CLEARY, JACOB ALEXANDER, M.S., Transgenerational and Epigenetic Effects of Developmental Exposure to Atrazine on Medaka (*Oryzias latipes*). (2018)
Directed by Dr. Ramji K. Bhandari. 57pp.

Exposure to environmental chemicals can cause epigenetic transgenerational inheritance of altered phenotypes. Chemicals such as the plasticizer bisphenol-A (BPA), the fungicidal vinclozolin, and the oral contraceptive component 17α-ethinylestradiol (EE2) induce epigenetic changes in various species. Each of these chemicals is an environmentally pervasive endocrine disrupting compound, as is the case with the abundantly used herbicide atrazine, whose endocrine disrupting mechanisms still remain unclear. Atrazine (ATZ) is presently one of the most abundantly used herbicides in the United States, and a common contaminant of natural water bodies and drinking waters in high-use areas. ATZ belongs to the triazine herbicides which specifically target photosynthetic tissues, yet ATZ can affect animal health. Fish exposed to ATZ and EE2 display dysregulation of reproductive processes, which include alterations to the hypothalamic-pituitary-gonadal (HPG) axis pathways. However, the potential for ATZ-induced transgenerational inheritance of reproductive dysfunction has not been investigated in fish. The transgenerational reproductive consequences of ATZ exposure have been explored in rats, but fish are among the species at greatest risk from ATZ exposure, therefore, I chose a model species to represent this environmentally relevant scenario. In the present study I analyzed the effects of ATZ and EE2 exposures during early development on transgenerational reproductive dysregulation in Japanese medaka (*Oryzias latipes*). F0 medaka were exposed to ATZ, EE2, and a solvent during the first twelve days of development with no exposure over the subsequent three generations.

These early developmental exposures overlap with the critical windows of embryonic germ cell development, gonadogenesis, and sex determination when gonadal DNA methylation is being reprogrammed. Exposed males and females of the F0 treatment lineages were bred to produce the F1 generation, the F1 offspring were bred to produce the F2 generation, and the F2 offspring were bred to produce the F3 generation. Neither ATZ nor EE2 altered sperm parameters, gonadosomatic, or hepatosomatic indices in the treated F0 generation. However, the hepatosomatic index was reduced in F2 females derived from F0 fish treated with either ATZ or EE2. Hepatosomatic and gonadosomatic indices are ratios of liver and gonad weights to the total weight of the fish, which are cursory metrics for evaluating energetic and reproductive health. The fecundity of F0 and F2 fish was unaffected by exposure to ATZ or EE2; however, the fertilization rate was decreased among the F2 fish derived from the low ATZ and low EE2 treated F0 generation. Moreover, there were significant transgenerational differences in the expression of reproductive regulatory genes and genes required for DNA methylation. Genomic methylation patterns are catalyzed by DNA methyltransferase enzymes (*dnmt*s) that regulate the heritable transcriptional activity of specific regions of DNA essential to development. Present results suggest that early life exposure to ATZ and EE2 cause no significant effects in the immediate generation, but that future generations of fish are at greater risk of reproductive dysfunction.

TRANSGENERATIONAL AND EPIGENETIC EFFECTS OF DEVELOPMENTAL EXPOSURE TO ATRAZINE ON MEDAKA (*ORYZIAS LATIPES*)

by

Jacob Alexander Cleary

A Thesis Submitted to
the Faculty of The Graduate School at
The University of North Carolina at Greensboro
in Partial Fulfilment
of the Requirements for the Degree
Master of Science

Greensboro
2018

Approved by

_____
Committee Chair

APPROVAL PAGE

This thesis written by Jacob Alexander Cleary has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

Committee Chair _____

Committee Members _____

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Date of Acceptance by Committee

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Date of Final Oral Examination

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CHAPTER I

INTRODUCTION

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) is an abundantly used pre-emergent herbicide that is commonly applied throughout the Midwestern United States and other agricultural regions of the world (Gilliom and others, 2006; Thelin and Stone, 2010). In the United States (US) alone, up to 82 million pounds of ATZ may be applied annually (Gilliom and others, 2006; Thelin and Stone, 2010). Atrazine (ATZ) is commonly used on corn, sorghum, and sugarcane crops, but is also a common additive within many residential herbicides. The compound's structure renders it environmentally stable and mobile, which indicates that it will likely persist and translocate between habitats once introduced into the environment (Jayachandran *et al*., 1994; Lerch *et al*., 2010).

ATZ is a chlorinated symmetrical triazine that was found to control the growth of broadleaf weeds and grasses (Solomon *et al.*, 2008). Once ATZ enters plants it is transmitted to the foliage by way of transpiration from the roots, and it is in the photosynthetic tissues of the foliage where ATZ begins to affect weed growth. ATZ's mode-of-action is to bind to the D1 protein of the multiprotein core of Photosystem II to disrupt the electron transfer to the final electron acceptor, oxygen (Gardner, 1989; Fuerst and Norman, 1991). The combination of light and photosynthesis blockers causes an accumulation of electrons that can lead to damaged chlorophyll molecules and oxidative

chemical species (Fuerst and Norman, 1991). While ATZ is considered far more toxic to plants than animals because photosynthetic systems are not found in animal cells, there is evidence that ATZ affects animal health (Hayes *et al*., 2011). As previously mentioned, the combination of ATZ's long half-life, water solubility, and the rainfall that accompanies the seasonal use of ATZ enables it to contaminate potable and ecological water sources (Jayachandran *et al*., 1994).

The primary route of aquatic exposure to ATZ is runoff from crops such as corn, making it a common contaminant of ground water, surface water, and human drinking water, especially due to its water solubility and structural integrity. However, there are seasonal surges, or periods when growing regions receive high amounts of rainfall after an ATZ field application. For most of the Midwestern US corn growers this seasonal surge occurs approximately 120 days after planting occurs from mid-March to June (Giddings *et al.*, 2005). The seasonal ATZ applications generally differ between the types of crops and can be affected by climatic conditions and other regionally specific abiotic factors that determine when those crops are planted. Although mean annual concentrations of ATZ in fresh water bodies typically conform to the US Environmental Protection Agency (USEPA) limit of 3 μg/L, there are however, seasonal surges which may exceed 55 μg/L (Battaglin *et al*., 2005; Thelin and Stone, 2010; Lerch *et al*., 2010). The bodies of water commonly evaluated are ponds, streams, and watersheds adjacent to agricultural fields, but other aquatic habitats may also have detectable levels of ATZ. As a potential contaminant to humans and wildlife, any effects induced by ATZ would be pertinent to both human and ecosystem health (USGS, 2007; USEPA, 2015). Moreover,

ATZ is a known endocrine disrupting compound (EDC) capable of dysregulating various aspects of endocrine related pathways, and is associated with increasing feminization in many lower vertebrate species including multiple frog and fish species (Hayes *et al*., 2011). However, the endocrine disrupting mechanisms of ATZ are poorly understood (Kucka *et al*., 2012).

Endocrine disruptors are chemicals that interfere with the body's endocrine system, producing adverse developmental, reproductive, neurological, and immune effects in both humans and wildlife (Vandenberg *et al*., 2012). A wide range of substances, both natural and synthetic, are EDCs including pharmaceuticals, dioxin and dioxin-like compounds, polychlorinated biphenyls, plasticizers such as bisphenol-A, insecticides, and herbicides such as ATZ. The plastic additive BPA is commonly found in the environment and human bodily fluids, and has been experimentally linked to reproductive impairment, diabetes, obesity, and cancer (Mathieu-Denoncourt *et al*., 2015). Along with the phenotypic consequences of exposure, many EDCs modify the epigenome, which is a heritable molecular consequence that can be transmitted to offspring if the epigenomic change occurs in the exposed organism's germ cells (Skinner *et al*., 2011).

The epigenome can be defined as a suite of epigenetic modifications, or attachments, found on and within the genome. Epigenetic changes are molecular modulations to the epigenome which subsist after the responsible stimulus is removed. Moreover, these may be mitotically and meiotically heritable alterations which cause changes in gene expression without simultaneously modifying the nucleic acid primary

sequence (Skinner *et al*., 2010). These changes include histone modifications, DNA methylation, and various types of non-coding RNAs. How changes to the epigenome are induced by EDC insult is unclear, but other studies have associated EDC associated alterations in the epigenome to abnormal phenotypes and other adverse health outcomes in various species (Rager *et al*., 2011; Kundakovic *et al.*, 2013; Markunas *et al.*, 2014). Among the readily detectable epigenetic modifications is DNA methylation, or the enzymatic addition of a methyl group to the cystosine residues of cytosine-phosphate-guanine dinucleotide (CpG) regions in the genome (Razin and Cedar, 1991). The addition of these methyl groups can modulate transcription factor and polymerase binding to local gene regions, subsequently enhancing expression if the affected region is a repressor, or repressing expression if the methyl group is attached to a promoter region. For my project I investigated DNA methylation because changes in genome wide DNA methylation are easily quantifiable and the genes responsible for catalyzing the addition of methyl groups to DNA are characterized in my model species, medaka.

An interesting feature of epigenetic modifications is that they are reprogrammed twice during development, at which point all epigenetic modifications are reset and DNA methylation is erased, then subsequently rewritten (Jacobs *et al*., 2017). The first event occurs at fertilization for zygotic development, and the second event is exclusive to the primordial germ cells (PGCs) which become the adult germ cells (Skinner *et al*., 2010). Consequently, if epigenetic modifications escape erasure, or are induced during these periods of reprogramming, they will be inherited by the next generation in a phenomenon referred to as transgenerational epigenetic inheritance (Skinner, 2011).

Transgenerational epigenetic inheritance is the transmission of heritable epigenetic modifications within the germline that are associated with phenotypic changes in subsequent generations (Skinner *et al*., 2010; Anway *et al*., 2005; McBirney *et al*., 2017). Several environmental chemicals and non-chemical stressors can induce transgenerational inheritance of phenotypic abnormalities. Exposure of ancestral *Oryzias melastigma* to hypoxic conditions impaired gonadal development and reduced sperm quality in unexposed F1 and F2 generations (Wang *et al*., 2016). A component of oral birth control, EE2, has been associated with reduced fertilization rates and embryonic survival in the grand-offspring of embryonically exposed fish (Bhandari *et al*., 2015). Wirbisky *et al*., 2016 demonstrated that adult zebrafish exposed only during embryonic development exhibited endocrine related transcriptomic changes, and produced offspring with morphological alterations in body indices compared to controls. In this study EE2 was chosen as a positive control for transgenerational epigenetic inheritance, and to observe if any transgenerational effects of ATZ appear similar to those of EE2 because it has been debated that ATZ has estrogenic properties. This thesis describes the results of investigations into the transgenerational effects of embryos exposed to either ATZ or EE2 on adult reproduction and phenotype-associated molecular alterations in the reproductive axis of medaka.

The following paragraphs outline why I chose certain genes and metric data points to evaluate the reproductive status of mature medaka fish. Phenotypic data, including length, weight, gonadosomatic and hepatosomatic indices, fecundity,

fertilization rate, and various sperm parameters, were used as potential indicators for reproductive health. Molecular data were used to assess any changes in gene expression that elucidate reproductive perturbations. It is important to note that gross phenotypes may not be solely indicative of reproductive health or dysregulation; therefore, molecular analyses were necessary to corroborate any significant gross phenotypes.

The condition of fishes is often cursorily evaluated by measuring their bodily weight and length; however, the weights of the gonads and the livers of the fishes relative to their body weights are considered useful indicators of reproductive and energetic health (Lambert and Dutil, 1997). Physical condition is integral to the reproductive capacity of many fishes. Fish in poor condition (i.e. low weight, or stunted growth) may experience reduced fecundity or produce unhealthy offspring (Morgan, 2004).

The relationship between the size of the liver and the body, as well as that of the gonad and the body provide two useful metrics when evaluating fish reproductive health. Hepatosomatic and gonadosomatic indices (HSI and GSI) are ratios of the liver and the gonad weights to the total weight of the fish, which are used to assess energetic and reproductive health. The GSI is related to the reproductive condition and frequency of spawning events in fishes. The HSI indicates the energetic and metabolic health of fish; however, in females the liver further indicates reproductive success as it is the site of egg yolk precursor protein synthesis (Lambert and Dutil, 1997).

I chose the set of genes based on their importance to reproductive development and maturation, as well as their suspected involvement in epigenetic regulated transgenerational inheritance. In the testes I measured the expression of androgen

receptor alpha (*ar*α), follicle stimulating hormone receptor (*fsh-r*), steroidogenic acute regulatory protein (*star*), DNA methyltransferase 1 (*dnmt1*), and DNA methyltransferase 3aa (*dnmt3aa*). In the ovaries I measured estrogen receptor alpha (*er*α), luteinizing hormone receptor (*lh-r*), cytochrome P450, family 19, subfamily A, polypeptide 1a (*cyp19a1a*, gonadal specific aromatase), DNA methyltransferase 1 (*dnmt1*), and DNA methyltransferase 3aa (*dnmt3aa*). In the brains of both male and female medaka fish I measured kisspeptin I (*kiss1*), kisspeptin II (*kiss2*), G-protein-coupled-receptor 54-1 (*gpr54-1*), G-protein-coupled-receptor 54-2 (*gpr54-2*), gonadotropin releasing hormone I (*gnrh 1*), gonadotropin releasing hormone II (*gnrh 2*), and DNA methyltransferase 1 (*dnmt1*).

Star gene expression controls the production of the transport protein StAR which is found on outer mitochondrial membranes. This protein controls the rate-limiting step of steroidogenesis by regulating the transfer of cholesterol into the inner mitochondrial membrane (Manna and Stocco, 2005). A series of enzymatic conversions taking place in the mitochondrion and endoplasmic reticulum will further transform the cholesterol into steroid hormones such as testosterone. Consequently, *star* expression in the vertebrate gonads is required due to the interrelatedness of hormones and sexual development and reproduction (Clark *et al.*, 1995). Furthermore, *cyp19a1a* (gonadal aromatase) is an enzyme that converts androgens to estrogens in single step conversion process that is crucial to sexual maturation and gonadal development in both sexes, even in fish (Wu *et al.*, 2008).

Arα and *er*α are nuclear receptors the mediate the genomic response in androgen and estrogen responsive cells (Katsu *et al*., 2008). The steroid hormones diffuse across the plasma membrane of cells and enter the cytosol where the nuclear receptors are located. Once a steroid hormone binds to its cognate nuclear receptor, the receptor and hormone dimerize and translocate to the nucleus where they behave as transcription factors to hormone responsive elements of the genome (Beato *et al*., 1996). While estrogens, such as estradiol-17β, regulate oogenesis, oocyte growth and maturation, and likely sexual differentiation in female medaka fish (Chakraborty *et al*., 2011), the androgens, primarily 11-ketotestosterone, are involved in testicular development, spermatogenesis, and steroidogenesis (Rolland *et al*., 2013).

The neuroendocrine signaling that controls the reproductive axis in medaka, and in other tetrapod vertebrates, begins in the hypothalamus (Zohar *et al.*, 2010). In the fish hypothalamus there are gonadotropin releasing hormone (GnRH) positive neurons whose dendritic projections reach the anterior pituitary gland (APG) gonadotrophic cells (Karigo *et al*., 2012). The discharge of GnRH stimulates the APG gonadotrophs to release the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH). Furthermore, a more recently discovered neuropeptide known as kisspeptin and its affiliated receptor G-protein-coupled receptor 54 (GPR54) regulate GnRH secretion and concomitantly LH and FSH release (Tena-Sempere, 2006).

While there are receptors for LH and FSH in various locations within medaka, I focused on the receptors located on the gonads for their relation to sexual development. I evaluated *fsh-r* in medaka testes for its role in mediating gonadal growth and

spermatogenesis through the Sertoli cells. In medaka ovaries I measured *lh-r* because it regulates the production of steroid hormones in the theca cells, but also promotes oocyte development and ovulation (Takahashi *et al*., 2016).

One of the epigenetic mechanisms that influences gene expression, DNA methylation, is catalyzed by the DNA methyltransferases (*dnmt*s). *Dnmt1* is responsible for the faithful transmission of the pattern of DNA methylation to daughter strands during DNA replication. *Dnmt3aa* produces *de novo* methylation during development and possibly in response to endocrine disrupting activity (Damelin and Bestor, 2007). DNA methylation dysregulation can alter sexual development when the insult occurs during gonadal development (Ribas *et al*., 2017), which may have further implications for transgenerational epigenetic inheritance if there is an impact on the germ cells.

CHAPTER II

MATERIALS AND METHODS

Experimental Design

The study design, maintenance and exposure of medaka fish, collection of tissues, measurements of morphometric parameters, egg collection, and ATZ dosing and uptake quantification experiments were performed at the USGS Columbia Environmental Research Center (CERC) in Columbia, Missouri. All of the RNA and DNA extractions, subsequent molecular experiments, and statistical analyses were performed at the University of North Carolina at Greensboro (UNCG) by the author, Jacob Cleary.

The study was designed to determine if there were any significantly different direct and transgenerational reproductive effects in medaka fish exposed to ATZ at an environmentally realistic concentration (5 μg/L) and a concentration reached during seasonal surges of herbicide use (50 μg/L). For comparison, we used two concentrations of EE2 at standard environmental (0.002 μg/L) and higher environmental (0.05 μg/L) concentrations. Significant differences were evaluated between the ATZ groups and controls, EE2 groups and controls, between the ATZ Low and EE2 Low, and the ATZ High and EE2 High. This was done to compare the transgenerational effects of both ATZ and EE2 to controls, as well as to each other given that ATZ has been argued to be estrogenic. EE2 induces transgenerational reproductive phenotypes in medaka, therefore, we chose to compare it to any transgenerational differences observed due to ATZ

exposure (Bhandari *et al.*, 2015), so EE2 was used as a positive control in my experiment. Fish were exposed throughout early embryonic development during the critical periods for gonadogenesis and germ cell differentiation. The gonadal primordium which gives rise to either ovaries or testes forms between 6-8 days after fertilization (daf), and differentiation of somatic gonadal structures begins approximately 10 daf (Siegfried, 2010). No outbreeding was performed between control and experimentally treated fish. Rather, fish were bred within the treatment groups to maintain a situation similar to that occurring in natural pond and stream habitats. The fish that were exposed as embryos and then developed to adulthood were designated as F0 adults. The F0 offspring were the F1 generation, and the F1 offspring were the F2 generation. Thus, the F2 generation was not directly exposed to test chemicals, rather any effects occurred because of ancestral exposure. The required tissues were collected and measured from animals sacrificed each generation at approximately 100 daf, or once sexual maturity is established. This was done to ensure that a sufficient amount of fertilized eggs could be collected to maintain each treatment lineage. Other morphological data including fish length, total fish wet weight, wet liver weight, and wet gonadal weight, were taken from sacrificed individuals as well. Fecundity and fertilization data were collected each day throughout the experiment until each generational sacrifice. Sperm parameters such as sperm count, sperm motility, and sperm progression were measured at each generation sacrifice after the testes were collected from reproductively mature fish. All F2 data that were significantly different from the control results were deemed transgenerational

Animal Care and Handling

All animal procedures were conducted in accordance with the methods described by the American Institute of Fishery Research Biologists (AIFRB) (Jenkins *et al*., 2014); and with all US Geological Survey CERC guidelines for the humane treatment of test organisms during culture and experimentation. Experimental protocols and the study plan was approved by the USGS-CERC Institutional Animal Care and Use Committee and the Institutional Animal Care and Use Committee (IACUC) of the University of Missouri.

Exposure and Maintenance of Fish

The Hd-rR inbred wildtype strain of medaka were cultured at the USGS-CERC. Ten adult medaka, 2 males and 8 females, were placed into each of four broodstock tanks so that each tank possessed a breeding group ratio of one male to four females. These breeding groups were used to produce embryos for exposure. For the current study, fertilized eggs (F0) were collected from approximately 40 total reproductively mature medaka. The fertilized eggs were pooled and randomly assigned to 15 glass petri dishes. 50 fertilized eggs were transferred to each of these 15 petri dishes, all of which contained 50 mL of well water. Each petri dish became one of three replicates within one of five treatment groups, thus 750 fertilized eggs were distributed among fifteen petri dishes.

Both test chemicals, ATZ and EE2, were dissolved in conditioned water obtained from the CERC deep tube well. The four experimental concentrations were 5 μg/L and 50 μg/L of ATZ (ATZ Low and ATZ High), and 0.002 μg/L and 0.05 μg/L of EE2 (EE2 Low and EE2 High), with one CERC well water control. Approximately 8 hours after fertilization (haf), embryos to be treated had their well water replaced with a

corresponding solution of ATZ or EE2 dissolved in well water. The low concentrations (ATZ Low and EE2 Low) are environmentally realistic contaminant levels and the high concentrations (ATZ High and EE2 High) are those reached within seasonal surges, during peak crop application season of ATZ from mid-March until early or mid-June, depending on the crop. Embryos were exposed from 8 haf until 12 daf so that the treatment spanned both gonadogenesis and germ cell differentiation (Kondo *et al*., 2009). 40 mL of control and treatment solutions were replaced daily until 12 daf, when most embryos had hatched. Upon cessation of the chemical exposures for F0 fish at 12 daf, no further exposures were performed for this or any subsequent generation. All juvenile fish, or fry, from each replicate were transferred to a corresponding floating meshed container after 12 daf, and all three replicates of a treatment regimen were placed in the same 10 L aquarium with flow-through water and aeration. Thus, there were approximately 50-70 fry per treatment with 17-23 fry per replicate in floating meshed containers. 30 daf the fry were transferred from the floating meshed containers to aquaria and maintained separately based on their exposure. The mature fish of each replicate were split into two tanks to ensure maximal egg production and prevent overcrowding. Thus, each replicate had a tank of approximately 4 fish and another tank consisting of approximately 12 fish. The 4 treatment lineages (ATZ Low, EE2 Low, ATZ High, and EE2 High) and the control lineage were cultured until reproductively active adults were produced in the F3 generation (Fig. 1).

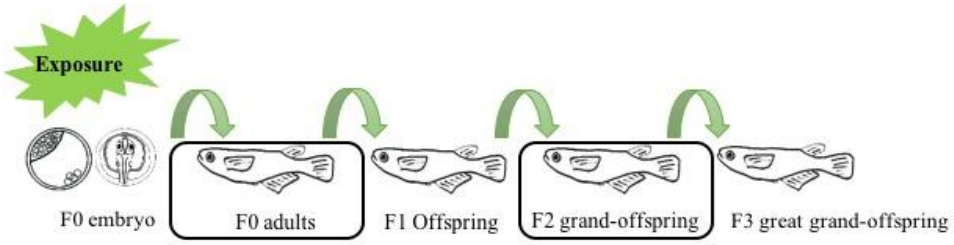

Figure 1. A Schematic Diagram Outlining the Maintenance of the Various Treated or Control Medaka Lineages. Only F0 embryos were exposed to treatment solutions. The F0 embryos were reared into reproductively mature adults and their fertilized eggs were collected from each treatment lineage to produce the subsequent generations as indicated above. Boxes indicate the adults produced from exposed embryos (F0) and the first generation in our experiments to demonstrate transgenerational phenotypes (F2).

Atrazine Dosing Solution and Uptake Measurement

The concentration of ATZ in the exposure solution at the onset of and after the exposure was quantified by enzyme linked immunosorbent assay (ELSIA) accordingly to Papoulias *et al*., 2014. The amount of EE2 uptake by medaka embryos, using the same nominal concentrations, was previously quantified using the same methods before my experiment began (Bhandari *et al*., 2015). In my investigation, we radioactively labeled ^{14}C-ATZ to measure ATZ uptake in medaka embryos by scintillation counting. Fertilized eggs were collected from the same breeding groups that produced the embryos for ATZ and EE2 exposure experiments. ^{14}C-ATZ dosing solutions were prepared at 5 μg/L (ATZ Low) and 50 μg/L (ATZ High) in CERC well water. 50 fertilized eggs were designated as control, ATZ Low, and ATZ High treatments. Each treated group was split into three replicates so that each replicate contained approximately 16 embryos in a 100 mL petri dish filled with 50 mL of the appropriate dosing solution. 25 mL of each dosing solution was replaced daily throughout the 12 day exposure period. At 24 hours, 8 days, and 12

days, the dosing solutions and embryos (some were fry by this period) were transferred to scintillation vials and measured separately to account for all radioactively labeled ^{14}C-ATZ. The combined embryos and fry were washed three times with a scintillation cocktail and the washes were also collected for scintillation counting. The scintillation cocktail was a formulation of solvents and scintillators which fluoresce when excited with ionizing radiation (REF) After the washes, embryos and fry were transferred into 5 mL of scintillation cocktail and homogenized. The homogenizer was also washed with water and 1 mL from each wash was mixed with 5 mL of the scintillation cocktail. ^{14}C-ATZ concentrations were then measured by a scintillation counter from the exposure media, embryos, fry, and the washes from the embryos and homogenizer. Final uptake (pg/mg egg or embryo) was calculated at three time points; at 1, 8, and 12 daf. These time points were chosen because they are at 24 hours after treatment, at standard hatching time for medaka (8 days), and the final day of exposure in our other experiments.

Medaka Lineage Maintenance and Phenotype Characterization

All the fish were maintained on a 14L:10D photoperiod with water temperature at 25 ± 0.5°C. Each tank was equipped with an overflow outlet and air supply. After collection of fertilized eggs for the next generation, the reproductively active adults were sacrificed for tissue collection (Fig. 1). Fecundity, fertilization rate, and embryo survival were recorded daily for the F0 through F3 generations. Fecundity was calculated as the total egg production by each breeding group/day, and the fertilization rate was calculated as the percentage of fertilized eggs within the combined daily egg masses removed from female medaka and eggs collected at the bottom of the tanks. The total number of eggs

from each tank was counted, and the number of eggs was divided by the number of females that produced eggs on the day they were collected. Embryo survival was determined as the number of eggs that successfully completed development through hatching at 10 daf. Body length, wet body weight, wet gonad weight, and wet liver weight were recorded when the fish were sacrificed. The weight of the fish and their tissues was recorded in milligrams (mg) to the nearest thousandth after the decimal (i.e, 0.001). Tissue samples of the gonads and brains with the pituitaries attached were collected and preserved in 500 μL RNA*later* ™ Stabilization Solution (ThermoFisher Scientific, SKU # AM7021) for nucleic acid isolation, according to the manufacturer's instructions. Sperm parameter analysis was performed at the laboratory of Dr. Yuksel Agca at the Department of Veterinary Pathobiology, University of Missouri. Collected testes were immediately transported to the Agca Laboratory which is approximately 5 miles from the CERC, and the sperm were activated with a buffer solution before computer assisted sperm analysis (CASA) was performed. CASA measures the number of sperm, motility, and forward progression using a high resolution microscope connected to a computer equipped with software to analyze the data. The sperm analysis was completed within 2-4 hrs of isolation of the testes.

RNA and DNA Isolation from Whole Tissue

We extracted the gonads and brains with the pituitaries attached from the largest fish in each treatment group with 5 males and 5 females used from each treatment replicate during the second spawning event. Whole tissues were collected from each generation from F0 through F3. All fish were euthanized with an MS-222 solution prior

to tissue extraction as required by the IACUC protocol. Gonads and brains were extracted from fish and transferred into RNA*later* ™ Stabilization Solution and the samples were kept at -20°C until nucleic acid isolation.

All whole tissue samples were homogenized for RNA and DNA isolation following the Zymo Research Z-R Duet™ MiniPrep Kit (Zymo Research, SKU# D7003) protocol. Samples were treated with DNAse I and proteinase K with the following amendments: tissues were transferred from RNA*later* ™ Stabilization Solution to 300 µl of lysis buffer in a 1.5 mL microcentrifuge tube and minced manually with a pestle before homogenization with the Benchmark D1000 handheld homogenizer, utilizing three 4 second pulses. Samples were briefly centrifuged after homogenization and subjected to RNA and DNA analysis. The RNA concentration and quality were assessed with a NanoDrop™ ND-2000 spectrophotometer and bleach gel electrophoresis (Aranda *et al*., 2012), respectively. The DNA concentration and quality were evaluated using only the NanoDrop™ ND-2000 spectrophotometer.

Reverse Transcription and Quantitative Gene Expression

The isolated RNA was reverse transcribed into complementary DNA (cDNA) using an Applied Biosystems' High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, CAT# 4368814) by following the standard manufacturer's standard protocol. cDNA synthesis was performed using an Applied Biosystems' SimpliAmp Thermal Cycler. Power-Up™ SYBR® Green Master Mix reagents (Applied Biosciences, CAT# A25742) were used to assess gene expression with primers specific to medaka

genes of interest. Messenger RNA (mRNA) levels were quantified by the quantitative polymerase chain reaction (qPCR) $^{\Delta\Delta}$Ct method and the expression data were presented as fold-change against the control.

Enzyme Linked Immunosorbent Assay (ELISA) and 5-Methylcytosine Quantification

The isolated genomic DNA from the ovaries and testes was assayed for total genome methylation with the Zymo Research 5-mC DNA ELISA Kit (Zymo Research, SKU# D5326) following the manufacturer's standard protocol. DNA isolated from the gonads of each of the 5 fish in a treatment replicate were pooled together and 100 ng aliquots of pooled DNA were tested twice, so that each treatment required six wells on a 96-well ELISA plate. Color was developed for approximately forty minutes before absorbance was measured with a BioTek™Microplate Reader at 405-450 nm.

Statistical Analyses

This study was designed to examine the exposure-induced transgenerational differences in phenotypes and molecular data points, including gene expression and DNA methylation (Table 1). Statistical analysis of ATZ uptake, gonadosomatic index (GSI) and hepatosomatic index (HSI), fecundity, fertilization, sperm parameters, and gene expression was compared between the control and treatment groups within the same generation, as well as between the high treatments and between the low treatments of ATZ and EE2 (i.e. ATZ Low vs. EE2 Low) of the same generation. Each replicate (tank) contained the data from five individual fish. Altogether, three biological replicates were used, each containing fifteen fish per treatment. The responses of the five fish within each tank were averaged for gene expression analysis, so that each tank was a replicate for

statistical purposes (n=3, per treatment). Means were analyzed using two-sample t-tests assuming unequal variance between independent treatment and control groups, the two high treatment groups (ATZ High vs. EE2 High), and the two low treatment groups (ATZ Low vs. EE2 Low) (Microsoft Excel, Data Analysis ToolPak). The confidence level of statistical analysis was $\alpha = 0.05$, and the data are presented graphically as the means ± standard error of the means (SEM). Importantly, the low n=3 value for analysis affected the statistical power and increased the SEM, thus if two SEM bars on a graph do not overlap it is not indicative of statistically different means. Differences between two generations were not taken into account. The graphs were generated using GraphPad Prism Software.

Table 1. Table of the Phenotypic and Molecular Data Points that were Measured in the F0 and F2 Generation Medaka. Next to each type of datum is an explanation of how it was characterized.

Experimental Measurements and Data	Measurement and Data Points
Atrazine Uptake	pg/mg egg or embryo
Gonadosomatic Index (GSI)	mg gonad/mg body
Hepatosomatic Index (HIS)	mg liver/mg body
Fecundity	# of eggs per mass/female/day+ # of eggs on tank floor/day
Fertilization Rate	# of fertilized eggs within total # of eggs/day
Sperm Count	Total sperm per sample
Motile Sperm	Number of motile sperm per sample
Percent Sperm Motility	Percentage of motile sperm per sample
Sperm Progression	Number of Sperm moving forward rather than in a spinning motion or immobile
Gene Expression	$^{\Delta\Delta}$Ct method: fold change difference in gene expression compared between each treatment and the control.
Enzyme Linked Immunosorbent Assay (ELISA)	Spectrophotometric values of absorbance indicating global DNA methylation.

CHAPTER III

RESULTS

Exposure Concentration and Uptake

The ATZ concentrations in each exposure medium were measured (Fig. 2A). These concentrations ranged from 3.41 to 4.79 μg/L for the ATZ Low group (5 μg/L) and from 48.50 to 58.01 μg/L for the ATZ High group (50 μg/L). These data indicated that the 5 μg/L exposure concentrations were actually 18-32% below nominal concentration while the 50 μg/L treatments were 5-16% greater than the nominal concentration (Fig. 2A). ATZ uptake by the embryos during the experimental period was between 15.80 and 22.94 pg/mg for the ATZ Low group and between 207.96 and 221.62 pg/mg for ATZ High group (Fig. 2B). These data suggest that ATZ uptake by the embryo reaches a maximal concentration within 24 hours of exposure and remains stable thereafter. There were no differences in solvent well water control or uptake of ATZ between the replicates.

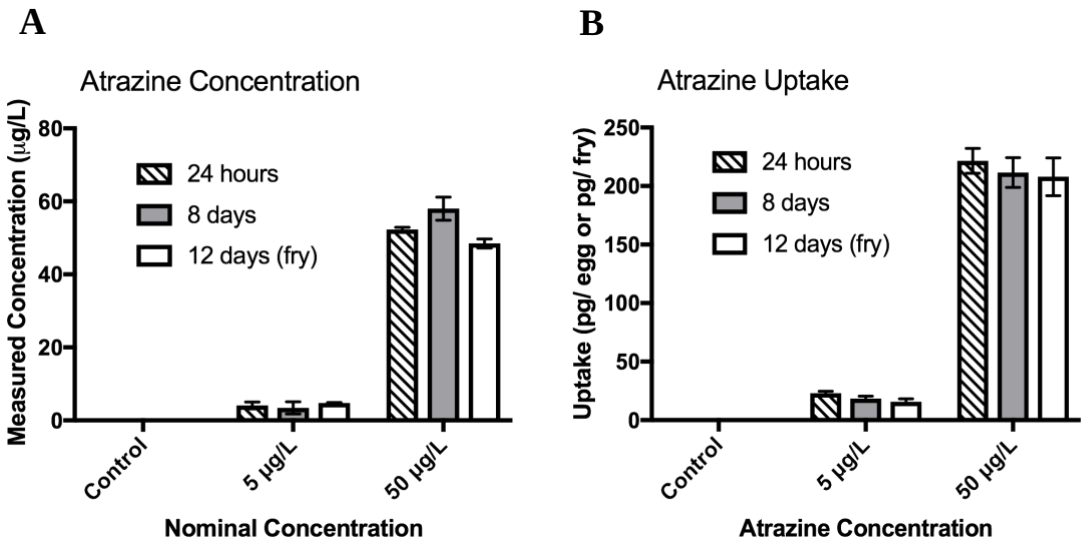

Figure 2. **Exposure Concentration of ATZ at the Onset of and After Exposure (A) and Uptake of ATZ by Embryos or Fry at Three Different Time Points (B).** The actual concentration in the ATZ Low group (5 µg/L) was below nominal concentration while the ATZ High group (50 µg/L) was above nominal concentration, and neither changed throughout the experiment. Maximal ATZ uptake for both the ATZ Low and ATZ High groups was reached at 24 hours and did not significantly change during the 12-day exposure period. The error bars represent the mean ± SEM, n=3 per treatment.

Fish Length and Weight

The final lengths and weights of sacrificed fish were measured after euthanization but prior to tissue removal. No significant differences were observed in the bodily weights or lengths of either male or female medaka fish due to any treatments when compared to the control groups in both generations (Fig.3A-D). Furthermore, there were no significant differences between the lengths and weights in low concentration treatment groups, EE2 Low vs. ATZ Low, or between the high concentration treatment groups, EE2 High vs. ATZ High (Fig.3A-D).

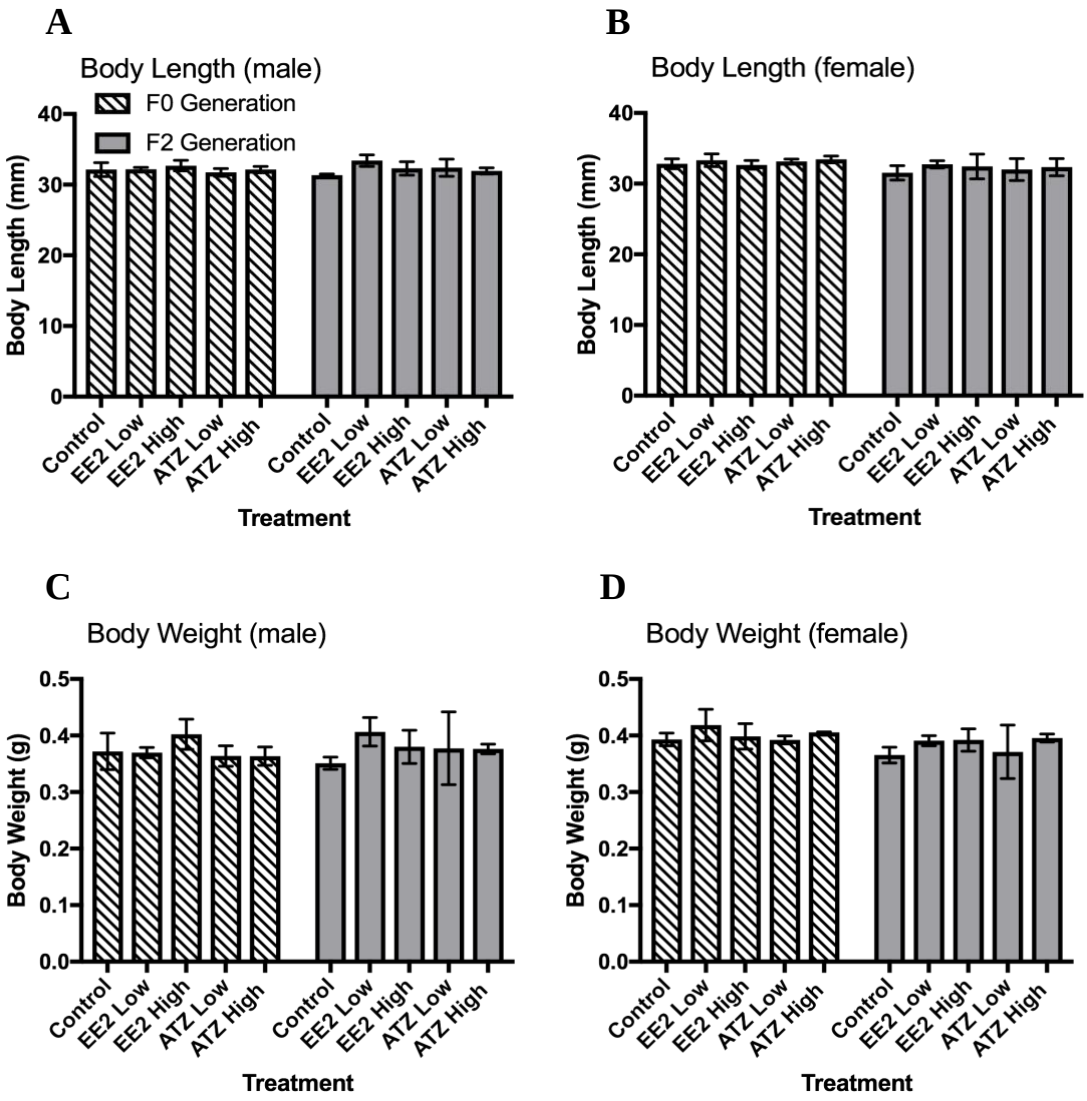

Figure 3. Morphological Assessment of Sexually Mature Medaka Body Length for Males (A) and Females (B), and Total Body Weight for Males (C) and Females (D). Length was measured from the rostral snout to the end of the caudal fin. No significant differences were observed in body length or weight for male or female medaka in either generation due to any treatments compared to the control groups. The error bars represent the mean ± SEM, n=3 per treatment except for the F2 generation ATZ Low (n=2).

Body Somatic Indices

The gonadosomatic index (GSI) was not significantly different among any treatment lineage for F0 or F2 generations in male or female medaka (Fig. 4B, D). Similarly, the hepatosomatic index (HSI) was not significantly different in F0 or F2 male fish for any treatment lineages (Fig. 4A). There were significant reductions in the HSI of the EE2 Low ($p < 0.05$), ATZ Low ($p < 0.05$), and ATZ High ($p < 0.01$) treatment lineages when compared to the control group within the female F2 generation (Fig. 4C). Neither HSI nor GSI were significantly different within the male F0 and F2 generations between any treatment lineage and the control (Fig. 4A, B). There were no significant differences in the males or the females for either the HSI or GSI between the low concentration treatment groups, EE2 Low vs. ATZ Low, or between the high concentration treatment groups, EE2 High vs. ATZ High (Fig.4A-D).

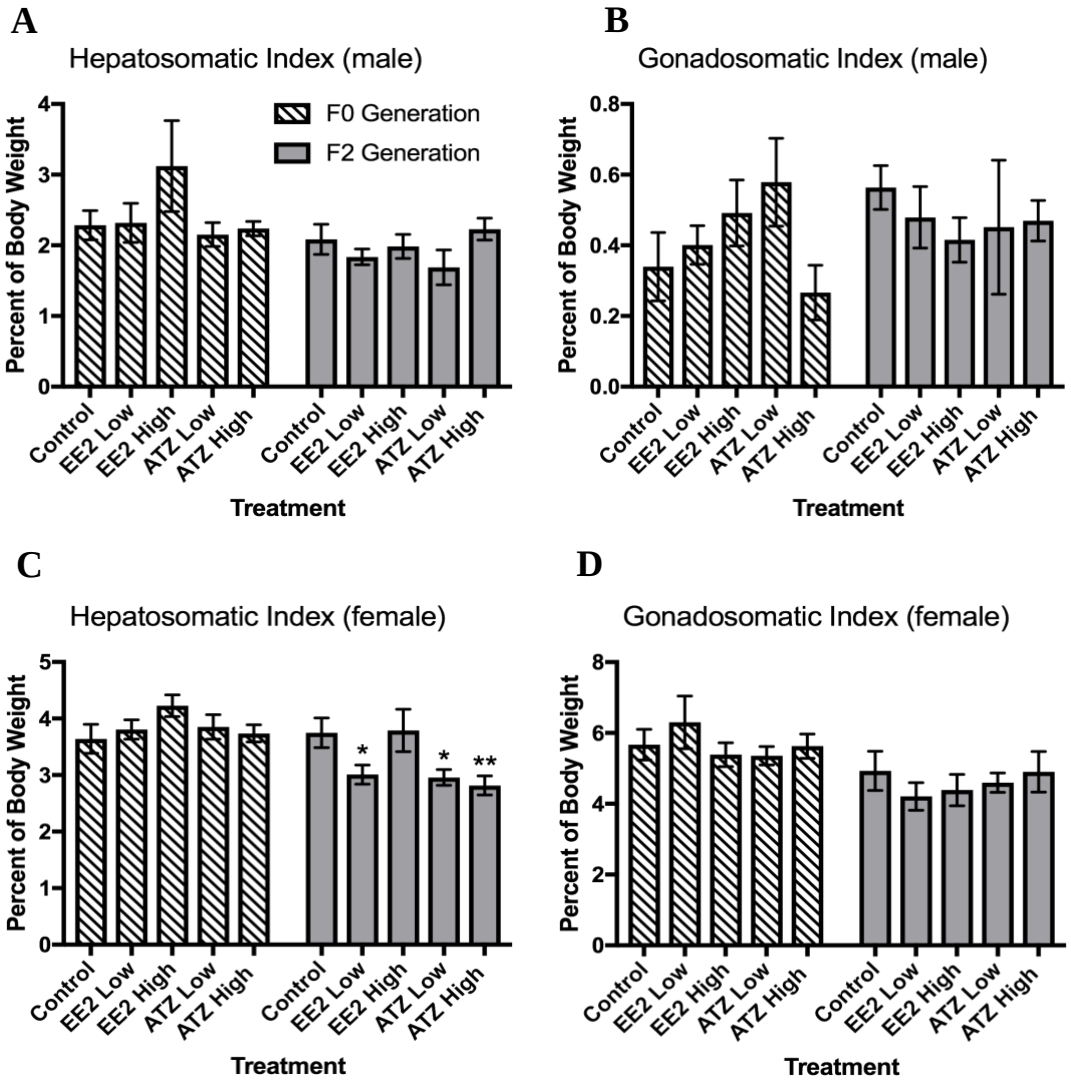

Figure 4. Transgenerational Differences in Hepatosomatic and Gonadosomatic Indices of Male (4A and 4B) and Female (4C and 4D) Medaka. The F0 and F2 generation males did not demonstrate significant differences in HSI or GSI between the control and treatment lineages (4A and 4B). The F0 and F2 female GSI did not exhibit changes (4D), but there were significant HSI (4C) decreases in the low treatment lineages ($p<0.05$), and in the ATZ High lineage ($p<0.01$). Asterisks indicate statistical significance compared to the control (*, $p<0.05$; **, $p<0.01$). The error bars represent the mean ± SEM, n=3 per treatment except for the F2 generation ATZ Low (n=2).

Fecundity and Fertilization

The fecundity, or total number of eggs, of medaka exposed to ATZ was statistically similar to the fecundity of unexposed control medaka (Fig. 5A). This was observed in both the F0 and F2 generations. The fertilization rate was only significantly increased in the ATZ High treatment lineage ($p < 0.05$) compared to the control group within the F0 generation (Fig. 5B). The difference in the mean fertilization success between the F0 generation control and ATZ High lineages was approximately 14.31 fertilized eggs/day, or a 15.27% increase in the number of fertilized eggs in the F0 generation ATZ High lineage. The number of fertilized eggs was significantly reduced in the F2 generation in the EE2 Low ($p < 0.05$) and ATZ Low ($p < 0.05$) lineages compared to the control lineage (Fig. 5B), indicating the existence of transgenerational impacts on fertilization success of future generations. The EE2 Low lineage produced 20.65 fewer fertilized eggs/day, or a decrease of 22.42%, while the ATZ Low lineage produced 16.93 fewer fertilized eggs/day, or a decrease of 18.38%, compared to the control lineage. The fecundity and fertilization rate were statistically similar between the low concentration treatment groups, EE2 Low vs. ATZ Low, and between the high concentration treatment groups, EE2 High vs. ATZ High (Fig.5A, B).

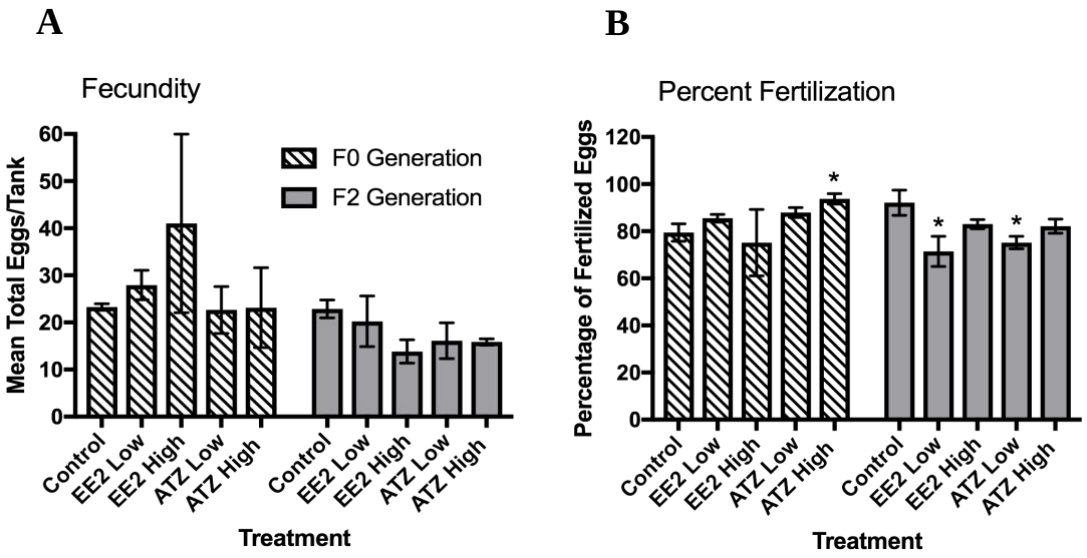

Figure 5. Transgenerational Differences in Fecundity (A) and Fertilization Rate (B) of the F2 Generation Adults Caused by ATZ Exposure to the Grandparent F0 Embryo. Fertilization success was increased by 15.27% in the F0 generation ATZ High treatment and declined by 22.42% and 18.38% in the F2 generation EE2 Low and ATZ Low lineages compared to the F2 control lineage. Asterisks indicate statistical significance compared to the control (*, $p<0.05$). The error bars represent the mean ± SEM, n=3 per treatment except for the F2 generation ATZ Low (n=2).

Sperm Parameters

Sperm count, number of motile sperm, sperm progression, and percentage of sperm motility were unaffected by all of the treatments in the F0 generation (Fig. 6A-D). There were significant reductions in the total sperm count in the F2 EE2 Low ($p < 0.05$) and in the ATZ Low treatment lineages ($p < 0.01$) by 32.49% and 48.82%, respectively, when compared to the F2 control lineage (Fig. 6A). The number of motile sperm was reduced in the F2 generation EE2 Low ($p < 0.05$), ATZ Low ($p < 0.05$), and ATZ High lineages ($p < 0.05$) by 55.15%, 57.79%, and 55.28% (Fig. 6B). Sperm forward progression was significantly reduced in the F2 generation EE2 Low ($p < 0.05$) and ATZ

Low treatment lineages ($p < 0.05$) by 53.50% and 50.21% (Fig. 6C). The percentage of sperm motility was significantly reduced in the F2 generation EE2 Low ($p < 0.05$) and ATZ High ($p < 0.05$) treatment lineages by 32.14% and 38.27% (Fig.6D).

The sperm count was calculated as the number of sperm per sample in millions of sperm per milliliter (Fig. 6A). The number of motile sperm was determined as the number of sperm in millions per milliliter that were moving, but the type of movement was not characterized (i.e, wiggling or forward movement) (Fig. 6B). Sperm progression is the number of sperm in millions per milliliter that are moving forward, rather than in circles or in an irregular pattern of movement (Fig. 6C). Forward movement does not indicate that there were particular directions the sperm moved in, but rather they were flagellating forward instead of left or right from their individual points-of-origin. The percentage of sperm motility is the number of motile sperm within the sample divided by the total number of sperm (Fig. 6D).

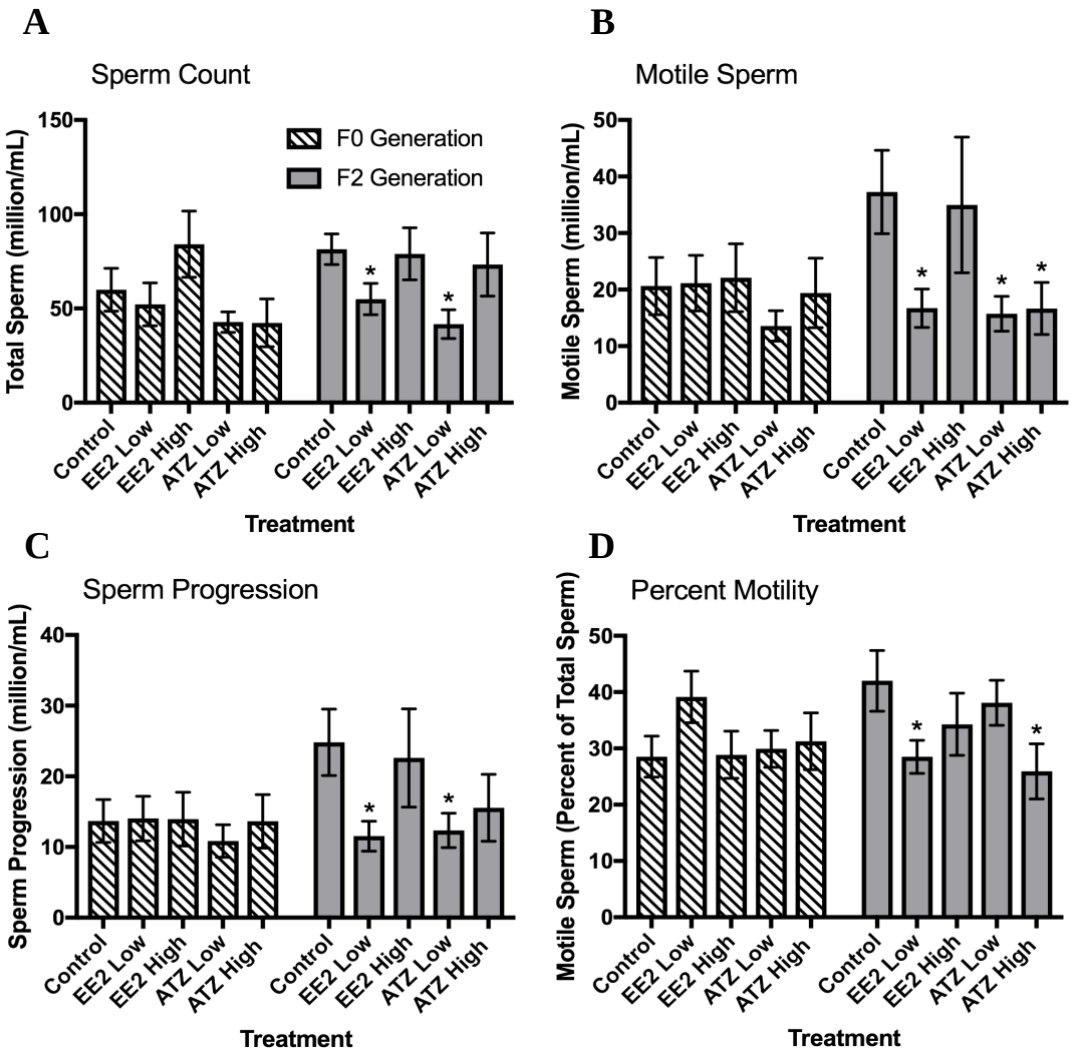

Figure 6. **Transgenerational Differences in Total Sperm Count (A), Motile Sperm (B), Sperm Progression (C), and Percent Sperm Motility (D) in Adult Males of the F2 Generation Caused by ATZ Exposure to the Grandparent F0 Embryos.** The decrease in mean sperm count in the F2 generation of the EE2 Low and ATZ Low treatment groups was between 26.46 and 39.76 million sperm per milliliter (A). There were mean decreases ranging from 20.55 to 21.54 million motile sperm per milliliter in the F2 generation EE2 Low, ATZ Low, and ATZ High lineages (B). The decreased mean number of sperm demonstrating forward progression in the F2 generation EE2 Low and ATZ Low lineages was 13.27 and 12.46 million per milliliter (C). The percentage of motile sperm per milliliter was also decreased in the F2 generation EE2 Low and ATZ High lineages by 32.14% and 38.27% (D). Asterisks indicate statistical significance compared to the control (*, $p<0.05$). The error bars represent the mean ± SEM, n=3 per treatment except for the F2 generation ATZ Low (n=2).

Gonadal Gene Expression Changes

The transgenerational gene expression profiles for male and female gonads (Fig. 7, 8). *Fsh-r* expression was reduced in each treatment group in the male F0 generation compared to the controls, while expression was increased in the male F2 generation EE2 Low ($p < 0.05$) and EE2 High ($p < 0.05$) lineages (Fig. 7A). The F0 generation EE2 Low and ATZ Low treatment groups were also statistically different ($p < 0.05$) (Fig. 7A). All of the other comparisons of *fsh-r* gene expression between treatment groups were statistically similar in both the F0 and F2 generations (Fig, 7A). *Ar*α expression was upregulated in the male F0 generation EE2 Low treatment ($p < 0.001$) and downregulated in the male F0 generation ATZ High treatment ($p < 0.05$) (Fig. 7B). In the F0 generation, *ar*α gene expression among the EE2 Low and ATZ Low treatment groups was statistically different ($p < 0.05$) (Fig. 7B). The *ar*α gene expression in the F2 generation was statistically similar between all of the treatment lineages and the control lineage, as well as between the low treatment lineages and between the high treatment lineages (Fig. 7B). *Star* expression was significantly reduced in the male F0 generation ATZ High treatment ($p < 0.05$), but the expression was upregulated in the male F2 generation ATZ Low ($p < 0.05$) and ATZ High lineages ($p < 0.05$) when compared to the controls (Fig. 7C). The *star* gene expression profiles were statistically similar between the low concentration treatment groups, EE2 Low vs. ATZ Low, and between the high concentration treatment groups, EE2 High vs. ATZ High (Fig. 7C).

Star expression was statistically similar between all treatment groups and the controls in the F0 and F2 generation females (Fig. 8A). The *star* expression was significantly different between the F2 generation EE2 Low and ATZ Low treatment lineages ($p < 0.05$) (Fig. 8A). There was an upregulation in *cyp19a1a* expression in the female F0 generation EE2 High ($p < 0.05$) and ATZ High ($p < 0.05$) treatments, while there were no transgenerational differences in the F2 generation between treatment and control groups (Fig. 8B). *Cyp19a1a* gene expression was statistically similar between the low concentration treatment groups, EE2 Low vs. ATZ Low, and between the high concentration treatment groups, EE2 High vs. ATZ High, in both the F0 and F2 generations (Fig. 8B). *Er*α was significantly downregulated in the ATZ High ($p < 0.05$) treatment group of the F0 generation females (Fig. 8C). There were no other significant differences in *er*α expression between high and low treatment groups or between treatment and control groups (Fig. 8C). All *lh-r* gene expression profiles were statistically similar between treatment and control groups within the F0 and F2 generation females (Fig. 8D).

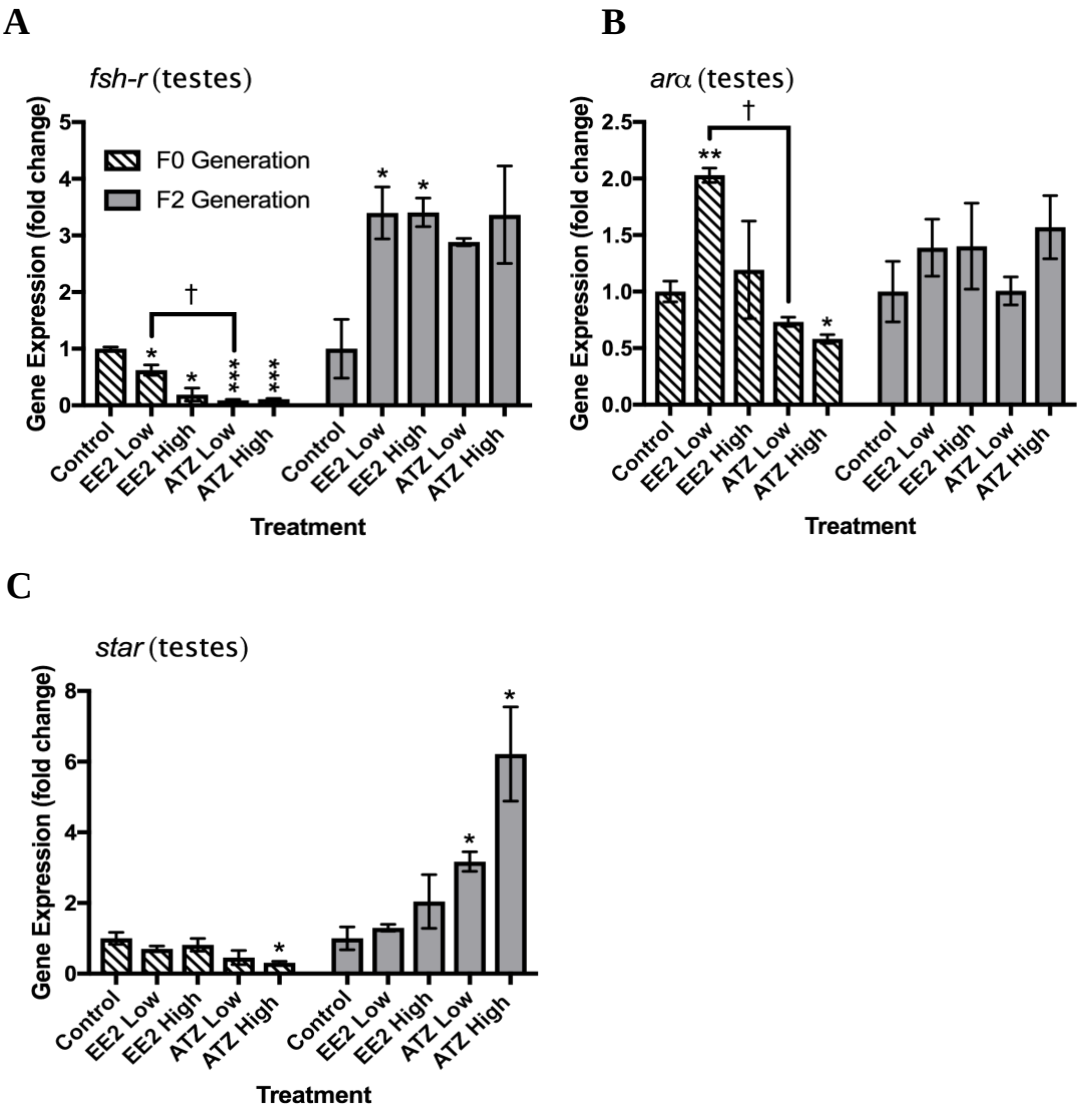

Figure 7. Transgenerational Differences in *fsh-r* (A), *arα* (B), and *star* (C) Gene Expression in Adult Male Testes. *Fsh-r* expression was reduced in the EE2 Low ($p<0.05$), EE2 High ($p<0.05$), ATZ Low ($p<0.001$), and ATZ High ($p<0.001$) treatment groups compared to the F0 controls (Fig. 7A). Asterisks indicate statistical significance compared to the control (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). Crosses indicate statistically significant differences between low concentration treatments (EE2 Low vs. ATZ Low) or between high concentration treatments (EE2 High vs. ATZ High) (†, $p<0.05$). The error bars represent the mean ± SEM, n=3 per treatment except for the F2 generation ATZ Low (n=2).

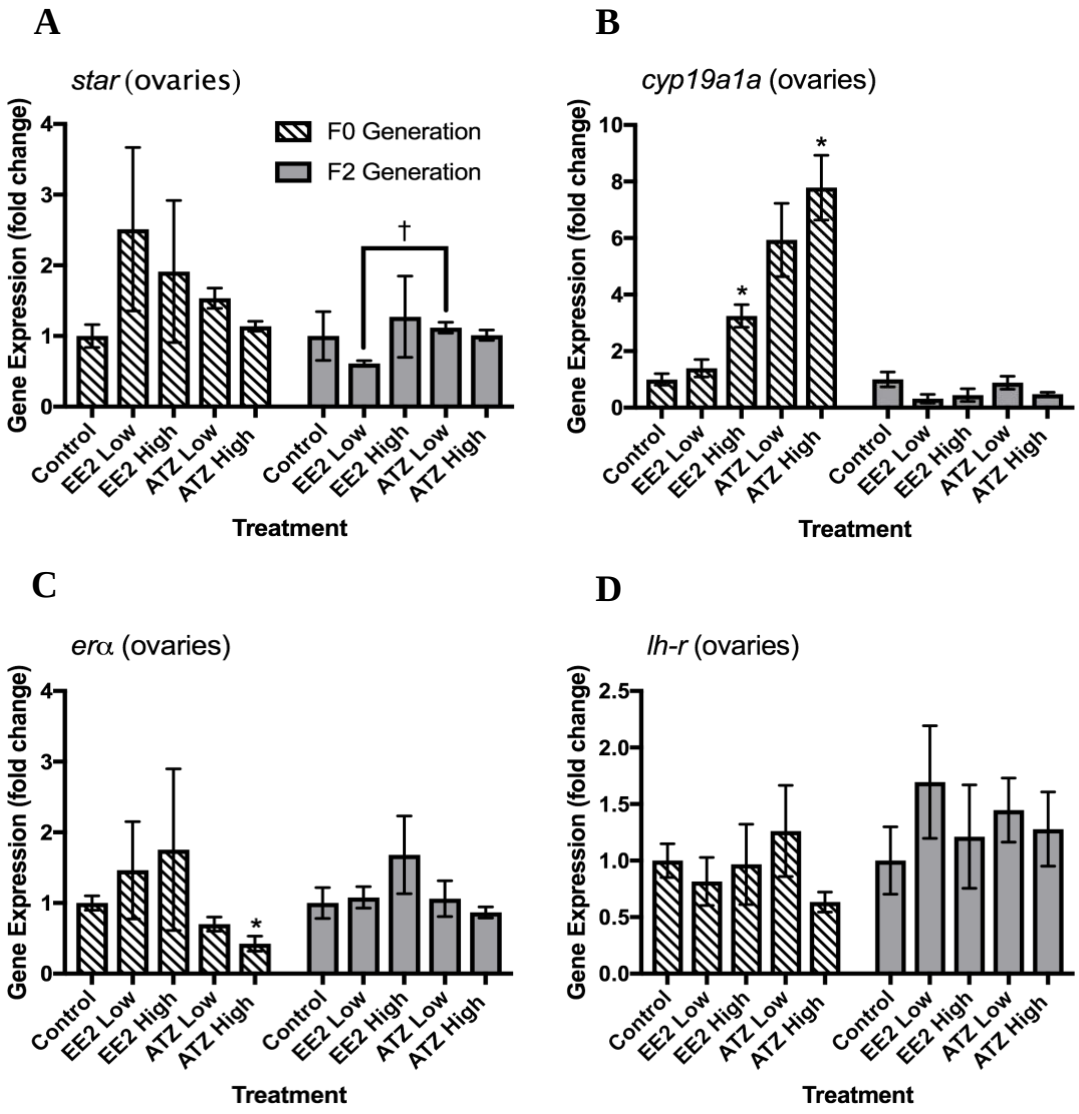

Figure 8. Transgenerational Differences in *star* (A), *cyp19a1a* (B), *erα* (C), and *lh-r* (D) Gene Expression in Adult Female Ovaries. There were no transgenerational changes in gene expression in the analyzed ovarian genes (Fig. 8). Asterisks indicate statistical significance compared to the control (*, $p<0.05$). Crosses indicate statistically significant differences between low concentration treatments, EE2 Low vs. ATZ Low, or between high concentration treatments, EE2 High vs. ATZ High, (†, $p<0.05$). The error bars represent the mean ± SEM, n=3 per treatment except for the F2 generation ATZ Low (n=2).

Brain Gene Expression Changes

The transgenerational gene expression profiles for male and female brains (Fig. 9, 10). *Kiss1* was significantly upregulated in the male F0 generation EE2 Low ($p < 0.01$) and ATZ Low ($p < 0.01$) treatment groups compared to the control group (Fig. 9A). The male *kiss1* gene expression profiles were also statistically different between the EE2 Low and ATZ Low treatment groups ($p < 0.01$) (Fig. 9A). *Kiss2* expression was increased in the male F2 generation EE2 High ($p < 0.05$) and ATZ Low ($p < 0.05$) treatment lineages compared to the control lineage, while there were no significant differences between the high concentration and low concentration treatment lineages (Fig. 9B). Similar to *kiss1*, *gpr54-1* expression was significantly increased in the male F0 generation EE2 Low ($p < 0.05$) and ATZ Low ($p < 0.01$) treatments compared to the control group, and the EE2 Low and ATZ Low treatment groups ($p < 0.001$) were also statistically different (Fig. 9C). There was only an increase in male F2 generation *gpr54-1* gene expression in the EE2 High ($p < 0.01$) treatment lineage compared to the control lineage (Fig. 9C). *Gpr54-2* expression was unaffected by all treatments in the male F0 generation compared to the control group (Fig. 9D) However, the *gpr54-2* expression in the EE2 High and ATZ High treatments of the male F0 generation were statistically different from each other ($p < 0.05$) (Fig. 9D). The F2 generation male *gpr54-2* expression was upregulated in the EE2 High ($p < 0.05$) and ATZ Low ($p < 0.01$) treatment lineages compared to the control lineage, and the EE2 Low and ATZ Low lineages were also significantly different from one another ($p < 0.05$) (Fig. 9D). There were no significant differences between treatment lineages and the control, high concentrations, or low concentrations in either *gnrh1* or

gnrh2 in the male F2 generation (Fig. 9E, F). *Gnrh1* was upregulated in the male EE2 Low ($p < 0.05$) and ATZ High ($p < 0.05$) treatment groups compared to the control (Fig. 9E). *Gnrh2* expression was increased in the male F0 generation EE2 Low ($p < 0.05$), ATZ Low ($p < 0.05$), and ATZ High ($p < 0.01$) treatments, but there were no significant transgenerational changes in *gnrh2* expression in the male F2 generation (Fig. 9F). The *gnrh2* EE2 High and ATZ High treatment lineages were also significantly different ($p < 0.05$) from each other (Fig. 9F).

Gnrh2 expression in the female F0 generation EE2 High ($p < 0.05$) treatment was significantly upregulated compared to the control (Fig. 10F). *Gpr54-2* expression was statistically different between the female EE2 Low and ATZ Low treatment lineages in the F2 generation, but there were no significant differences between treatment and control lineages (Fig. 10F). There were no other significant changes in gene expression in the female brain for either generation due to any of the treatments (Fig. 10A-E).

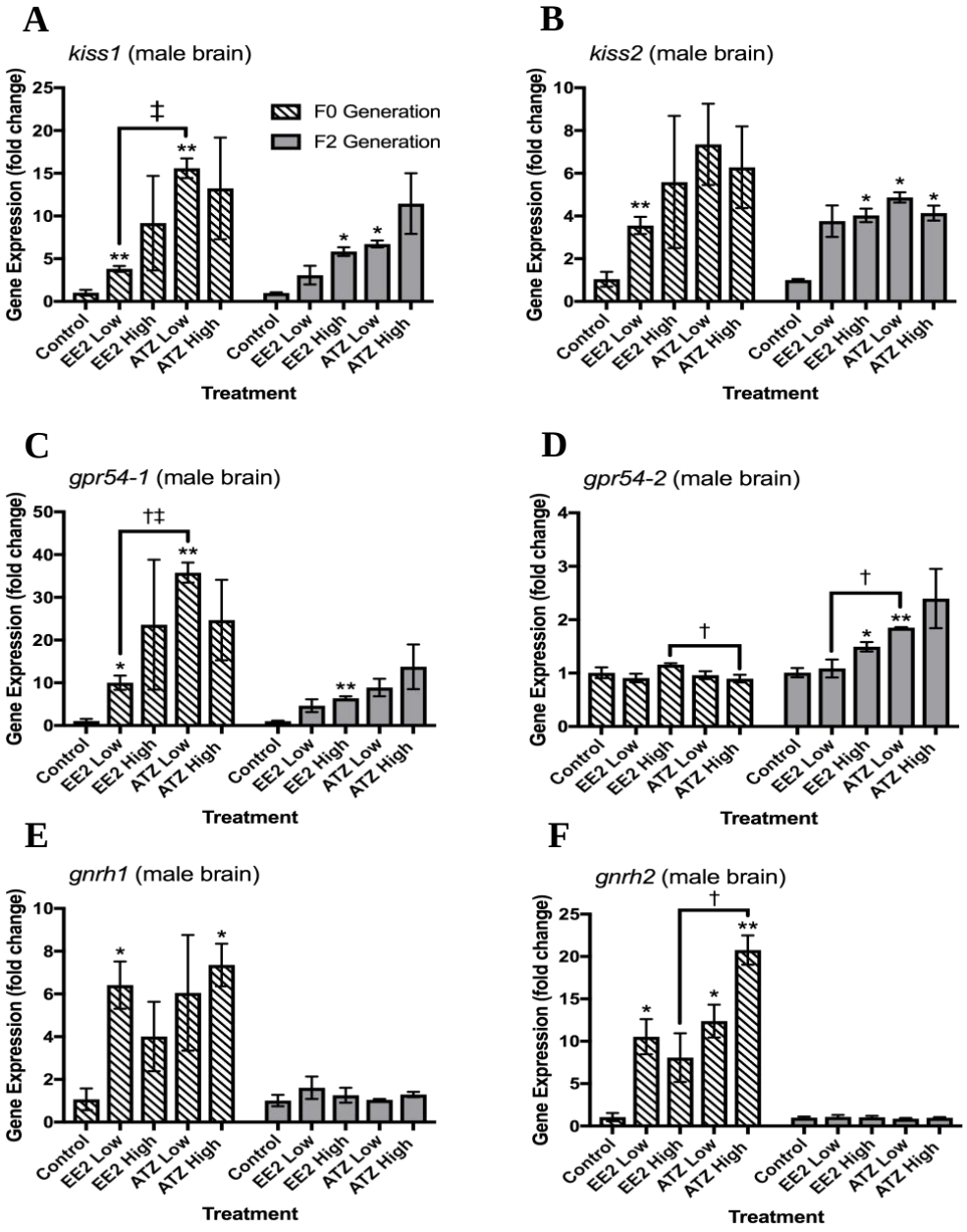

Figure 9. Transgenerational Differences in *kiss1* (A), *kiss2* (B), *gpr54-1* (C), *gpr54-2* (D), *gnrh1* (E), and *gnrh2* (F) Gene Expression in Adult Male Brains. Asterisks indicate statistical significance compared to the control (*, $p<0.05$, **, $p<0.01$). Crosses indicate statistically significant differences between low concentration treatments (EE2 Low vs. ATZ Low) or between high concentration treatments (EE2 High vs. ATZ High) (†, $p<0.05$; ‡, $p<0.01$, †‡, $p<0.001$). The error bars represent the mean ± SEM, n=3 per treatment except for the F2 generation ATZ Low (n=2).

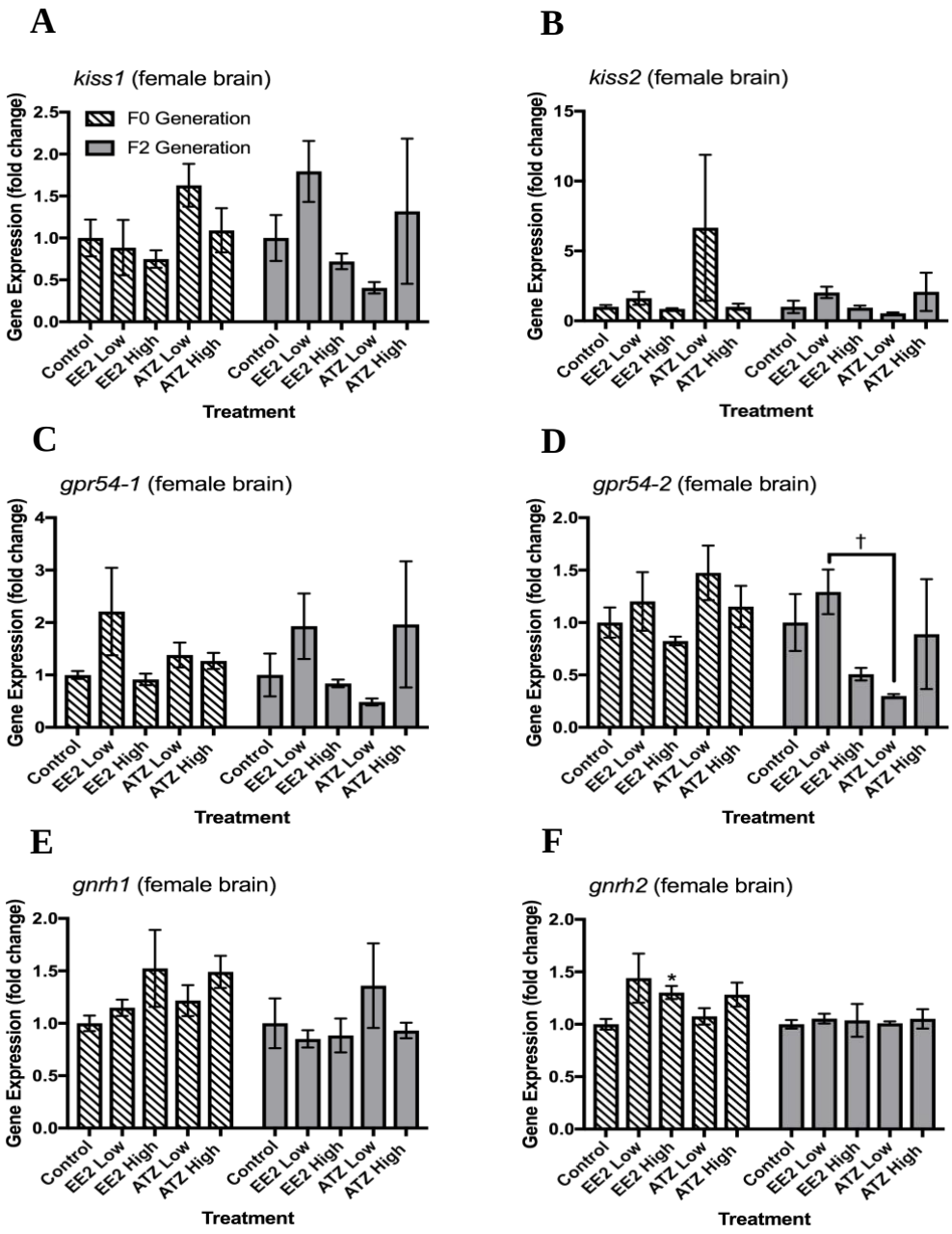

Figure 10. Transgenerational Differences in *kiss1* (A), *kiss2* (B), *gpr54-1* (C), *gpr54-2* (D), *gnrh1* (E), and *gnrh2* (F) Gene Expression in Adult Female Brains. Asterisks indicate statistical significance compared to the control (*, $p<0.05$). Crosses indicate statistically significant differences between low concentration treatments (EE2 Low vs. ATZ Low) or between high concentration treatments (EE2 High vs. ATZ High) (†, $p<0.05$). The error bars represent the mean ± SEM, n=3 per treatment except for the F2 generation ATZ Low (n=2).

Epigenetic (DNA Methylation) Genes

Dnmt3aa and *Dnmt1* expression was quantified as a measure of epigenetic changes in the brains and gonads of male and female medaka fish (Fig. 11A-F). *Dnmt1* expression was unaffected in the testes by any treatment group in the F0 generation, but there was an increase in expression in the testes of the F2 generation EE2 Low treatment ($p < 0.05$) (Fig. 11A). The expression of *dnmt1* was statistically different between the EE2 Low and ATZ Low ($p < 0.01$) treatment groups in the F0 generation testes (Fig. 11A). *Dnmt3aa* expression was significantly downregulated in the ATZ Low ($p < 0.001$) and ATZ High ($p < 0.01$) treatments in F0 generation testes, but it was unaffected by all treatments by the F2 generation (Fig. 11B). The F0 generation testes exhibited significant differences in *dnmt3aa* expression between the EE2 Low and ATZ Low ($p < 0.001$) treatment groups (Fig. 11B).

Dnmt1 expression in the ovaries of the F0 generation was significantly downregulated in the EE2 High ($p < 0.01$) and ATZ High ($p < 0.05$) treatments, while the downregulation in the ovaries of the F2 generation was in the ATZ Low ($p < 0.05$) lineage (Fig. 11C). *Dnmt3aa* expression in the ATZ Low ($p < 0.01$) and ATZ High ($p < 0.05$) F0 generation ovaries was significantly downregulated, and there were no observed transgenerational changes in *dnmt3aa* expression in the F2 generation ovaries (Fig. 11D). The EE2 Low and ATZ Low ($p < 0.05$) treatment groups in the F0 generation ovaries were also significantly different (Fig. 11D). *Dnmt1* expression in the male brain was unaffected by treatment in the F0 generation, but was significantly downregulated in the

male brain F2 generation EE2 Low ($p < 0.01$), EE2 High ($p < 0.01$), ATZ Low ($p < 0.05$), and ATZ High ($p < 0.01$) lineages (Fig. 11E). The *dnmt1* expression in the male brains was significantly different between the EE2 Low and ATZ Low ($p < 0.05$) treatment groups in the F0 generation (Fig. 11E). There were no significant changes in *dnmt1* expression in the female brain in either generation due to treatments (Fig. 11F).

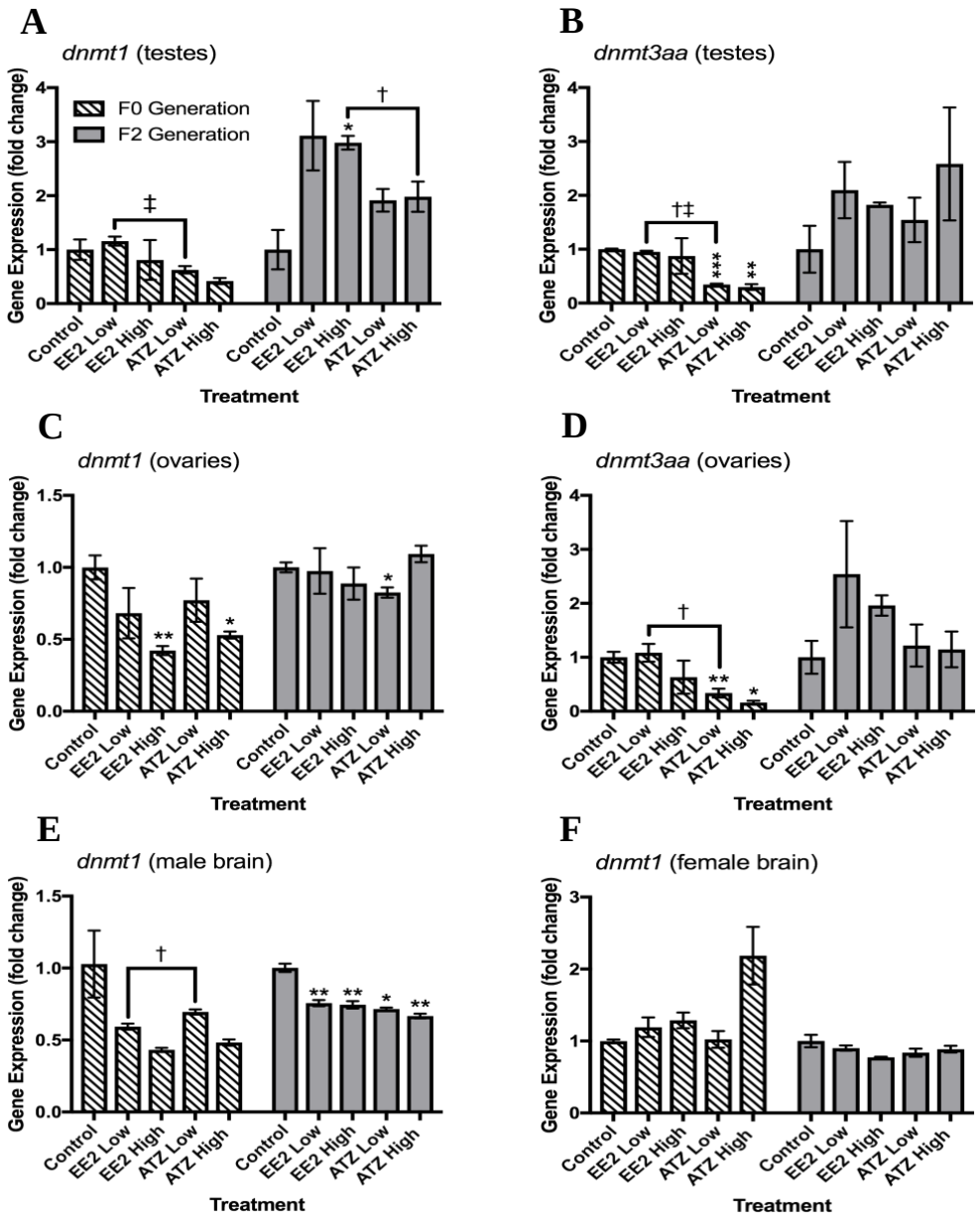

Figure 11. Transgenerational Differences in *dnmt1* Gene Expression in the Testes (A), Ovaries (C), Male Brain (E), and Female Brain (F), and *dnmt3aa* Expression in the Testes (B), and Ovaries (D). Asterisks indicate statistical significance compared to the control (*, $p<0.05$, **, $p<0.01$, ***, $p<0.001$). Crosses indicate statistically significant differences between low concentration treatments (EE2 Low vs. ATZ Low) or between high concentration treatments (EE2 High vs. ATZ High) (†, $p<0.05$; ‡, $p<0.01$, †‡, $p<0.001$). The error bars represent the mean ± SEM, n=3 per treatment except for the F2 generation ATZ Low (n=2).

Global DNA Methylation

Global DNA methylation of the testes and ovaries was quantified to determine if changes in the total gonadal methylome were observable (Fig.12A, B). Global methylation was reduced in the ATZ high treatment testes of the F0 generation ($p < 0.01$) (Fig. 12A). The global DNA methylation was statistically similar between all of the treatment groups and controls, as well as between the high concentration and low concentration groups (Fig. 12B). There were no significant transgenerational differences in global DNA methylation in either the testes or ovaries (Fig. 12A, B).

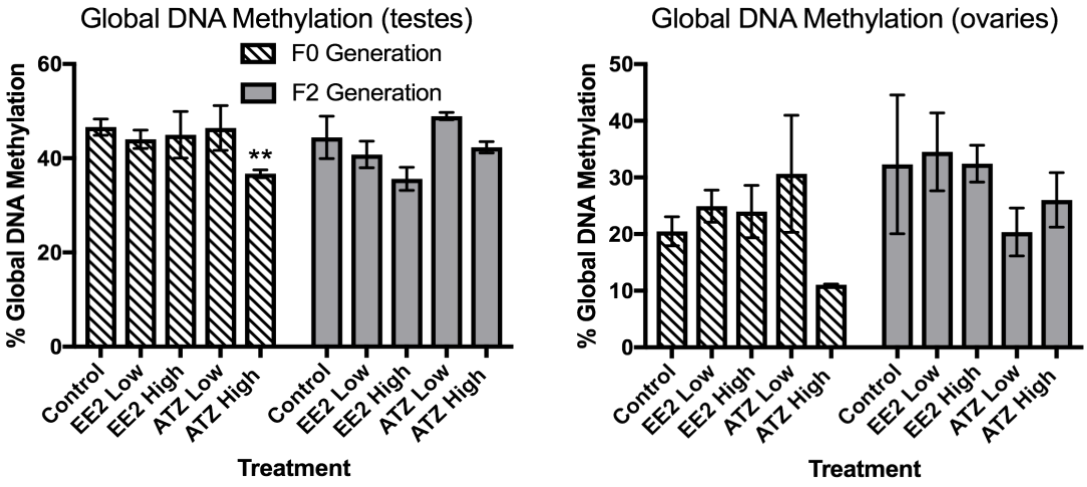

Figure 12. Transgenerational Differences in DNA Methylation in the Whole Testes (A) or Ovaries (B) in Medaka Fish. Asterisks indicate statistical significance compared to the control (*, $p<0.05$). The error bars represent the mean ± SEM, n=3 per treatment except for the F2 generation ATZ Low (n=2).

CHAPTER IV

DISCUSSION

In the United States, ATZ can be detected in surface water, with the highest concentration during the period of its application in the field. While it has been previously demonstrated that direct exposure to ATZ may pose minor risk to animals, it has not been assessed whether an environmentally relevant exposure model, such as a teleost fish, will transmit reproductive impacts to subsequent generations due to their early life exposure to ATZ. The present study, therefore, studied ATZ exposure effects in medaka embryos using two concentrations: one environmentally relevant and the other the concentration that can reach during seasonal surge. My results demonstrate that ATZ exposure to medaka embryos does not induce phenotypic abnormalities in the directly exposed generations, but impacts future generations by altering the reproductive capacity of fish at both the whole sperm and the molecular levels. Additionally, alterations in the expression of DNA methyltransferase genes (*dnmt3aa* and *dnmt1*) suggest the involvement of epigenetic transgenerational effects in the gonads.

An early life exposure to ATZ did not alter GSI (relative gonad weight) and fecundity in females in both immediate and future generations; very few studies have demonstrated developmental exposure effects in adulthood or in future generations. In zebrafish, ATZ exposure at a concentration of 30 parts per billion (30 ng/L) during the first 72 hours of life caused a significant decrease in spawning and a significant increase

in follicular atresia at adulthood, with structural defects in immediate offspring (Wirbisky *et al*, 2016). No change in body or testes weight, gonadosomatic index, testes histology, or levels of 11-ketotestosterone or testosterone were observed in the males with the same treatment (Wirsbisky *et al*., 2016). Because fewer studies focused on early developmental exposure effects in adults and offspring, it is difficult to compare discrepancies in effects of exposure that could be due to ATZ concentration differences, species-specificity and differences in early life history stages of the test animal.

There were no observed differences in fecundity between any treatment lineages and controls within either the F0 or F2 generation. However, grand-offspring (F2) of the EE2 Low and ATZ Low lineage medaka fish exhibited significantly reduced fertilization success compared to control lineage fish of the same generation. It is interesting to note that the transgenerational differences in fertilization success coincide with a transgenerational decrease in sperm quality within the same treatment lineages while there were no transgenerational impacts on fecundity. Because the exposed fish exclusively copulated with fish from the same exposure regimen, it may be possible that the observed decrease in fertilization success in the two affected treatment lineages is due to male reproductive impairment. My data demonstrate that the sperm count, number of motile sperm, and sperm progression were all significantly decreased in the F2 generation EE2 Low and ATZ Low lineage males. These data are in accordance with recent studies that suggest male reproductive function is more sensitive to endocrine disruptor insult (Nordkap *et al*., 2012). The Transgenerational impairment of fertilization success has

been experimentally demonstrated in marine medaka fish exposed to hypoxic conditions (Wang *et al*., 2016), freshwater medaka embryos exposed to BPA or EE2 (Bhandari *et al*., 2015), and juvenile zebrafish exposed to 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD) (Baker *et al*., 2014). These results suggest that early developmental exposure to endocrine disruptors, such as ATZ, may not pose reproductive risk into adulthood of exposed fish, but rather induces molecular changes in the gametes in such a way that it produces transgenerational phenotype in the offspring two generations later.

Previous studies characterizing ATZ's impact on steroidogenesis have demonstrated significant alterations in steroid hormone synthesis, but few studies examined the effects of an embryonic exposure on adult steroid hormone activity, or the activity of the subsequent generation. Steroidogenic acute regulatory protein (StAR) is a rate limiting protein in the synthetic steroidogenic pathway which is required to sequester cholesterol in the mitochondria (Kiriakidou *et al*., 1996). Without proper expression of this transport protein gene, the available amount of steroid hormones and regulation of lipid accumulation is affected (LaVoie, 2017). My F0 results demonstrated a downregulation of *star* expression due to a developmental exposure to a high concentration of ATZ. *Star* expression has been found to both increase (Suzawa and Ingraham, 2008) and decrease (Pogrmic *et al*., 2009) after ATZ exposures. Interestingly, in the present study, the F2 generation exhibited increases in both ATZ Low and ATZ High treatment lineages, suggesting that the consequences of early developmental exposures pose a dissimilar risk to the grand-offspring of the exposed generation than it

does to the exposed generation. Transgenerational effects appear to differ from direct toxic effects of exposure and require careful explanation as the germline effects at F0 generation can be inherited by both the germ cells and the somatic cells of the subsequent generation. These effects may be elucidated only when the ATZ-induced reprogramming of germ cells, including the gametes in adult males, is characterized step-by-step across three generations as demonstrated previously for mice (Hao *et al*, 2016) and rats (McBirney *et al*, 2017).

While *ar*α expression was unaffected in the grand-offspring (F2) males, there was a significant increase in the EE2 Low and decrease in the ATZ High treated F0 generation. Similarly, there were no transgenerational changes in *er*α expression. Nuclear receptors such as *ar*α and *er*α translate the condition of the lipid environment of cells and tissues into a genetic response that modulates the transcription of endocrine and developmental gene pathways (Ozgyin *et al*., 2015). Therefore, if EDCs such as ATZ affect the lipid environment in an organism, it is likely the nuclear receptors will be involved in the subsequent endocrine response. While the nuclear receptor may mediate the response of an exogenous agent within an organism, the effect of concomitant DNA methylation associated with the exogenous agent's presence may further exacerbate that response (Ozgyin *et al*., 2015). Previous studies demonstrated that synthetic estrogens like BPA modify the methylation state of various promoter regions in the genome, including some estrogen responsive promoters in the first generation of offspring produced by exposed organisms (Bromer *et al*., 2010). My results demonstrate dissimilar

patterns of altered nuclear receptor expression and simultaneous changes in *dnmt1* or *dnmt3aa* expression. ATZ did not significantly impact the transgenerational expression of *ar*α, however, the ATZ High treatment group did downregulate *er*α, *dnmt1,* and *dnmt3aa* expression in the F2 generation female medaka.

Furthermore, ATZ is an alleged estrogenic compound, and the use of EE2 in my study was to compare the effects of ATZ to a known estrogenic substance. My data indicate that the responses between EE2 Low and ATZ Low, and sometimes between EE2 High and ATZ High, treatment groups often differ from each other in both direct and transgenerational effects. Therefore, my data do not demonstrate that ATZ produces an estrogenic response in the neuroendocrine system or throughout the HPG axis.

Our study was the first to evaluate the transgenerational changes in brain gene expression profiles in a fish model after embryonic ATZ exposure. There are few studies that have characterized the effect of ATZ on the brain in the offspring of exposed organisms, and fewer that have characterized transgenerational consequences in the brain in any model. Of those studies, none of them quantify the effects on key HPG axis gene expression such as kisspeptin (Kiss I and Kiss II) and its cognate receptors (Gpr54-I and Gpr54-II), or gonadotropin releasing hormones (GnRHI1 and GnRH II).

Neuroendocrine regulation in the brain begins with kisspeptin (kiss 1, kiss 2) agonizing the Gpr54-1 and Gpr54-2 receptors located on GnRH positive neurons that stimulate GnRH release (Jin and Yang, 2014; Nejad *et al*., 2017). GnRH (gnrh 1 and gnrh 2) stimulates the gonadotropes of the anterior pituitary gland (APG) to relaease

luteinizing hormone (LH) and follicle stimulating hormone (FSH), which act on discrete cells within the testes and ovaries, as well as maintain the regulatory feedback loop of gonadotropin release (Jin and Yang, 2014; Nejad *et al*., 2017; Wirbisky *et al*., Freeman, 2016).

Snapping turtles exposed to 2ppb ATZ during embryonic development exhibited an increase in kisspeptin 1 in male and female hatchlings (Russart and Rhen, 2016), with similar effects on kisspeptin 1 observed in female rats exposed to ATZ (Goldman *et al*., 2013). These results are consistent with our observations of kiss1 in the brains of male medaka after exposure to low concentrations of EE2 and ATZ. Conversely, both kiss1 and kiss 2 were downregulated in the F2 generation males of the EE2 high and ATZ low treatment lineages, and a decrease in kiss 2 was observed in the ATZ high treatment lineage F2 males. These data indicate that kisspeptin, a neuropeptide necessary for pubertal onset, reproductive maturity, and fertility, is affected by ATZ treatment in both directly exposed organisms and in their grandoffspring.

Gnrh 1 and gnrh 2 were unaffected in males and females by all treatment groups in the F2 generations. The F0 generation males exhibited increases in gnrh 1 and gnrh 2 in both EE2 and ATZ treatments, with no changes in F0 females except for the EE2 high treatment lineage. Other studies on GnRH and ATZ are limited and inconsistent as ATZ did not affect GnRH in rats (Foradori *et al*., 2014), or snapping turtles (Russart and Rhen, 2016), but was decreased in exposed quails (Qin *et al*., 2015). While our data do not

indicate whether ATZ directly affects GnRH or kisspeptin neurons, it is apparent that ATZ can alter the neuroendocrine system in a transgenerational manner.

Interestingly, Dnmt1 expression was decreased in both male and female medaka gonads. The decreased expression was observed in female medaka F0 EE2 High and ATZ High treatment lineages, and F2 ATZ Low lineage. However, only male medaka in the F2 ATZ Low lineage exhibited decreased Dnmt1 expression. These F0 results parallel those of Xing *et al*., 2015 who found that ATZ decreased the DNA methylation and various *dnmts* expression in the brain and gonad of common carp.

Transgenerational alterations in sperm differential DNA methylation regions (DMR) have also been demonstrated after embryonic ATZ exposure in mice, which promote the transmission of transgenerational phenotypes (McBirney *et al*., 2017). Changes in sperm DMRs, possibly promoting transgenerational inheritance, was recently reported in human males treated with chemotherapy during adolescence compared to males that have not received chemotherapeutics (Shnorhavorian *et al*., 2017).

Exposure to environmental contaminants during early developmental periods of sex determination and gonadogenesis may imprint novel patterns of DNA methylation on the primordial germ cell epigenome (Jirtle and Skinner, 2007; Skinner *et al*., 2011). If an aberration of DNA methylation occurs during this period, it is more likely to avoid DNA methylation erasure events prior to epigenetic reprogramming in the germ line (Jirtle and Skinner, 2007; Skinner, 2015). When new epigenetic information, such as a change in DNA methylation, occurs in the primordial germ line, it will be transmitted to the

offspring and subsequent generations in what is known as transgenerational epigenetic inheritance (Jirtle and Skinner, 2007; Skinner *et al*., 2011; Xin *et al*., 2015). Phenotypic deficits, disease susceptibility, and genomic instability may be inherited across generations once this epigenetic change has occurred in the ancestral germ line, even without further chemical exposure in the subsequent generations (Bernal and Jirtle, 2010; Xin *et al*., 2015).

Our results are the first to demonstrate transgenerational differential DNA methyltransferase activity in the gonads of both male and female medaka. The present study is contributing to the growing body of evidence suggesting that chemical exposure during critical developmental windows can induce heritable aberrations in DNA methylation.

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