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# Dioxin Induction of Transgenerational Inheritance of Disease in Zebrafish

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## Abstract

Dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDD) is an aryl hydrocarbon receptor (AHR) agonist, an endocrine disruptor, and a potent global pollutant. TCDD exposure is associated with diseases of almost every organ system, and its toxicity is highly conserved across vertebrates. While the acute developmental effects of dioxin exposure have been extensively studied, the ability of early sublethal exposure to produce toxicity in adulthood or subsequent generations is poorly understood. This type of question is difficult to study because of the time frame of the effects. With human subjects, such a study could span more than a lifetime. We have chosen zebrafish (*Danio rerio*) as a model because they are vertebrates with short generation times and consistent genetic backgrounds. Zebrafish have very modest housing needs, facilitating single and multigenerational studies with minimal time and expense. We have used this model to identify transgenerational effects of TCDD on skeletal development, sex ratio, and male-mediated decreases in reproductive capacity. Here we compare these findings with transgenerational effects described in laboratory rodent species. We propose that the zebrafish is a cost-effective model system for evaluating the transgenerational effects of toxic chemicals and their role in the fetal basis of adult disease.

### Keywords

TCDD; AHR; zebrafish; rodent; transgenerational; epigenetic

## Introduction

Mounting evidence suggests that environmental factors can alter developmental programming, resulting in the adult onset of latent diseases, including but not restricted to cancer, diabetes, cardiovascular disease and reproductive disorders (Gluckman and Hanson, 2004; Lau and Rogers, 2004; Heindel, 2005; Marczylo *et al.*, 2012; Veenendaal *et al.*,



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2013). The etiology of some diseases is now linked to tissue- and developmental stage-specific epigenetic alterations in gene expression, resulting from nutritional deficits or exposure to contaminants *in utero*. Exposure to endocrine disruptors is of concern due to the roles that hormones play in regulating transient and irreversible developmental processes. Evidence is mounting that developmental exposure to chemicals, including endocrine disruptors, results in adult disease (Heindel, 2008; Corrales *et al.*, 2014b).

TCDD is a toxic environmental contaminant that impacts growth and development in vertebrates and is associated with several diseases. It is the prototypical member of a family of dioxin-like compounds (DLCs), and is generally produced as a byproduct of industrial processes and waste incineration. TCDD is stable in the environment, highly lipophilic and bioaccumulative, and human exposure comes mostly through dietary sources. TCDD acts primarily through activation of the AHR/ARNT transcriptional regulator to alter gene expression, but cross talk with other signal transduction systems is suspected (Poland and Bradfield, 1992; Swanson and Bradfield, 1993; Schmidt and Bradfield, 1996; Puga *et al.*, 2009). AHR activation by TCDD leads to altered expression of hormone receptors, receptor activators and repressors, metabolic enzymes needed for metabolism of xenobiotics and hormone synthesis and degradation, and other gene products required for normal development and endocrine function (Abbott *et al.*, 1994; Gierthy *et al.*, 1996; Safe *et al.*, 1998; Massaad *et al.*, 2002; Beischlag *et al.*, 2008)

Diseases in humans that have been associated with exposure to TCDD include cancer as well as chloracne, porphyria, and defects in the cardiovascular, skeletal, immune, central nervous system, hepatic and reproductive systems (Eskenazi *et al.*, 2000; Guo *et al.*, 2000; Pelclova *et al.*, 2006; Warner *et al.*, 2007; NAS-IOM, 2011; Warner *et al.*, 2011). Recent epidemiologic evaluation following a major industrial release of TCDD revealed that exposure to TCDD *in utero* leads to reduced sperm quality, feminized sex ratio, and altered thyroid function in the offspring (Mocarelli *et al.*, 2000; Baccarelli *et al.*, 2008; Mocarelli *et al.*, 2011).

Laboratory studies confirm the potential for TCDD to cause disease later in life. Direct exposure to TCDD leads to infertility in many vertebrate species, including humans, and is associated with down-regulation of enzymes in the estrogen synthesis pathway, decreased egg release, increased number of atretic ovarian follicles, and decreased fertilization success (DeVito and Birnbaum, 1994; King-Heiden *et al.*, 2006; Yoshizawa *et al.*, 2009; King-Heiden *et al.*, 2012; Baker *et al.*, 2013). Toxicity in adults following TCDD exposure during early development suggests that physiologic systems are being mis-programmed and that exposure to TCDD can potentially initiate irreversible and permanent modifications in gene expression and cell lineages. However, the molecular mechanisms that underlie latent and transgenerational disease caused by developmental exposure to TCDD are not well understood.

Our recent work has focused on studying the latent and transgenerational effects of TCDD exposure during critical periods of development, using zebrafish (*Danio rerio*) as a model system. In this review, we compare our findings with effects observed in rodent studies to

highlight the usefulness of this model system for evaluating the potential for chemicals to cause disease in adults and subsequent generations.

## Zebrafish as a Model for Multigenerational Studies

To study transgenerational effects, we need a vertebrate model that has a short time to sexual maturity so that we can study successive generations. From this perspective, humans are not ideal subjects for study (Heindel, 2007; Skogen and Overland, 2012). In addition, the diverse genetics of the human population, confounded by individual variations in exposures make studies with human subjects difficult. The zebrafish is well established as a model for investigating human disease, especially as it pertains to altered development. Attributes that make the zebrafish outstanding in this arena are: short time to sexual maturity (about 3–4 months), transparent embryos that allow observation of organ development without disturbing the embryo, the ability to obtain large groups of synchronously developing embryos, low cost for exposure chemicals since volumes are small, and the ease of housing multiple generations of fish. This last point means that one can expose the first $F_0$ generation and maintain offspring across many generations inexpensively and compactly.

While small rodent models are more common than small fish models for studying human disease, rodents have a number of disadvantages for studying the fetal basis of adult disease. Rats and mice for example have far fewer offspring per pair, and maintenance costs are considerably greater. While zebrafish reach sexual maturity in a similar timeframe to some rodents, their small size allows for the ability to house and maintain large groups of synchronously developing fish over multiple generations inexpensively and compactly. Similar to human populations, laboratory zebrafish are less isogenic than laboratory rodent strains, which decreases inbreeding effects when studying changes in the zebrafish genome/epigenome. Zebrafish developmental processes are well characterized, and many organs and cell types have been marked with fluorescent reporters in transgenic lines. Due to complete sequencing of the zebrafish genome, technologies that include specific antibodies, genetic/epigenetic markers, and high throughput sequencing also can be readily utilized. MicroRNAs may be involved in the transgenerational inheritance of disease (Wagner *et al*., 2008; Grandjean *et al*., 2009) and there is a rapidly growing microRNA literature in zebrafish. Finally, developing zebrafish are very small and transparent, so development can be readily followed with microscopy and automated screening techniques (Kaufman *et al*., 2009; Wittmann *et al*., 2012; Westhoff *et al*., 2013).

Even though zebrafish are oviparous, the reproductive system of fish and mammals is similar. The testis and ovary in zebrafish contain the same germ cells that are found in mammals, and hormonal regulation of spermatogenesis and oogenesis is highly conserved across vertebrates, occurring via the hypothalamic-pituitary-gonadal axis (Segner, 2009; Liu *et al*., 2011; Lohr and Hammerschmidt, 2011).

## Defining Transgenerational Toxicity: Zebrafish vs. Rodents

Chemical exposures that affect subsequent generations are now well documented. An epigenetic mechanism is likely for cases of multigenerational disease, in which neither the parent nor the offspring have been directly exposed. This is a transgenerational effect

because there is no direct connection to chemical exposure (Skinner, 2008). In rodent models, exposure during early development requires prenatal exposure in an $F_0$ generation mother during pregnancy (Figure 1, left column). This leads to $F_1$ offspring that developed in an exposed environment. The $F_2$ offspring then develop in parents that were exposed *in utero*, so only effects in the $F_3$ generation can be due to epigenetic alterations in gametes.

In contrast, zebrafish eggs are fertilized in water, embryos develop externally, and are subsequently exposed at the juvenile stage of development (Figure 1, right column). Thus, $F_0$ fish are equivalent to $F_1$ mice because they develop in an exposed environment. The $F_1$ zebrafish generation originates from gametes produced by exposed fish, similar to the $F_2$ mouse generation. The gametes producing the $F_2$ zebrafish generation have not been exposed so the effects seen in $F_2$ zebrafish are transgenerational. Thus, the $F_2$ zebrafish is equivalent to the exposure-free $F_3$ mouse.

## Using Zebrafish to Identify Transgenerational Effects of TCDD

Sublethal TCDD exposure *in utero* and in early development leads to adverse health effects in adulthood and subsequent generations. Adverse effects have included increased congenital abnormalities, decreased survival, differences in sex ratios of offspring, and decreased reproductive function and fertility in both males and females (Wolf *et al.*, 1999; Nomura *et al.*, 2004; Ikeda *et al.*, 2005a and b; King-Heiden *et al.*, 2009; Ding *et al.*, 2011). Transgenerational effects of TCDD exposure have now been observed in mice, rats and zebrafish (Bruner-Tran and Osteen, 2011; Manikkam *et al.*, 2012a and b; Nilsson *et al.*, 2012; Baker *et al.*, 2014). The TCDD-induced transgenerational defects identified in these species involve skeletal development, sex ratio, ovary, and reproductive success and are summarized in Table 1.

## Skeletal Development

Direct TCDD exposure causes skeletal, cartilage, and bone abnormalities in several animal models (Peterson *et al.*, 1993; Hornung *et al.*, 1999; Xiong *et al.*, 2008; Bursian *et al.*, 2013). *Spina bifida*, a developmental abnormality that is caused by incomplete closing of the neural tube and malformed vertebrae, occurs in human offspring following exposure to Agent Orange, a TCDD-contaminated herbicide (NAS-IOM, 2011). In mink, skeletal abnormalities were observed in $F_1$ offspring of TCDD-exposed adults (Bursian *et al.*, 2013). TCDD exposure during development altered craniofacial structures in adult zebrafish, and produced scoliosis-like kinks in the axial skeletons of adult $F_0$ parents as well as in $F_1$ and $F_2$ offspring (Table 1; King-Heiden *et al.*, 2009; Baker *et al.*, 2013; Baker *et al.*, 2014). Transgenerational skeletal abnormalities have not yet been reported in mammals. This response might be idiopathic to fish, but the effects may also be easier to observe in zebrafish. It is also possible that zebrafish are more sensitive to skeletal toxicity compared to mammals. If so, a zebrafish model may be useful in developing screens for transgenerational skeletal effects of chemical exposure.

## Sex Ratio

One effect of human exposure to TCDD is a change in the sex ratio towards a higher percentage of girls born to parents exposed during an industrial accident in Seveso, Italy (Clapp and Ozonoff, 2000; Eskanazi *et al*., 2004; Mocarelli *et al*., 2000 and 2008). Some studies in rats and zebrafish have also produced a shift in the sex ratio of offspring toward females (Ikeda *et al*., 2005b; Baker *et al*., 2013). The shift towards females was also observed in the $F_1$ and $F_2$ offspring of $F_0$ zebrafish exposed to TCDD during sexual differentiation and maturation (Table 1; Baker *et al*., 2014). While not as well understood in fish as in mammals, sex determination in zebrafish is primarily genetic, with environmental factors influencing sex secondarily (Liew and Orban, 2014). Although, zebrafish would be useful in screening for the ability of environmental contaminants to influence sex ratios, zebrafish do not have a pair of highly differentiated sex chromosomes. Until we know more about what determines male and female sex in zebrafish, mechanistic interpretation will be difficult.

## Ovary

While sex determination is not well understood in zebrafish, the pathways and genes involved in gonad differentiation are conserved between zebrafish and other vertebrates (Wilkins, 1995; Marshall-Graves and Peichel, 2010). Zebrafish ($F_0$) exposed to TCDD as embryos or adults show similar ovarian toxicities (King-Heiden *et al.*, 2005 and 2006; Hutz *et al.*, 2006; Daouk *et al.*, 2011; Baker *et al*., 2013). A group of studies from Skinner and colleagues reported several transgenerational phenotypic changes in $F_3$ female rats following *in utero* exposure of the $F_1$ generation to TCDD, including: early onset of puberty, reduced numbers of total follicles, reduced numbers of primordial follicles, and increased numbers of small cysts within the ovary (Table 1; Manikkam *et al*., 2012a and b; Nilsson *et al*., 2012). As in the rodent studies, $F_1$ female zebrafish had abnormal ovarian structure, with atretic follicles (King-Heiden *et al*., 2009; Baker *et al*., 2014). However, this effect diminished with time, and was not statistically significant by the $F_2$ generation. This steady diminution of effects over time may help provide insight into the mechanism of transgenerational effects, and will be an important part of assessing the long-term impact of developmental exposure on human populations.

## Reproductive Success

Studies with mice have demonstrated transgenerational effects of TCDD exposure on fertility. Offspring of TCDD exposed mice were less likely than control to become pregnant, and those that did become pregnant were less likely than control to deliver at full term (Table 1, Bruner-Tran and Osteen, 2011). We observed similar results with $F_0$ zebrafish exposed to TCDD during sexual development in the $F_0$ generation. Both $F_1$ and $F_2$ offspring showed decreased reproductive capacity, with significantly decreased egg release and reduced percentage of eggs fertilized (Table 1; Baker *et al*., 2013 and 2014). A simple explanation for decreased egg release could be adverse effects on ovarian development, but interestingly, transgenerational reduction in egg release was linked to males rather than females. Egg release during spawning involves both a female releasing the eggs and a male

eliciting egg release. In spawnings of TCDD-lineage $F_0$, $F_1$, and $F_2$ zebrafish males with control females, fewer eggs were produced compared to controls. Similar results have been reported with control females that avoid mating with vinclozolin-lineage $F_3$ male rats (Crews *et al*., 2007). In fact, decreased reproductive capacity was observed in spawnings of TCDD-lineage $F_1$ and $F_2$ zebrafish males with control females (Table 1; Baker *et al*., 2014), suggesting that transgenerational reduction in fertility can be attributed to effects on males in the lineage. These findings indicate that some transgenerational effects can be sex-specific. Decreased sperm release could explain these fertility deficits, but as with rodent studies, the testes of TCDD-lineage zebrafish appeared normal on histological examination (Manikkam *et al*., 2012a and b; Baker *et a*l., 2014). Exposure to TCDD or PCBs *in utero* also decreased masculine, while increasing feminine sexual behavior in rats (Mably *et al.*, 1992; Colciago *et al.*, 2009). Thus, TCDD does something transgenerationally to alter reproductive success in the male; however at present we are limited to trying to link what is known about AHR and what we know about reproductive function. Further studies are needed to elucidate whether alterations in male zebrafish spawning behavior, pheromone production, and/or other aspects of male reproductive biology are causing decreased fertility and what mechanisms are responsible.

## Epigenetic Effects of Chemical Exposure

As we search for the mechanisms that allow a chemical exposure to have effects that persist through multiple generations, epigenetic changes via covalent DNA and chromatin modification come to the forefront. Epigenetic modifications can be carried in the gametes, ultimately modifying gene expression to produce phenotypic changes. Heritable natural epigenetic changes producing a phenotype have been documented in plants, worms and insects (Cubas *et al.*, 1999; Manning *et al.*, 2006; Ruden and Lu, 2008; Greer *et al.*, 2011). Kuroki and colleagues (2013) discovered that mice lacking the H3K9 demethylase, regulating histone function in the chromatin, were subject to male to female sex reversal, demonstrating that changes in chromatin can play a pivotal role in sex determination.

Several attempts have been made to identify epigenetic changes in DNA and chromatin in individuals displaying transgenerational effects of toxic chemicals. Skinner and colleagues have shown that DNA methylation is altered in many places throughout the genome in the affected generations compared to controls (Anway *et al*., 2005; Manikkam *et al*., 2012a and b). In other cases this group has also focused on altered gene expression patterns as biomarkers of the exposure (Nilsson *et al*., 2012). Dolinoy and colleagues (2006) showed an effect of genistein on coat color in mice that was associated with altered methylation upstream of the agouti gene, a regulator of coat color.

Studies on transgenerational effects of AHR agonists in zebrafish have just begun. Although no specific epigenetic change has been shown to produce the transgenerational effects caused by a toxicant, it appears likely that epigenetic changes play a role in producing and transmitting these effects through generations. Changes in DNA methylation and gene expression patterns have been identified in $F_0$ generation zebrafish following exposure to benzo(a)pyrene and 7,12-dimethylbenz(a)anthracene (Mirbahai *et al.*, 2011; Fang *et al.*, 2013; Corrales *et al.*, 2014a and b). The transgenerational phenotypic effects identified in

TCDD lineage zebrafish (Table 1, Baker *et al*., 2014) are similar to TCDD effects observed in the $F_0$ generation. Whether the transgenerationally altered reproductive and skeletal phenotypes are due to epigenetic modifications in the regulation of AHR-ARNT signaling in these tissues will require further research.

In addition to clarifying mechanism, it will be important to assess the stability of the toxic effects across generations. While the effects on egg release persisted through the $F_2$ generation in our zebrafish experiments, the effects on ovarian structure waned with each generation such that it was no longer observed in the $F_2$ generation. The mechanism for such waning effects may be similar to the evolved multi-generational resistance to dioxin-like compound toxicity in wild fish populations (Wirgin *et al., 2011*). How long these effects last are vitally important. In the past we have been concerned about the persistence and chemical stability of the environmental contaminants themselves. However, if contaminants are capable of producing adverse effects that can be passed across generations, we also will want to know if these effects are reversible and how many generations will be affected.

## Conclusion

Transgenerational toxicity due to TCDD exposure has been observed in mice, rats and zebrafish (Bruner-Tran and Osteen, 2011; Manikkam *et al*., 2012a and b; Baker *et al.*, 2014). Remarkably, several of the phenotypic effects are similar across vertebrate classes, especially the reduction of reproductive capacity in unexposed TCDD-lineages. In zebrafish, unexposed TCDD-lineage $F_2$ offspring have reproductive, skeletal, and sex ratio abnormalities. More specifically, the decrease in fertility and egg release in control female zebrafish is due to the unexposed, TCDD-lineage $F_2$ male zebrafish. Thus, ancestral TCDD exposure reduces reproductive success of male zebrafish across multiple generations. This is most likely an epigenetic effect since TCDD has been shown not to be mutagenic (Poland and Glover, 1979). Epigenetic changes provide an avenue for better understanding how these heritable changes occur. Zebrafish are a promising model because many generations can be produced and studied in relatively little time and space. Also, reproduction and development are easy to assess in zebrafish, and this model is broadly available.

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## Abbreviations

| | |
|---|---|
| **TCDD or dioxin** | 2,3,7,8-tetrachlorodibenzo-*p*-dioxin |
| **DLC** | dioxin-like compound |
| **AHR** | aryl hydrocarbon receptor |
| **ARNT** | aryl hydrocarbon receptor nuclear translocator |

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## Highlights

- We review transgenerational effects of dioxin in fish and other vertebrate species
- Zebrafish model is ideal for investigating multigenerational effects of chemicals
- Dioxin induces transgenerational skeletal and reproductive phenoytpes in zebrafish

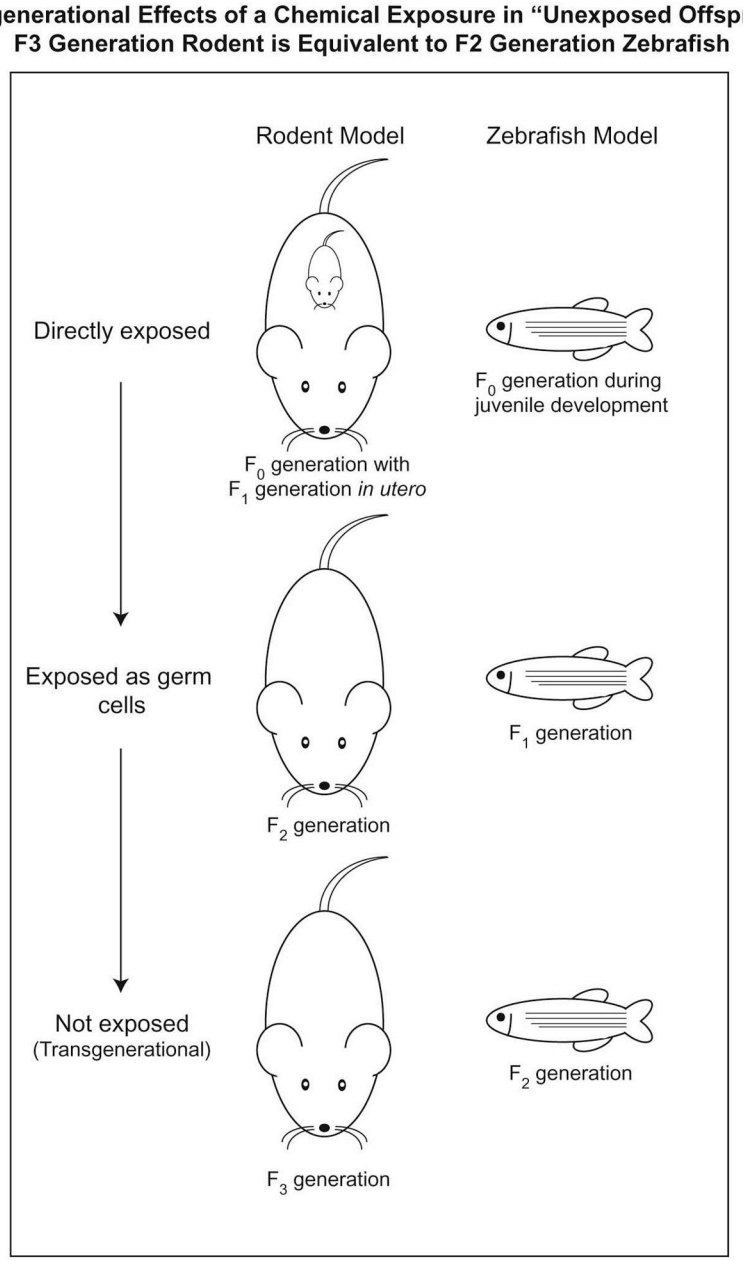


**Figure 1. Comparison of the experimental design of a transgenerational study in the laboratory rodent and zebrafish**

In the rodent model, *in utero* exposure to a chemical leads to direct exposure of the $F_0$, $F_1$ and $F_2$ generations and the $F_3$ generation is not exposed. In the zebrafish model, juvenile developmental exposure leads to direct exposure of the $F_0$ and $F_1$ generations to the chemical and the $F_2$ generation is not exposed.

## Table 1

Sex-Specific, Transgenerational Effects of TCDD in Rodents and Zebrafish

| Species | Sex | Transgenerational Effect | TCDD Exposure of $F_0$ Generation | Reference |
|---|---|---|---|---|
| Mice | Female | Decreased pregnancy rate<br>Increased preterm birth | 10 μg/kg, po, at E15.5 | Bruner-Tran and Osteen, 2011 |
| Rats | Female | Decreased follicle number<br>Decreased primordial follicles<br>Early puberty<br>Increased small cysts in ovary | 100 ng/kg/day, ip, E8 to E14 | Nilsson *et al*., 2012<br>Manikkam *et al*., 2012a,b |
| | Male | Increased kidney disease | | |
| Zebrafish | Female and Male | Changed in sex ratio<br>Increased skeletal malformations | 50 pg/ml, 1 hr, static waterborne at 3 and 7 wpf[a] | Baker *et al*., 2014 |
| | Male[b] | Decreased egg release<br>Decreased egg fertilization | | |

[a] Weeks post fertilization

[b] Decreased egg release and fertilization is observed in control, female zebrafish mated with TCDD lineage $F_2$ males. It does not occur when TCDD lineage $F_2$ females are mated with control males. Thus, the transgenerational effect of TCDD, in decreasing reproductive capability in zebrafish, is male-mediated.