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Research Article

Ancestral TCDD Exposure Induces Multigenerational Histologic and Transcriptomic Alterations in Gonads of Male Zebrafish

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ABSTRACT

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), the classic aryl hydrocarbon receptor (AhR) agonist, is a potent environmental toxicant and endocrine-disrupting chemical (EDC) with known developmental toxicity in humans, rodents, and fish. Early life exposure to some EDCs, including TCDD, is linked to the occurrence of adult-onset and multigenerational disease. Previous work exposing juvenile F_0 zebrafish (*Danio rerio*) to 50 ppt (parts per trillion) TCDD during reproductive development has shown male-mediated transgenerational decreases in fertility (F_0–F_2) and histologic and transcriptomic alterations in F_0 testes. Here, we analyzed male germline alterations in F_1 and F_2 adult fish, looking for changes in testicular histology and gene expression inherited through the male lineage that could account for decreased reproductive capacity. Testes of TCDD-lineage F_1 fish displayed an increase in spermatogonia (immature germ cells) and decrease in spermatozoa (mature germ cells). No histological changes were present in F_2 fish. Transcriptomic analysis of exposed F_1 and F_2 testes revealed alterations in lipid and glucose metabolism, oxidation, xenobiotic response, and sperm cell development and maintenance genes, all of which are implicated in fertility outcomes. Overall, we found that differential expression of reproductive genes and reduced capacity of sperm cells to mature could account for the reproductive defects previously seen in TCDD-exposed male zebrafish and their descendants, providing insight into the distinct multigenerational effects of toxicant exposure.

Key words: toxicity; transgenerational; dioxin; zebrafish; testicular tissue; microarray.

The long-term outcomes of exposure to endocrine-disrupting chemicals (EDCs), natural or synthetic compounds that interfere with hormonal signaling pathways, are a heavy burden on society in terms of health outcomes and financial impact. The World Health Organization (WHO) and Endocrine Society have released statements of concern regarding the increasing role of exposure to environmental EDCs in disease outcomes, including rises in male infertility, genital malformations, and adverse pregnancy outcomes (Bergman *et al.*, 2013; Gore *et al.*, 2015), and recent estimates put the cost of EDC-linked disease at $340 billion (2.33% GDP) in the United States, and the equivalent of $217 billion (1.28% GDP) in the European Union (Attina *et al.*, 2016). Although EDCs are usually detected at very low levels in the environment, these quantities are enough to potentially alter the highly sensitive endocrine system (Welshons *et al.*, 2003). Many of these chemicals are bioaccumulative and have long half-lives in the human body (ASTDR, 1998; Steele *et al.*, 1986) with potential for additive or synergistic effects with the milieu of other chemicals in the environment (Kortenkamp, 2014). Exposure to many of these compounds, particularly those that are lipophilic, during pregnancy or infancy may disrupt critical windows of development (Brouwer *et al.*, 1999; Eckstrum *et al.*, 2018; Zoeller *et al.*, 2012), leading to life-long and multigenerational disease outcomes (Baker *et al.*, 2014a,b; Brehm *et al.*, 2018; Bruner-Tran

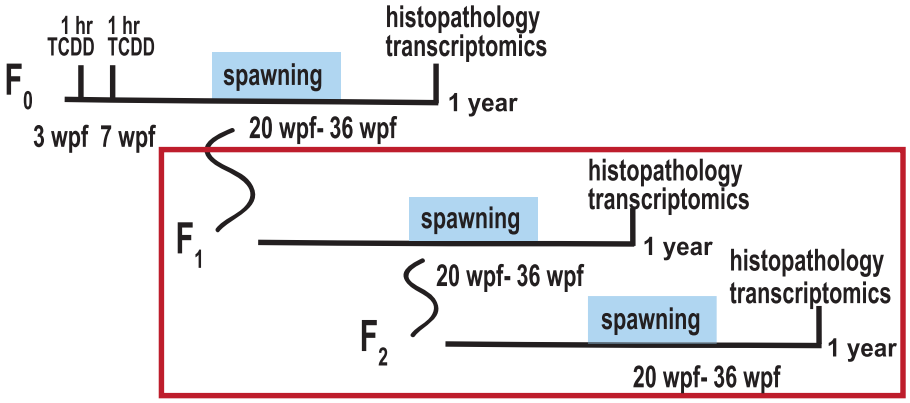


Figure 1. TCDD exposure paradigm. F_0 zebrafish were exposed to 50 pg/ml TCDD for 1 h at 3 and 7 wpf (weeks post fertilization). Fish were spawned for fertility assessment at 20–36 wpf, and offspring from spawns were raised to create the next generation. Fish were assessed for testicular histology and transcriptomics at 1 year. F_1 and F_2 fish were not directly exposed to TCDD but were spawned and analyzed as described above. TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

and Osteen, 2011). Altering the endocrine system during these crucial timeframes can result in altered reprogramming of the epigenome (Manikkam *et al.*, 2012; Skinner *et al.*, 2013). As the methylome is heritable through the male germline in zebrafish (Jiang *et al.*, 2013b), this has implications for inheritance of disease-causing epimutations across generations.

TCDD, or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, is an EDC and environmental contaminant known as the most potent ligand for the aryl hydrocarbon receptor (AhR) (Denison and Nagy, 2003). Highly bioaccumulative and lipophilic (ATSDR, 1998), TCDD is persistent in the environment and affects a vast variety of physiological systems and model organisms (Bruner-Tran and Osteen, 2011; Hermsen *et al.*, 2008; Hornung *et al.*, 1999). Although environmental levels of TCDD have declined over recent decades (Aylward and Hays, 2002), its persistence in humans (ASDTR, 1998), as well as its ability to model effects of other environmental AhR ligands (Okey, 2007), make it a useful and informative chemical to study. Of particular interest are the reproductive effects of early TCDD exposure, which extend for multiple generations (Baker *et al.*, 2014a,b; Bruner-Tran and Osteen, 2011; Sanabria *et al.*, 2016).

In zebrafish (*Danio rerio*), an NIH-validated model organism with relatively rapid turnover time, high fecundity, and endocrine signaling similar to that of humans (Peterson and Macrae, 2012; Teraoka *et al.*, 2003), exposure to low-level dioxin (50 pg/ml) during gonadal differentiation and maturation (at 3 and 7 weeks postfertilization [wpf]) in the F_0 generation resulted in transgenerational decreases in number of eggs elicited and fertilized (Baker *et al.*, 2013, 2014b). Outcrosses with control fish for each generation (F_0–F_2) revealed that these effects were attributable to the male lineage. In fact, testicular histopathology and transcriptomics for the F_0 generation indicated delayed spermiation and alteration in several genes involved in fertility, spermatogenesis, or lipid metabolism, pathways implicated in this male-mediated reduction in fertility (Baker *et al.*, 2016).

In this study, we perform analysis on testes from subsequent generations to interrogate the persistence of delayed spermiation in the F_1 TCDD-lineage generation and transcriptomic alterations in both F_1 and F_2 TCDD-lineage generations. Thus, we can characterize the cross-generational impact of early life F_0 generation EDC exposure, as well as the physiological and genetic alterations potentially underlying these reproductive outcomes. The initial study was the first instance of transgenerational inheritance of disease using zebrafish as a model organism, and our further investigation of these findings will enhance the use of this organism in modelling human disease.

MATERIALS AND METHODS

Fish husbandry. AB lineage zebrafish were maintained on a 14:10 h light/dark cycle (Westerfield, 2000) in reverse osmosis water buffered with Instant Ocean salts (60 mg/l, Aquarium Systems, Mentor, Ohio), with temperatures maintained at 27°C–30°C. Fish were fed 2 times daily and euthanized with an overdose of tricaine methanesulfonate (MS-222; 1.67 mg/ml). Adult fish were raised on a recirculating system at a maximum density of 5 fish per liter. F_0 fish were raised in beakers with daily water changes of 40%–60% at a density of 5 fish per 400 ml beaker between 3 and 6 weeks, and 5 fish per 800 ml beaker between 6 and 9 weeks postfertilization (wpf). Animal use protocols were approved by the Institutional Animal Care and Use Committees at Wayne State University and the University of Wisconsin-Madison, according to the National Institutes of Health Guide to the Care and Use of Laboratory Animals (Protocol No. M00489).

TCDD exposure and spawning. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (>99% purity, Chemsyn) was dissolved in dimethyl sulfoxide (DMSO) as vehicle. Exposures occurred according to Baker *et al.* (2013). In brief, F_0 fish were exposed to 50 pg/ml waterborne TCDD or 0.1% DMSO control in small beakers for 1 h at both 3 and 7 weeks post fertilization (wpf), during sexual differentiation and maturation. As indicated in Baker *et al.* (2016), 3 replicate blocks were exposed. Each block consisted of 8 vials, which each held 5 fish. Fish were raised to 6 months and group-spawned to create the F_1 generation, which was indirectly exposed as germ cells within juvenile F_0 fish (2 cohorts/block). F_1 fish were subsequently spawned at six months to create the unexposed F_2 generation (2 cohorts/block). At 1 year of age, F_1 and F_2 male fish were euthanized and collected for histologic and transcriptomic analysis. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin exposure and spawning schema are diagrammed in Figure 1.

Histology. Sample processing was performed according to Baker *et al.* (2016). For F_1 and F_2 generations, 6 male fish at 1 year of age from both TCDD and DMSO lineages were euthanized, fixed in 10% Zn-formalin, decalcified with Cal-ExII, and sagittally bisected. Fish were dehydrated in ethanol, cleared in xylene,

paraffin-embedded, and sectioned at 5 μm thickness. Sections were mounted and hematoxylin and eosin (H&E) stained. Testicular tissue was imaged using a Nikon SMZ18 stereomicroscope at 21.6× magnification with a Nikon DS-Qi2 camera. From at least 3 images, 9 seminiferous tubules with well-defined boundaries were selected per fish for quantification purposes. Germ cells within these seminiferous tubules were visually sorted into the four main stages of spermatogenic differentiation as follows: Spermatogonia (least mature), spermatocytes, spermatids, and spermatozoa (most mature). Supplementary Figure 1 demonstrates the categorization of these cell types within seminiferous tubules. ImageJ version 1.5 (http://imagej.nih.gov; last accessed January 6, 2016) was used to assess each tubule for the area occupied per cell type and for germinal epithelium width.

RNA isolation. Five male fish each from TCDD and DMSO lineages from F_1 to F_2 generations were euthanized, and testes were extracted, flash-frozen in liquid nitrogen, and stored at −80°C. Testes were homogenized in QIAzol lysis reagent (Qiagen), and RNA was isolated with the Qiagen RNEasy mini kit. RNA was quantified and purity was assessed with Nanodrop-1000 (ThermoFisher Scientific). The Agilent RNA 6000 Nano kit was used to assess RNA quality using an Agilent Bioanalyzer, as performed in Baker *et al.* (2016). RNA integrity number (RIN) values were between 7.1 and 8.2 for F_1 and between 7.2 and 8.7 for the F_2 (Supplementary Table 1).

Microarray. Whole genome transcriptomic changes in the testes of TCDD-lineage F_1 and F_2 male fish were examined through the use of Zebrafish Gene 1.0 ST Genome Arrays (Affymetrix). Procedure was carried out as previously described in Baker *et al.* (2016) for F_0 fish. Briefly, RNA was isolated as described above from five TCDD-lineage and five control-lineage fish per generation (F_1 and F_2) and processed with the Ambion WT Expression Kit. Processing, labeling, and hybridization was carried out according to MIAME guidelines by the University of Wisconsin Biotechnology Gene Expression Center. Samples were end-terminus labeled according to the protocol from the Affymetrix GeneChip WT terminal labeling and hybridization user manual target (P/N 702808 Rev. 7), then fragmented and hybridized to independent Zebrafish Gene 1.0 ST Genome Arrays per each sample. Hybridization and analysis occurred according to Baker *et al.* (2016). In short, 10 μg cRNA was hybridized at 45°C for 16 h, and GeneChips were, respectively, washed and stained in the Affymetrix Fluidics Station 450 and scanned using the Affymetrix GeneArray Scanner GC3000 G7.

The Transcriptome Analysis Console (TAC, Affymetrix) was used to normalize gene expression data by calculating individual gene intensity per sample using the Tukey's Biweight average (log 2 scale) for all eligible exon (probe selection regions [PSRs]) intensities in that gene. Then each PSR was normalized using the calculated gene intensity for that sample. Affymetrix Expression Console Software was used to assess data for outliers; none were found or removed from analysis. Condition 1 (TCDD-lineage) and Condition 2 (DMSO-lineage) normalized intensities were combined across PSRs and junctions within a gene. Genes of interest were defined as those with a p-value $<.05$ and an absolute fold change >1.5. Data were uploaded to NCBI GEO database (GSE111446). Pathway analysis was performed on genes of interest (as defined above) using Ingenuity Pathway Analysis (IPA). All F_0 transcriptomic data included in this study for multigenerational comparison was reported in a previous study (Baker *et al.*, 2016), and uploaded to NCBI GEO database (GSE77335).

qRT-PCR. qRT-PCR was run on a sampling of genes in order to validate microarray results. RNA from testes was isolated as described above and used to validate nine genes of interest using TaqMan Gene Expression Assays (Life Technologies). Initially, RNA concentration was re-verified using RNA High Sensitivity assays on the Qubit Fluorometer (ThermoFisher Scientific). The High Capacity cDNA Reverse Transcription kit with MultiScribe Reverse Transcriptase was used to reverse-transcribe 10 μl of 50 ng/μl RNA into 20 μl of 25 ng/μl cDNA. The TaqMan Preamp Mastermix Kit (ThermoFisher Scientific) was then used to preamplify 250 ng of cDNA for genes of interest. The preamplification reaction was 14 cycles long at a reaction volume of 50 μl. Probes used were either predesigned Taqman Gene Expression Assay probes [*actb1* (Dr03432610_m1), *aste1* (Dr03425048_m1), *mhc1uba* (Dr03144312_m1), *si: dkeyp-72h1.1* (Dr03092415_m1), *smyd1a* (Dr03438580_m1), *spata4* (Dr03421991_m1), and *zgc: 158731* (Dr03149463_m1)], probes custom-designed to input sequences using the ThermoFisher Scientific Custom TaqMan Assay Design bioinformatics tool (*mcf2la* [Genomic Position: 1: 46675304–46697602; Assay ID: ARZTDZ7]), or probes custom-designed to input sequences by ThermoFisher Scientific bioinformatics design technicians (*socs1a* [NCBI RefSeq ID: NM_001003467; Custom ID: APYMJJM]).

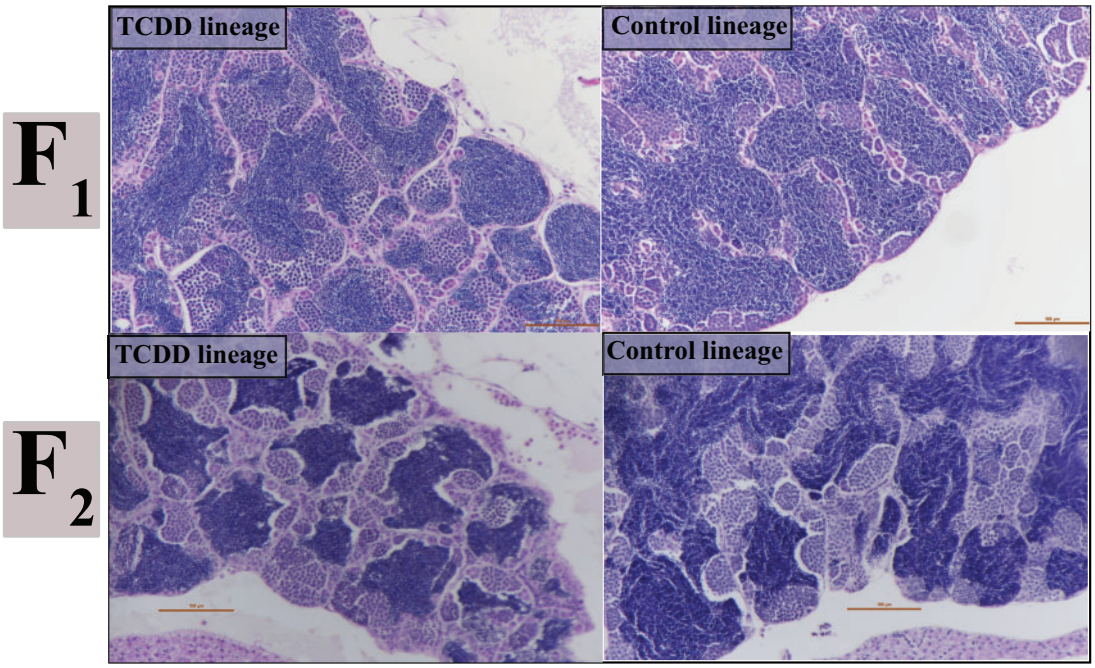
qRT-PCR was analyzed using a QuantStudio 5. qRT-PCR reactions were plated using a Gilson 268 PIPETMAX liquid handling platform and were run in triplicate on 384-well plates. Reactions were performed using TaqMan Universal Master Mix (ThermoFisher Scientific), and reaction volumes were 20 μl, 2 μl of which consisted of preamplified cDNA. Manufacturers' protocol was followed to determine thermal cycling parameters. Probes and qRT-PCR protocols are MIQE-compliant. Probes were selected or designed to provide best coverage according to the ThermoFisher database. $2^{-\Delta\Delta Ct}$ (cycle threshold) methods were used to analyze qRT-PCR data by normalizing all transcripts to the reference gene *actb1* (β-actin), which did not show alteration due to TCDD on either the microarray or qPCR.

Statistical analysis. Histologic quantification and qPCR gene expression data were analyzed using Student's t-test in Microsoft Excel, and microarray data were analyzed with one-way between subject ANOVA comparing each generation independently (ie F_1-TCDD vs F_1-DMSO and F_2-TCDD vs F_2-DMSO) using the TAC. For all analyses, significant difference between treated and control was determined by p-value $<.05$.

RESULTS

Histology

Based on our results in the F_0 generation, we assessed ratios of germ/sperm cells within the testes in F_1 and F_2 testicular tissue. Quantification of germ/sperm cells revealed spermatogenic dysregulation in F_1 TCDD-lineage fish. Seminiferous tubule size was not significantly different between the F_1 TCDD and F_1 DMSO (control) lineages. Within the seminiferous tubules, F_1 TCDD-lineage fish demonstrated an increase in area occupied by spermatogonia (undifferentiated germ cells; $p = .014$) and a simultaneous decrease in area occupied by spermatozoa (mature sperm cells; $p = .021$) when compared with control fish (Figure 2). No differences were found in area between control and exposed fish for spermatocytes ($p = .73$) or spermatids ($p = .55$), both intermediate cell types, and no differences were noted in germinal epithelium width ($p = .41$) (Figure 2).



	F_1 males		F_2 males	
	TCDD	*Control*	*TCDD*	*Control*
Testicular Cell Type (μm²)				
Spermatogonia	1222 ± 129 *	837 ± 85	836 ± 84	971 ± 64
Spermatocytes	4685 ± 366	4893 ± 472	3344 ± 203	3796 ± 308
Spermatids	909 ± 141	802 ± 106	918 ± 90	1136 ± 133
Spermatozoa	5264 ± 588 *	7379 ± 683	5054 ± 358	6186 ± 502
Tubule Area	13616 ± 983	15253 ± 1086	11311 ± 658	13136 ±771
Germinal Epithelium Thickness (μm)				
	36.1 ± 1.1	37.5 ± 1.3	34 ± 1.3	36.3 ± 1.4

Figure 2. Quantification of germ cell types within the testes (ImageJ). H&E images of testicular tissue at ×21.6 magnification indicate that TCDD-lineage F_1 fish show a decreased area occupied by spermatozoa and an increased area of spermatogonia within seminiferous tubules. F_2 fish did not demonstrate differences in germ cell area due to ancestral TCDD exposure compared with controls. *Top row* consists of F_1 TCDD and control (DMSO) lineage testes. *Second row* consists of F_2 TCDD and control (DMSO) lineage testes. Scale bars in images are 100 μm length. Values in the *table below* indicate the mean area (μm²) ± SEM of each cell type within seminiferous tubules or the mean width of the germinal epithelium (μm) ± SEM for TCDD or control lineages. *Significant difference from control, as defined by $p < .05$. H&E, Hematoxylin and eosin; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; DMSO, dimethyl sulfoxide; SEM, Standard Error of the Mean.

In the F_2 generation, which demonstrated decreased reproductive capacity (Baker *et al.*, 2014b), there was a trend toward a decrease in spermatozoa in TCDD-lineage F_2 fish. No significant differences were observed in seminiferous tubule size, germinal epithelium width ($p = .23$), or any of the four germ/sperm cell types [spermatogonia ($p = .20$), spermatocytes ($p = .22$), spermatids ($p = .17$), and spermatozoa ($p = .07$)] (Figure 2).

Microarray

Whole-genome microarray analysis on F_1 and F_2 adults showed that 634 genes were differentially expressed in F_1 TCDD-lineage fish when compared with DMSO-lineage fish; 560 of those genes were uniquely altered in the F_1 generation (Figure 3). When compared with controls, 68% of these genes were significantly downregulated and 32% were upregulated. In the F_2 generation of TCDD-lineage fish, 1105 genes were differentially expressed, with 1010 of those genes unique to the F_2 generation. Of those genes, 46% were downregulated, and 54% were upregulated. Table 1 indicates several differentially expressed genes of interest from F_0 to F_2 generations, and the 100 most differentially regulated annotated genes in either direction are included in Supplementary Table 2. The most highly upregulated gene from the F_1 generation was *acyl-Coenzyme A oxidase 1, palmitoyl* (*acox1*), a peroxisomal β-oxidation enzyme involved in lipid metabolism (Rakhshandehroo *et al.*, 2010), with a fold change of 10.65 and a *p*-value of .0071. *Spermatogenesis associated 4* (*spata4*), a gene enriched in the testes and suspected to play a role in regulating germ cell apoptosis (Jiang *et al.*, 2015), was also highly downregulated (−19.12). Though the microarray *p*-value was just above the cutoff for significance ($p = .050589$), qRT-PCR analysis successfully validated the gene as significantly downregulated. Other dysregulated genes with roles in infertility included *gcga* (7.96,

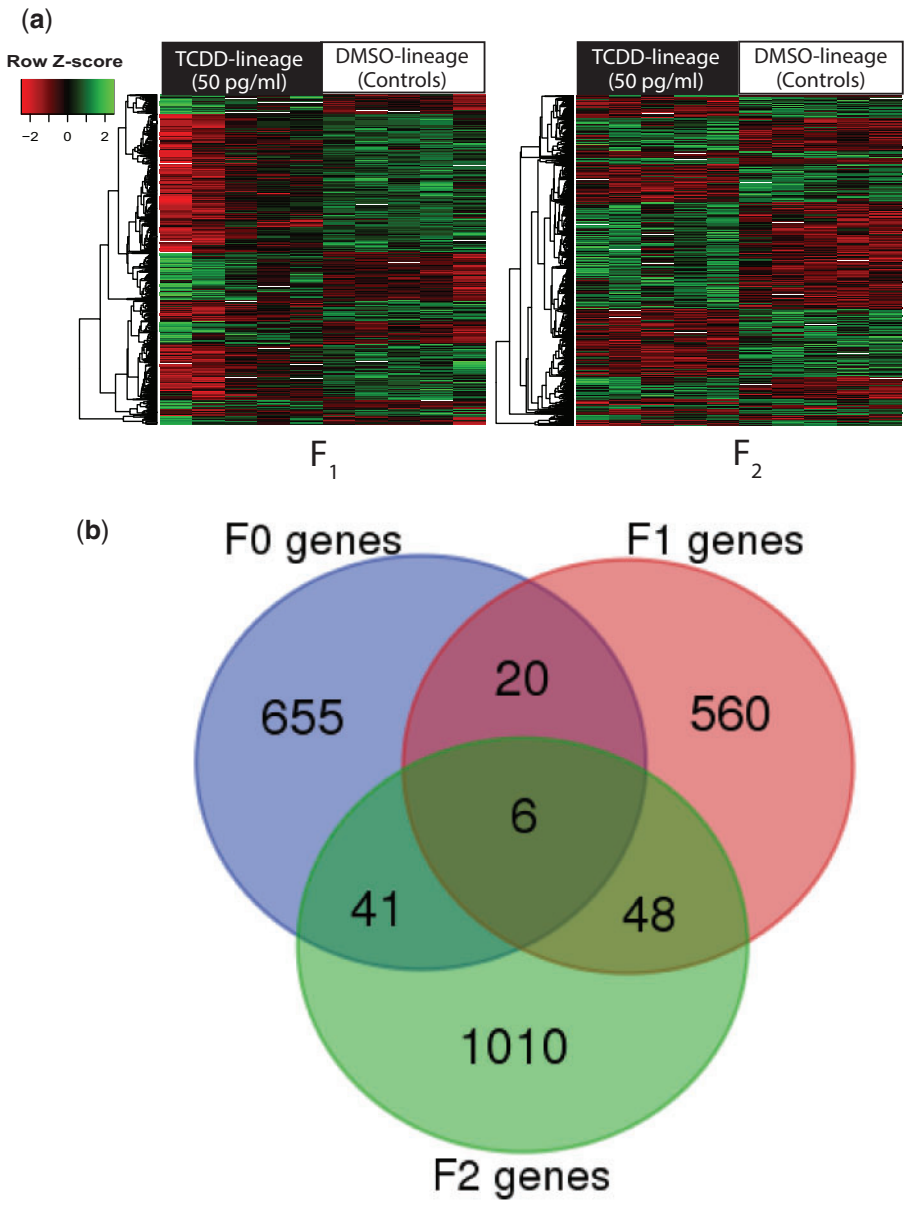


Figure 3. Differentially expressed transcripts in TCDD-lineage F_1–F_2 generations (microarray) and multigenerational (F_0–F_2) transcriptomic overlap. A, Microarray results for F_1 and F_2 generations. Heatmapper (http://www1.heatmapper.ca; last accessed February 18, 2018) (Babicki *et al.*, 2016). was used to hierarchically cluster differentially expressed (p-value $<.05$, FC >1.5) transcripts by similar expression level (row clustering) using Euclidean distance. Individual fish are grouped by treatment (TCDD-lineage vs DMSO-lineage) and each column indicates an individual. The scale indicates normalized (z-score) transcript expression levels. B, Venn diagram indicating overlap among differentially expressed transcripts in TCDD-lineage F_0–F_2 generations. Differentially expressed transcripts (FC >1.5; p-value $<.05$) in the TCDD lineage for each generation were uploaded into the Bioinformatics and Evolutionary Genomics custom Venn diagram generator (http://bioinformatics.psb.ugent.be/webtools/Venn/; last accessed January 7, 2018) and assessed for overlap across generations. Minimal transgenerational overlap is present. TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; FC, fold change; DMSO, dimethyl sulfoxide.

$p = .034$) and *gcgb* (2.08, $p = .030$), or *glucagon a* and *b*, *ins*, or *preproinsulin* (3.86, $p = .038$), and *sgk1*, or *serum/glucocorticoid regulated kinase 1* (2.22, $p = .039$). In the F_2 generation, one of the most differentially regulated genes was *ftr50*, or *finTRIM (tripartite motif) family, member 50* (−131.88, $p = .038$), a member of a gene family implicated in antiviral immunity (Luo *et al.*, 2017). Similar to the F_1 generation, several genes involved in spermatogenesis and fertility were also upregulated, including *spata4* (8.22, $p = .000078$), *acox1* (6.12, $p = .000522$), *nudt15*, or *nudix (nucleoside diphosphate linked moiety X)-type motif 15* (3.68, $p = .002318$), a gene involved in xenobiotic response and mediating oxidative toxicity (Cai *et al.*, 2003) and *hsp70*, or *heat shock protein 70* (1.98, $p = .002$).

To verify that effects on gene expression were not due to any direct exposure to TCDD, *cyp1a* transcripts were assessed; no *cyp1a* induction was found in F_1 or F_2 TCDD-lineage testicular tissue (Supplementary Table 3). In a similar vein, a variety of hormone synthesis, hormone response, and classical AhR battery response transcripts were not altered by TCDD exposure in the F_1 or F_2 generations (Supplementary Table 3).

Ingenuity pathway analysis was performed on differentially regulated genes, as defined above (FC >1.5, p-value $<.05$) using GenBank IDs; in the F_1 generation, 139 molecules were accessible for analysis with IPA, and 164 molecules were available in the F_2 generation. A table of the most enriched disease and

Table 1. Microarray Values for Differentially Expressed Genes Involved in Fertility or Spermatogenesis in TCDD-lineage F_0–F_2 Testes

		F_0		F_1		F_2	
Symbol	Name	FC	*p*-value	FC	*p*-value	FC	*p*-value
acox1	*Acyl-coenzyme A oxidase 1*	−1.32	>0.1	**10.65**	**0.0071**	**6.12**	**0.000522**
gcga	*Glucagon a*	1.3	>0.1	**7.96**	**0.034**	3.14	>0.1
ins	*Preproinsulin*	1.17	>0.1	**3.86**	**0.038**	3.06	0.099364
sgk1	*Serum/glucocorticoid regulated kinase 1*	**1.32**	**0.03904**	**2.22**	**0.039**	−1.17	>0.1
gcgb	*Glucagon b*	−1.09	>0.1	**2.08**	**0.03**	2.52	>0.1
spata4	*Spermatogenesis associated 4*	−1.77	0.073348	−19.12	0.050589	**8.22**	**0.000078**
nudt15	*Nudix (nucleoside diphosphate linked moiety X)-type motif 15*	**−3.03**	**0.0086**	−2.48	0.094414	**3.68**	**0.002318**
hsp70	*Heat shock protein 70*	**−1.49**	**0.022**	−1.21	>0.1	**1.98**	**0.002**
ftr50	*finTRIM(tripartite motif) family, member 50*	1.14	>0.1	−18.24	>0.1	**−131.88**	**0.038**

Fold changes and *p*-values for microarray data are listed per gene; *p*-value is determined using ANOVA. Bolded text indicates significant difference from control (FC >1.5, *p*-value <0.05).

TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; FC, fold change; ANOVA, analysis of variance.

biologic functions is included as Supplementary Table 4 with specific pathways involved in reproductive function listed in Table 2. As indicated in Supplementary Table, endocrine system disorders and metabolic disease were 2 of the top 5 enriched diseases and disorders for the F_1 generation. Several of the specific enriched pathways in the F_1 generation (Table 2) included reproductive system disease, metabolic disease, and lipid metabolism, which are all functions implicated in fertility and spermatogenesis. For pathways that were enriched in the IPA analysis of the F_2 generation, inflammatory and immunological disease were among the most upregulated pathways, which corresponds with the large number of immune (finTRIM family, *major histocompatibility complex class I UBA*, granulin family) genes differentially regulated in the F_2 generation (Supplementary Table 2). The affected pathways related to fertility and spermatogenesis in the F_2 generation included cell-to-cell signaling and interaction, cellular movement, reproductive system disease, cellular compromise, lipid metabolism, and metabolic disease.

We compared differentially expressed transcripts from F_0 to F_2 generations and found minimal overlap (Figure 3); only 6 transcripts are consistently altered in the TCDD exposure lineage across all three generations (Table 3). Three of the 6 transcripts are predicted to code for the *novel immune-type receptor 3, related 1-like* (*nitr3r.1l*), and 1 is predicted to code for the *gastrula zinc finger protein* (*XlCGF52.1*). No homologous sequences/genes for the remaining two transcripts were identified despite a search using Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi; last accessed January 13, 2018). Five out of the 6 transcripts were downregulated in both the F_0 and F_1 but upregulated in the F_2 generation, whereas the 6th was downregulated in all three generations. In both the F_0 and F_1 generations, 20 differentially expressed transcripts were selectively altered. In the F_1 and F_2 generations, 48 transcripts were altered including *acox1*, which is upregulated in both generations, and 41 transcripts were altered in both the F_0 and F_2 generations, including *nudt15*, which is downregulated in the F_0 but upregulated in the F_2 generation.

qRT-PCR

Genes from the F_1 and F_2 generations were selected for qRT-PCR validation of microarray results based on microarray values (high fold change or low *p*-values) or relevance to spermatogenesis (Supplementary Table 5). The direction of fold change was consistent between the two methods for all genes.

DISCUSSION

Our results correspond with other studies that found dysregulation in spermatogenic cells and multigenerational infertility as a result of TCDD exposure (Baker *et al.*, 2014b; Bruner-Tran *et al.*, 2014; Chahoud *et al.*, 1992; Sanabria *et al.*, 2016). Histological analysis of F_1 and F_2 testes revealed an altered germ/sperm cell ratio in TCDD-lineage F_1 fish, with increases in the area of immature spermatogonia and decreases in mature spermatozoa, whereas no changes were present in intermediate cell types. Additionally, though testicular germinal epithelium was reduced in width in the F_0 generation (Baker *et al.*, 2016), this did not persist into the F_1 or F_2 generations. Ablation/reduction of germinal epithelium is a well-established endpoint of reproductive toxicity (Baker *et al.*, 2016; Chahoud *et al.*, 1992; Cordeiro *et al.*, 2018), but may be an effect of direct toxicant-induced damage to seminiferous tubule structure. Intriguingly, the shift in germ/sperm cell ratio persisted from the F_0 parental generation, which were directly exposed to TCDD as juveniles (Baker *et al.*, 2016), to the indirectly exposed F_1 generation, and trended towards significance in the unexposed F_2 generation.

According to the OECD Guidance Document for the Diagnosis of Endocrine-Related Histopathology of Fish Gonads (Johnson *et al.*, 2009), such a shift in germ cell ratio is considered both a primary (increase in spermatogonia) and secondary (decrease in spermatozoa) indication of EDC-induced histopathological disruption. Also known as hypospermatogenesis, this shift is a major category of testicular dysgenesis syndrome that is impacted by environmental factors during early programming events (Bhattacharya and Majumdar, 2015; Mocarelli *et al.*, 2007). Thus, the persistence of this shift in germ/sperm cell ratio is likely explained by inheritance of epimutations, a hypothesis that is further supported by our previous finding that transgenerational infertility following TCDD exposure in zebrafish is transmitted through the male germline (Baker *et al.*, 2014b), as well as the sex-specific mechanism of zebrafish genome reprogramming, ie the male epigenetic lineage controls the reprogramming process, with the female lineage silenced (Jiang *et al.*, 2013b). Additionally, accumulation of TCDD in lipophilic reservoirs (Lanham *et al.*, 2012) and the germ line would be minimal, if any, given the short period of TCDD exposure (1 h), time between exposure and spawning (4 months), small size of the juvenile fish, and lack of *cyp1a* induction as assessed by microarray in F_1 and F_2 testicular tissues, thus limiting the possibility of direct exposure of subsequent generations.

Table 2. Ingenuity Pathway Analysis-Generated List of Select Pathways Implicated in Reproduction and Fertility in TCDD-Lineage F_1 and F_2 Zebrafish Testes

	p-value	# of genes
F1 IPA Genes		
Vitamin and mineral metabolism		
Synthesis of glucocorticoid	9.85E−03	2
Synthesis of steroid hormone	**1.17E−02**	**3**
Metabolism of aldosterone	6.27E−03	1
Reproductive system disease		
Disorder of pregnancy	**4.61E−03**	**12**
Abortion	**6.37E−03**	**3**
Impotence	1.07E−02	2
Female infertility	1.15E−02	2
Panhypopituitarism-X-linked	5.77E−04	2
Metabolic disease		
Glucose metabolism disorder	**1.60E−04**	**26**
Disorder of lipid metabolism	**9.75E−03**	**6**
Hypoinsulinemia	7.59E−03	2
Lipid metabolism		
Quantity of steroid hormone	**9.00E−05**	**7**
Quantity of steroid	**2.26E−03**	**11**
Synthesis of lipid	**3.60E−03**	**14**
Synthesis of fatty acid	**3.85E−03**	**8**
Synthesis of long chain fatty acid	**5.02E−04**	**3**
Conversion of phospholipid	6.23E−03	2
Reduction of palmitoyl-coenzyme A	6.27E−03	1
Concentration of corticosterone	**1.16E−03**	**5**
F_2 IPA Genes		
Cell-to-cell signaling and interaction		
Adhesion of spermatids	1.60E−02	1
Cellular movement		
Movement of spermatids	2.40E−02	1
Reproductive system disease		
Antley-Bixler syndrome with genital anomalies and disordered steroidogenesis	8.05E−03	1
Abortion	**1.26E−02**	**3**
Cytotoxicity of gonadal cell lines	2.40E−02	1
Cell viability of gonadal cell lines	1.46E−02	2
Metabolic disease		
Combined oxidative phosphorylation deficiency 5	8.05E−03	1
Disorder of lipid metabolism	**8.95E−03**	**7**
High density lipoprotein deficiency type 2	1.60E−02	1
Cytochrome P450 oxidoreductase deficiency	8.05E−03	1
Lipid metabolism		
Attachment of cholesterol	8.05E−03	1
Oxidation of long chain fatty acid	**1.90E−02**	**3**
Loss of sterol	1.60E−02	1
Abnormal quantity of phospholipid	1.58E−02	
Cellular compromise		
Depletion of lipid droplets	8.05E−03	1

Number of differentially expressed genes involved in each pathway is noted. Bolded rows indicate comparatively enriched pathways (≥3 genes). TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

Whole genome microarray analysis on TCDD-lineage F_1 and F_2 testicular tissue revealed dysregulation of glucose regulation, lipid metabolism, and germ cell development genes heavily implicated in fertility and spermatogenesis. In the F_1 generation, *glucagon a/b* and *preproinsulin*, both involved in glucose regulation, were upregulated despite their conflicting role in glucose metabolism: Glucagon upregulates and insulin downregulates glucose levels (Zhao *et al.*, 2018). Upregulation of both genes may indicate that one is primarily altered by TCDD, whereas the other is upregulated as a feedback mechanism for maintaining glucose homeostasis. Tightly regulated glucose metabolism is essential for the maintenance of spermatogenic germ cells and subsequent functionality of sperm cells (Alves *et al.*, 2013). Therefore, any disruption can lead to reduced fertility outcomes. Additionally, the cell survival regulator *sgk1* (serum glucocorticoid-regulated kinase 1) was upregulated in the F_1 generation. *Sgk1*, induced in response to hormonal stimuli, has been linked with coordination and maintenance of reproductive function in females, and was upregulated in a model of testicular torsion (Cho *et al.*, 2011), an injury that leads to ischemia in the testes, subsequent germ cell apoptosis, and infertility (Filho *et al.*, 2004). In the F_2 generation, *hsp70*, a chaperone and quality control protein (Mayer and Bukau, 2005) that is also dysregulated in the F_0 generation (Baker *et al.*, 2016) and is positively correlated with infertility in human sperm samples (Erata *et al.*, 2008), was upregulated, suggesting a compensatory mechanism to counteract germ cell apoptosis.

Despite minimal overlap in differentially expressed transcripts across generations, several genes with importance in reproductive function were differentially expressed in more than one generation. *Acox1*, for instance, was upregulated across both F_1 and F_2 generations. Normal peroxisomal function as mediated by *acox1* is critical for fertility (Fan *et al.*, 1996). The upstream regulator of *acox1*, PPARα (peroxisomal proliferator-activated receptor alpha), engages in crosstalk with the AhR (Ernst *et al.*, 2014; Shaban *et al.*, 2004), and thus TCDD and dioxin-like compounds, as AhR ligands, may misregulate PPARα signaling pathways. Increases in *acox1* activity also correspond with an overall increase in peroxisomal activity, and therefore can be associated with the presence of increased oxidative stress (Zeng *et al.*, 2017), a TCDD-induced outcome (Latchoumycandane *et al.*, 2003) that can result in infertility through damaging sperm membrane and DNA (Tremellen, 2008).

Spata4, a testis-specific gene closely associated with spermatogenic fate and regulation of Sertoli cell proliferation (Jiang *et al.*, 2013a, 2015), was altered in both F_1 and F_2 generations. *Spata4* was downregulated in the F_1 generation, which corresponds with our histopathologic findings of hypospermatogonia; previous studies also found decreases in *spata4* expression accompanied by inhibition of spermatogenesis in fish exposed to flame retardants (Li *et al.*, 2014). Conversely, *spata4* was greatly upregulated in the F_2 generation. Likewise, *nudt15*, a xenobiotic response gene involved in mediating oxidative stress (Cai *et al.*, 2003), was upregulated in the F_2 generation, whereas in the F_0, it was downregulated. The upregulation of both genes in the F_2 may indicate lingering epigenetic changes that compensated for TCDD-induced pathway dysregulation in the F_1 generation, ie F_2 generation is adapting to a toxicant that is no longer present. Supporting this idea, a greater percentage of differentially expressed genes shift from downregulation to upregulation between the F_1 and F_2 (from 32% to 54% upregulated, respectively).

Several families of genes involved in immune response were also dysregulated. Specifically, multiple finTRIM family and granulin genes were downregulated in the F_2 generation. Furthermore, *nitr3r.1l*, the gene associated with 3 of the 6 differentially expressed transcripts across all generations, is an immune receptor gene, indicating that immune response is disrupted across generations, but particularly in the F_2.

Table 3. Microarray Transcripts Differentially Expressed in TCDD-Lineage Fish Across All 3 Generations (F_0–F_2)

F_0–F_2 Microarray Overlap

Computer-Predicted				F_0		F_1		F_2	
Transcript No.	**Gene ID**	**Symbol**	**Name**	**FC**	***p*-Value**	**FC**	***p*-Value**	**FC**	***p*-Value**
13280478	XM_005166663	*nitr3r.1l*	*novel immune-type receptor 3, related 1-like*	−1.59	0.030	−2.09	0.039	1.52	0.015
13284886	XM_005166663	*nitr3r.1l*	*novel immune-type receptor 3, related 1-like*	−1.59	0.030	−2.09	0.039	1.52	0.015
13280442	XM_005166663	*nitr3r.1l*	*novel immune-type receptor 3, related 1-like*	−1.59	0.030	−2.09	0.039	1.52	0.015
13273229	XM_003201642	*XlCGF52.1*	*gastrula zinc finger protein*	−1.71	0.005	−1.52	0.046	1.98	0.012
Unidentified				F_0		F_1		F_2	
Transcript No.	**Gene ID**		**Genomic Location**	**FC**	***p*-Value**	**FC**	***p*-Value**	**FC**	***p*-Value**
13201649	–		chr5: 7194748–7196383	−1.72	0.035	−2.16	0.026	2.69	< .001
13022462	–		chr15: 43367712–43368245	−3.58	0.009	−2.99	0.002	−4.21	0.002

TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; BLAST, Basic Local Alignment Search Tool; FC, fold change.

Disruption of immune function on a broad scale can have implications for fertility, as several inflammatory cytokines and factors involved in immune response are also involved in supporting and signaling within germ cell populations (Loveland *et al.*, 2017). Misregulation of the immune system in reproductive tissues can lead to inflammation or infection, well-established causal factors for impaired spermatogenesis and decreased sperm count and quality (Schuppe *et al.*, 2008).

Transcriptomic analysis in all three generations showed dysregulation in many of the same pathways, including lipid and steroid metabolism, oxidative stress, glucose metabolism, spermatogenesis, and germ cell development, although overlap in specific genes was minimal. A greater number of genes involved in germ cell development and maturation were differentially regulated in the F_0 when compared with subsequent generations (Baker *et al.*, 2016). Overall, however, a similar number of genes were differentially altered between the F_0 (Baker *et al.*, 2016) and F_1 generations. The transcriptome of the F_2 generation was evidently more altered due to ancestral TCDD exposure with almost twice the number of differentially expressed transcripts as the previous two generations. This comparative abundance of differentially expressed transcripts in the F_2 runs counterintuitive to expected results, as effects of direct exposure to TCDD would reasonably be more severe than subsequent generations. However, certain outcomes only present transgenerationally; previously, exposure to a plastics mixture during gestation resulted in a variety of disease outcomes across generations, with some (obesity) only present in the rodent F_3 generation (Manikkam *et al.*, 2013). The mechanisms underlying these multigenerational findings deserve further interrogation, particularly concerning the type and genomic location of potential epimutations.

Transgenerational studies of endocrine disruption have raised public and academic concern on a population-wide level, but the possibility that EDC exposure may induce generationally distinct transcriptomes, due to the interplay of differential mechanisms of toxicity across generations, complicates the clinical effort to rescue such phenotypes. As a whole, this multigenerational response gives insight into the nature of EDC-induced reproductive disease, and raises many potential ideas concerning the mechanism of epigenetic heritability of this disease, the genes to which it might be linked, and the mechanisms through which direct exposure, indirect exposure, and unexposed generations have their own unique, often opposing transcriptomic footprint, despite all exhibiting similar phenotypes of infertility.

SUPPLEMENTARY DATA

Supplementary data are available at *Toxicological Sciences* online.

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