# The Investigation of Multigenerational and Transgenerational Trichloroethylene Toxicity using the Zebrafish (*Danio Rerio*)

by

Taylor Elizabeth Abernathy

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Approved by

Katharine Horzmann, [Assistant Professor] Pathobiology
Russell Cattley, [Tyler and Francis Young Professor] Pathobiology
Jennifer Panizzi, [Associate Professor] Anatomy, Physiology, and Pharmacology

## Abstract

Multigenerational and transgenerational toxicology studies evaluate the persistence of toxicity in progeny of individuals exposed to a toxicant. For many toxicants, non-genetic mechanisms, especially the alteration of the epigenome, are suspected to cause transgenerational toxicity. TCE is a legacy chemical with known adverse effects; however, the epigenetic toxicity is not well characterized. This thesis reviews the concepts of multi- and transgenerational toxicity, mechanisms of epigenetic toxicity, and the role of zebrafish in studying epigenetic, multigenerational, and transgenerational toxicity. In the second chapter, the zebrafish (*Danio rerio*) model is used to characterize changes in progeny directly and indirectly exposed to TCE. The multigenerational progeny (F1), or those who had direct exposure to TCE as gametes, demonstrated changes in morphology, had an increased heart rate, and changes in locomotion and turning behaviors as compared to the controls. The transgenerational progeny (F2), those with no direct exposure to TCE, demonstrated changes in morphology, had decreased heart rate and changes in locomotion, path shape, and turning behavior. These results support the need for further investigation into the epigenetic, multigenerational, and transgenerational effects of TCE exposure.

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Dedication

*Delta,*
*Let's go get that Doctorate*
*I love you, T*

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| | |
|---|---|
| ADME | Absorption, distribution, metabolism, elimination |
| DNA | Deoxyribonucleic acid |
| DNMT | DNA methyltransferase |
| F0 | Parental generation |
| F1 | First filial generation |
| F2 | Second filial generation |
| GWAS | Genome wide association studies |
| HPF | Hours post fertilization |
| miRNA | MicroRNA |
| MPF | Months post fertilization |
| PPB | Parts per billion |
| PPT | Parts per trillion |
| RNA | Ribonucleic acid |
| RNS | Reactive nitrogen species |
| ROS | Reactive oxygen species |
| TCE | Trichloroethylene |
| VMR | Visual Motor Response |

# Chapter 1. Swimming Through the Generations: Using Zebrafish (*Danio rerio*) as a Model for Epigenetic, Multigenerational, and Transgenerational Toxicity Studies

## 1.1 Abstract

Exposure to toxicants can cause complex and far-reaching effects including to progeny of exposed organisms. Awareness of the multi- and transgenerational effects of toxicant exposures is increasing and the concern is that the health of a given generation is affected by the toxic exposures of their parents and even grandparents. It is believed that changes in the epigenome are one of the primary mechanisms that cause these transgenerational effects. Epigenetic toxicants can alter the normal processes of DNA methylation and histone modification, and cause the inappropriate expression of microRNAs. Each of these epigenetic alterations effects the expression of organism's genome without altering the actual sequence of the DNA. This review aims to briefly discuss each of these mechanisms and then describe the benefits of using the zebrafish (*Danio rerio*) biomedical model to study epigenetic, multigenerational, and transgenerational toxicity.

## 1.2 Multi- and Trans-generational Studies

Multi- and trans- generational studies are an emerging area of toxicologic research. These studies evaluate the offspring of the originally exposed organism (Horzmann *et al.*, 2018). Multi- and transgenerational effects have public health importance, as often there are no or minimal acute consequences to the initial exposure; however, changes in the individual's germ

cells affect the health of subsequent generations (Baker *et al.*, 2014). Multigenerational toxicity studies evaluate organisms that received direct exposure to the toxicant; in mammals, this exposure could occur in an adult (F0), fetus (F1), or primordial germ cell (F2). Transgenerational studies evaluate the subsequent generations that did not have direct exposure to a toxicant (F3 and beyond) (Horzmann *et al*., 2018).

## 1.3 Mechanisms of Toxicity

### 1.3.1 Xenobiotic Toxicity

A xenobiotic is any biologic or chemical compound that is foreign to an organism. It is important to remember that not all xenobiotics are innately toxic; rather the disposition of the xenobiotic within the body can induce toxicity. Disposition within the body can be further defined as the absorption, distribution, metabolism, and excretion (ADME) that occurs through cellular pathways within the organism (Peterson *et al.*, 2013). As Paracelsus, the father of toxicology stated: the dose is what makes the poison. However, once the xenobiotic has been absorbed and distributed throughout the body, what is it exactly that causes it to become toxic?

While some xenobiotics are toxic prior to biotransformation, it is the process of metabolism of the xenobiotic that often results in toxicity (Park *et al.*, 2014). When the body begins to metabolize the xenobiotic, toxicity can occur in various ways. The xenobiotic itself can require transformation in order to become activated and thus be toxic or the metabolites of the xenobiotic can be toxic (Lehman-McKeeman, 2013). The byproduct of the xenobiotic metabolism is known as the ultimate toxicant. This toxicant could act through multiple mechanisms of action, such as free radical formation (reactive oxygen/nitrogen species- ROS or RNS, respectively), electrophile or nucleophile formation, or intuitive active species

(Lehman-McKeeman, 2013). These by-products of xenobiotic metabolism often result in changes in gene expression, cellular dysfunction, or even genetic or epigenetic alterations.

Xenobiotic metabolism occurs in two phases. Phase I of metabolism adds functional groups to the xenobiotic via hydrolysis, reduction, or oxidation (Parkinson *et al.*, 2013). The second phase of xenobiotic metabolism is the conjugation phase. The Phase II reaction adds an additional molecule to the intermediate metabolite formed in Phase I; the most common additions are: glucuronic acid, glutathione, sulfonic acid, amino acids, acetyl group, or a methyl group (Parkinson, et al., 2013). The ultimate goal of Phase I and Phase II metabolism is to create a highly hydrophilic molecule that can be easily eliminated from the body. While Phase I and Phase II often create a chemical that can be excreted, they sometimes produce metabolites which are highly reactive with DNA (Heflich, 1991). This interaction with DNA can cause genetic toxicity.

### 1.3.2 Genotoxicity

Genotoxicity can be defined as harmful changes to the genetic material within an organism (Ren *et al.*, 2017). Examples of these harmful changes include: unregulated DNA synthesis, sister chromatin combinations, base removal, nucleotide crosslinking, and even DNA strand breaks (Gupta, 2016; Lee *et al.*, 2013). The difference between mutagens and genotoxicants is important, as mutagens cause permanent alterations in DNA sequence; thus indicating that not all mutagens are genotoxic. Furthermore, somatic genotoxic effects are not heritable (Gupta, 2016).

Genotoxicity can occur as a result of industrial chemical toxicants (Claxton *et al.*, 1998), plants (Marabini *et al.*, 2019), pharmaceuticals (Brambilla *et al.*, 2006; Novak *et al.*,

2017), as well as heavy metals (De Boeck *et al.*, 2003; Winder *et al.*, 1993). While genotoxicity often has a negative connotation, it is most often the mechanism by which anti-cancer drugs work (Kfoury *et al.*, 2013; Swift *et al.*, 2014; Zhang *et al.*, 2013). The same mechanisms which cause cell mutations associated with cancer, are also capable of being used as anti-cancer drugs.

Genotoxicity not only includes the alterations to the DNA sequence, but also the ability of the cellular repair mechanisms to correct changes in DNA sequence or repair other damage (World Health Organization, 2020). Transcription and translation are highly regulated processes in a normal functioning cell. When the DNA sequence or the proteins that repair the DNA are altered, malignancies and cellular stress are more likely to occur within the cell. The three possibilities that result from genetic damage within the cell are DNA repair, the insertion of a mutation, or the initiation of the apoptosis pathway (DeMarini, 2019). In addition to the six hallmarks of cancer, there are enabling characteristics. One of the enabling characteristics is genome instability and mutation (Hanahan *et al.*, 2011). Although not all genotoxicity results in cancer, it is a grossly visible result of genotoxicity.

### 1.3.3 Epigenetic Toxicity

Genotoxicity and epigenetic toxicity differ in two ways: epigenetic alterations do not directly involve the DNA sequence and the alterations can be inheritable (Kadhim, 2017). Nilsson et al. further describes epigenetic transgenerational inheritance as alterations in germline cells in the absence of continued toxic exposure (Nilsson *et al.*, 2018). Molecular alterations that can occur are DNA methylation, histone modifications, and altered expression of the microRNAs (Figure 2). Below is a brief review of each of those molecular alterations.

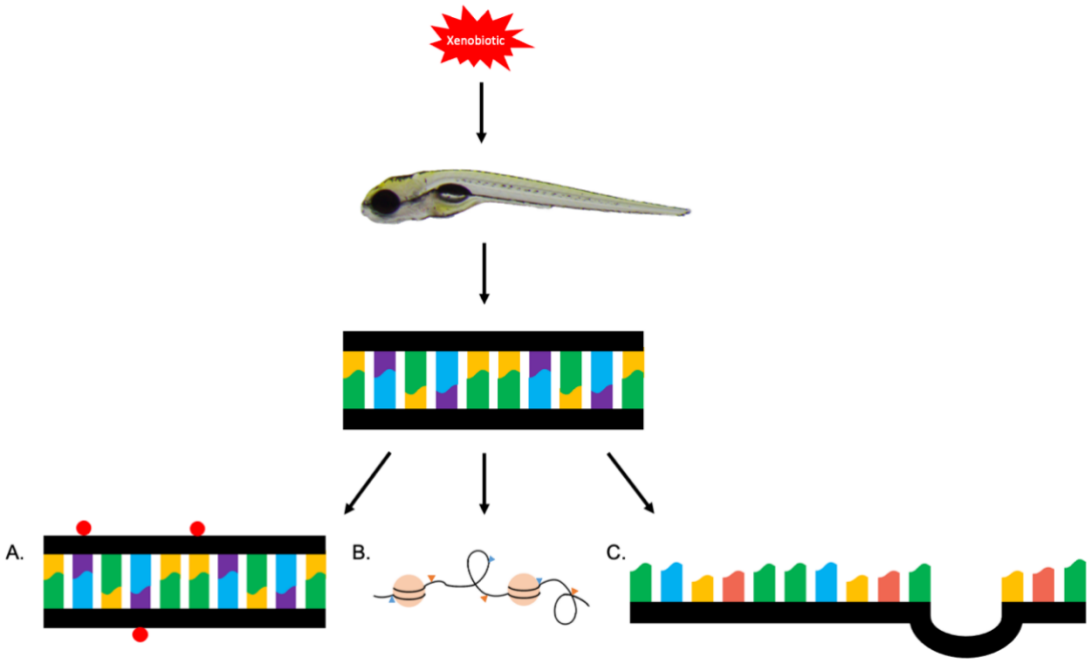


Figure 1.2 Mechanisms of Epigenetic Toxicity. DNA methylation (A), histone modifications (B), and alterations to microRNA (C).

## 1.4 Mechanisms of Epigenetic Toxicity

### 1.4.1 DNA Methylation

DNA methylation is the process by which a methyl group is added to the DNA strand, most commonly at the fifth carbon of the cytosine nucleotide (Moen *et al.*, 2015; Rider *et al.*, 2019). Due to the attachment of the methyl group at the cytosine nucleotide, these areas are often referred to as CpG islands within the genome. One of the proposed functions of these CpG islands is a start site for replication (Delgado *et al.*, 1998; Jones *et al.*, 2001). The methylation of CpG rich areas at transcription start sites has been shown to cause gene silencing (Holliday *et al.*, 1975; Phillips, 2008). Methylation does occur at non-CpG sites; however, the function of these methylation sites is unknown (Jones, 2012). Furthermore, CpG methylation contributes chromosomal stability in areas rich with repeats (Moarefi *et al.*, 2011).

It has been noted that the majority of the CpG islands in somatic cells are unmethylated, thus indicating the majority of methylation occurs within the germline cells (Borchiellini *et al.*, 2019). The methylation of the DNA strand results in inappropriate placement of transcription factors along with the attraction of methyl binding proteins, which are associated with chromatin condensation and gene silencing (Fazzari *et al.*, 2004).

DNA methyltransferases (DNMTs) are the enzyme families involved in the addition of the methyl group. These enzymes are known to be semi-conservative during DNA replication, meaning they allow the methyl group to remain on the daughter DNA strand at the completion of duplication (Jones, 2012; Szyf, 2011). DNMT1 acts to maintain methylation status, while DNMT3A and DNMT3B control *de novo* methylation during development (Portela *et al.*,

2010). CpG methylation is shown to have little if any effect in the adult organism (Edwards *et al.*, 2017; Louie *et al.*, 2003).

If methylation has minimal effect on the adult organism, what is the concern, especially considering toxicity? It is well recognized that the DNMTs are intimately involved in embryonic development (Moore *et al.*, 2013). Early embryonic exposure to toxicants can alter the normal *de novo* methylation process. However, changes occurring in the adult epigenome might not cause phenotypic alterations until the production of offspring. This generational delay complicates the evaluation of toxicity, but highlights the need for multi- and transgenerational studies.

### 1.4.2 Histone Modification

Histones are responsible for the condensation of DNA in chromatin, which can influence the expression of genes (Smith, 1991; Tordera *et al.*, 1993). Post-translation modifications of histones are essential in the structure and function of chromatin, thus the methylation and acetylation of the histone results in inappropriate structure and function of both the DNA strand and chromatin (Gong *et al.*, 2019). Histone octamers wrapped with DNA form nucleosomes, which serve as the fundamental subunit of chromatin (Mariño-Ramírez *et al.*, 2005).

Histone modifications include methylation, acetylation, phosphorylation, and ubiquitylation. The amino acid lysine on the histone protein is often methylated, acetylated, or ubiquitinated, while arginine is primarily acetylated and serine is phosphorylated (Peterson *et al.*, 2004). Histone methylation is important in the regulation of transcription (Gong, et al., 2019; Kouzarides, 2002). Furthermore, the methylation of one histone protein might lead to upregulation of transcription, while methylation of the neighboring protein leads to down

regulation (Barski *et al.*, 2007). Inappropriate methylation due to xenobiotic toxicity could cause the inappropriate expression of genes.

The acetylation of histone tails results in an overall change in charge, making it neutral and decreasing the binding capacity of the DNA to the histone (Kouzarides, 2002). The failure of the DNA to wrap around the histone creates gaps in the DNA, which previously would have been absent, allowing transcription factors to bind to the DNA (Guo *et al.*, 2018; Khan *et al.*, 2012). The acetylation of histones has been linked to an increased rate of cancer, due to the neutralization of the binding properties (Audia *et al.*, 2016). In addition to the initiation of cancer, histone acetylation has been shown to be a contributor to neural tube defects in fetal development (Li *et al.*, 2019; Shyamasundar *et al.*, 2013; Yu *et al.*, 2016). This is just one example of the effects of histone modification.

When considering multi- and trans-generational studies, alteration of the germline cells are most heavily studied. During spermatogenesis, histones are removed and replaced by protamines in order to condense DNA into the head of the sperm (Bao *et al.*, 2016; Ben Maamar *et al.*, 2018). This condensation requires the up- and down- regulation of numerous genes (Burlibaşa *et al.*, 2019; Hao *et al.*, 2019; Meng *et al.*, 2019; Zhang *et al.*, 2019). The condensation of genetic material during spermatogenesis is quite complex, increasing the likelihood of errors in the genetic code. Since oogenesis, the development of an oocyte prenatally, is not a consistently occurring processing after birth, unlike spermatogenesis, the alterations in maternal genetic material must occur during the developmental period of the organism. The disturbance of spermatogenesis is an emerging area in toxicologic studies, more specifically its role in epigenetic alterations.

### 1.4.3 microRNA

microRNAs (miRNA) are noncoding RNAs approximately 22 bases long, whose primary function is to regulate mRNA (Simonson *et al.*, 2015). When functioning normally, miRNAs act to control the translation of proteins, most often by decreasing expression of mRNA but without elimination (Baek *et al.*, 2008; Selbach *et al.*, 2008). Determining the exact function of miRNAs can be quite difficult as a single miRNA can have multiple roles. For example, miRNA 125b is upregulated in hematopoietic tumors and colon cancer, yet down regulated in hepatocellular carcinoma and mammary tumors (Banzhaf-Strathmann *et al.*, 2014). For a more thorough review of the various types of xenobiotic induced miRNA alterations, see the review by Ahkin Chin Tai and Freeman in *Toxicology Reports* (Ahkin Chin Tai *et al.*, 2020).

## 1.5 Zebrafish

### 1.5.1 Zebrafish as a biomedical model

The zebrafish (*Danio rerio*) is a well-known and widely accepted biomedical model organism. Zebrafish are useful in studies including, but not limited to: toxicology, reproduction and development, and various physiological systems (Horzmann, et al., 2018; Teame *et al.*, 2019). These small freshwater fish bridge the gap between cell culture and higher mammalian models. In regards to toxicity evaluations, there are many advantages to using the zebrafish as a model organism. Some of these advantages include ex vivo fertilization, translucent embryos, well-characterized development, and genetic similarities to humans.

The external fertilization of zebrafish embryos allows for easy manipulation. Due to the small size of zebrafish embryos, multiple embryos are able to be treated with the toxicant of interest and multiple experimental replicates are easily attainable, as compared to higher

mammalian models (Hill *et al.*, 2005). The fecundity of the adult zebrafish in conjunction with the small sized embryos allows for variety in technique of toxicant exposure. Embryos can be dosed in a Petri dish (Souder *et al.*, 2017), glass beaker (Yang *et al.*, 2021), multi-well plates (Truong *et al.*, 2017), and even glass vials (Wirbisky *et al.*, 2016), with either static exposures or daily renewed exposures. The embryonic developmental period in zebrafish is considered 0 hours post fertilization (hpf) through 72 hpf, or 3 days (Kimmel *et al.*, 1995; Parichy *et al.*, 2009). However, it is not until 5 day post fertilization (dpf) that the developing zebrafish will require supplemental nutrition or be considered a free living organism. From fertilization through 5 dpf, the developing zebrafish will absorb nutrients from its yolk sac (MacRae *et al.*, 2003). This allows investigators to expose embryos to the toxicant of interest during critical developmental time points.

In addition to adult fecundity and variety in mechanism of toxicant exposure, the optical clarity of the embryo allows for tissue-specific labeling. One way to create transgenic zebrafish is to insert fluorescently labeled RNA into the embryo (Lee *et al.*, 2015). There are many benefits for transgenic zebrafish, especially in toxicology studies, including tissue specific staining and gene labeling (Hill, et al., 2005; Lee, et al., 2015). Transgenic zebrafish have been used to study Myc-induced leukemia (Langenau *et al.*, 2003), induce a biosensor used to analyze ecologically relevant levels of heavy metals (Liu *et al.*, 2016), and evaluate vasotoxicity through the measurement of the length of fluorescently labeled blood vessels (Delov *et al.*, 2014). For a more complete review of transgenic zebrafish in toxicology, the Lee et al. article in *Critical Reviews in Toxicology* provides an in depth analysis of the various uses of transgenic zebrafish (Lee, et al., 2015).

### 1.5.2 Zebrafish genomics

Zebrafish have been used as a research model since the late 1970's. During this time, it was noted that they could be a valuable tool for studying genetics; however, it was not until 2001 that zebrafish genome sequencing was begun at the Wellcome Sanger Institute (Howe *et al.*, 2013). The first edition of the referenced genome was released in 2013, yet an updated reference was released in 2017 after the correction of 400 genome wide issues (Genome Reference Consortium b). Having the zebrafish genome completely referenced allows for manipulation of the genome, as well as comparison of the zebrafish and human genome.

The haploid zebrafish genome contains 25 chromosomes, as compared to the 23 in the human genome. Within those 25 chromosomes, there are 1,679,186,873 bases (Genome Reference Consortium b), and approximately 26,000 protein coding genes (Collins *et al.*, 2012; Howe, et al., 2013). This compares with the human genome of 3,272,116,950 base pairs (Genome Reference Consortium a). Of these 3 billion base pairs, approximately 1% of the genome consists of protein coding genes (Zhao, 2012). Although the human genome contains double the number of base pairs, compared to the zebrafish genome, there is ~70% similarity (Howe, et al., 2013). Using genome wide association studies (GWAS), Howe et al. found that 76% of the human genes that cause disease have a zebrafish ortholog.

The known similarities, especially in disease causing genes, allow the zebrafish to be a superb model for toxicologic studies. The teleost genome underwent two duplication events, resulting in the copying of a large number of human genes (Braasch *et al.*, 2016; Smith *et al.*, 2015; Taylor *et al.*, 2003). Despite this double duplicate of the genome, the genetic material contained within remains highly conserved, and the paralogous genes in zebrafish allow for investigation into sub- and neofunctionalization and can rescue what could be lethal knockout

mutations in mammals. It has been noted that the non-coding regions of the genome are what lead to evolutionary divergence, allowing the coding regions to remain intact and provide similarity between zebrafish and humans (The ENCODE Project Consortium, 2007; Kwon *et al.*, 2019; Taher *et al.*, 2011).

### 1.6.3 Zebrafish in Epigenetic, Multigenerational, and Transgenerational Toxicology

The use of zebrafish (*Danio rerio*) for multi- and transgenerational studies necessitates a change in generational definitions. Zebrafish embryos are fertilized externally, which means that when zebrafish embryos are exposed to a toxicant, the embryonic (F0) and germline (F1) exposure represent the multigenerational, or direct exposure, toxicity. Finally, the F2 progeny and beyond are representative of transgenerational toxicity (Figure 1.2).

The number of epigenetic toxicity studies involving zebrafish is quite small. However, there is great evidence that zebrafish are an appropriate *in vivo* model for the analysis of DNA methylation patterns and those effects (McGaughey *et al.*, 2014). The aforementioned study demonstrates that zebrafish DNA methylation patterns are similar to that of humans and that similarly to humans, methylation patterns are tissue specific. The use of zebrafish as a model for examining miRNA function is growing, however the number of toxicity studies are still quite small (Shen *et al.*, 2020; Wang *et al.*, 2019; Wienholds *et al.*, 2005; Zayed *et al.*, 2019). The previously mentioned studies observe the expression of miRNAs in normal embryonic development, ovarian cells, craniofacial development, and cardiac development. Histone modification studies in zebrafish are extremely limited. The use of zebrafish as a model organism for the investigation of epigenetic toxicity studies is highly under used and

underappreciated. The striking similarities between the human and zebrafish genome generate a compelling argument for the use of zebrafish a model for comparative research.

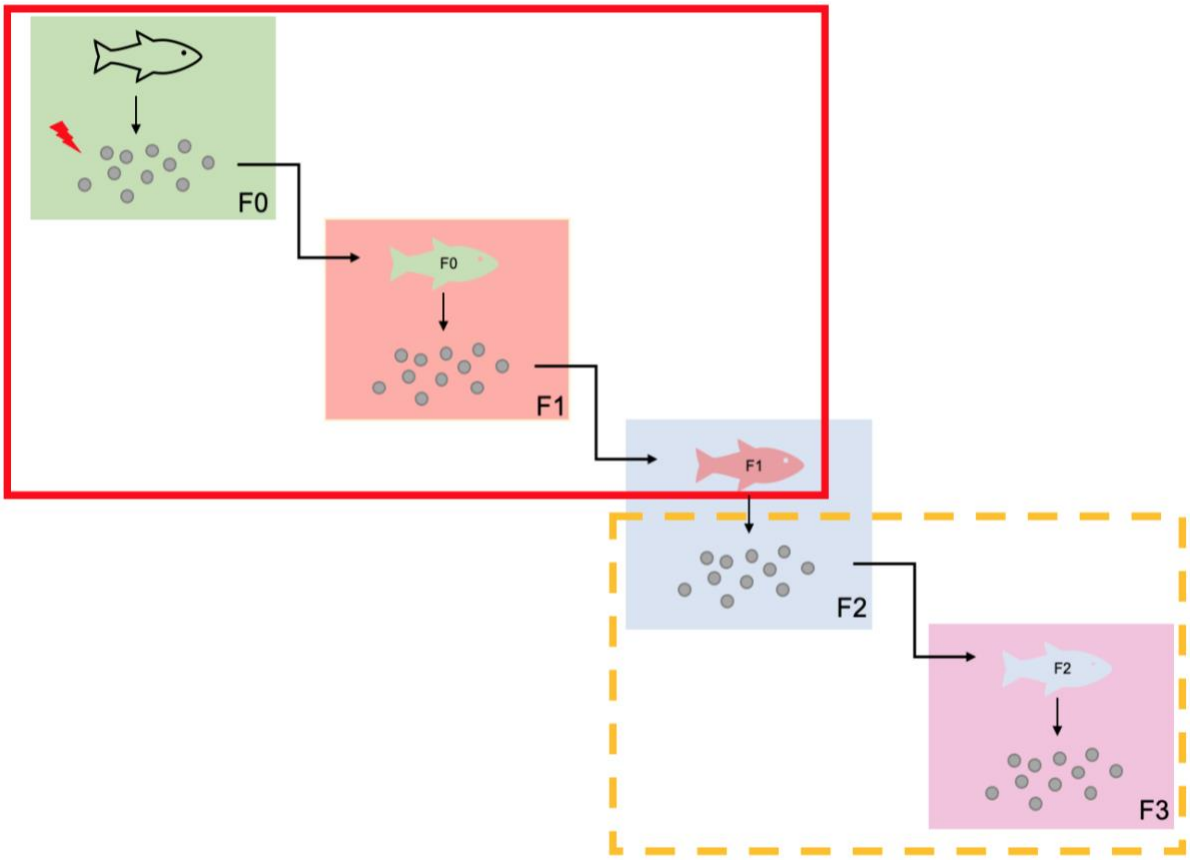


Figure 1.2 Multi- vs. Trans- generational toxicity in zebrafish. Multigenerational is represented by the solid red line. Transgenerational represented by the dashed orange line.

## 1.6 References

Ahkin Chin Tai JK, Freeman JL. 2020. Zebrafish as an integrative vertebrate model to identify mirna mechanisms regulating toxicity. Toxicol Rep. 7:559-570.

Audia JE, Campbell RM. 2016. Histone modifications and cancer. Cold Spring Harb Perspect Biol. 8(4):a019521-a019521.

Baek D, Villén J, Shin C, Camargo FD, Gygi SP, Bartel DP. 2008. The impact of microrna on protein output. Nature. 455(7209):64-71.

Baker TR, Peterson RE, Heideman W. 2014. Using zebrafish as a model system for studying the transgenerational effects of dioxin. Toxicol Sci. 138(2):403-411.

Banzhaf-Strathmann J, Edbauer D. 2014. Good guy or bad guy: The opposing roles of microrna 125b in cancer. Cell Commun Signal. 12:30-30.

Bao J, Bedford MT. 2016. Epigenetic regulation of the histone-to-protamine transition during spermiogenesis. Reproduction. 151(5):R55-R70.

Barski A, Cuddapah S, Cui K, Roh TY, Schones DE, Wang Z, Wei G, Chepelev I, Zhao K. 2007. High-resolution profiling of histone methylations in the human genome. Cell. 129(4):823-837.

Ben Maamar M, Sadler-Riggleman I, Beck D, Skinner MK. 2018. Epigenetic transgenerational inheritance of altered sperm histone retention sites. Sci Rep. 8(1):5308-5308.

Borchiellini M, Ummarino S, Di Ruscio A. 2019. The bright and dark side of DNA methylation: A matter of balance. Cells. 8(10):1243.

Braasch I, Gehrke AR, Smith JJ, Kawasaki K, Manousaki T, Pasquier J, Amores A, Desvignes T, Batzel P, Catchen J et al. 2016. The spotted gar genome illuminates vertebrate evolution and facilitates human-teleost comparisons. Nat Genet. 48(4):427-437.

Brambilla G, Martelli A. 2006. Genotoxicity and carcinogenicity studies of antihypertensive agents. Mutat Res. 612(2):115-149.

Burlibaşa L, Ionescu AC, Dragusanu DM. 2019. Histone hyperacetylation and DNA methylation interplay during murine spermatogenesis. Zygote. 27(5):305-314.

Claxton LD, Houk VS, Hughes TJ. 1998. Genotoxicity of industrial wastes and effluents. Mutation Research/Reviews in Mutation Research. 410(3):237-243.

Collins JE, White S, Searle SMJ, Stemple DL. 2012. Incorporating rna-seq data into the zebrafish ensembl genebuild. Genome Res. 22(10):2067-2078.

De Boeck M, Kirsch-Volders M, Lison D. 2003. Cobalt and antimony: Genotoxicity and carcinogenicity. Mutat Res. 533(1-2):135-152.

Delgado S, Gómez M, Bird A, Antequera F. 1998. Initiation of DNA replication at cpg islands in mammalian chromosomes. The EMBO Journal. 17(8):2426-2435.

Delov V, Muth-Köhne E, Schäfers C, Fenske M. 2014. Transgenic fluorescent zebrafish tg(fli1:Egfp)y¹ for the identification of vasotoxicity within the zfet. Aquat Toxicol. 150:189-200.

DeMarini DM. 2019. The role of genotoxicity in carcinogenesis. In: Baan R, Stewart B, Straif K, editors. Tumour Site Concordance and Mechanisms of Carcinogenesis. International Agency for Research on Cancer.

Edwards JR, Yarychkivska O, Boulard M, Bestor TH. 2017. DNA methylation and DNA methyltransferases. Epigenetics Chromatin. 10:23-23.

Fazzari MJ, Greally JM. 2004. Epigenomics: Beyond cpg islands. Nature Reviews Genetics. 5(6):446-455.

Genome Reference Consortium (a). Human genome assemby grch38.P13. National Institutes of Health.

Genome Reference Consortium (b). Zebrafish genome overview. National Institutes of Health.

Gong F, Miller KM. 2019. Histone methylation and the DNA damage response. Mutation research. 780:37-47.

Guo P, Chen W, Li H, Li M, Li L. 2018. The histone acetylation modifications of breast cancer and their therapeutic implications. Pathol Oncol Res. 24(4):807-813.

Gupta PK. 2016. Chapter 13 - genotoxicity. In: Gupta PK, editor. Fundamentals of toxicology. Academic Press. p. 151-164.

Hanahan D, Weinberg Robert A. 2011. Hallmarks of cancer: The next generation. Cell. 144(5):646-674.

Hao SL, Ni FD, Yang WX. 2019. The dynamics and regulation of chromatin remodeling during spermiogenesis. Gene. 706:201-210.

Heflich RH. 1991. Genetic toxicology. Li AP, Heflich RH, editors. Caldwell, New Jersery: Telford Press.

Hill AJ, Teraoka H, Heideman W, Peterson RE. 2005. Zebrafish as a model vertebrate for investigating chemical toxicity. Toxicol Sci. 86(1):6-19.

Holliday R, Pugh JE. 1975. DNA modification mechanisms and gene activity during development. Science. 187(4173):226-232.

Horzmann KA, Freeman JL. 2018. Making waves: New developments in toxicology with the zebrafish. Toxicol Sci. 163(1):5-12.

Howe K, Clark MD, Torroja CF, Torrance J, Berthelot C, Muffato M, Collins JE, Humphray S, McLaren K, Matthews L et al. 2013. The zebrafish reference genome sequence and its relationship to the human genome. Nature. 496(7446):498-503.

Jones PA. 2012. Functions of DNA methylation: Islands, start sites, gene bodies and beyond. Nature Reviews Genetics. 13(7):484-492.

Jones PA, Takai D. 2001. The role of DNA methylation in mammalian epigenetics. Science. 293(5532):1068.

Kadhim, M. 2017. Introduction to epigenetic effects and ionising radiation. MK-Epige Seminar; November 2017; Oxford, UK. Department of Biological and Medical Sciences

Oxford Brookes University.

Kfoury A, Le Corf K, El Sabeh R, Journeaux A, Badran B, Hussein N, Lebecque S, Manié S, Renno T, Coste I. 2013. Myd88 in DNA repair and cancer cell resistance to genotoxic drugs. J Natl Cancer Inst. 105(13):937-946.

Khan SI, Aumsuwan P, Khan IA, Walker LA, Dasmahapatra AK. 2012. Epigenetic events associated with breast cancer and their prevention by dietary components targeting the epigenome. Chem Res Toxicol. 25(1):61-73.

Kimmel C, Ballard W, Kimmel S, Ullmann B, Schilling T. 1995. Stages of embryonic development of the zebrafish. Developmental Dynamics. 203:253-310.

Kouzarides T. 2002. Histone methylation in transcriptional control. Curr Opin Genet Dev. 12(2):198-209.

Kwon RY, Watson CJ, Karasik D. 2019. Using zebrafish to study skeletal genomics. Bone. 126:37-50.

Langenau DM, Traver D, Ferrando AA, Kutok JL, Aster JC, Kanki JP, Lin S, Prochownik E, Trede NS, Zon LI et al. 2003. Myc-induced t cell leukemia in transgenic zebrafish. Science. 299(5608):887-890.

Lee O, Green JM, Tyler CR. 2015. Transgenic fish systems and their application in ecotoxicology. Crit Rev Toxicol. 45(2):124-141.

Lee SJ, Yum YN, Kim SC, Kim Y, Lim J, Lee WJ, Koo KH, Kim JH, Kim JE, Lee WS et al. 2013. Distinguishing between genotoxic and non-genotoxic hepatocarcinogens by gene expression profiling and bioinformatic pathway analysis. Sci Rep. 3(1):2783.

Lehman-McKeeman LD. 2013. Casarett and doull's toxicology: The basic science of poisons. Klaassen CD, editor. McGraw Hill Education.

Li D, Wan C, Bai B, Cao H, Liu C, Zhang Q. 2019. Identification of histone acetylation markers in human fetal brains and increased h4k5ac expression in neural tube defects. Mol Genet Genomic Med. 7(12):e1002-e1002.

Liu L, Yan Y, Wang J, Wu W, Xu L. 2016. Generation of mt:Egfp transgenic zebrafish biosensor for the detection of aquatic zinc and cadmium. Environ Toxicol Chem. 35(8):2066-2073.

Louie E, Ott J, Majewski J. 2003. Nucleotide frequency variation across human genes. Genome Res. 13(12):2594-2601.

MacRae CA, Peterson RT. 2003. Zebrafish-based small molecule discovery. Chem Biol. 10(10):901-908.

Marabini L, Galli CL, La Fauci P, Marinovich M. 2019. Effect of plant extracts on the genotoxicity of 1'-hydroxy alkenylbenzenes. Regul Toxicol Pharmacol. 105:36-41.

Mariño-Ramírez L, Kann MG, Shoemaker BA, Landsman D. 2005. Histone structure and nucleosome stability. Expert Rev Proteomics. 2(5):719-729.

McGaughey DM, Abaan HO, Miller RM, Kropp PA, Brody LC. 2014. Genomics of cpg methylation in developing and developed zebrafish. G3 (Bethesda). 4(5):861-869.

Meng C, Liao J, Zhao D, Huang H, Qin J, Lee TL, Chen D, Chan WY, Xia Y. 2019. L3mbtl2 regulates chromatin remodeling during spermatogenesis. Cell Death Differ. 26(11):2194-2207.

Moarefi AH, Chédin F. 2011. Icf syndrome mutations cause a broad spectrum of biochemical defects in dnmt3b-mediated de novo DNA methylation. Journal of Molecular Biology. 409(5):758-772.

Moen EL, Mariani CJ, Zullow H, Jeff-Eke M, Litwin E, Nikitas JN, Godley LA. 2015. New themes in the biological functions of 5-methylcytosine and 5-hydroxymethylcytosine. Immunol Rev. 263(1):36-49.

Moore LD, Le T, Fan G. 2013. DNA methylation and its basic function. Neuropsychopharmacology. 38(1):23-38.

Nilsson EE, Sadler-Riggleman I, Skinner MK. 2018. Environmentally induced epigenetic transgenerational inheritance of disease. Environ Epigenet. 4(2):dvy016.

Novak M, Žegura B, Modic B, Heath E, Filipič M. 2017. Cytotoxicity and genotoxicity of anticancer drug residues and their mixtures in experimental model with zebrafish liver cells. Sci Total Environ. 601-602:293-300.

Parichy DM, Elizondo MR, Mills MG, Gordon TN, Engeszer RE. 2009. Normal table of postembryonic zebrafish development: Staging by externally visible anatomy of the living fish. Dev Dyn. 238(12):2975-3015.

Park Y-C, Lee S, Cho M-H. 2014. The simplest flowchart stating the mechanisms for organic xenobiotics-induced toxicity: Can it possibly be accepted as a "central dogma" for toxic mechanisms? Toxicol Res. 30(3):179-184.

Parkinson A, Ogilvie BW, Buckley DB, Kazmi F, Parkinson O. 2013. Casarett and doull's toxicology: The basic science of poisons. Klaassen CD, editor. McGraw Hill Education.

Peterson CL, Laniel M-A. 2004. Histones and histone modifications. Current Biology. 14(14):R546-R551.

Peterson ME, Talcott PA. 2013. Small animal toxicology. Third ed. St. Louis, Missouri: Elsevier. https://books.google.com/books?hl=en&lr=&id=BLkPFlB15v0C&oi=fnd&pg=PP1&ots=TxarLTEQLf&sig=4htHp7sNbM1k6HjiWs9e8Hc6zFs#v=onepage&q&f=false.

Phillips T. 2008. The role of methylation in gene expression. Nature Education.

Portela, A., and Esteller, M. (2010). Epigenetic modifications and human disease. *Nature Biotechnology* **28**(10), 1057-1068.

Ren N, Atyah M, Chen W-Y, Zhou C-H. 2017. The various aspects of genetic and epigenetic toxicology: Testing methods and clinical applications. J Transl Med. 15(1):110-110.

Rider CF, Carlsten C. 2019. Air pollution and DNA methylation: Effects of exposure in humans. Clin Epigenetics. 11(1):131-131.

Selbach M, Schwanhäusser B, Thierfelder N, Fang Z, Khanin R, Rajewsky N. 2008. Widespread changes in protein synthesis induced by microrna. Nature. 455(7209):58-63.

Shen Y, Lu H, Chen R, Zhu L, Song G. 2020. Microrna-29c affects zebrafish cardiac development via targeting wnt4. Mol Med Rep. 22(6):4675-4684.

Shyamasundar S, Jadhav SP, Bay BH, Tay SSW, Kumar SD, Rangasamy D, Dheen ST. 2013. Analysis of epigenetic factors in mouse embryonic neural stem cells exposed to hyperglycemia. PloS one. 8(6):e65945-e65945.

Simonson B, Das S. 2015. Microrna therapeutics: The next magic bullet? Mini Rev Med Chem. 15(6):467-474.

Smith JJ, Keinath MC. 2015. The sea lamprey meiotic map improves resolution of ancient vertebrate genome duplications. Genome Res. 25(8):1081-1090.

Smith MM. 1991. Histone structure and function. Current Opinion in Cell Biology. 3(3):429-437.

Souder JP, Gorelick DA. 2017. Quantification of estradiol uptake in zebrafish embryos and larvae. Toxicological sciences : an official journal of the Society of Toxicology. 158(2):465-474.

Swift LH, Golsteyn RM. 2014. Genotoxic anti-cancer agents and their relationship to DNA damage, mitosis, and checkpoint adaptation in proliferating cancer cells. Int J Mol Sci. 15(3):3403-3431.

Szyf M. 2011. The implications of DNA methylation for toxicology: Toward toxicomethylomics, the toxicology of DNA methylation. Toxicological sciences : an official journal of the Society of Toxicology. 120(2):235-255.

Taher L, McGaughey DM, Maragh S, Aneas I, Bessling SL, Miller W, Nobrega MA, McCallion AS, Ovcharenko I. 2011. Genome-wide identification of conserved regulatory function in diverged sequences. Genome Res. 21(7):1139-1149.

Taylor JS, Braasch I, Frickey T, Meyer A, Van de Peer Y. 2003. Genome duplication, a trait shared by 22000 species of ray-finned fish. Genome Res. 13(3):382-390.

Teame T, Zhang Z, Ran C, Zhang H, Yang Y, Ding Q, Xie M, Gao C, Ye Y, Duan M et al. 2019. The use of zebrafish (danio rerio) as biomedical models. Animal Frontiers. 9(3):68-77.

The ENCODE Project Consortium., Analysis Coordination., Birney, E. *et al.* 2007. Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project. Nature 447:799–816.

Tordera V, Sendra R, Pérez-Ortín JE. 1993. The role of histones and their modifications in the informative content of chromatin. Experientia. 49(9):780-788.

Truong L, Tanguay RL. 2017. Evaluation of embryotoxicity using the zebrafish model. Methods Mol Biol. 1641:325-333.

Wang D, Weng Y, Guo S, Qin W, Ni J, Yu L, Zhang Y, Zhao Q, Ben J, Ma J. 2019. Microrna-1 regulates ncc migration and differentiation by targeting sec63. Int J Biol Sci. 15(12):2538-2547.

Wienholds E, Kloosterman WP, Miska E, Alvarez-Saavedra E, Berezikov E, de Bruijn E, Horvitz HR, Kauppinen S, Plasterk RH. 2005. Microrna expression in zebrafish embryonic development. Science. 309(5732):310-311.

Winder C, Bonin T. 1993. The genotoxicity of lead. Mutat Res. 285(1):117-124.

Wirbisky SE, Damayanti NP, Mahapatra CT, Sepulveda MS, Irudayaraj J, Freeman JL. 2016. Mitochondrial dysfunction, disruption of f-actin polymerization, and transcriptomic alterations in zebrafish larvae exposed to trichloroethylene. Chem Res Toxicol. 29(2):169-179.

World Health Organization. 2020. Genotoxicity. Hazard Identification and Characterization: Toxicological and Human Studies, of Environmental Health Criteria. Second ed.: World Health Organization.

Yang H, Liang X, Zhao Y, Gu X, Mao Z, Zeng Q, Chen H, Martyniuk CJ. 2021. Molecular and behavioral responses of zebrafish embryos/larvae after sertraline exposure. Ecotoxicology and Environmental Safety. 208:111700.

Yu J, Wu Y, Yang P. 2016. High glucose-induced oxidative stress represses sirtuin deacetylase expression and increases histone acetylation leading to neural tube defects. J Neurochem. 137(3):371-383.

Zayed Y, Qi X, Peng C. 2019. Identification of novel micrornas and characterization of microrna expression profiles in zebrafish ovarian follicular cells. Front Endocrinol (Lausanne). 10:518.

Zhang P, Zhao Y, Zhang H, Liu J, Feng Y, Yin S, Cheng S, Sun X, Min L, Li L et al. 2019. Low dose chlorothalonil impairs mouse spermatogenesis through the intertwining of estrogen receptor pathways with histone and DNA methylation. Chemosphere. 230:384-395.

Zhang X, Jia D, Liu H, Zhu N, Zhang W, Feng J, Yin J, Hao B, Cui D, Deng Y et al. 2013. Identification of 5-iodotubercidin as a genotoxic drug with anti-cancer potential. PLoS One. 8(5):e62527.

Zhao RF. 2012. Encode: Deciphering function in the human genome. Genome Advance of the Month. November 8, 2012 ed. Bethesda, MD: National Human Genome Research Institute.

# Chapter 2. Multigenerational and Transgenerational Toxicity in Progeny of Zebrafish (*Danio rerio*) with Developmental Trichloroethylene (TCE) Exposure

## 2.1 Abstract

Trichloroethylene (TCE) is a volatile organic compound that has been used as a metal degreaser and as an industrial solvent. It is a significant legacy environmental toxicant and has been found at over half of the sites on the US EPA's National Priorities List. TCE is a known carcinogen and has been linked to central nervous system abnormalities and congenital defects. TCE is thought to alter DNA methylation; however, the epigenetic effects are not well characterized. This study uses the zebrafish model (*Danio rerio*) to test the hypothesis that developmental exposure to ecologically relevant levels of TCE causes multigenerational and transgenerational toxicity. F0 zebrafish were exposed as embryos to 0, 5, 50, or 500 parts per billion (ppb; µg/L) TCE from 1-120 hours post fertilization; their F1 and F2 progeny were assessed for developmental toxicity at 120 hours post fertilization through morphologic measurements, heart rate analysis, and a visual motor response behavior assay. The F1 morphologic assessment demonstrated significant differences in head width, head width to body length ratio, eye diameter, jaw length, and pericardial area between the progeny groups. The 500 ppb F1 progeny had an increased heart rate compared to controls. The F1 50 and 500 ppb progeny had a dose dependent decrease in distance moved, velocity, and time spent moving and decreased turning frequency on the behavioral assay. The F2 morphologic assessment demonstrated significant differences in body length, head length, head width, head

length to body length ratio, head width to body length ratio, eye diameter, jaw length, pericardial area, and head to trunk angle. The 500 ppb F2 progeny had a significant decrease in heart rate compared to controls. In the behavioral assay, the F2 500 ppb progeny had a decrease in time spent moving and velocity, the 500 ppb progeny had increased heading degrees, and the 50 and 500 ppb progeny had decreased turning frequency. The morphologic, physiological, and behavioral changes in F1 and F2 progeny underscore the need for further investigation of epigenetic toxicity following developmental TCE exposure.

## 2.2 Introduction

Trichloroethylene (TCE) is a legacy chemical commonly used throughout the mid-twentieth century as a metal degreaser and industrial solvent (International Agency for Research on Cancer, 2014). TCE is a clear liquid that is non-flammable, non-corrosive, and lipophilic and is classified as a volatile organic compound (VOC). Due to its historical use as an industrial chemical, TCE is now a significant environmental toxicant with possible exposure through ingestion of contaminated drinking water or food, inhalation, or from dermal contact (Agency for Toxic Substances and Disease Registry, 2019). Drinking water is most commonly contaminated via inappropriate industrial waste release or leaching from containment sites into ground water sources (Rusyn *et al.*, 2014).

As of 2019, TCE is listed 16$^{th}$ on the Agency for Toxic Substances and Disease Registry (ATSDR) Priority List (Agency for Toxic Substances and Disease Registery, 2020). Of the over 85,000 chemicals listed by the United States Environmental Protection Agency (US EPA) in the Toxic Substances Control Act Chemical Substance Inventory (United States Environmental Protection Agency, 2017), the listed chemicals are identified as a priority

due to frequency and likelihood of human exposure. Additionally, TCE is found at 1,051 of the 1,854 National Priority Sites within the United States (Agency for Toxic Substances and Disease Registry, 2019). Currently, the US EPA has set a maximum contaminant level (MCL) of 5ppb (0.005 mg/L); however the maximum contaminant level goal (MCLG) is zero (Agency for Toxic Substances and Disease Registry, 2019). The 1999–2000 National Health and Nutrition Examination Survey (NHANES) found that 12% of the population had measurable levels of TCE in their blood, with the minimum detection limit being 50 parts per trillion (ppt) (Jia *et al.*, 2012).

TCE is an important chemical in regards to public health due to the known adverse health effects. TCE is considered to be a group 1 carcinogen by the International Agency for Research on Cancer (IARC), based on epidemiologic evaluation (International Agency for Research on Cancer, 2014). TCE is also considered a developmental toxicant, with reports of morphologic alterations, most commonly decreased birth weight, in both humans and rats exposed *in utero* (Forand *et al.*, 2012; Loch-Caruso *et al.*, 2019; Ruckart *et al.*, 2014), neurotoxicity (Blossom *et al.*, 2017; Goodman *et al.*, 2012; Guehl *et al.*, 1999; Kaneko *et al.*, 1997), and congenital cardiac defects (Goldberg *et al.*, 1990; Harris *et al.*, 2018; Horzmann *et al.*, 2020; Wirbisky, et al., 2016). Furthermore, TCE is suspected to cause epigenetic toxicity (Cooney, 2014; Cui *et al.*, 2016; Marczylo *et al.*, 2016), and may act via altered DNA methylation (Blossom *et al.*, 2020; Jiang *et al.*, 2014; Lai *et al.*, 2021; Phillips *et al.*, 2019). Epigenetic toxicity has the potential to cause adverse health effects in subsequent generations after an initial exposure through an inherited epigenomic changes (Marczylo *et al.*, 2016). Multigenerational toxicity refers to adverse effects seen in organisms (adult, fetus, or primordial germ cells) which have had direct exposure to the toxicant of interest; however,

transgenerational toxicity refers to adverse effects observed in organisms with no direct exposure to the toxicant (Horzmann, et al., 2018a).

Zebrafish (*Danio rerio*) serve as a useful model for epigenetic studies due to their external fertilization and well characterized development (Kimmel, et al., 1995), fecund reproduction with a short generational period (Hoo *et al.*, 2016), sequenced genome with 70-80% similarity to the human genome (Howe, et al., 2013). Zebrafish share similar epigenetic machinery but without the requirement of imprinting for viability (Goll *et al.*, 2011), allowing for the investigation into the role of methylation in developmental toxicity. This study will use the zebrafish biomedical model to test the hypothesis that developmental exposure to ecologically relevant levels of TCE causes multigenerational and transgenerational toxicity in the progeny of exposed zebrafish. Multi- and transgenerational toxicity will be evaluated through an assessment of morphologic features, heart rate analysis, and a visual motor response behavioral test.

## 2.3 Materials and Methods

### 2.3.1 Zebrafish Husbandry

F0 embryos were collected from a breeding colony of SPF AB wild-type laboratory zebrafish (*Danio rerio*). All adult zebrafish are maintained on a ZS660 system (Aquaneering Inc., San Diego, CA) with a 14:10 light dark schedule. The aquaria water is maintained at 26-28 °C, pH 7.3-7.5, and a conductivity of 500-600 μS. The pH probe is calibrated on a bi-weekly basis to ensure accuracy. All fish and the aquaria system are monitored twice daily. Adult fish are fed a mixture of brine shrimp (*Artemia franciscana*; Artemia International LLC. Fariview, Texas), Golden Pearls 500-800 mm (Artemia International LLC. Fariview, Texas,

and Zeigler adult zebrafish food (Zeigler Bros Inc., Gardners, Pennsylvania) twice daily. All zebrafish were treated humanely with utmost consideration to avoid and eliminate suffering in accordance with the guidelines set forth by the United States (National Research Council, 2011) and Auburn University under the approved Auburn University Institution Animal Care and Use Committee protocol #2019-3525.

### 2.3.2 Chemical TCE Treatment and Multi- and Transgenerational Embryo Collection

F0 embryos were exposed to a 120 hour static treatment of 0, 5, 50, or 500 ppb (ug/L) of TCE (CAS registry number 79- 01-6, >99.5% purity, BeanTown Chemical, Hudson, NH) in glass screw top vials as previously described (Horzmann, et al., 2020). F0 zebrafish were rinsed with artificial aquaria water at 120 hpf and grown into adults under normal laboratory conditions. F0 zebrafish were bred at 7-months post fertilization according to standard practice (Horzmann, et al., 2020; Westerfield, 2007) for the collection of F1 embryos and the assessment of multigenerational toxicity. F0 Males and females of each developmental TCE exposure were inbred within the same exposure to maintain separate progeny groups (0, 5, 50, and 500 ppb). The F1 progeny were used to assess multigenerational toxicity, as they were exposed to TCE as germ cells. A subset of F1 progeny were reared into adults under normal laboratory conditions and bred similarly to produce F2 embryos. The F2 progeny were used to evaluate transgenerational toxicity as the generation had no exposure to TCE but could have inherited an altered epigenome.

For both F1 and F2 progeny, embryos from each progeny group were collected at approximately 1 hour post fertilization (hpf), and 50 embryos per progeny group per replicate were transferred to separate petri dishes with 20 mL of artificial system water (RO water with

marine salt added to a conductivity of 500-600 μS and a pH of 7.3-7.5). A replicate included all embryos from each progeny group collected on a single synchronized breeding day. Embryos were incubated at 28.5°C for 120 hpf. Embryonic survival was checked daily, and only replicates with greater than 80% survival were used for analysis.

### 2.3.3 Morphologic Assessment

Larval zebrafish from the 0, 5, 50, and 500 progeny groups were assessed for morphologic alterations at 120 hpf. After being anesthetized with 0.4 mg/mL buffered tricaine-S (ethyl m-amino benzoate methanesulfonate; Western Chemical Inc., Ferndale, Washington), larvae were observed and photographed via an Olympus SZX10 stereomicroscope with an Olympus DP22 camera and cellSense Entry software (Olympus Corporation, Center Valley, Pennsylvania). After placing the fish in lateral recumbency, the following parameters were evaluated: otolith diameter, jaw length, pericardial area, and head to trunk angle (Martínez *et al.*, 2019). The fish were placed in a dorsal-ventral view to evaluate the following parameters: body length, head length, head width, head length to body length ratio, and head width to body length ratio. Six biologic replicates (n=6) with 20 subsamples per progeny group per replicate (total of 120 subsamples total per progeny group) were measured.

### 2.3.4 Heart Rate Analysis

Heart rate was measured in 120 hpf larval zebrafish to evaluate for cardiotoxicity. Larval zebrafish from each progeny group (0, 5, 50, 500 ppb TCE) were anesthetized with buffered tricaine-S (ethyl m-amino benzoate methanesulfonate; Western Chemical Inc., Ferndale, Washington), with 6 mL of 0.4 mg/mL added to each petri-dish resulting in a final

concentration of 0.1mg/mL (Horzmann, et al., 2020). Using the Olympus SZX10 stereomicroscope and cellSens Entry software, zebrafish were placed in lateral recumbency, and 30 second videos were recorded. The Noldus DanioScope (Noldus Information Technology, Wageningen, Netherlands) cardiology module was used to measure beats per minute. Six biologic replicates (n=6) with 20 subsamples per progeny group per replicate (total of 120 subsamples total per progeny group) were recorded. When heartrate was not detected by software due to larval movement, position, or video quality, videos were discarded.

### 2.3.5 Larval Visual Motor Response Assessment

A Visual Motor Response (VMR) test was utilized to evaluate larval behavior as previously described (Horzmann *et al.*, 2017; Horzmann, et al., 2020). Larvae from each progeny group (0, 5, 50, and 500 ppb TCE) were placed in individual wells in a 96-well plate with 0.5 mL of artificial system water per well. Progeny groups were arranged in a balanced manner to eliminate any potential bias. Using the Noldus DanioVision Observation Chamber (Noldus Information Technology, Wageningen, Netherlands), infrared technology tracked the activity of the larvae. Within the observation chamber, the water temperature was maintained at 28.5 °C with the Noldus Temperature Control Unit. The larvae were given a 10-minute acclimation period inside the observation chamber prior to beginning the VMR test. Behavior was evaluated through the Noldus White Light routine. The specified routine involves 10-minute intervals alternating between white light and no light for a total of 50 minutes. The movement was traced via Basler GenICam acA I300-60gm camera and further analyzed with Noldus EthoVision XT Software. All behavior experiments were performed between the hours of 11:00 am and 1:00 pm to minimize potential variability in circadian rhythm. The final

parameters evaluated were total distance moved, velocity, time spent moving, turn angle, angular velocity, meander, and turn direction. There were ten biologic replicates (n=10) with 24 subsamples per progeny group per replicate (240 subsamples total per progeny group).

### 2.3.6 Statistical Analysis

An analysis of variance (ANOVA) via a General Linear Model (GLM; Minitab 19) was used to test for differences among progeny groups for all morphologic parameters, heart rate, and behavioral endpoints. The ANOVA was blocked by replicate. When the ANOVA p-value was less than 0.05, a Fisher's Least Significant Difference (LSD) post hoc test with $\alpha$=0.05 was used to determine differences between progeny groups.

## 2.4 Results

### 2.4.1 Morphologic Assessment

The dorsal measurements of the 120 hpf F1 larval zebrafish revealed a significant difference ($p<0.001$) in head width, with the 50 ppb progeny having increased head width, and head width to body length ratio ($p<0.001$), with the 50 and 500 ppb progeny having an increased head width to body length ratio as compared to the controls. Body length, head length, and head length to body length ratio were not statistically different between progeny groups (Table 2.1).

Table 2.1. F1 Dorsal Morphological Measurements.

| F0 TCE Exposure (ppb) | Body Length (um) | Head Length (um) | Head Width (um) | Head Length/ Body Length | Head width/ Body Length |
|---|---|---|---|---|---|
| 0 | 4014.95(123.063) | 792.48 (74.189) | 653.00 (21.450) | 0.1974 (0.01765) | 0.1627(0.00562) |
| 5 | 3993.15 (126.822) | 801.07(32.747) | 653.94 (24.932) | 0.2006 (0.00636) | 0.1638(0.00589) |
| 50 | 4011.53 (115.062) | 800.016 (31.958) | * 664.98 (28.352) | 0.1994(0.00700) | * 0.1658(0.00678) |
| 500 | 3982.63 (126.225) | 788.86 (33.580) | 655.88 ( 23.753) | 0.1981(0.00581) | * 0.1648(0.00580) |

Values listed as mean ± standard deviation. n=6, with 20 subsamples per progeny group per replicate. * Denotes significant difference from controls with $p<0.05$.

Lateral recumbency was used to measure eye diameter, jaw length, otolith diameter, pericardial area, and head to trunk angle. In F1 progeny, eye diameter was significantly different ($p<0.001$) between groups with the 5, 50, and 500 ppb progeny all having an increased eye diameter compared to controls. Jaw length was significantly different ($p<0.001$) between progeny groups with the 5 and 500 ppb groups having increased jaw length compared to controls. The pericardial area was significantly different ($p=0.03$) between groups, with the 5 and 50 ppb progeny having decreased pericardial area. Otolith diameter and head to trunk angle were not significantly different compared to the controls (Table 2.2).

Table 2.2. F1 Lateral Morphological Measurements.

| F0 TCE Exposure (ppb) | Eye Diameter (um) | Jaw Length (um) | Otolith Diameter (um) | Pericardial Area (um²) | Head to Trunk Angle |
|---|---|---|---|---|---|
| 0 | 345.73 (17.772) | 198.79 (26.819) | 54.10 (10.506) | 17143.89 (6890.689) | 154.61 (3.303 |
| 5 | * 363.07 (17.032) | * 205.68 (22.838) | 51.35 (10.207) | * 16072.68 (6025.402) | 155.14 (3.464) |
| 50 | * 354.25 (25.826) | 196.15 (18.203) | 52.18 (8.651) | * 15848.48 (5374.863) | 154.69 (3.423) |
| 500 | * 355.11 (18.586) | * 210.51 (18.770) | 54.22 (9.618) | 16597.64 (5748.002) | 154.71 (3.304) |

Values listed as mean ± standard deviation. n=6, with 20 subsamples per progeny group per replicate. * Denotes significant difference from controls with $p<0.05$.

The F2 larval zebrafish demonstrated several alterations in morphology. Body length was significantly different ($p=0.006$) between progeny groups, with both the 50 and 500 ppb groups having significantly increased body length as compared to the controls. Head length and head length to body length ratio were significantly different ($p<0.001$, $p<0.001$), with the 50 and 500 ppb progeny having decreased head length and decreased head length to body length ratios. Head width and head width to body length ratio were significantly different ($p<0.001$, $p<0.001$) with the 5 and 50 ppb progeny having decreased head width and the 50 ppb progeny having a decreased head width to body length ratio (Table 2.3).

In the F2 lateral measurements, all exposure groups (5, 50, and 500 ppb) had significantly decreased eye diameter ($p<0.001$) as compared to the control group. Jaw length was significantly different ($p=0.006$) between groups, with the 500 ppb progeny having decreased jaw length as compared to the controls. Pericardial area was also significantly different ($p<0.001$) among progeny groups, with the 5 ppb progeny having decreased area and

the 50 ppb and 500 ppb progeny having increased pericardial area. Finally, the head to trunk angle was significantly decreased (p=0.029) in the 5, 50, and 500 ppb progeny (Table 2.4).

Table 2.3. F2 Dorsal Morphological Measurements.

| F0 TCE Exposure (ppb) | Body Length (um) | Head Length (um) | Head Width (um) | Head Length/ Body Length | Head Width/ Body Length |
|---|---|---|---|---|---|
| 0 | 3984.74 (100.997) | 798.61 (28.245) | 664.97 (22.239) | 0.2004 (0.00722) | 0.1670 (0.00656) |
| 5 | 3953.99 (133.106) | 797.20 (32.916) | * 657.20 (28.976) | 0.2017 (0.00804) | 0.1663 (0.00672) |
| 50 | * 3994.69 (163.985) | * 774.18 (45.461) | * 650.03 (27.176) | * 0.1940 (0.01113) | * 0.1629 (0.0077) |
| 500 | * 4008.24 (125.193) | * 787.14 (27.048) | 666.93 (22.951) | * 0.1964 (0.00586) | 0.1665 (0.00696) |

Values listed as mean ± standard deviation. n=6, with 20 subsamples per progeny group per replicate. * Denotes significant difference from controls with $p<0.05$.

Table 2.4. F2 Lateral Morphological Measurements.

| F0 TCE Exposure (ppb) | Eye Diameter (um) | Jaw Length (um) | Otolith (um) | Pericardial Area ($um^2$) | Head to Trunk Angle (degrees) |
|---|---|---|---|---|---|
| 0 | 352.92 (14.905) | 238.53 (22.198) | 61.84 (9.762) | 18134.93 (3261.508) | 152.42 (34.366) |
| 5 | * 346.21 (13.615) | 232.28 (24.440) | 102.65 (485. 166) | * 14717.74 (1600.139) | * 146.92 (5.730) |
| 50 | * 345.92 (15.300) | 237.87 (32.338) | 62.24 (10.742) | * 21534.05 (2488.368) | * 146.73 (5.316) |
| 500 | * 345.81 (9.880) | * 228.58 (24.906) | 561.52 (5507.012) | * 19402.56 (2081.857) | * 146.63 (5.740) |

Values listed as percent survival ± standard deviation. n=6, with 20 subsamples per progeny group per replicate. * Denotes significant difference from controls with $p<0.05$.

### 2.4.2 Heart Rate Analysis

In the F1 generation, there was a significant difference ($p<0.001$) in heart rate between progeny groups, with the 500 ppb progeny having a significantly increased beats per minute (BPM) as compared to the control progeny (Figure 2.1). The F2 generation also had a significant difference between progeny groups ($p<0.001$), with the 500 ppb progeny having a significantly decreased heart rate compared to that of the controls.

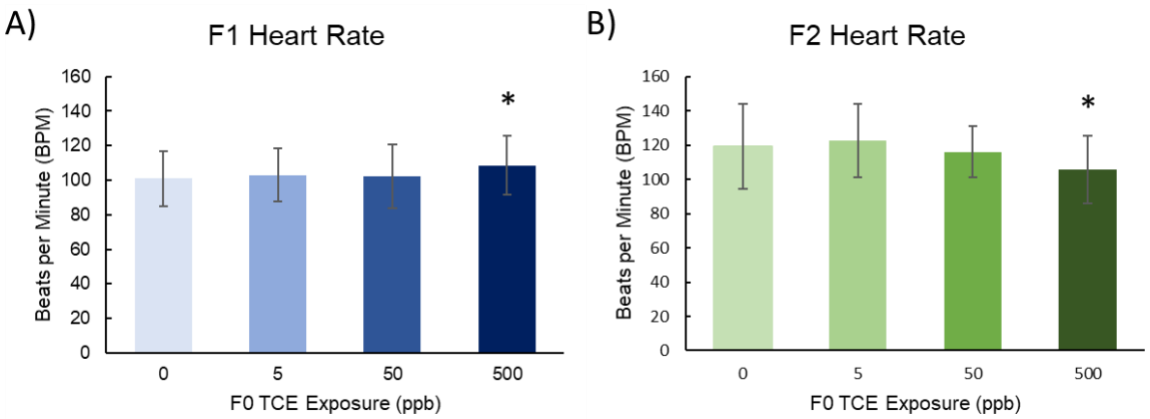


Figure 2.1. Alterations in heart rate in F1 and F2 progeny. The 500 ppb F1 progeny had significantly increased BPM compared to controls (A) while the 500 ppb F2 progeny had significantly decreased BMP compared to controls (B). n=6, with 20 subsamples per progeny group per replicate. Error bars represent standard deviation. * = $p < 0.05$.

### 2.4.3 Larval Visual Motor Response Assessment

The VMR test demonstrated changes in F1 larval locomotion. The 50 and 500 ppb progeny had decreased distance moved ($p<0.001$), velocity ($p<0.001$), and time spent moving ($p<0.001$) with increased time spent not moving compared to controls (Figure 2.2). The F1 progeny did not have any significant difference between groups with respect to path shape parameters

(Figure 2.3); however, the F1 larval zebrafish had changes in rotational behavior. The 50 and 500 ppb progeny had decreased frequency of clockwise rotations ($p=0.001$) while counterclockwise rotations were decreased in the 5, 50, and 500 ppb progeny ($p<0.001$) (Figure 2.4).

The F2 larval zebrafish also demonstrated alterations in locomotion, with decreased distance moved ($p=0.018$) and velocity ($p=0.018$) in the 500 ppb progeny (Figure 2.5). With regard to path shape parameters, there was no difference in turn angle, angular velocity and mean meander between progeny groups, but the heading was increased in the 500ppb progeny ($p=0.018$) compared to controls (Figure 2.6). Decreased rotational activity was seen in the 5 and 500 ppb progeny groups, with both clockwise ($p=0.001$) and counterclockwise ($p=0.001$) frequency decreased compared to controls (Figure 2.7).

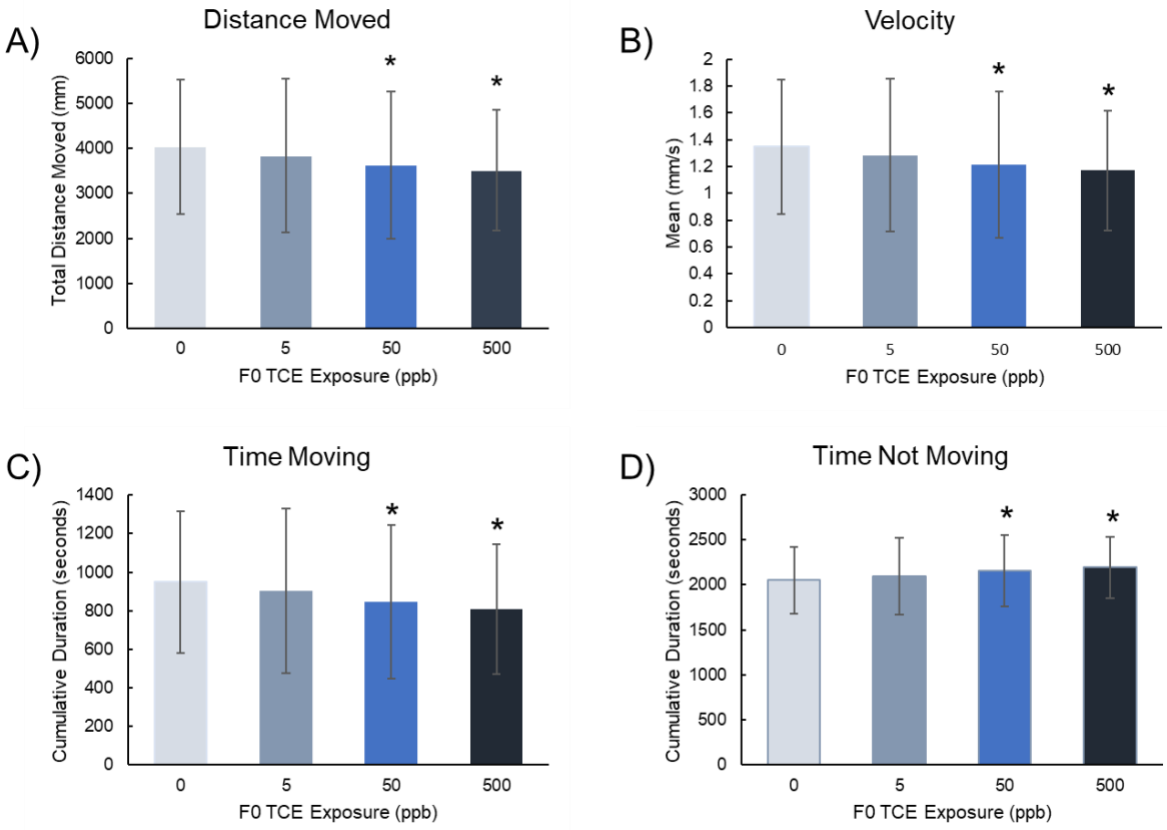


Figure 2.2. Alterations in locomotion in F1 progeny. The 50 and 500 F1 progeny groups had significantly decreased distance moved (A), velocity (B), time moving (C), and significantly increased time not moving (D) compared to controls. n=10, with 24 subsamples per progeny group per replicate. Error bars represent standard deviation. *$p < 0.05$.

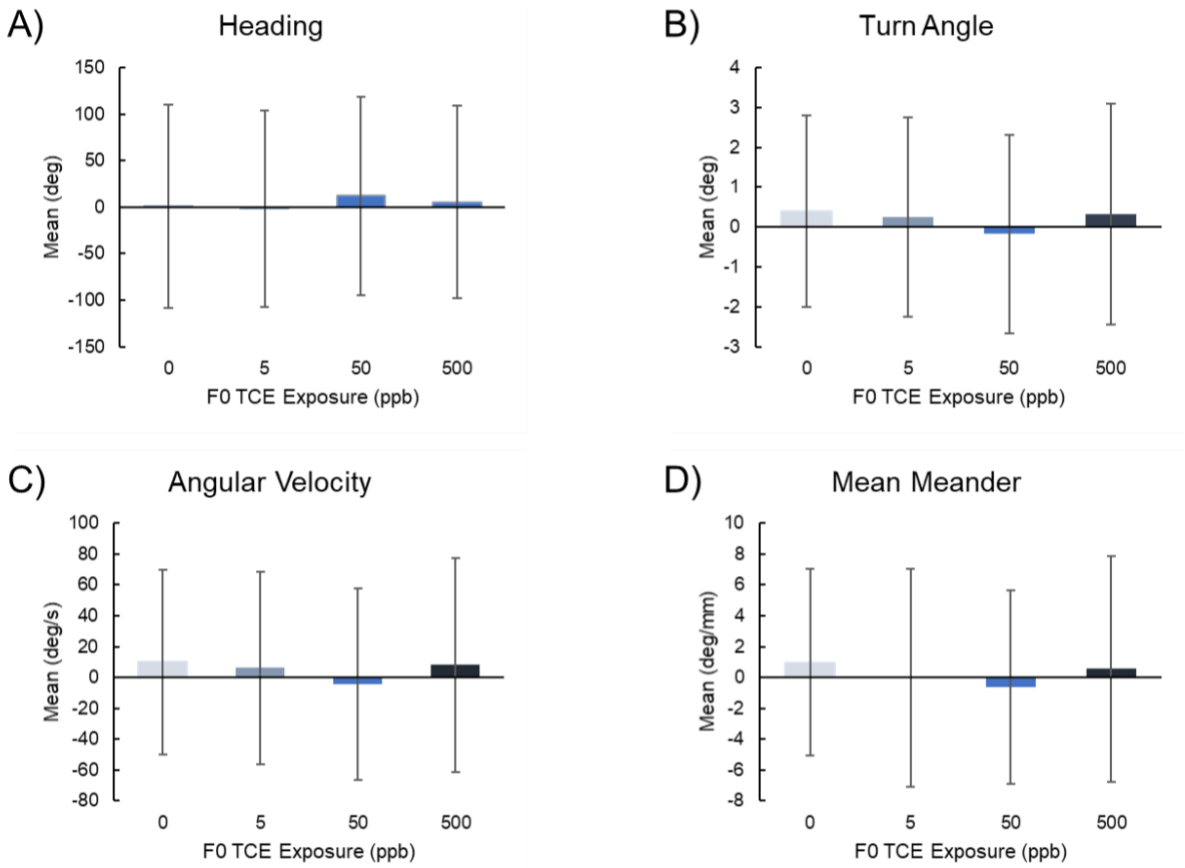


Figure 2.3. Alterations in path shape in F1 progeny. There were no significant differences between progeny groups with respect to heading (A), turn angle (B), angular velocity (C), or mean meander (D). n=10, with 24 subsamples per progeny group per replicate. Error bars represent standard deviation. $p < 0.05$.

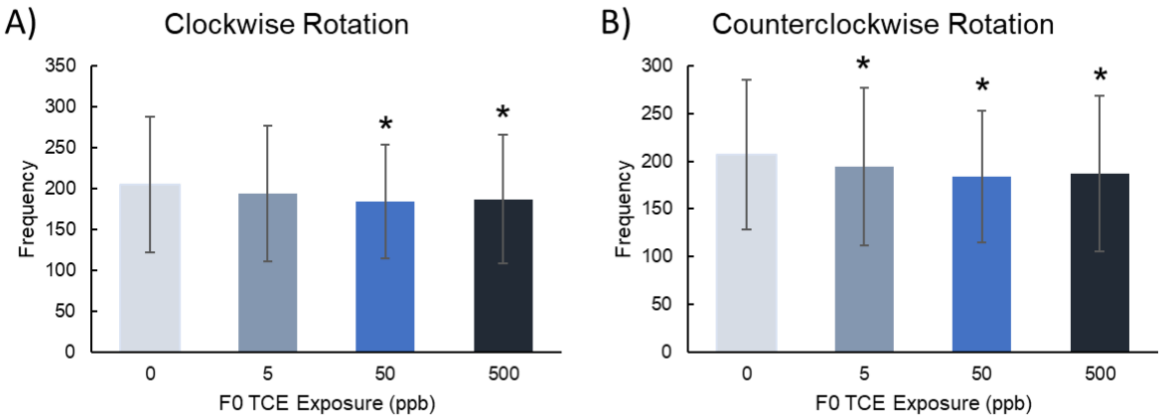


Figure 2.4. Alterations in rotation in F1 progeny. The 50 and 500 ppb F1 progeny groups had significantly decreased frequency of clockwise rotation (A) while the 5, 50, and 500 ppb progeny all had decreased frequency of counterclockwise rotation compared to controls (B). n=10, with 24 subsamples per progeny group per replicate. Error bars represent standard deviation. * $p < 0.05$.

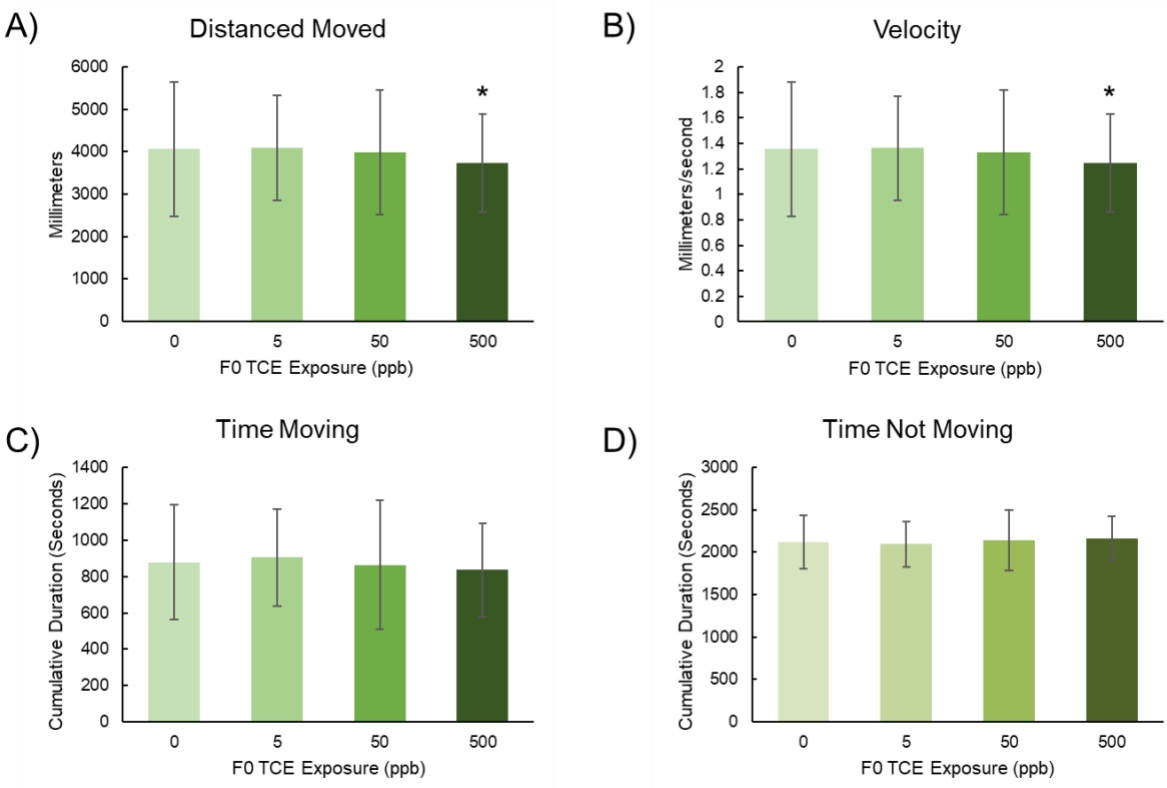


Figure 2.5. Alterations in locomotion in F2 progeny. The 500 ppb F2 progeny had significantly decreased distance moved (A) and velocity (B) compared to controls. There was no difference in time spent moving (C) or not moving (D) between progeny groups. n=10, with 24 subsamples per progeny group per replicate. Error bars represent standard deviation. * $p < 0.05$.

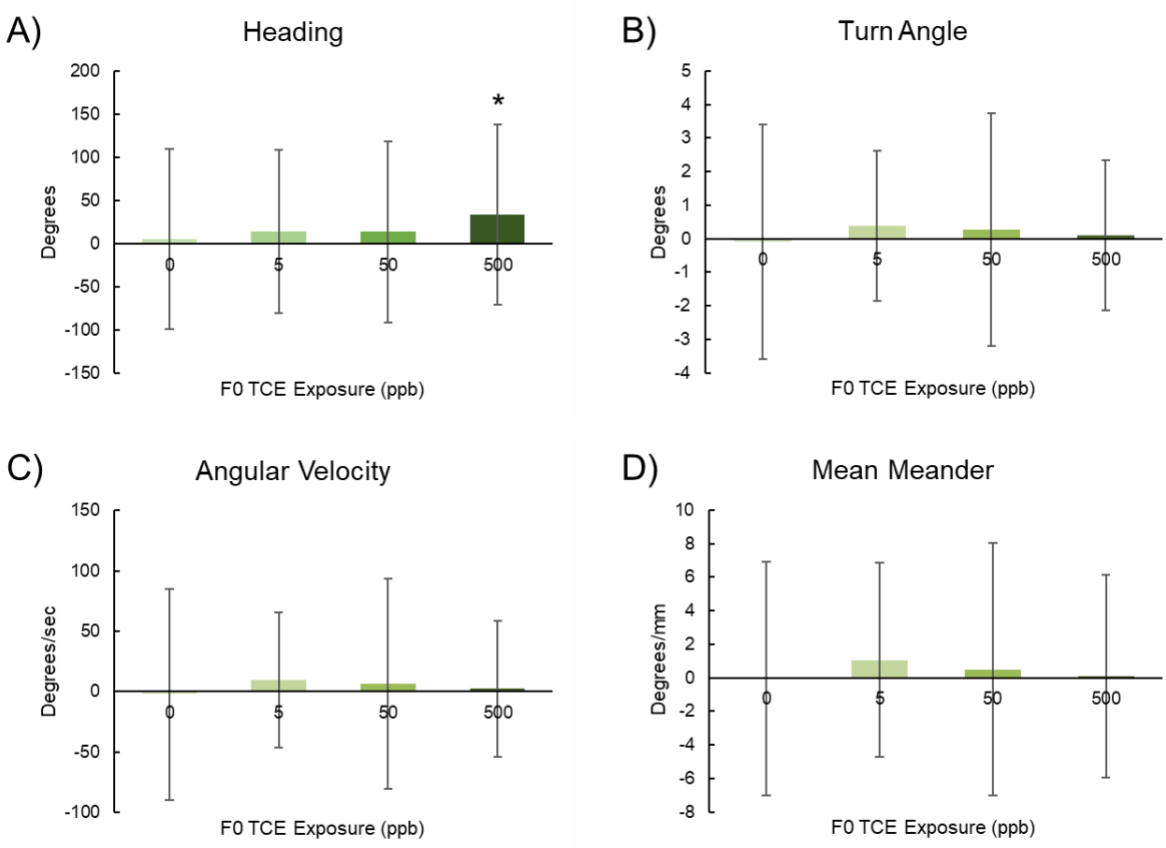


Figure 2.6. Alterations in path shape in F2 progeny. The 500 ppb progeny had a significantly increased heading compared to controls (A), but there were no significant differences between progeny groups with respect to turn angle (B), angular velocity (C), or mean meander (D). n=10, with 24 subsamples per progeny group per replicate. Error bars represent standard deviation. * $p < 0.05$.

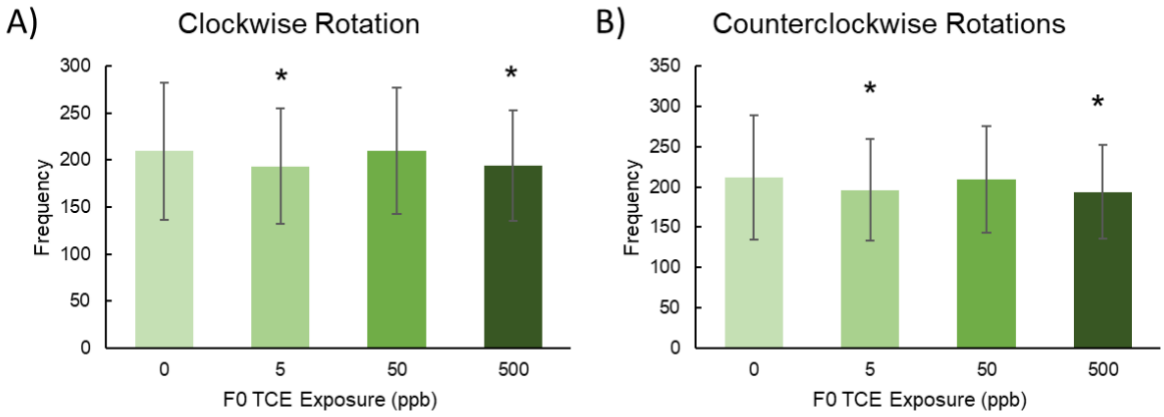


Figure 2.7. Alterations in rotation in F2 progeny. The 5 and 500 ppb F2 progeny groups had significantly decreased frequency of clockwise rotation (A) and counterclockwise rotation (B) compared to controls. n=10, with 24 subsamples per progeny group per replicate. Error bars represent standard deviation. * $p < 0.05$.

## 2.5 Discussion

This study investigated the multi- and transgenerational effects in F1 and F2 generations following a developmental TCE exposure in the F0 generation. In a previous study, F0 zebrafish with 1-120 hpf exposure to TCE had alterations in morphologic measurements, heart rate, and behavioral path shape (Horzmann, et al., 2020). In this study, similar morphologic, physiologic, and behavioral endpoints were evaluated to determine if TCE causes persistent changes that are passed through the generations.

In order to investigate if TCE caused physical developmental changes in subsequent generations, basic morphologic parameters were evaluated on 120 hpf larval F1 and F2 progeny zebrafish. Previous studies have noted that the end of development, 120 hpf, is the time period when teratogenic effects are the most observable in larval zebrafish (Brannen *et al.*, 2010; Horzmann, et al., 2020). In this study, head width was decreased in both the F1 (multigenerational) and F2 (transgenerational) larval zebrafish. Head width to body length ratio was altered in both F1 and F2 zebrafish along with head length to body length ratio being altered in the F2 larval zebrafish. Recent data from this lab, demonstrated an alteration in both head length to body length ratio and head width to body length ratio in the F0 generation (Horzmann, et al., 2020). The F0 embryos received static exposure to 0 ppb, 5 ppb, 50 ppb, or 500 ppb for 5 days, or 120 hpf. The persistent alteration in head size to body length ratio is suggests a dysregulation in craniofacial development. Craniofacial development in the embryonic/larval zebrafish is quite complex, as it involves 43 bones, 30 of which undergo intramembranous ossification (Cubbage *et al.*, 1996; Mork *et al.*, 2015). In addition to head size, body length was increased in the F2 larval zebrafish. In the F0 120 hpf exposure trial, body length was actually decreased. The reason for this dissimilarity is

unclear, but over-correction of epigenetic developmental adaptation is one potential mechanism (Tian *et al.*, 2018). Nonetheless, the altered body length in both the F0 and F2 generation supports epigenetic TCE toxicity, as the F2 progeny were not directly exposed to TCE. The results of this study are significant considering TCE has been associated with cleft palate in children whose mothers had occupational or residential exposure to TCE (Brender *et al.*, 2014; Lorente *et al.*, 2000). It is currently unknown if the children of affected individuals would have increased risk of similar craniofacial defects.

In zebrafish with direct developmental exposure to 10 ppb TCE for 1-72 hpf, transcriptomic analysis indicated altered expression of genes related to organismal development, organ morphology, and cellular growth, proliferation, and development (Wirbisky, et al., 2016). It is possible that TCE acts on the epigenetic regulation of these pathways in the F0 larvae to cause inheritable altered development in progeny. A potential epigenetic mechanism associated with craniofacial defects is the dysregulation of histone deacetylases. Histone deacetylases are involved in the removal of acetyl groups placed on histones, a known epigenetic alteration, while they are involved in multiple cellular processes they are known to be involved in bone development and remodeling (Bradley *et al.*, 2015). Zebrafish studies have shown that alterations in histone deacetylase function led to developmental alterations (Cunliffe *et al.*, 2006; Farooq *et al.*, 2008; He *et al.*, 2014; Yamaguchi *et al.*, 2005). While there are not zebrafish specific studies showing alterations in histone deacetylases causing abnormal craniofacial developmental, this has been demonstrated in mouse models (Haberland *et al.*, 2009; Singh *et al.*, 2013; Zimmermann *et al.*, 2007). Though the human literature is minimal, the studies in mouse models are consistent in showing alterations in histone deacetylases leading to craniofacial deformities (Williams *et al.*, 2010;

Wu *et al.,* 2013). The epigenetic mechanism causing the multi- and transgenerational morphologic abnormalities in zebrafish with TCE exposure require further investigation.

In addition to examining morphologic alterations with the zebrafish positioned in a dorsoventral view, zebrafish were also examined while they were laterally recumbent. From this view, the F1 and F2 larval zebrafish had significant changes in eye diameter and jaw length. In the F1 progeny, the eye diameter was increased; however, the F2 progeny had decreased eye diameter. Studies in Sprague-Dawley and Fisher rats have shown TCE exposure can cause eye deformities (Narotsky *et al.*, 1995; Warren *et al.*, 2006). Considering previous studies in zebrafish have shown the retina is capable of regeneration following damage (Wan *et al.*, 2016), we hypothesize that there is a compensatory mechanism occurring in the F1 generation, however it is not seen within the next generation. Due to the minimal studies on TCE exposure and eye development, further studies are required to determine the mechanism of action.

One reason zebrafish serve as a beneficial model for the investigation of cardiotoxicity is the ability to visualize the heart through the developmental period (Heideman *et al.*, 2005). In both the F1 and F2 progeny, heart rate was altered in the 500 ppb progeny, although in different directions. Pericardial area was also examined while the larval zebrafish were laterally recumbent and in the F1 progeny, the 5 ppb and 50 ppb exposure groups had a decreased pericardial area. Interestingly, the F2 progeny demonstrated a decreased pericardial area in the 5 ppb exposure group; however, the 50 ppb and 500 ppb exposure groups had increased pericardial area. Previous studies using zebrafish, chick embryos and rat myocytes have demonstrated the cardiotoxic effects seen on TCE exposure during developmentally critical periods (Caldwell *et al.*, 2008; Drake *et al.*, 2006; Horzmann, et al., 2020; Makwana *et*

*al.*, 2013; Makwana *et al.*, 2010). Possible mechanisms identified include disruption of calcium homeostasis and an increased number of cardiac cells, cardiomyocytes and endocardiocytes. Due to the consistent findings of altered heart rate in the 500 ppb in both F1 and F2 progeny, yet varying response in pericardial area, we hypothesize there is an epigenetic dysregulation, again possibly related to adaptive epigenetic modifications (Tian, et al., 2018).

Zebrafish exposed to TCE demonstrated alterations in microRNA expression, a known epigenetic mechanism. Investigators found 19 miRNAs and 48 miRNAs to be downregulated and upregulated, respectively in zebrafish with developmental exposure to 10 ppb TCE (Huang *et al.*, 2020). The downregulation of miR-133a causes a cascade of additional reactions; the downregulation of miR-133a leads to the overexpression of oxidative stress genes, ultimately increasing the number of reactive oxygen species (ROS) in the heart. Furthermore, atrial and ventricular hyperplasia was noted in the hearts of affected larval zebrafish, as compared to the controls. While this study confirms TCE can cause epigenetic alterations, it only evaluates the F0 zebrafish. Studies involving human stem cells have shown that TCE exposure causes disruption in development of cardiac cells (Jiang *et al.*, 2016). Retrospective epidemiologic studies have shown TCE exposure causes cardiac abnormalities in humans (Bove *et al.*, 2002; Forand, et al., 2012; Gilboa *et al.*, 2012; Shaw *et al.*, 1990; Wilson *et al.*, 1998; Yauck *et al.*, 2004). Based on the data presented from laboratory and epidemiologic studies, we know TCE exposure can induce cardiac abnormalities; however, the consequence and mechanism of epigenetic alterations require further investigation.

Behavior was assessed via the VMR test. Behavioral assays are often used to evaluate visual, neurologic, and locomotive responses in larval zebrafish (Haug *et al.*, 2010; Horzmann *et al.*, 2018b; Kalueff *et al.*, 2013; MacPhail *et al.*, 2009; Portugues *et al.*, 2009). In both the

F1 and the F2 generations, the distance moved and the average velocity were decreased. Furthermore, turning frequency was altered in both the F1 and F2 generations. It has been shown in zebrafish that most of the brain is developed with the major axon tracts present by 96 hpf (Orger *et al.*, 2017). A study using mice demonstrated that TCE exposure during development produced alterations in glutathione homeostasis, a measure of oxidative stress, within the cerebellum (Blossom, et al., 2020). Disruption in glutathione homeostasis was also shown to alter DNA methyltransferases-leading to DNA hypomethylation, an epigenetic alteration. The cerebellum not only controls smooth muscle movements, but also sensory inputs (D'Angelo, 2018; Hibi *et al.*, 2012; Hsieh *et al.*, 2014; Therrien *et al.*, 2019). Thus, the alterations in gene expression within the cerebellum provide supporting evidence that TCE can create epigenetic alterations that affect neurologic function. The knowledge gap on TCE related epigenetic neurotoxicity in laboratory species and humans demonstrates a desperate need for evaluation of these mechanisms.

This study is not without limitations. As our lab has previously noted, the absorption, metabolism, and tissue dose are not known in the F0 generation. Thus, it is difficult to know how well these exposures transcend generations and how it relates to other species. Additionally, our study is representative of a one-time static exposure in the F0 generation. This is likely not as representative of epigenetic exposure, as drinking water contamination would likely happen directly and indirectly in each generation. However, the results suggest that even indirect TCE exposure does cause alterations in morphology, heart rate, and behavior.

## 2.6 Conclusions

TCE is a significant environmental toxicant that is suspected to cause epigenetic toxicity. In the present study, altered morphology, heart rate, and locomotor and turning behavior in F1 and F2 progeny of F0 zebrafish with developmental TCE exposure suggest that environmentally relevant levels of TCE can cause multi- and transgenerational toxicity. This further supports TCE's inclusion on the ATSDR's National Priority list and reinforces the need for further investigation into mechanisms of TCE related epigenetic toxicity.

## 2.7 References:

Agency for Toxic Substances and Disease Registery (2020). *ATSDR's Substance Priority List.* Available at: https://www.atsdr.cdc.gov/spl/. Accessed.

Agency for Toxic Substances and Disease Registry (2019). Toxicological profile for Trichloroethylene (TCE).

Blossom, S. J., Melnyk, S. B., Li, M., Wessinger, W. D., and Cooney, C. A. (2017). Inflammatory and oxidative stress-related effects associated with neurotoxicity are maintained after exclusively prenatal trichloroethylene exposure. *Neurotoxicology* **59**, 164-174 (Article; Proceedings Paper).

Blossom, S. J., Melnyk, S. B., and Simmen, F. A. (2020). Complex epigenetic patterns in cerebellum generated after developmental exposure to trichloroethylene and/or high fat diet in autoimmune-prone mice. *Environ Sci Process Impacts* **22**(3), 583-594.

Bove, F., Shim, Y., and Zeitz, P. (2002). Drinking water contaminants and adverse pregnancy outcomes: a review. *Environ Health Perspect* **110 Suppl 1**(Suppl 1), 61-74.

Bradley, E. W., Carpio, L. R., van Wijnen, A. J., McGee-Lawrence, M. E., and Westendorf, J. J. (2015). Histone Deacetylases in Bone Development and Skeletal Disorders. *Physiol Rev* **95**(4), 1359-81.

Brannen, K. C., Panzica-Kelly, J. M., Danberry, T. L., and Augustine-Rauch, K. A. (2010). Development of a zebrafish embryo teratogenicity assay and quantitative prediction model. *Birth Defects Research Part B: Developmental and Reproductive Toxicology* **89**(1), 66-77.

Brender, J. D., Shinde, M. U., Zhan, F. B., Gong, X., and Langlois, P. H. (2014). Maternal residential proximity to chlorinated solvent emissions and birth defects in offspring: a case-control study. *Environ Health* **13**, 96.

Caldwell, P. T., Thorne, P. A., Johnson, P. D., Boitano, S., Runyan, R. B., and Selmin, O. (2008). Trichloroethylene disrupts cardiac gene expression and calcium homeostasis in rat myocytes. *Toxicological sciences : an official journal of the Society of Toxicology* **104**(1), 135-143.

Cooney, C. A. (2014). Epigenetic Alterations due to Trichlorotheylene. In *Trichloroethylene: Toxicity and Health Risks* (K. Gilbert, and S. J. Blossom, Eds.), pp. 185-208. Springer London, London.

Cubbage, C. C., and Mabee, P. M. (1996). Development of the cranium and paired fins in the zebrafish Danio rerio (Ostariophysi, Cyprinidae). *J Morphol* **229**(2), 121-160.

Cui, Y., Choudhury, S. R., and Irudayaraj, J. (2016). Epigenetic toxicity of trichloroethylene: a single-molecule perspective. *Toxicol. Res.* **5**(2), 641-650 (Article).

Cunliffe, V. T., and Casaccia-Bonnefil, P. (2006). Histone deacetylase 1 is essential for oligodendrocyte specification in the zebrafish CNS. *Mechanisms of Development* **123**(1), 24-30.

D'Angelo, E. (2018). Chapter 6 - Physiology of the cerebellum. In *Handb Clin Neurol* (M. Manto, and T. A. G. M. Huisman, Eds.), Vol. 154, pp. 85-108. Elsevier.

Drake, V. J., Koprowski, S. L., Hu, N., Smith, S. M., and Lough, J. (2006). Cardiogenic Effects of Trichloroethylene and Trichloroacetic Acid Following Exposure during Heart Specification of Avian Development. *Toxicological Sciences* **94**(1), 153-162.

Farooq, M., Sulochana, K. N., Pan, X., To, J., Sheng, D., Gong, Z., and Ge, R. (2008). Histone deacetylase 3 (hdac3) is specifically required for liver development in zebrafish. *Developmental Biology* **317**(1), 336-353.

Forand, S. P., Lewis-Michl, E. L., and Gomez, M. I. (2012). Adverse birth outcomes and maternal exposure to trichloroethylene and tetrachloroethylene through soil vapor intrusion in New York State. *Environ Health Perspect* **120**(4), 616-21.

Gilboa, S. M., Desrosiers, T. A., Lawson, C., Lupo, P. J., Riehle-Colarusso, T. J., Stewart, P. A., van Wijngaarden, E., Waters, M. A., and Correa, A. (2012). Association between maternal occupational exposure to organic solvents and congenital heart defects, National Birth Defects Prevention Study, 1997-2002. *Occup Environ Med* **69**(9), 628-35.

Goldberg, S. J., Lebowitz, M. D., Graver, E. J., and Hicks, S. (1990). An association of human congenital cardiac malformations and drinking water contaminants. *J Am Coll Cardiol* **16**(1), 155-64.

Goll, M. G., and Halpern, M. E. (2011). DNA methylation in zebrafish. *Prog Mol Biol Transl Sci* **101**, 193-218.

Goodman, M., Naiman, J. S., Goodman, D., and LaKind, J. S. (2012). Cancer clusters in the USA: what do the last twenty years of state and federal investigations tell us? *Crit Rev Toxicol* **42**(6), 474-90.

Guehl, D., Bezard, E., Dovero, S., Boraud, T., Bioulac, B., and Gross, C. (1999). Trichloroethylene and parkinsonism: a human and experimental observation. *Eur J Neurol* **6**(5), 609-11.

Haberland, M., Mokalled, M. H., Montgomery, R. L., and Olson, E. N. (2009). Epigenetic control of skull morphogenesis by histone deacetylase 8. *Genes Dev* **23**(14), 1625-1630.

Harris, A. P., Ismail, K. A., Nunez, M., Martopullo, I., Lencinas, A., Selmin, O. I., and Runyan, R. B. (2018). Trichloroethylene perturbs HNF4a expression and activity in the developing chick heart. *Toxicol. Lett.* **285**, 113-120.

Haug, M. F., Biehlmaier, O., Mueller, K. P., and Neuhauss, S. C. F. (2010). Visual acuity in larval zebrafish: behavior and histology. *Frontiers in Zoology* **7**(1), 8.

He, Y., Mei, H., Yu, H., Sun, S., Ni, W., and Li, H. (2014). Role of histone deacetylase activity in the developing lateral line neuromast of zebrafish larvae. *Experimental & Molecular Medicine* **46**(5), e94-e94.

Heideman, W., Antkiewicz, D. S., Carney, S. A., and Peterson, R. E. (2005). Zebrafish and cardiac toxicology. *Cardiovasc Toxicol* **5**(2), 203-14.

Hibi, M., and Shimizu, T. (2012). Development of the cerebellum and cerebellar neural circuits. *Dev Neurobiol* **72**(3), 282-301.

Hoo, J. Y., Kumari, Y., Shaikh, M. F., Hue, S. M., and Goh, B. H. (2016). Zebrafish: A Versatile Animal Model for Fertility Research. *Biomed Res Int* **2016**, 9732780-9732780.

Horzmann, K. A., de Perre, C., Lee, L. S., Whelton, A. J., and Freeman, J. L. (2017). Comparative analytical and toxicological assessment of methylcyclohexanemethanol (MCHM) mixtures associated with the Elk River chemical spill. *Chemosphere* **188**, 599-607.

Horzmann, K. A., and Freeman, J. L. (2018a). Making Waves: New Developments in Toxicology With the Zebrafish. *Toxicol Sci* **163**(1), 5-12.

Horzmann, K. A., Portales, A. M., Batcho, K. G., and Freeman, J. L. (2020). Developmental toxicity of trichloroethylene in zebrafish (Danio rerio). *Environmental Science: Processes & Impacts* **22**(3), 728-739 (10.1039/C9EM00565J).

Horzmann, K. A., Reidenbach, L. S., Thanki, D. H., Winchester, A. E., Qualizza, B. A., Ryan, G. A., Egan, K. E., Hedrick, V. E., Sobreira, T. J. P., Peterson, S. M., *et al.* (2018b). Embryonic atrazine exposure elicits proteomic, behavioral, and brain abnormalities with developmental time specific gene expression signatures. *J Proteomics* **186**, 71-82.

Howe, K., Clark, M. D., Torroja, C. F., Torrance, J., Berthelot, C., Muffato, M., Collins, J. E., Humphray, S., McLaren, K., Matthews, L., *et al.* (2013). The zebrafish reference genome sequence and its relationship to the human genome. *Nature* **496**(7446), 498-503.

Hsieh, J.-Y., Ulrich, B., Issa, F. A., Wan, J., and Papazian, D. M. (2014). Rapid development of Purkinje cell excitability, functional cerebellar circuit, and afferent sensory input to cerebellum in zebrafish. *Front Neural Circuits* **8**, 147-147.

Huang, Y., Jiang, B., Xia, Y., Wang, J., Ji, C., Tong, J., Chen, T., and Jiang, Y. (2020). Downregulation of miR-133a contributes to the cardiac developmental toxicity of trichloroethylene in zebrafish. *Chemosphere* **251**, 126610.

International Agency for Research on Cancer (2014). *Trichloroethylene.*, Vol. 106. IARC, Lyon, France. Monographs on the Evaluation of Carcinogenic Risks to Humans.

Jia, C., Yu, X., and Masiak, W. (2012). Blood/air distribution of volatile organic compounds (VOCs) in a nationally representative sample. *Sci. Total Environ.* **419**, 225-232.

Jiang, Y., Chen, J., Tong, J., and Chen, T. (2014). Trichloroethylene-induced gene expression and DNA methylation changes in B6C3F1 mouse liver. *PloS one* **9**(12), e116179-e116179.

Jiang, Y., Wang, D., Zhang, G., Wang, G., Tong, J., and Chen, T. (2016). Disruption of cardiogenesis in human embryonic stem cells exposed to trichloroethylene. *Environmental Toxicology* **31**(11), 1372-1380.

Kalueff, A. V., Gebhardt, M., Stewart, A. M., Cachat, J. M., Brimmer, M., Chawla, J. S., Craddock, C., Kyzar, E. J., Roth, A., Landsman, S., *et al.* (2013). Towards a comprehensive catalog of zebrafish behavior 1.0 and beyond. *Zebrafish* **10**(1), 70-86.

Kaneko, T., Wang, P. Y., and Sato, A. (1997). Assessment of the health effects of trichloroethylene. *Ind Health* **35**(3), 301-24.

Kimmel, C., Ballard, W., Kimmel, S., Ullmann, B., and Schilling, T. (1995). Stages of Embryonic Development of the Zebrafish. *Developmental Dynamics* **203**, 253-310.

Lai, C., Wu, F., Wang, Y., Wang, W., Li, Y., Zhang, G., Gao, J., Zhu, Z., Yuan, J., Yang, J., *et al.* (2021). Specific epigenetic microenvironment and the regulation of tumor-related gene expression by trichloroethylene in human hepatocytes. *Ecotoxicology and Environmental Safety* **208**, 111453.

Loch-Caruso, R., Hassan, I., Harris, S. M., Kumar, A., Bjork, F., and Lash, L. H. (2019). Trichloroethylene exposure in mid-pregnancy decreased fetal weight and increased placental markers of oxidative stress in rats. *Reprod Toxicol* **83**, 38-45.

Lorente, C., Cordier, S., Bergeret, A., De Walle, H. E., Goujard, J., Ayme, S., Knill-Jones, R., Calzolari, E., and Bianchi, F. (2000). Maternal occupational risk factors for oral clefts. Occupational Exposure and Congenital Malformation Working Group. *Scand J Work Environ Health* **26**(2), 137-45.

MacPhail, R. C., Brooks, J., Hunter, D. L., Padnos, B., Irons, T. D., and Padilla, S. (2009). Locomotion in larval zebrafish: Influence of time of day, lighting and ethanol. *Neurotoxicology* **30**(1), 52-8.

Makwana, O., Ahles, L., Lencinas, A., Selmin, O. I., and Runyan, R. B. (2013). Low-dose trichloroethylene alters cytochrome P450-2C subfamily expression in the developing chick heart. *Cardiovascular toxicology* **13**(1), 77-84.

Makwana, O., King, N. M., Ahles, L., Selmin, O., Granzier, H. L., and Runyan, R. B. (2010). Exposure to low-dose trichloroethylene alters shear stress gene expression and function in the developing chick heart. *Cardiovasc Toxicol* **10**(2), 100-7.

Marczylo, E. L., Jacobs, M. N., and Gant, T. W. (2016). Environmentally induced epigenetic toxicity: potential public health concerns. *Crit Rev Toxicol* **46**(8), 676-700.

Martínez, R., Navarro-Martín, L., Luccarelli, C., Codina, A. E., Raldúa, D., Barata, C., Tauler, R., and Piña, B. (2019). Unravelling the mechanisms of PFOS toxicity by combining morphological and transcriptomic analyses in zebrafish embryos. *Science of The Total Environment* **674**, 462-471.

Mork, L., and Crump, G. (2015). Zebrafish Craniofacial Development: A Window into Early Patterning. *Curr Top Dev Biol* **115**, 235-269.

Narotsky, M. G., Weller, E. A., Chinchilli, V. M., and Kavlock, R. J. (1995). Nonadditive Developmental Toxicity in Mixtures of Trichloroethylene, Di(2-ethylhexyl) Phthalate, and Heptachlor in a 5 × 5 × 5 Design. *Fundamental and Applied Toxicology* **27**(2), 203-216.

National Research Council (2011). *Guide for the care and use of laboratory animals*, 8th ed. National Academies Press.

Orger, M. B., and Polavieja, G. G. d. (2017). Zebrafish Behavior: Opportunities and Challenges. *Annual Review of Neuroscience* **40**(1), 125-147.

Parkinson, A., Ogilvie, B. W., Buckley, D. B., Kazmi, F., and Parkinson, O. (2013). *Casarett and Doull's Toxicology: The Basic Science of Poisons*, Ninth Edition ed. McGraw Hill Education Biotransformation of Xenobiotics.

Phillips, R. V., Rieswijk, L., Hubbard, A. E., Vermeulen, R., Zhang, J., Hu, W., Li, L., Bassig, B. A., Wong, J. Y. Y., Reiss, B., *et al.* (2019). Human exposure to trichloroethylene is associated with increased variability of blood DNA methylation that is enriched in genes and pathways related to autoimmune disease and cancer. *Epigenetics* **14**(11), 1112-1124.

Portugues, R., and Engert, F. (2009). The neural basis of visual behaviors in the larval zebrafish. *Current Opinion in Neurobiology* **19**(6), 644-647.

Ruckart, P. Z., Bove, F. J., and Maslia, M. (2014). Evaluation of contaminated drinking water and preterm birth, small for gestational age, and birth weight at Marine Corps Base Camp Lejeune, North Carolina: a cross-sectional study. *Environ Health* **13**, 99.

Rusyn, I., Chiu, W. A., Lash, L. H., Kromhout, H., Hansen, J., and Guyton, K. Z. (2014). Trichloroethylene: Mechanistic, epidemiologic and other supporting evidence of carcinogenic hazard. *Pharmacology & Therapeutics* **141**(1), 55-68.

Shaw, G. M., Swan, S. H., Harris, J. A., and Malcoe, L. H. (1990). Maternal Water Consumption During Pregnancy and Congenital Cardiac Anomalies. *Epidemiology* **1**(3), 206-211.

Singh, N., Gupta, M., Trivedi, C. M., Singh, M. K., Li, L., and Epstein, J. A. (2013). Murine craniofacial development requires Hdac3-mediated repression of Msx gene expression. *Dev Biol* **377**(2), 333-44.

Therrien, A. S., and Bastian, A. J. (2019). The cerebellum as a movement sensor. *Neuroscience Letters* **688**, 37-40.

Tian, F.-Y., and Marsit, C. J. (2018). Environmentally Induced Epigenetic Plasticity in Development: Epigenetic Toxicity and Epigenetic Adaptation. *Current Epidemiology Reports* **5**(4), 450-460.

United States Environmental Protection Agency (2017). *Toxic Substances Control Act Chemical Substance Inventory*. US Environmental Protection Agency, Washington, DC.

Wan, J., and Goldman, D. (2016). Retina regeneration in zebrafish. *Curr Opin Genet Dev* **40**, 41-47.

Warren, D. A., Graeter, L. J., Channel, S. R., Eggers, J. S., Goodyear, C. D., Macmahon, K. L., Sudberry, G. L., Latendresse, J. R., Fisher, J. W., and Baker, W. H. (2006). Trichloroethylene, trichloroacetic acid, and dichloroacetic acid: do they affect eye development in the Sprague-Dawley rat? *Int J Toxicol* **25**(4), 279-84.

Westerfield, M. (2007). *The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (Danio rerio)*, 5th ed. University of Oregon Press, Eugene, Oregon.

Williams, S. R., Aldred, M. A., Der Kaloustian, V. M., Halal, F., Gowans, G., McLeod, D. R., Zondag, S., Toriello, H. V., Magenis, R. E., and Elsea, S. H. (2010). Haploinsufficiency of HDAC4 causes brachydactyly mental retardation syndrome, with brachydactyly type E, developmental delays, and behavioral problems. *Am J Hum Genet* **87**(2), 219-228.

Wilson, P. D., Loffredo, C. A., Correa-Villasenor, A., and Ferencz, C. (1998). Attributable Fraction for Cardiac Malformations. *American Journal of Epidemiology* **148**(5), 414-423.

Wirbisky, S. E., Damayanti, N. P., Mahapatra, C. T., Sepulveda, M. S., Irudayaraj, J., and Freeman, J. L. (2016). Mitochondrial Dysfunction, Disruption of F-Actin Polymerization, and Transcriptomic Alterations in Zebrafish Larvae Exposed to Trichloroethylene. *Chem. Res. Toxicol.* **29**(2), 169-179 (Article).

Wu, Z., Ma, C., Shan, Z., Ju, Y., Li, S., and Zhao, Q. (2013). Histone deacetylase inhibitors suppress the growth of human osteosarcomas in vitro and in vivo. *J buon* **18**(4), 1032-7.

Yamaguchi, M., Tonou-Fujimori, N., Komori, A., Maeda, R., Nojima, Y., Li, H., Okamoto, H., and Masai, I. (2005). Histone deacetylase 1 regulates retinal neurogenesis in zebrafish by suppressing Wnt and Notch signaling pathways. *Development* **132**(13), 3027-43.

Yauck, J. S., Malloy, M. E., Blair, K., Simpson, P. M., and McCarver, D. G. (2004). Proximity of residence to trichloroethylene-emitting sites and increased risk of offspring congenital heart defects among older women. *Birth Defects Res A Clin Mol Teratol* **70**(10), 808-14.

Zimmermann, S., Kiefer, F., Prudenziati, M., Spiller, C., Hansen, J., Floss, T., Wurst, W., Minucci, S., and Göttlicher, M. (2007). Reduced body size and decreased intestinal tumor rates in HDAC2-mutant mice. *Cancer Res* **67**(19), 9047-54.