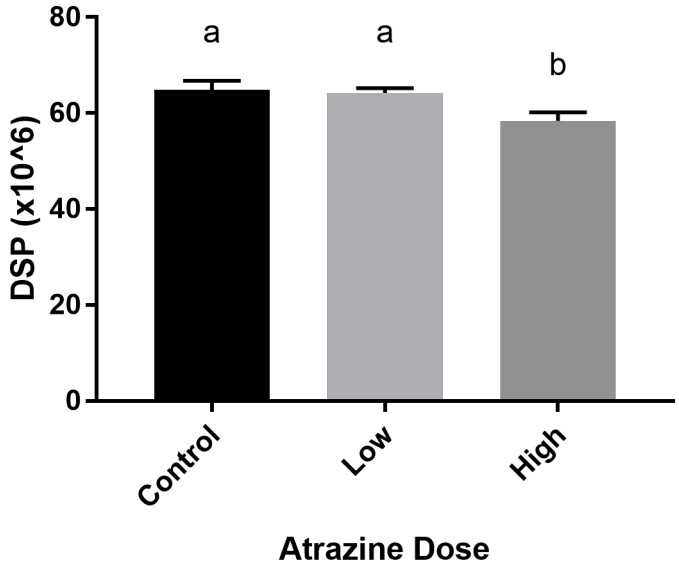

Figure 7. Daily Sperm Production (Paired Testis)
There was a significant difference between the control and high dose groups, as well as between the low dose and high dose groups. There was no significant difference between the control and low dose groups. Columns labeled with different letters (a vs. b) indicate a significant difference based on $p \leq 0.05$.

Since spermatogenesis and testicular function depend on hormone signaling, we next looked at serum hormone levels of gonadotropin hormones to determine if the effect of atrazine was altered via pituitary hormone secretion. The concentrations of LH within the blood were numerically higher in the high dose group, but LH was not significantly different between the three groups (Figure 8). The control group had an average of 0.24 ng/ml ± 0.05, the lose dose group had an average of 0.27 ng/ml ± 0.06 and the high dose group had an average of 0.33 ng/ml ± 0.05. Serum FSH levels were significantly different between the three groups (Figure 9). The control group had an average of 4.47 ng/ml ± 0.40, the low dose group had an average of 4.80 ng/ml ± 0.42 and the high dose group had an average of 5.96 ng/ml ± 0.36. There were no significant differences between the control group and the low group, or the low group and the high group. FSH was significantly higher in the high dose group compared to the control group. Thus, increased secretion of FSH from the pituitary may be contributing to the changes in DSP induced by atrazine.

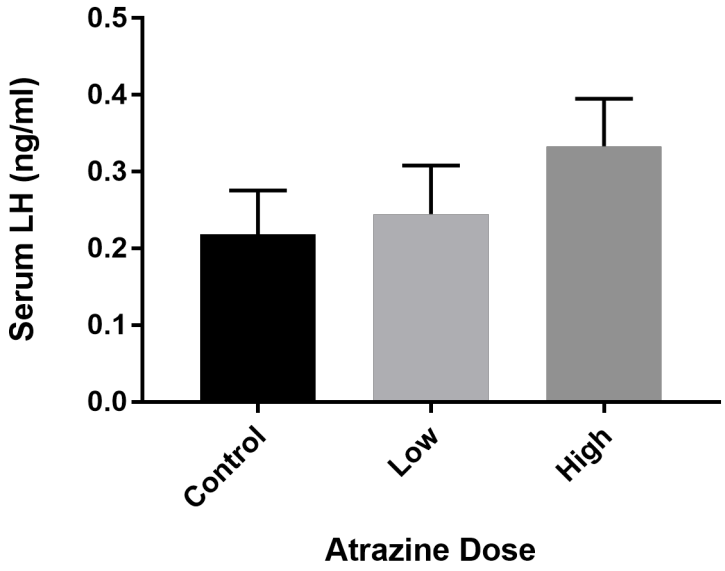

Figure 8. Serum LH
Serum LH levels were not significantly different between the control, low dose, and high dose groups.

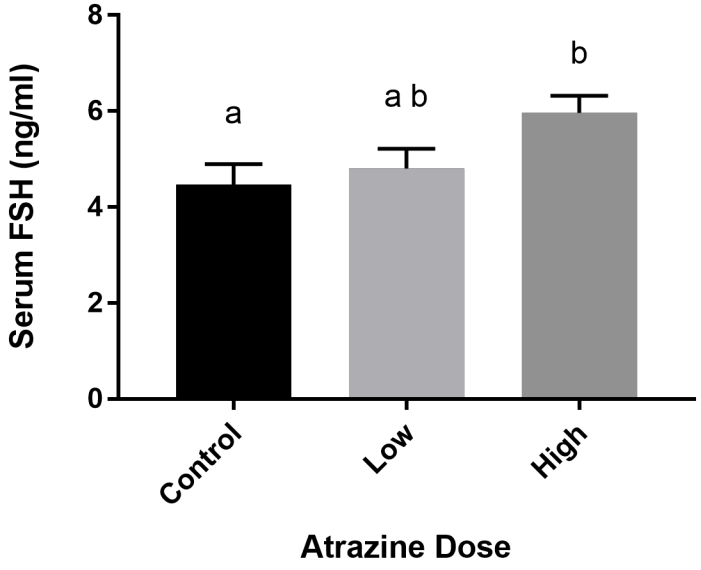

Figure 9. Serum FSH

Serum FSH levels were significantly different between the control and the high dose groups. There were no significant differences between the control and the low dose group, or between the low dose and the high dose groups. Columns labeled with different letters (a vs. b) indicate a significant difference based on $p \leq 0.05$.

Spermatogenesis is also regulated by gonadal steroids so we next looked at concentrations of steroid hormones in both the serum and testis tissue. There were no significant differences in serum testosterone levels between the three groups (Figure 10). The control group had an average of 3.32 ng/ml ± 0.66, the low dose group had an average of 3.51 ng/ml ± 0.83, and the high dose group had an average of 2.55 ng/ml ± 0.43 . There was a significant difference in serum estradiol levels between the three groups (Figure 11). The control group had an average of 3.45 pg/ml ± 0.20, the low dose group had an average of 3.72 pg/ml ± 0.20, and the high dose group had an average of 4.48 pg/ml ± 0.29. Similar to FSH levels, estradiol was significantly higher in the high dose group compared to the control group (Figure 8).

Since the testis produces testosterone and estradiol, we also looked at local/tissue hormone concentrations. There were no significant differences in testosterone levels within the testis (per gram of tissue) between the three groups. The control group had an average of 41.74 ng/g ± 5.92, the low dose group had an average of 49.61 ng/g ± 5.33, and the high dose group had an average of 43.64 ng/g ± 5.98 (Figure 12). Interestingly, testis estradiol was reasonably different, but not significantly different, between the three groups, even though serum levels of estradiol were significantly different. The control group had an average of 616.65 pg/g ± 85.2, the low dose group had an average of 684.44 pg/g ± 56.3, and the high dose group had an average of 718.22 pg/g ± 81.2 (Figure 13). In regards to steroid hormones it appears atrazine alters serum estradiol and thus, in addition to FSH, could be a mechanism to explain the altered sperm production parameters.

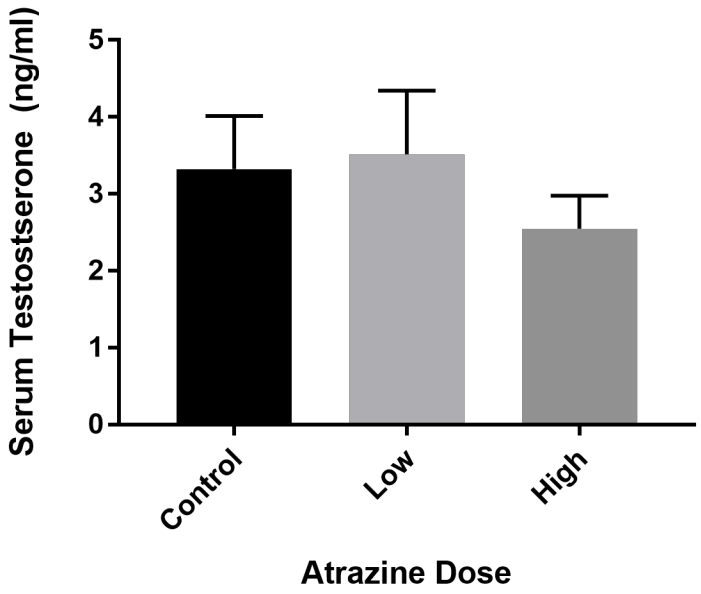

Figure 10. Serum Testosterone
Serum testosterone levels were not significantly different between the control, low dose, and high dose groups.

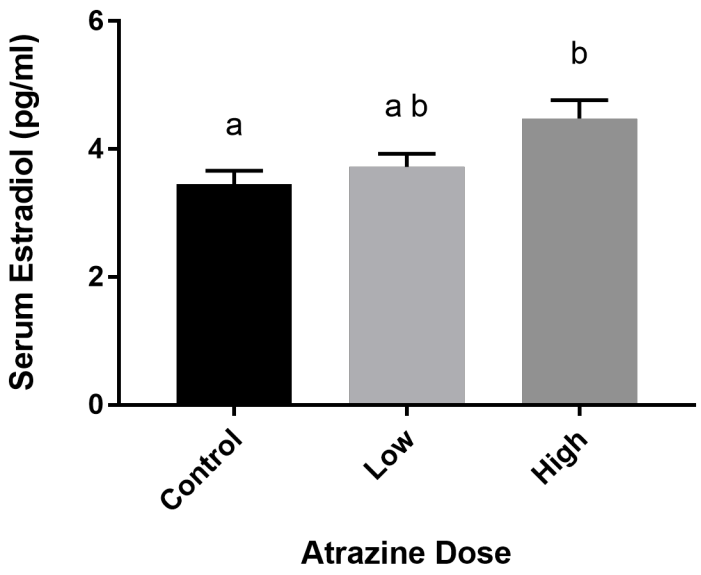

Figure 11. Serum Estradiol
There was a significant difference in the serum estradiol levels between the control and high dose groups. There were no significant differences between the control and low dose groups, or between the low dose and high dose groups. Columns labeled with different letters (a vs. b) indicate a significant difference based on $p \leq 0.05$.

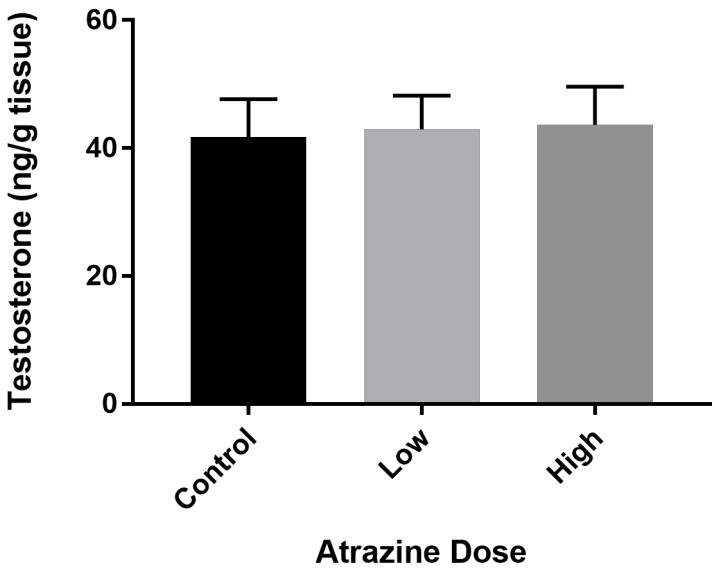

Figure 12. Testis Testosterone
Testis testosterone levels were not significantly different between the control, low dose, and high dose groups.

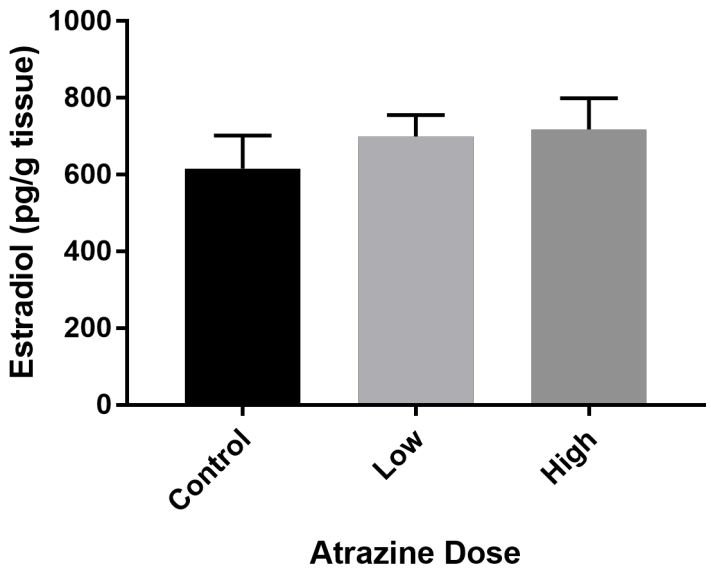

Figure 13. Testis Estradiol
Testis estradiol levels were not significantly different between the control, low dose, and high dose groups.

Since estradiol was increased, the testicular expression of the enzyme CYP19, commonly known as aromatase, which converts testosterone into estradiol, was next investigated via immunohistochemistry. Aromatase is localized in Leydig cells in-between the tubules in the male testis. As shown in Figure 14 in the interstitial space where the Leydig cells reside, the expression pattern of the aromatase has a much higher intensity in the low and high dose groups (panels B and C, respectively) compared to the control (panel A), suggesting a much higher expression of aromatase in the testes exposed to either low or high doses of atrazine.

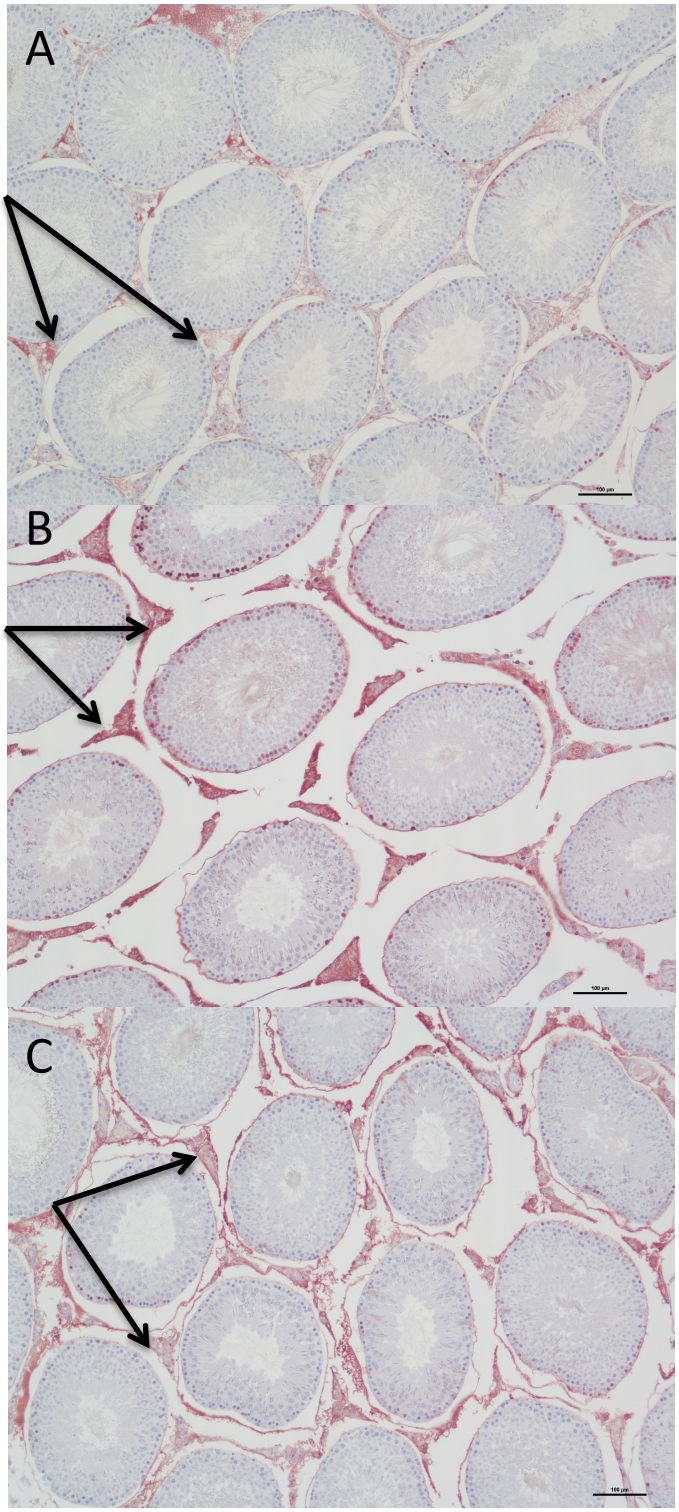

Figure 14. Immunohistochemistry – CPY19

CYP19, commonly known as aromatase, stained with NovaRed. From top to bottom, panels are arranged control group, low dose, and high dose. IHC intensity increased from the control group (panel A) to both the low dose (panel B) and high dose (panel C). Arrows pointing to positive Leydig cells. Bar = 100 µm**.**

To further investigate a potential involvement of androgen signaling, we localized androgen receptors (AR). AR's are localized in both Leydig cells in the interstitial space as well as in Sertoli cells within the tubules. The NovaRed expression pattern for AR's were distributed evenly among all three groups suggesting atrazine did not have a significant effect on AR expression within the male testis (Figure 12). If we noticed a difference in androgen receptor expression, we could attribute changes in DSP in part to androgen signaling. Since testosterone levels were not shown to be significantly different in the blood or testis, this reinforces our observation that no role was played by testosterone in this process, and therefore we attribute the observed changes to differences in estradiol.

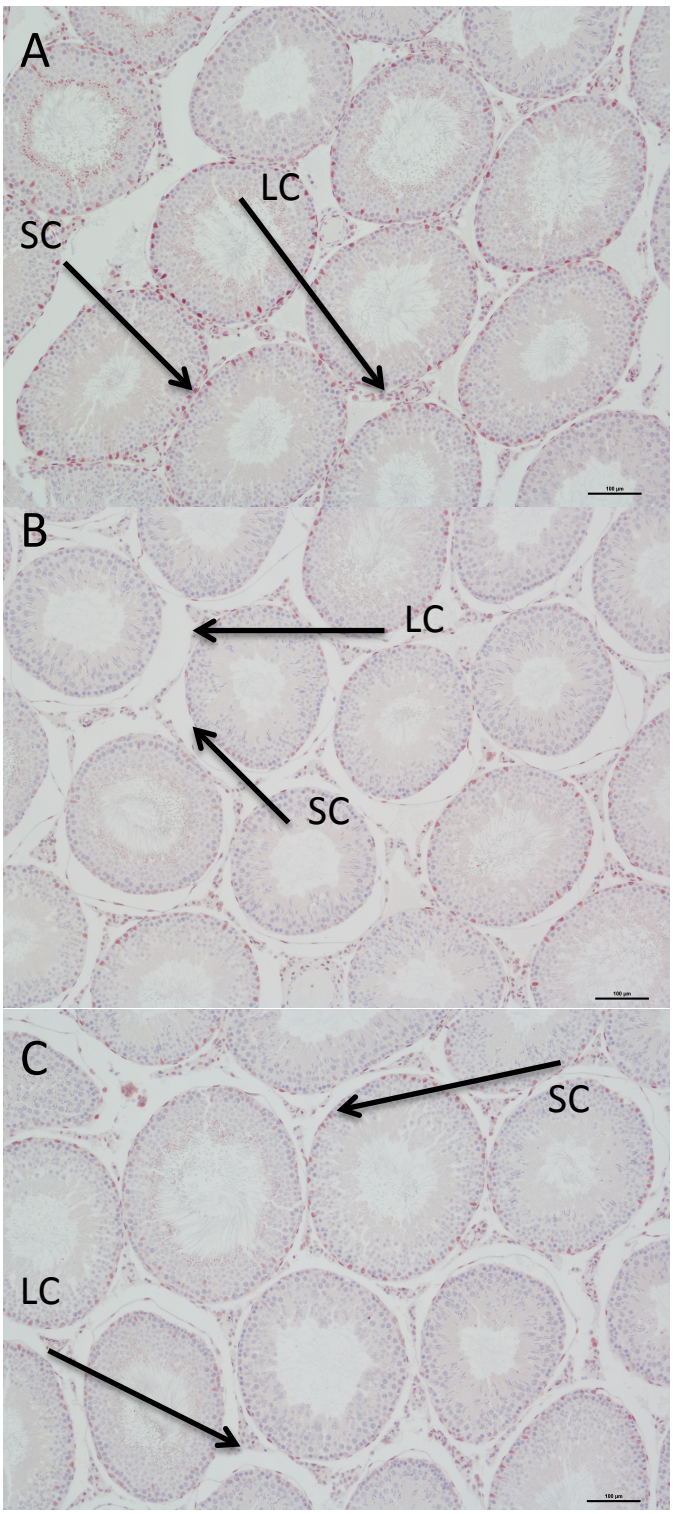

Figure 15. Immunohistochemistry – Androgen Receptor
Androgen Receptor (AR) was stained using NovaRed. From top to bottom, panels are arranged control group (panel A), low dose (panel B), and high dose (panel C). There was no observable difference in expression patterns between any of the groups. Arrows pointing to positive Leydig cells (LC) and Sertoli cells (SC). Bar = 100 µm**.**

Chapter 5. Discussion and Conclusion

The purpose of this study was to investigate the effects of the endocrine disrupting chemical atrazine on the reproductive health of 7-month-old male Sprague Dawley rats. It was hypothesized that atrazine would have a negative effect on reproductive function. This study demonstrated that atrazine adversely affects male Sprague Dawley rat fertility and hormone levels. At 7 months of age, rats raised on the high dose of atrazine did not differ from the control group in testis weight or sperm/gram, but their daily sperm production (DSP) was significantly lower than both the control and the low dose groups. DSP for the rats raised in the low dose group did not differ from the control, but testis weight was significantly higher than the control and high dose groups, and the sperm/gram was significantly lower than the control and high dose groups. Therefore, these results show that two differing doses of atrazine have adverse effect on DSP, sperm/gram, and overall male reproductive function.

Both FSH and estradiol concentrations in the blood were significantly increased, as well as the concentration of the enzyme CYP19, commonly known as aromatase, responsible for converting testosterone into estradiol. This suggests atrazine indirectly increases estradiol concentrations and subsequently decreasing fertility via promoting the synthesis of aromatase among other mechanisms. Analysis of LH and testosterone in the serum as well as in the testis revealed these hormone levels did not change in response to atrazine. Also, immunohistochemistry in the testis showed no changes in androgen receptor expression, the primary receptor for testosterone in mammalian males and further confirmed that the androgen pathway did not play a role in the decreases in daily sperm production in response to atrazine.

Sertoli cells are among the most essential components of reproductive physiology in males. The cells support germ cell development throughout the entire seminiferous epithelium and help maintain the structural integrity of the blood-testis barrier (França et al., 2016). This blood-testis barrier prevents leukocytes or antibodies from encountering developing germ cells and triggering an autoimmune response (Johnson and Setchell, 1968). Sertoli cell numbers directly correlate with the efficiency of sperm production and are the primary location of androgen receptors in the testis (Orth et al., 1988). Deficiency in either production or action of testosterone will certainly cause infertility in males, and this is true for excessively high or low levels (Kliesch, 2010). Since testosterone has the most direct role of maintaining fertility within mammalian males, and Sertoli cells are the primary target for testicular testosterone as well as the maintenance and development of sperm, another factor must be at play in the testis due to the observed unchanged levels of serum and testicular levels of LH, testosterone, and androgen receptors in all groups.

Immunohistochemistry revealed that estrogen receptor α (ERα) was present in Leydig cells, Sertoli cells, spermatogonia, spermatocytes, round spermatids and elongated spermatids/spermatozoa, while estrogen receptor β (ERβ) was present in the same cell types except spermatogonia and Sertoli cells (Carreau and Hess, 2010). This study demonstrates ERα mRNA expression in human testis and describes its localization in somatic and germ cell subtypes. These findings suggest that both ER isoforms are involved in the control of testicular function (Cavaco et al., 2009). Both ERα and ERβ bind 17β-estradiol with high affinity and they bind to classical estrogen response elements in a similar if not identical fashion (Dechering et al., 2000). In the testes, spermatogenesis is modulated at every level by estradiol, starting with

the hypothalamic-pituitary-gonadal axis, followed by the Leydig, Sertoli, and germ cells, and finishing with the ductal epithelium, epididymis, and mature sperm. Regulation of testicular cells by estradiol shows both an inhibitory and a stimulatory influence, indicating an intricate symphony of dose-dependent modulation (Schulster et al., 2016). The fairest interpretation of the data presented is that no single factor is responsible for the decrease in overall fertility in atrazine exposed rats, but that a complex interaction between both positive (increase in FSH, estradiol and aromatase) and negative (decrease in testis weight and DSP) factors ultimately determines the level of quality sperm production.

Body weights of the low dose groups only increased slightly, yet the paired testis weights of the low dose group were significantly higher than both the control and the high dose group. Interestingly, the sperm/g of the low dose group were shown to be significantly lower than both the control and the high dose group. This peculiarity led the DSP for the low dose group to remain statistically insignificant, though the combination of increased testis weight and decrease in sperm/g hid this discrepancy. Conversely, a past study found neonatal treatment of three separate exogenous estrogens resulted in significant decreases in adult testis weight, but similarly to the current study sperm/g also decreased significantly in response to exposure to various estrogens and were largely dose dependent (Atanassova et al., 1999).

Control of the endocrine system is a result of proper hormone production, circulation, and clearance within the HPG-axis. Normally, estradiol acts to maintain fertility. For example, it has been shown that FSH in combination with estradiol is necessary for the mRNA transcription of N-cadherin, the protein responsible for cell-to-cell adhesion. Thus, the dynamic process of spermatogenesis involving Sertoli cells separating and reforming tight junctions via N-cadherins

is, at least in part, regulated by estradiol (MacCalman et al., 1994). Estradiol can also modulate nuclear transcription to trigger either proapoptotic or antiapoptotic genes (Royer et al., 2012).

The most likely issue related to significantly decreased sperm counts in the high dose group or low dose group is the increase in levels of estradiol and a disruption of normal endocrine function and maintenance within the testis *in utero* and throughout development. Estradiol has exhibited inhibitory effects emphasizing the level of estradiol sensitivity of reproductive mechanisms within the testis. In a previous study concerning healthy men and possible male hormonal contraception, administration of estradiol produced a dose-dependent increase in peak plasma estradiol at one month and prolonged suppression of plasma LH and FSH leading to significantly enhanced suppression of sperm output (Handelsman et al., 2000). Furthermore, these effects of estradiol appear to manifest via central inhibition of gonadotropin secretion. It is unreasonable to fully rule out additional direct effects of estradiol on testicular function. The limitations of estradiol illustrated in the present study were that while the lower dose (10 mg) had negligible effects, only a two-fold higher dose (20 mg) produced dose-limiting adverse effects. Hence estradiol has a narrow therapeutic window (Handelsman et al., 2000). The current study observed similar effects of spermatogenic suppression in response to increases in serum estradiol, but conversely observed significant increases in FSH as well, rather than decreases. Thus, atrazine may be working under multiple different mechanisms throughout the endocrine system.

Estradiol has also been shown to modulate the expression of specific ion transporters and higher levels can disrupt Sertoli cell metabolic function, which could partially explain the disruption and decrease in sperm counts observed in this study (Martins et al., 2013;Bernardino

et al., 2016). In clinical settings, sperm retrieval rates were seen to increase 1.4-fold either by decreasing estradiol directly and normalizing the ratio or testosterone to estrogen with aromatase inhibitors or indirectly blocking estradiol centrally with clomiphene citrate, in turn, increasing gonadotropin secretion (Schulster et al., 2016).

Body weights of rats in the high group remained unchanged relative to the control. Similarly, the paired testis weights of both the control and high dose group remained unchanged, but DSP was significantly lower than both the control and the low dose groups. FSH levels were not significantly different between the control and the low dose groups, or between the low dose and the high dose groups. However, FSH levels in the high dose group were significantly higher than the control group. This suggests atrazine had a significant effect on FSH synthesis and secretion when in high concentrations. Following the same pattern, serum estradiol levels were not significantly different between the control and low dose groups, nor between the low dose and high dose groups. However, serum levels of estradiol in the high dose group were significantly higher than the control. This suggests the elevated FSH levels in the high dose group had a significant effect on estradiol synthesis or secretion, which resulted in significantly elevated estradiol in the serum. Furthermore, estradiol levels in the testis were reasonably elevated for the high dose group relative to the control, but not enough to make it statistically significant. Taken altogether, these results further emphasize the relationship between increasing estradiol levels and decreased male reproductive function.

It is clear that exposure of the developing male to exogenous estrogens (or increased endogenous estrogens) either in utero or neonatally can result in a range of abnormalities of reproductive development and function (Sharpe et al., 1998;Atanassova et al., 1999). The latter

include effects on the developing Sertoli cells, which express ERβ, and suggest that inappropriately high estrogen exposure during neonatal life can reduce final Sertoli cell number and impair Sertoli cell functional maturation, one possible mechanism for how atrazine might adversely affect overall male fertility (Sharpe et al., 1998). Albatino et al. recently reported that atrazine was able to exert an estrogen-like proliferative activity in various cell models, including ovarian and breast cancer cells and cancer-associated fibroblasts, by inducing the expression of several estrogen target genes without binding or activating the classical ERα (Chevalier et al., 2016). Specifically, after one hour of treatment with 1 μmol/L of atrazine, increased levels of c-fos, CTGF, cyclin-A, PR, BG-1, and pS2 were detected— all target genes of estrogen and associated growth and cell-cycle factors. These results in ovarian cancer cells suggest that atrazine triggered an increase through ERα and the EGFR-MAPK transduction pathway (Albanito et al., 2015), another possible mechanism in which atrazine could disrupt normal male reproductive function. One other relevant atrazine study stated that an endocrine basis for mammary tumors in Sprague Dawley rats is likely that atrazine leads to the maintenance of elevated blood levels of 17β-estradiol (E2) and prolactin; the mechanism for tumor development may include the induction of aromatase (CYP19) and/or other P450 oxygenases, or antagonist action at the estrogen feedback receptor in the hypothalamus (Gammon et al., 2005).

Aromatase is present largely in the mature Leydig cells, producing a significant amount of the estradiol in the testis (Schulster et al., 2016). The observed increase in aromatase concentration within the testis suggests a means by which estradiol significantly increased within the blood in the high dose group and reasonably in the low dose group. Aromatase

synthesizes testosterone into estradiol, and it is possible that atrazine could upregulate the expression of aromatase within the testis itself; indirectly increasing estradiol levels and it's associated effects on male reproductive function and development.

Non-monotonic dose-response (NMDR) relationships are more frequently reported today in experimental studies than they were 10 years ago (Vandenberg et al., 2013). The term "NMDR" describes a dose-response relationship characterized by a curve whose slope changes direction within the range of tested doses. Non-monotonicity represents a challenge to fundamental concepts in toxicology and risk assessment. Endocrine disrupting chemicals (EDCs) are regularly associated with NMDR relationships (Lagarde et al., 2015). Of the 231 publications related to EDCs assessed in a qualitative literature review, 51 experimental studies described one or more NMDR profile concerning EDCs and/or natural hormones, or 22%. From these 51 studies, 170 dose-effect relationships were claimed by authors as NMDR, and the most often-cited substances were BPA and 17β- estradiol (Lagarde et al., 2015). Of the same 51 studies, 17 involved male reproductive organs including: testes, epididymis, seminal vesicle, preputial glands, and prostate. All of which involved BPA or 17β- estradiol (Lagarde et al., 2015).

As clearly stated by Vandenberg et al., "if a non-monotonic relationship occurs between the doses tested in traditional toxicology studies and the calculated "safe" or reference dose, this would still have serious implications for risk assessment," (Vandenberg et al., 2013). Further studies should aim to explore ad identify the mechanisms through which atrazine works within the male testis. Specifically, whether or not atrazine acts as a modulator of estrogenic activity and synthesis, an upregulator of aromatase, or perhaps further upstream in the pituitary from where FSH is secreted. An area of interest beyond the scope of this study could

be the analysis of why aromatase would increase in the presence of atrazine and what levels of estradiol are destructive in the developing and adult male reproductive tract.

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