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Reproductive and Developmental Toxicity of Dioxin in Fish[1]

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Abstract

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD or dioxin) is a global environmental contaminant and the prototypical ligand for investigating aryl hydrocarbon receptor (AHR)-mediated toxicity. Environmental exposure to TCDD results in developmental and reproductive toxicity in fish, birds and mammals. To resolve the ecotoxicological relevance and human health risks posed by exposure to dioxin-like AHR agonists, a vertebrate model is needed that allows for toxicity studies at various levels of biological organization, assesses adverse reproductive and developmental effects and establishes appropriate integrative correlations between different levels of effects. Here we describe the reproductive and developmental toxicity of TCDD in feral fish species and summarize how using the zebrafish model to investigate TCDD toxicity has enabled us to characterize the AHR signaling in fish and to better understand how dioxin-like chemicals induce toxicity. We propose that such studies can be used to predict the risks that AHR ligands pose to feral fish populations and provide a platform for integrating risk assessments for both ecologically relevant organisms and humans.

Keywords

TCDD; AHR; development; reproduction; toxicity; zebrafish; trout

Introduction

Halogenated aromatic hydrocarbons (HAHs) constitute a class of global environmental contaminants that include polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs). HAHs cause toxicity at

[1]In this review the same nomenclature is used for both fish and mammals in naming genes, mRNAs, and proteins (*ahr*, gene; *Ahr*, mRNA; and AHR, protein).

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environmentally relevant levels; and aquatic ecosystems such as the Great Lakes tend to act as repositories for such compounds, putting fish and wildlife that depend on these ecosystems at risk of exposure. Impacts of HAH exposure on inhabitants of the Great Lakes ecosystem was first documented in avian populations due to the availability of close population monitoring (Gilbertson et al., 1991; Jones et al., 1994; Kubiak et al., 1989). Effects of these chemicals on wild fish populations proved more difficult to evaluate as they are less readily observable and effects can be direct (i.e., adverse effects on survival, growth and reproduction) and indirect (i.e., secondary to habitat destruction).

Initial suspicions that HAHs may be affecting wild fish populations occurred when, despite over four decades of re-stocking efforts, lake trout populations were no longer able to sustain themselves (for review see Tillitt et al., 2008). Several lines of evidence suggested that HAH contamination might be responsible for this recruitment failure. The greatest concentrations of HAHs were found in lake trout compared to other fish species (Clark et al., 1984; Hickey et al., 2006) and areas lacking evidence for natural lake trout reproduction contained the highest HAH contamination levels (Baumann and Whittle, 1988; Maack and Sonzogni, 1988). Also, dioxin-like chemicals (PCDDs, PCDFs and coplanar PCBs) act through the aryl hydrocarbon receptor (AHR) pathway and trout express the AHR and its dimerization partner the aryl hydrocarbon nuclear translocator (ARNT) and are capable of responding to AHR agonist exposure with toxicity (Abnet et al., 1999; Pollenz et al., 1996).

Since 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is the most potent AHR agonist among HAHs, it has become a “prototype” compound for investigating their toxicity. TCDD has the potential to interfere with several biological processes that impair growth and development of various organs and function of the liver and cardiovascular, immune, skeletal, reproductive and central nervous systems (Peterson et al., 1993). AHR signaling mediates the toxicity of HAHs such as TCDD (Schmidt and Bradfield, 1996). In fish, one of the first biological responses to TCDD activation of the AHR pathway is induction of xenobiotic metabolizing enzymes. These are phase I and phase II enzymes, which are crucial for biotransformation of lipophilic compounds. Of these xenobiotic-metabolizing enzymes, induction of cytochrome P450 1A1 (CYP1A1) has become a hallmark response to AHR activation (Hahn and Stegeman, 1994; Hahn et al., 1998; Stegeman et al., 1995) and was initially thought to play an integral role in TCDD toxicity. Since HAHs such as TCDD are poorly metabolized, it was thought that induction of CYP1 A1 might result in toxicity through the generation of reactive oxygen species or modulation of normal signaling cascades. After five decades of intensive research, the mechanisms by which TCDD induces toxicity are not completely understood. However, we now know that developmental toxicity of TCDD is not likely secondary to CYP1A induction as initially thought and that other genes are involved (Carney et al., 2004; Xiong et al., 2008).

Fish larvae are among the most sensitive vertebrates to the toxic effects of TCDD (Peterson et al., 1993); and exhibit similar signs of toxicity as other vertebrates including decreased food intake, wasting syndrome, delayed mortality, lesions in epithelial and lymphomyeloid tissues, cardiovascular dysfunction, edema, hemorrhages, craniofacial malformations, impaired reproductive success and mortality (Carney et al., 2006b; Peterson et al., 1993; Spitsbergen et al., 1991; Tanguay et al., 2003; Walker and Peterson, 1992, 1994a; Walker et al., 1991, 1994). However, while the endpoints of TCDD toxicity are similar among bony fishes, sensitivity to TCDD toxicity can vary greatly between species and the endpoints of toxicity are age-dependent.

We propose that zebrafish can be used as a model to predict the risks that exposure to dioxin-like compounds pose to feral fish populations. Furthermore, since the zebrafish is now an accepted model for human health and disease, we believe that the zebrafish can

provide the framework for integrating assessments of risks to both ecologically relevant organisms and humans. Here we present a brief historical perspective of TCDD toxicity in fishes at different life stages, the potential risks that exposure to HAHs such as TCDD pose to feral fish populations, and document how using the zebrafish as a model to investigate TCDD toxicity and AHR signaling has enabled us to make great strides in better understanding how exposures to dioxin-like chemicals induce certain endpoints of reproductive and developmental toxicity.

TCDD Toxicity in Adult and Juvenile Feral Fish

Adult fish are less susceptible to TCDD-induced toxicity compared to earlier life stages, requiring considerably higher body burdens to elicit adverse effects (Lanham et al., 2011; Peterson et al., 1993; Walker and Peterson, 1992, 1994a; Walker et al., 1991, 1994). Lethal potencies of TCDD range from 3–16 μg/kg, with yellow perch, carp and bullhead being less sensitive to TCDD toxicity than adult rainbow trout, bluegill and largemouth bass (Kleeman et al., 1988; van der Weiden et al., 1994). The hallmark signs of overt TCDD toxicity in adult fish is a wasting syndrome and fin necrosis with loss of body weight being both species- and dose-dependent (Kleeman et al., 1988). Yearling rainbow trout exposed to TCDD became hypophagic, exhibited fin necrosis, developed ascites and showed reduced hematopoiesis (Spitsbergen et al., 1986); while adult female lake trout primarily exhibited liver toxicity and displayed reduced hepatocellular glycogen, nuclear chromatin clumping in the liver along with TCDD-induced lesions in the liver and spleen (Walter et al., 2000). Similarly, adult medaka exhibit fatty liver, mild hepatocellular atrophy and glycogen depletion in the liver (Volz et al., 2005, 2006) following exposure to TCDD. Histopathological lesions in adult fish caused by TCDD exposure are also found in the spleen, gill, heart, thymus and gonad (Kleeman et al., 1988; Spitsbergen et al., 1988a, 1988b; van der Weiden et al., 1994).

While TCDD induces several overt toxicities in adult fishes, it also exerts detrimental effects on reproductive success. Reproductive toxicity of TCDD varies among different fish species, and certain species (*e.g.*, salmonids) are more vulnerable than other fish species (Cook et al., 1993; Peterson et al., 1993; Walker and Peterson, 1992, 1994a; Walker et al., 1994). Acute TCDD exposure impairs female reproduction via reduced steroid hormone secretion, depressed capacity for ovarian steroid biosynthesis, altered ovarian growth and development, fecundity with age, age to maturation, secondary sexual characteristics and egg and larval size, reduced egg production and early life stage toxicity of offspring (Giesy et al., 2002; Hutz et al., 1999; Munkittrick et al., 1991, 1998; Palstra et al., 2006; Peterson et al., 1993; Thomas, 1990; Van der Kraak et al., 1992; Walker and Peterson, 1992; Wu et al., 2001). Less is known regarding TCDD-induced reproductive toxicity in male fishes; however, it has been demonstrated in medaka that TCDD causes lesions in the testis characterized by a disorganization of spermatogenesis at the testis periphery, disruption of the interstitium, Leydig cell swelling and Sertoli cell vacuolation (Volz et al., 2005, 2006). Effects of chronic, sublethal TCDD exposure on reproductive success of fish are a growing concern. While this has not been studied extensively, some studies suggest that sublethal exposure to TCDD impairs gonad development, ovulation and survival of offspring (recruitment) (Giesy et al., 2002; Jones et al., 2001; Tietge et al., 1998; Walker et al., 1994; Walter et al., 2000).

Juvenile fishes are also susceptible to TCDD-induced wasting and fin necrosis; however, it is thought that wasting is not ultimately responsible for lethality (Walker and Peterson 1994a). In juvenile fishes, endpoints of TCDD toxicity include cutaneous hyperpigmentation, hemorrhage and ascites as well as histopathologic lesions in several tissues. As in adult fishes, susceptibility to TCDD toxicity in juveniles varies across species.

For example, fingerling rainbow trout fed TCDD for 13 weeks followed by 13 weeks of depuration showed cutaneous hemorrhage, reduced growth rate or lethality; while fingerling yellow perch exposed to similar concentrations of TCDD showed no signs of reduced growth rate, fin necrosis, cutaneous hemorrhage or lethality (Kleeman et al., 1986). However, signs of fin necrosis, petechial cutaneous hemorrhage, loss of body weight and ascites following exposure to greater concentrations of TCDD, as well as histopathologic lesions including thymic atrophy, decreased hematopoiesis, focal myocardial necrosis, fibrinous pericarditis, sub mucosal gastric edema and hyperplasia of gill filaments and lamellae were identified (Spitsbergen et al., 1988a). Juvenile rainbow trout administered TCDD intraperitoneally also demonstrated TCDD-induced histopathological lesions including multifocal necrosis of gastric cardiac glandular mucosa and morphological lesions in epithelial and lymphomyeloid tissue. The lymphomyeloid lesions included splenic lymphoid depletion, thymic involution and hypocellularity of hematopoietic tissues in the head and trunk kidney (Spitsbergen et al., 1988b). Peripheral leucopenia and thrombocytopenia were also observed in the juvenile Shasta strain of rainbow trout (Spitsbergen et al., 1988b).

TCDD Developmental Toxicity in Feral Fish

Fish are most sensitive to the toxic effects of TCDD during early stages of development (Peterson et al., 1993). Embryos develop normally through gastrulation and primary organogenesis with most toxic effects manifesting after hatching during larval sac fry development (Spitsbergen et al., 1991; Tillitt et al., 2008; Walker et al., 1991). Early life stage TCDD toxicities include yolk sac edema, hemorrhage, craniofacial malformations, stunted growth and post-hatch mortality (Carney et al., 2006b; Elonen et al., 1998; Johnson et al., 1998; Spitsbergen et al., 1991; Tanguay et al., 2003; Walker and Peterson, 1992, 1994a, 1994b; Walker et al., 1991, 1992, 1994; Yamauchi et al., 2006; Zabel et al., 1995b). These TCDD developmental toxicities (Figure 1A, lake trout larva; 1B, zebrafish larva) closely resemble “blue sac disease,” an edematous syndrome first reported in salmonid hatcheries (Wolf, 1969). The most distinguishing feature of this syndrome is the accumulation of fluid between the membranes surrounding the yolk sac (yolk sac edema) during the sac fry stage (Wolf, 1957, 1969). This edema can manifest as a milky blue color, giving the syndrome its name. In addition to yolk sac edema, other significant features of blue sac disease include pericardial edema, congestion of peripheral blood circulation, hemorrhage and craniofacial malformations (Symula, 1990; Wolf, 1957, 1969). Due to its similarity to blue sac disease, “blue sac syndrome” has since been assigned to describe the developmental defects induced by exposure to dioxin-like chemicals in these fish species during early life stage development (Symula, 1990; Walker et al., 1994).

Although the endpoints of TCDD toxicity in larvae are largely identical across various species of bony fish, there are major differences in TCDD potency (Figure 1C). Bull trout and lake trout are the most sensitive fish species to the developmental toxic effects of TCDD (Elonen et al., 1998; Tanguay et al., 2003) with bull trout larvae being three times more sensitive than lake trout (Cook et al., 2000). In laboratory studies, lake trout exposed to TCDD at the fertilized egg stage and observed through the fry stage exhibited the greatest mortality at the sac fry stage (Walker et al., 1991). TCDD-exposed lake trout sac fry exhibited signs of blue sac disease, necrosis of the brain, retina and spinal cord, craniofacial malformations, growth retardation, meningeal and subcutaneous hemorrhage and the severity of edema correlated with cumulative mortality (Guiney et al., 1997, 2000; Spitsbergen et al., 1991; Walker et al., 1991). TCDD exposure elicits similar responses in brook trout and rainbow trout larvae including signs of blue sac disease, craniofacial malformations that include reduced cranial length and shortened maxillas as well as a reduction in eye diameter, opercular defects, multifocal hemorrhages, liver vacuolation and

hepatocellular necrosis, pericardial edema, reduced heart size, and retarded development that preceded lethality (Carvalho et al., 2004; Helder, 1981; Hornung et al., 1996a, 1996b, 1999; Walker and Peterson, 1994a, 1994b; Walker et al., 1994). Retinal ganglion cell densities also appeared to be decreased in swim-up rainbow trout resulting in a reduction in visual and motor function, and at high doses reduced prey capture ability (Carvalho and Tillitt, 2004). Reduced circulation, pericardial edema, vascular hemorrhage, blood clots and bone malformations including dorsoventrally and laterally curved spines, deformed neural and haemal spines and caudal fins and alterations in spine calcification in the posterior region were prominent endpoints of TCDD toxicity in the medaka embryo (Cantrell et al., 1996, 1998; Kawamura and Yamashita, 2002). Thus, in fish early life stages the cardiovascular system and developing cartilaginous structures in the craniofacial region are particularly vulnerable to TCDD developmental toxicity.

Impact of TCDD-Like Chemicals on Great Lakes Lake Trout

In the Great Lakes Ecosystem, lake trout have historically been the top predator fish species (Balon, 1980) and constitute an important part of the region's economy. While populations of lake trout were self-sustaining in the Great Lakes since the late 1800s, populations crashed in the 1950s in all of the Great Lakes except Lake Superior (Tillitt et al., 2008). Overharvesting by commercial fishermen in conjunction with the introduction of the sea lamprey into the Great Lakes has long been thought to be the cause of this population decline.

However, it later became apparent that sac fry mortality caused by exposure to TCDD-like chemicals contributed to the failure of population recruitment of lake trout in the Great Lakes (Cook et al., 2003). Blue sac disease had been identified in feral fish populations, most notably among lake trout of the Great Lakes. Since laboratory exposure of lake trout embryos to waterborne TCDD or PCB 126 resulted in endpoints consistent with blue sac disease (Spitsbergen et al., 1991; Walker et al., 1991; Zabel et al., 1995a), exposure to such environmental toxicants was suspected as a potential cause of the disease, but no association was found between blue sac disease and contamination with chemicals such as 1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethylene (DDE) or total PCBs (Simonin, 1990). However, it was noted that lake trout from Lake Ontario had a higher incidence of blue sac disease than trout from the other Great Lakes, which coincided with higher concentrations of TCDD in Lake Ontario (DeVault et al., 1989; Zacharewski et al., 1989).

Concern about reproductive success and growth of wild lake trout populations combined with a greater appreciation for the high sensitivity of lake trout larvae to TCDD-induced blue sac syndrome mortality (Cook et al., 2003; Spitsbergen et al., 1991; Tillitt et al., 2008; Walker et al., 1991; Zabel et al., 1995a) led to increased interest in determining the molecular basis for the developmental toxicity of TCDD in aquatic species. Lake trout larvae are among the most sensitive of all fish species to the toxic effects of dioxin-like compounds. Accordingly, it was hypothesized that while the adult populations appear less affected, exposure of lake trout larvae to dioxin-like chemicals, from environmental exposure and maternal transfer, was causing sac fry mortality and impairing natural recruitment such that lake trout populations could no longer sustain themselves in the most contaminated Great Lakes (Cook et al., 2003; Tillitt et al., 2008). In support of this notion, it was subsequently determined that levels of dioxin-like chemicals were high enough in Lake Ontario to kill essentially 100% of lake trout larvae by blue sac syndrome that hatched there from the late 1940s to the late 1970s (Figure 2) (Cook et al., 2003). While environmental levels of AHR agonists have been reduced in recent years, these persistent organic pollutants still constitute a major risk to wild fish populations including lake trout that are highly sensitive to dioxin developmental toxicity. Furthermore, to integrate assessment risks to

wildlife and humans, it is important to demonstrate common mechanisms of toxicity across species.

Initial work used rainbow trout and lake trout embryos to study the early life stage developmental effects produced by TCDD (Peterson et al., 1993). However, lake trout proved not to be an amenable model to extensively study TCDD developmental toxicity because the opaque chorions made evaluation of early development difficult, and lake trout embryos and larvae develop slowly taking as much as 3–6 months for hatching and sexual differentiation. Trout species also require large facilities for general fish care and maintenance. Furthermore, as tetraploids lake trout have a higher incidence of duplicated genes, which when combined with slow advances in genome annotation and molecular techniques, limited this model for use in identifying genes and signaling pathways that mediate TCDD toxicity. In order to delineate the molecular mechanism(s) of developmental TCDD toxicity in fish, a different species, whose embryonic and larval development was easily observed, rapid, amendable to genetic manipulation, had available genome annotation and whose maintenance was more manageable than trout was required.

Zebrafish as a Model for Assessing TCDD Reproductive and Developmental Toxicity

Zebrafish have been used extensively as a model for studying development and genetics, and its use has recently expanded to the fields of pharmacology and toxicology (Grunwald and Eisen, 2002; Hill et al., 2005; Peterson et al., 2000; Tanguay et al., 2003). Zebrafish embryogenesis is complete within 72 hours post fertilization (hpf) and zebrafish reach sexual maturity within 3 months (Westerfield, 1993). Adult females readily produce hundreds of eggs on a regular basis, and embryos are transparent which allows for microscopic observation during early development. Most significant are the continuing advances in genetic and molecular tools that have allowed for the understanding of genes and their functions in early developmental processes. For instance, production of mutant and transgenic zebrafish and transient knockdown of gene translation or splicing with targeted morpholino oligonucleotides have resulted in better understanding of gene function during embryonic development (Kawakami, 2005; Mullins and Nusslein-Volhard, 1993; Nasevicius and Ekker, 2000). In addition, advances in the annotation of the zebrafish genome suggested high conservation of genes across zebrafish and mammals (Curwen et al., 2004). Exposure of zebrafish embryos to TCDD also induces the hallmark signs of blue sac disease, establishing this organism as a useful laboratory model for the analysis of TCDD toxicity in fishes (Henry et al., 1997; Walker et al., 1991).

Many of these same attributes make the zebrafish an excellent model organism for examining reproductive toxicity following exposure to TCDD-like chemicals in juveniles and adults (Carvan et al., 2005; Spitsbergen and Kent, 2003). Much is known about the processes of sexual differentiation, anatomy and function of the reproductive system, breeding behaviors, and environmental conditions that affect sexual differentiation in zebrafish (Ankley and Johnson, 2004; Ankley et al., 2009; Coe et al., 2008; Laan et al., 2002). Established protocols for evaluating histopathology in juveniles and adults are readily available (Leal et al., 2009), and several commercial assays exist for measuring serum steroid hormone concentrations. The zebrafish is commonly used for life-cycle toxicity assays, and there is a wealth of data regarding effects of reproductive toxicants in this species (Ankley and Johnson, 2004).

Zebrafish (Figure 1B) exhibit essentially the same profile of TCDD developmental toxicity responses as other larval fishes (Figure 1A), although zebrafish are significantly less sensitive to TCDD-induced early life stage mortality than other fish species (Figure 1C).

This is reflected in about a 120 fold difference in the lethal potency of TCDD between the most sensitive fish species, bull trout and least sensitive, zebrafish. Henry et al. (1997) were the first to characterize TCDD developmental toxicity in larval zebrafish, and Elonen et al. (1998) reported similar findings. Reproductive toxicity of TCDD is also similar in adult zebrafish compared with other fish species (King-Heiden et al., 2005, 2006, 2009; Wannemacher et al., 1992). Due to the extensive genetic characterization and rigorous examination of embryonic and larval development in zebrafish, this model is ideal for investigating the cellular and molecular basis of TCDD developmental and reproductive toxicity.

Understanding Mechanisms of TCDD Toxicity - AHR Signaling in Zebrafish

In order to fully utilize the zebrafish as a model to investigate the mechanisms of TCDD toxicity, it was essential to identify and characterize the function of the different AHR signaling pathway components. As previously mentioned, TCDD and other dioxin-like chemicals induce toxicity by binding to the AHR (Figure 3). The AHR protein is highly conserved across vertebrate species and is a basic helix-loop-helix, Per-Arnt-Sim (PAS) domain-containing, ligand-activated transcription factor. In the absence of ligand, the AHR resides in the cytoplasm where it is found in a complex with chaperone proteins including heat shock protein 90 (HSP90) and the AHR interacting protein (AIP, ARA9 or XAP2). Upon activation by a ligand such as TCDD, the AHR complex translocates into the nucleus where it binds to its heterodimer partner, the aryl hydrocarbon receptor nuclear translocator (ARNT). This heterodimer subsequently binds to DNA at AHR response elements (AHREs), also referred to as dioxin response elements (DREs) or xenobiotic response elements (XREs), and induces the transcription of target genes.

Members of the PAS domain-containing family are typically involved in responding to environmental stimuli such as light or oxygen tension (Taylor and Zhulin, 1999), and the AHR is no exception. Its most well-defined role is as an environmental sensor that mounts an adaptive response to xenobiotic exposure by up-regulating Class I and Class II xenobiotic metabolizing enzymes in a ligand-dependent manner, thereby facilitating detoxification of noxious compounds. However, multiple studies have suggested additional roles for the AHR in development (Fernandez-Salguero et al., 1995; Schmidt et al., 1996), cell-cell interactions (Cho et al., 2004; Ikuta et al., 2004), cell cycle control (Ge and Elferink, 1998; Kolluri et al., 1999; Puga et al., 2000a), circadian rhythms (Garrett and Gasiewicz, 2006; Mukai and Tischkau, 2007; Sato et al., 2008), and endocrine signaling (Ohtake et al., 2003). In addition, some studies indicate that AHR may not only be functioning as a mediator of transcription, but also as a modulator of transcription by interacting with and amplifying or repressing the activity of other transcription factors at non-AHRE sites [e.g., E2F (Marlowe et al., 2004), ER (Ohtake et al., 2003) and BRCA1 (Kang et al., 2008)]. It is thought that AHR-mediated transcriptional regulation underlies the mechanisms by which AHR agonists such as TCDD induce toxicity; however, some actions of TCDD may be AHR-independent and/or result from downstream transcriptional changes (King-Heiden et al., 2008; Pande et al., 2005; Puga et al., 2000b; Volz et al., 2005).

Fish express both AHR and ARNT, in addition to another protein known as the AHR repressor (AHRR) (Karchner et al., 2002), which can antagonize AHR function; however, fish possess multiple copies of AHR genes (Hahn, 2002). Initially cloned from *Fundulus heteroclitus* and *Mustelus canis* (Hahn et al., 1997; Karchner et al., 1999), the duplicated AHR genes were found to have diverged and were placed into separate clades denoted as AHR1 and AHR2. Multiple AHR genes were soon identified in other fish species, including zebrafish, *Danio rerio* (Andreasen et al., 2002; Karchner et al., 2005; Tanguay et al., 1999), Atlantic salmon, *Salmo salar* (Hansson et al., 2004), and rainbow trout, *Oncorhynchus*

mykiss (Abnet et al., 1999). Zebrafish have three known AHR genes, one from the AHR2 clade (Tanguay et al., 1999) and two from the AHR1 clade (AHR1a and AHR1b) (Andreasen et al., 2002; Karchner et al., 2005); however, the zfAHR1a cannot bind TCDD and appears to be non-functional as a mediator of transcription. The zebrafish also has two ARNT genes (ARNT1 and ARNT2) (Prasch et al., 2006; Tanguay et al., 2000) and two AHRR genes (AHRR1 and AHRR2) (Evans et al., 2005). Both ARNT1 and ARNT2 can produce multiple splice variants (ARNT1a, b, c and ARNT2a, b, c), and three of these, ARNT2b, ARNT1b and ARNT1c, can functionally heterodimerize with zfAHR2 *in vitro* (Prasch et al., 2006; Tanguay et al., 2000).

Once components of the AHR signaling pathway had been characterized, a series of studies using morpholino technology demonstrated, by knocking down the expression of several components of the AHR pathway, that TCDD toxicity is mediated by zfAHR2 and zfARNT1 dimerization partners but not by the zfCYP1 A in embryonic zebrafish (Antkiewicz et al., 2006; Carney et al., 2004; Prasch et al., 2003, 2004, 2006). Following these milestones, the zebrafish model was poised to investigate the more sensitive endpoints of TCDD developmental toxicity, namely, adverse effects on cardiovascular, craniofacial and ovarian development.

Using Zebrafish to Understand TCDD-Induced Cardiovascular Toxicity

While toxic effects on some organ systems may not result in mortality, the cardiovascular system is perhaps the most evident example of a close association between organ dysfunction and mortality (Heideman et al., 2005). Studies of HAH toxicity in mammals and birds had identified the cardiovascular system as a key target of TCDD toxicity. Also the first published study of TCDD developmental toxicity in lake trout in 1991, identified the cardiovascular system as the initial tissue affected in both the TCDD toxicity syndrome and in blue sac disease of developing lake trout (Spitsbergen et al., 1991). Six years later, Henry et al. (1997) demonstrated that TCDD exposure also adversely affected the cardiovascular system of developing zebrafish (Henry et al., 1997). Zebrafish embryos treated with TCDD shortly after fertilization developed malformed hearts and pericardial edema at 72 hpf (Figure 4A), followed by the onset of yolk sac edema (96 hpf) and mortality (132 hpf). Reduced blood flow in vascular beds of the trunk, head and gills and slowed heart rate also occurred in TCDD-treated larvae prior to or coincident with the onset of other signs of toxicity (Henry et al., 1997). This was the first report in zebrafish to demonstrate that the cardiovascular system was a target of TCDD developmental toxicity. This conclusion was reinforced by a subsequent study on TCDD developmental toxicity in rainbow trout, where arrested heart development and reduced perfusion of tissues with blood were identified as playing a primary role in the developmental toxicity of TCDD (Hornung et al., 1999). Thus, several lines of evidence, in three fish species, lake trout, rainbow trout and zebrafish, demonstrated that the developmental toxicity of TCDD in fish is characterized by cardiovascular toxicity.

TCDD Toxicity in the Vasculature

Since then several studies using TCDD as a prototypical AHR agonist reported toxicity in fish early life stages that could be attributed to adverse effects on the vascular system. Dioxin-treated lake trout embryos develop yolk sac, pericardial and meningeal edema as well as hemorrhage (Spitsbergen et al., 1991). These effects were associated with ultrastructural changes in the endothelium of affected vascular beds of lake trout fry (Guiney et al., 2000). Similar effects were found in medaka embryos where TCDD-induced DNA damage, apoptosis and loss of functional integrity of the vasculature (Cantrell et al., 1996, 1998). Atlantic killifish embryos exposed to TCDD developed edema and hemorrhage

accompanied by increased apoptosis in the heart and vasculature (Toomey et al., 2001). In another marine fish, seabream, early embryonic exposure resulted in several toxic effects including subcutaneous, yolk sac, pericardial and meningeal edema (Ortiz-Delgado and Sarasquete, 2004). Induction of CYP1A activity in the vascular endothelium of lake trout embryos was associated with increased vacuolation and cytoplasmic blebbing of the tissue (Guiney et al., 1997, 2000) and in medaka CYP1A induction was correlated with effects on the heart (Cantrell et al., 1998).

More recent studies are shedding additional light on TCDD vascular toxicity in zebrafish larvae. Malformation of certain blood vessels and reduced regional blood flow have been observed in TCDD exposed zebrafish larvae and been shown to be AHR2 and ARNT1 dependent (Dong et al., 2004; Kubota et al., 2011; Teraoka et al., 2009, 2010). Delayed regression of the common cardinal vein was found in TCDD exposed zebrafish and red seabream larvae (Bello et al., 2004; Yamauchi et al., 2006) and malformation of the mesencephalic vein and prosencephalic artery, was discovered in the head of zebrafish larvae exposed to dioxin (Teraoka et al., 2010). The mechanism of the TCDD-induced decrease in midbrain regional blood flow in zebrafish larvae has been the focus of recent research. It was discovered that a TCDD-induced increase in cyclooxygenase 2 (COX-2) is involved in the mesencephalic vein circulation failure and can be prevented by selective COX-2 inhibitors and rescued by knocking down COX-2 activity (Teraoka et al., 2009). TCDD induction of COX-2 has also been observed medaka embryos (Dong et al., 2010). The inhibitory effect of TCDD exposure on midbrain blood flow can also be blocked by selective antagonists of the thromboxane receptor and by suppression of thromboxane A synthetase 1 activity (Teraoka et al., 2009). Thus, COX-2, thromboxane A synthetase, and the thromboxane receptor axis are involved in the mesencephalic vein circulation failure in TCDD-exposed zebrafish larvae (Teraoka et al., 2009). A novel *cyp1* subfamily, the *cyp1cs*, was discovered a few years ago in fish and *cyp1c1* and *cyp1c2* were cloned and characterized in zebrafish (Godard et al., 2005; Jönsson et al., 2007). Zebrafish adults and embryos showed basal and TCDD-induced expression of both *Cyp1c* transcripts in the endothelial cells of blood vessels and they appear to play a role in the TCDD-induced decrease in midbrain blood flow (Kubota et al., 2011). This was suggested by gene knock-down of CYP1C1 and CYP1C2 blocking reduced mesencephalic vein blood flow in zebrafish larvae caused by TCDD (Kubota et al., 2011).

TCDD Toxicity in the Heart

The discovery of TCDD-induced vascular toxicity in fish by itself, however, does not provide a full understanding of the scale and character of the cardiovascular toxicity of TCDD in developing fish, nor does it strengthen the premise that the heart is a direct target of TCDD toxicity as suggested by Henry et al. (1997). As zebrafish became established as a model vertebrate for investigating TCDD toxicity, the zebrafish embryo and larva were selected for a detailed analysis of the effects of TCDD exposure on heart development and function (Carney et al., 2006a). Early studies of zebrafish embryos treated with a lethal dose of TCDD shortly after fertilization reported development of pericardial edema, reduced cardiac output (Figure 4B) and decreased peripheral blood flow at 72 hpf (Antkiewicz et al., 2005; Belair et al., 2001; Henry et al., 1997; Prasch et al., 2003). Antkiewicz et al. (2005) reported for the first time that the zebrafish heart was affected prior to the onset of both edema and a decrease in peripheral blood flow, with a TCDD-induced reduction in cardiac myocyte number occurring as early as 48 hpf. This demonstrated that the heart is particularly sensitive to dioxin; and sublethal exposure to TCDD was later shown to result in reduced cardiac output, even when pericardial edema was absent and gross morphology of the heart was unaffected (King-Heiden et al., 2009). Furthermore, exposure to 50 pg TCDD/ml from 0–7 weeks post fertilization resulted in persistent cardiovascular toxicity in adults

(King-Heiden et al., 2009). Thus, adverse effects on the heart are among the earliest occurring and most sensitive signs of TCDD developmental toxicity in zebrafish.

Results from two different TCDD dosing paradigms suggest that the TCDD-induced decrease in cardiac output is the cause of reduced peripheral blood flow in zebrafish rather than a consequence of it. First, in embryos treated shortly after fertilization, a decrease in cardiac output was detected at 60 hpf, prior to or concomitant with the onset of pericardial edema and decrease in peripheral blood flow (Antkiewicz et al., 2006). Second, larvae treated at 72 hpf exhibited a reduction in stroke volume and cardiac output at 80 hpf, prior to the decline in peripheral blood flow at 84 hpf (Antkiewicz et al., 2005; Carney et al., 2006a). Other TCDD-induced effects on the zebrafish heart included reduction in heart size at 72 hpf and altered heart morphology (Antkiewicz et al., 2005). At 96 hpf, the heart of TCDD-treated embryos was no longer looped, with the ventricle small and compacted and the atrium thin and elongated. These effects were AHR-dependent (Antkiewicz et al., 2006). TCDD also inhibited heart valve development (Figure 4C). This was evidenced by a failure of valve cushion and subsequent valve leaflet formation at the atrio-ventricular (AV) and bulbo-ventricular (BV) valve junctions, resulting in blood regurgitation between heart chambers (Figure 4D) (Mehta et al., 2008). TCDD exposed larvae also exhibited abnormal development of the bulbus arteriosus (Mehta et al., 2008). Finally, the same endpoints of cardiac toxicity observed in zebrafish larvae after exposure to TCDD (Antkiewicz et al., 2005; Carney et al., 2006a; Mehta et al., 2008) were seen after exposure to another potent AHR agonist, 3,3’,4,4’,5-pentachlorobiphenyl (PCB 126) (Grimes et al., 2008).

The cardiotoxic responses elicited by TCDD exposure suggest that signaling pathways responsible for normal heart development are disrupted (Figure 4E). By evaluating the TCDD-induced gene expression changes underlying the cardiotoxicity in larval zebrafish, we have learned much about its mechanism of action in causing this endpoint of toxicity. Carney et al. (2006a) performed a time course analysis of the transcriptional response to TCDD exposure that began at 72 hpf in the hearts of zebrafish larvae 1, 2, 4 and 12 h later. TCDD induced rapid expression changes of 42 genes (within 1 h), the majority were involved in xenobiotic metabolism, proliferation, contractility and regulation of heart development (Carney et al., 2006a). These rapidly induced gene expression changes preceded signs of TCDD cardiovascular toxicity, making them strong candidates as downstream targets of AHR signaling that contribute and/or mediate TCDD-induced cardiovascular toxicity. This immediate transcriptional response in the heart of zebrafish larvae suggests that the heart may be direct target of TCDD toxicity. Specifically, a cluster of genes that includes genes that promote cell division and growth by functioning in DNA replication, DNA repair, regulation of the cell cycle, cell division, transcription and chromosome assembly and maintenance are down regulated preceding TCDD-induced attenuation of heart function (Carney et al., 2006a; Chen et al., 2008). Taken together, downregulation of this cluster of genes, referred to as the cell cycle gene cluster (CCGC), is considered responsible for the reduction in number of cardiomyocytes in the TCDD-exposed zebrafish embryo heart (Figure 4E). TCDD also affects the expression of genes in zebrafish embryos that are important for heart function, and suggests altered expression of genes for both sarcomeric components and mitochondrial ROS production may play a role in TCDD-induced cardiac toxicity (Handley-Goldstone et al., 2005). Finally the failure of valve formation in the heart of zebrafish embryos exposed to TCDD is associated with increased ectopic expression, as well as mislocalized expression, of *Bmp4* and *Notch1b* in areas of the heart where valves would normally form (Mehta et al, 2008; Figure 4E).

Using Zebrafish to Understand TCDD-Induced Craniofacial Malformation

The skeleton is another example where organ dysfunction in fish negatively impacts survival. Maximal growth rate in fish larvae through the transition period from endogenous to exogenous feeding is necessary for survival with a depression in growth rate associated with high mortality (Cowan and Shaw, 2002). Exposure to TCDD has adverse effects on skeletal development and body growth in several species of fish, demonstrating that the skeleton is another target of TCDD toxicity (Carvalho et al., 2004; Cook et al. 2003; Helder, 1980, 1981; Henry et al., 1997; Hill et al., 2004; Hornung et al., 1999; Teraoka et al., 2002; Walker et al., 1991). Craniofacial cartilage development is particularly vulnerable to TCDD exposure, and much has been learned about the mechanisms by which this occurs using the zebrafish model. TCDD-induced jaw malformation appears to be independent of cardiovascular effects as this malformation occurs prior to decreases in peripheral blood flow (Henry et al., 1997; Teraoka et al., 2002).

Craniofacial cartilage malformations induced by TCDD are observed in zebrafish as early as 60 hpf (Teraoka et al., 2002) and are characterized by stunted upper and lower jaw cartilage growth that prevents anterior extension of the lower jaw beyond the eyes, resulting in a shortened jaw in TCDD-exposed larvae (Henry et al., 1997; Figure 5A). The most striking effects of TCDD on the lower jaw cartilages are the malformation of Meckel's, palatoquadrate and ceratohyal cartilages (Figure 5B). Meckel's cartilage fails to extend anteriorly as far as the ethmoid plate, and palatoquadrate cartilages are shorter. Furthermore, the acute angle formed at the intersection of the ceratohyal cartilages in vehicle control larvae is lost in TCDD-treated larvae (Teraoka et al., 2002, 2006; Xiong et al., 2008). Similar to the ceratohyal cartilages, the acute angles where branchial arches intersect are also disrupted. Closer examination of the upper jaw also identified a smaller and shorter ethmoid plate in TCDD-exposed larvae. Histological analysis of the head in zebrafish larvae exposed to TCDD revealed intact, albeit smaller, cartilage, bone and muscle (Henry et al., 1997). Overall, TCDD alters the shape and reduces the size of the head skeletal elements. This is illustrated by the reduction in lower jaw length in TCDD-exposed larvae (Figure 5C).

Development of craniofacial structures involves establishing lineages of skeletal precursors, re-arrangements and proliferation of cartilage condensations (chondrocyte differentiation) and cartilage organogenesis followed by endochondral ossification, all of which is tightly coordinated with the development of the vascular and nervous system (Yelick and Schilling, 2002). Alcian blue staining of cartilages in zebrafish embryos exposed to TCDD demonstrate that chondrocyte differentiation was not inhibited as many of the cartilages developed; however, the shape and size of the cartilages were altered (Teraoka et al., 2002). Subtle malformations of the lower jaw occur even when cardiovascular toxicity is not present (Hill et al., 2004; King-Heiden et al., 2009), and exposure to 25 pg TCDD/ml from 0–7 weeks post fertilization resulted in persistent craniofacial malformations in adults (King-Heiden et al., 2009). Together, these results suggest that altered blood flow does not result in craniofacial toxicity and that TCDD does not affect differentiation of the cranial components (i.e., chondrocytes, osteocytes and myocytes), but instead disrupts proliferation and/or expedites apoptosis of these cranial cell types resulting in jaw malformation.

As expected, TCDD-induced jaw malformation is AHR2-dependent in developing zebrafish embryos (Prasch et al., 2003). In addition, it was also determined that ARNT1, not ARNT2, was the critical dimerization binding partner with AHR2 that mediated TCDD-induced jaw toxicity in zebrafish embryos (Prasch et al., 2006). While initial chondrocyte differentiation is not affected by TCDD, it is possible that TCDD could disrupt the expression of genes important for regulating the initial patterning of craniofacial structures or the process of endochondrial ossification. Since hedgehog signaling plays such a prominent role in

craniofacial development (Eberhart et al., 2006), alterations in this signaling cascade may underlie TCDD-induced malformation of skeletal elements (Teraoka et al., 2006). Exposure to TCDD decreases *Sonic hedgehog a* and *b* transcript expression in an AHR2-dependent manner; however, subsequent downregulation of hedgehog receptors *Patched 1* and *2*, which are dependent upon hedgehog signaling, suggests that other pathways in addition to hedgehog impair jaw growth (Teraoka et al., 2006). Therefore, identification of an AHR-induced transcriptional response was necessary to elucidate the molecular mechanism(s) of TCDD-induced jaw malformation.

To identify other pathways important for jaw development disrupted by TCDD, Xiong et al. (2008) used microarray techniques to identify transcripts dysregulated in isolated jaw tissue 1, 2, 3 and 12 h, after 96 hpf larvae were exposed to TCDD for 1 h. As expected, TCDD increased expression of AHR-regulated genes (i.e., *cyp1a*, *cyp1b1*, and *sulfotransferase*), in addition to causing dysregulation of 24 genes involved with cartilage or bone development. Genes activated by TCDD included those important for promoting terminal chondrocyte differentiation and cartilage morphogenesis; while those suppressed included transcription factors important for chondrogenic cell specification, osteocyte adhesion molecules, and key components in cartilage extracellular matrix. Most notably, TCDD-induced a 15 fold decrease in the expression of *Sox9b* (Xiong et al., 2008), a transcription factor essential for regulating proper differentiation and proliferation of chondrocytes (Yan et al., 2002, 2005). It was subsequently demonstrated that heterozygous *sox9b* mutant zebrafish larvae were more susceptible to TCDD-induced jaw malformation than wild type larvae and that over expression of *Sox9b* in TCDD-treated larvae ameliorated TCDD effects on jaw development (Xiong et al., 2008). Downregulation of *Sox9b* by TCDD was first detected in the regenerating fin of zebrafish (Andreasen et al., 2006; Mathew et al., 2008), a process that also involves chondrogenesis. Xiong et al. (2008) hypothesize that disruption of *Sox9b* expression may expedite terminal chondrocyte differentiation, causing proliferation in chondrocytes to cease (Figure 5D). These findings strongly implicate *Sox9b* as a downstream effector of TCDD-induced jaw malformation.

Planchart and Mattingly (2010) uncovered a different developmental pathway important for regulating the formation of the lower jaw that also is sensitive to disruption by TCDD. *Foxq1a* (previously named *foxq1b*) is rapidly induced in zebrafish embryos exposed to TCDD during the earliest stages of development (0–24 hpf) and also is associated with disruption of Meckel's cartilage (Planchart and Mattingly, 2010; Figure 5D). *Foxq1a* is a gene in the family of forkhead box transcription factors important for the development of craniofacial structures. *Foxq1a* was identified to be upregulated prior to *cyp1a*, an AHR target gene, suggesting *Foxq1a* is likely a direct target of TCDD-activated AHR signaling. Supporting data suggests *Foxq1a* upregulation was dependent on AHR2 signaling, and *Foxq1a* expression was observed in the jaw primordium showing localization in Meckel's cartilage and other posterior arches affected by TCDD exposure.

Thus, exposure to TCDD during early embryonic and larval development disrupts two pathways that regulate both the development (*Foxq1a*) and growth (*Sox9b*) of lower jaw structures (Figure 5D). Importantly, these studies illustrate the usefulness of zebrafish in identifying multiple genes and signaling pathways that when disrupted by TCDD lead to a specific endpoint of developmental toxicity, jaw malformation.

Using Zebrafish to Understand TCDD-Induced Reproductive Toxicity

While most TCDD research in zebrafish has focused on developmental toxicity, another important endpoint of toxicity is impaired reproduction. The reproductive toxicity of TCDD in fish is of interest because TCDD is an endocrine disrupting compound (EDC). It inhibits

estrogen biosynthesis in fish most likely by inhibiting steroidogenic enzyme activity (Hutz et al., 2006). In contrast, in the rat TCDD inhibits the rate-limiting step in testicular steroidogenesis, mobilization of cholesterol to the inner mitochondria (Kleeman et al., 1990); but this possible mechanism of action has not yet been investigated in fish. Estrogens coordinate processes that are vital for population viability including regulation of embryonic development and all aspects of reproductive development from sex differentiation to gonad maturation, reproductive cycling and behaviors. In fish, periods of gonad differentiation and reproduction in mature adults offer two “windows” of enhanced susceptibility to disruption by TCDD. Since endocrine signaling is important for the regulation of early development and gonad differentiation, exposure to TCDD during critical ontogenetic periods may cause permanent functional changes that result in reduced fitness and reproductive capacity later in life (Bigsby et al., 1999; Guillette et al., 1995; Segner, 2006). This may occur by TCDD exposure altering reproductive behavior, population sex ratio and/or sexual selection in addition to decreasing reproductive capacity. Such alterations can affect community structure and genetic diversity and lower long-term fitness of feral fish populations (Guinand et al., 2003; Keller and Waller, 2002).

Reproductive toxicity of TCDD in zebrafish is similar to that reported in other fish species and consists of reduced fitness as both reproductive capacity and recruitment of offspring is reduced. Most research on TCDD reproductive toxicity in zebrafish has evaluated effects on female reproduction and demonstrated that ovarian development and egg release is impaired. In adults, dietary exposure to acutely toxic levels of TCDD induce overt toxicity associated with dose-dependent reductions in egg production and a complete suppression of spawning activity, corresponding with arrested gonad development and oocyte atresia (Wannemacher et al., 1992). Exposure to sublethal concentrations of TCDD also impairs female reproduction. Adults exposed via the diet show reduced egg release; however, even when ovarian development is not significantly impacted, subtle physiological changes induced by TCDD lead to altered follicular development and decreased serum 17β estradiol and vitellogenin concentrations that correlate with reduced reproductive capacity (King-Heiden et al., 2005, 2006).

The mechanisms by which TCDD modulates follicular development within a mature ovary are not understood. One study shows that exposure of adult female zebrafish to TCDD downregulates enzymes involved in estrogen biosynthesis and attenuates estrogen signaling pathways (King-Heiden et al., 2008). Histopathological analyses of the ovary also show that adverse effects of TCDD on follicular development and vitellogenesis probably result from a direct effect on the ovary by modulating follicular development and inducing follicular atresia (King-Heiden et al., 2006, 2009).

While TCDD has known effects on the adult ovary, less is known about its impacts on the developing gonad. Since reproductive hormones regulate gonad development, there is potential for TCDD to impact sexual differentiation and sex ratios as well as cause organizational effects that alter reproductive success later in life. Waterborne exposure of zebrafish to TCDD from the embryo stage of development through sex differentiation was discovered to impair reproductive capacity of not only the TCDD-exposed zebrafish when they reached adulthood (Figure 6A), but also their offspring (King-Heiden et al., 2009). Some TCDD exposed females had ovarian lesions (Figure 6B) while TCDD exposed males showed no lesions in the testis. Not all of the TCDD-exposed females showed reduced egg production. About half of the TCDD-exposed females produced only slightly fewer eggs per spawn compared to control females, while the other half exhibited complete reproductive failure (< 20 eggs/spawn) (King-Heiden et al., 2009). While King-Heiden et al. (2009) did not directly correlate ovary histopathology to reduced reproductive capacity of the TCDD-exposed females, the results show that ovaries from these TCDD-exposed females contained

significantly more atretic follicles, smaller vitellogenic follicles and approximately half of the TCDD-exposed females had malformed ovaries (Figure 6C). Since other aspects of reproduction (i.e., fertility, gamete quality and recruitment) were also impaired, it suggests that exposure to low concentrations of dioxin-like chemicals during early life stage development and sexual differentiation in fish could pose a threat to the sustainability of TCDD-exposed feral fish populations (King-Heiden et al., 2009).

Little is known regarding the effects of TCDD exposure on male fish reproduction. Despite a lack of observed pathology in the testis, male zebrafish exposed to TCDD during early stages of development appear to be a larger contributor to reduced female egg release during spawning than TCDD-exposed female zebrafish (Figure 6A) (King-Heiden et al., 2009). This finding highlights the need for a more careful evaluation of the impacts of TCDD exposure on the testis, sperm count, sperm motility and male spawning behavior.

While direct impacts of TCDD on gonad development, egg production and fertilization success have obvious impacts on reproductive capacity, it is also important to consider the effects of TCDD exposure on recruitment of offspring. Not surprisingly, maternal transfer of TCDD to eggs reduces offspring survival, induces the typical signs of larval toxicity (e.g., blue sac syndrome) and reduces recruitment (Cook et al., 2003; King-Heiden et al., 2005; Peterson et al., 1993; Tillitt et al., 2008). However, some effects on offspring recruitment may not be solely related to maternal transfer of TCDD to the eggs and subsequent uptake of TCDD from the eggs by the developing embryos and larvae. This point is illustrated by F1 generation offspring, derived from F0 zebrafish exposed to TCDD during early stages of development, also showing reduced reproductive success and ovarian malformations (King-Heiden et al., 2009). The F1 generation zebrafish were only exposed to TCDD as a germ cell. That is, germ cells giving rise to the F1 generation were directly exposed to TCDD during early development of the parental F0 generation. There was no other exposure of the F1 generation to TCDD. Yet the F1 generation still exhibited reproductive toxicity which demonstrates that their germ cell-only exposure to TCDD was sufficient to cause reproductive toxicity (King-Heiden et al., 2009). The significance of this finding is that it raises the possibility for TCDD reproductive toxicity in fish to be transgenerational in nature. If this hypothesis is supported by future studies, it would mean that the reproductive toxicity caused by transient exposure to TCDD during early development and sexual differentiation might span multiple generations, and if great enough, reduce the size of exposed wild fish populations.

Translating TCDD Toxicity in Zebrafish to Wild Fish Populations

High bioaccumulation of dioxin-like compounds by fish has the potential, if their larvae are sufficiently sensitive to TCDD-induced mortality, to prevent recruitment of offspring into the population (Cook et al., 2003; Tillitt et al., 2008). At lower levels of TCDD exposure than are required to produce blue sac syndrome associated mortality there is the potential for TCDD also to impair survival of adults, by decreasing feeding, predation avoidance and reproductive capacity or by shifting the population dynamics due to a change in sex ratio or effects on parentage (Coe et al., 2008; Kidd et al., 2007; Lange et al., 2008; Nash et al., 2004; Tyler et al., 1998). Evaluating population-level consequences requires careful examination of the effects of sublethal TCDD exposure on development, reproductive capacity and successful recruitment. Such studies are difficult to address in wild fish populations due to complexities of the ecosystem and inability to control multiple and concurrent influences that act upon feral fish populations.

Since multi-generation studies in wild fish populations are costly and pose numerous technical difficulties, laboratory studies using a small fish model such as TCDD-exposed

zebrafish can provide the framework for predicting risk to TCDD-exposed feral fish populations. Dioxin developmental toxicity in zebrafish larvae has been well characterized and is consistent with endpoints of toxicity observed in several wild fish species, substantiating its use as a model organism for assessing the impact of environmental toxicant exposures on feral fish populations. Recent work describing the territorial and competitive breeding behavior of zebrafish in relation to reproductive success, along with zebrafish being group spawners which is the most common reproductive strategy in fish, makes them ideal for laboratory-based reproductive and developmental toxicity studies. The use of TCDD-exposed zebrafish for life-cycle and transgenerational studies has recently been demonstrated (King-Heiden et al., 2009). Those results support the use of zebrafish for evaluating population relevant effects of TCDD-like chemical exposures in wild fish and their offspring, as has been done for estrogenic compounds (Coe et al., 2008; Kidd et al., 2007).

While zebrafish allow us to gain insight into endpoints and mechanisms of TCDD toxicity in fish, as with any laboratory fish model, it also has limitations when it comes to translating risk to feral fish populations. Population responses to pollution are highly dependent upon life history traits. Iteroparous fish that have short life spans and generation times tend to be less sensitive to chronic stressors than semelparous or seasonal iteroparous fish with longer life spans and moderate to low fecundity (Schaaf et al., 1987; Spromberg and Birge, 2005). Furthermore, while zebrafish are useful for predicting risk to many fish faunas such as the *Cyprinidaae* and *Percidae* families, because they share similar “opportunistic” life history strategies, many of the more TCDD sensitive species like the *Salmonids* tend to be “equilibrium strategists” (Mims et al., 2010), making comparisons and predictions from TCDD toxicity findings in zebrafish more challenging. Standard toxicity assays with zebrafish would benefit from incorporating toxicity effects into a modeling framework such as dynamic energy budget models (Muller et al., 2010) or other individually based population models (Miller et al., 2007; Van Winkle et al., 1993). This would allow toxicologists to go beyond determining the no observable effect concentration (NOEC) for TCDD in a particular fish species to identifying more ecologically-relevant measures that predict population level risks (Congdon et al., 2001; Muller et al., 2010; Spromberg and Birge, 2005). Additionally, cross species comparisons of TCDD reproductive toxicity in zebrafish and other fish species with different modes of reproduction and life history traits will help to identify target organs within the male and female reproductive system that are most susceptible to disruption by TCDD. This should further elevate utility of the zebrafish model in predicting reproductive risk to dioxin-like compounds for feral fish populations with different modes of reproduction.

Evolved Resistance in Wild Fish Populations to the Toxicity of Dioxin-Like Compounds

Long-term exposure to environmental contaminants over multiple generations has the potential to drive local adaptation (Wiens and Graham, 2005). Therefore it is not surprising that resistance to dioxin toxicity has been observed in Atlantic killifish, *Fundulus heteroclitus*, from three Atlantic coast estuaries highly contaminated with PCBs (VanVeld and Nacci, 2008) and in Atlantic tomcod from a region of the Hudson River where there is extensive PCB pollution (Wirgin et al., 2011). In most, but not all, of the PCB-exposed killifish populations resistance to PCB126-induced embryo toxicity was heritable between the F1 and F2 generation and was unrelated to altered expression of AHR signaling pathway components (Nacci et al., 2010; Powell et al., 2000). Greater insight into the mechanistic basis of resistance to the dioxin-like PCBs was obtained in Hudson River tomcod where variants in AHR2 were observed that were nearly absent in tomcod elsewhere (Wirgin et al., 2011). AHR2-1, common in resistant Hudson River tomcod, was impaired in both TCDD

binding ability and in driving expression in reporter gene assays (compared to the more prevalent AHR2-2). Since affinity of AHR2-1 for TCDD in Hudson River tomcod was five times lower than that of AHR2-2, it is considered the primary mechanism for evolved resistance to dioxin-like chemicals in this highly PCB-contaminated species. The reduced AHR2-1 binding affinity for TCDD in resistant tomcod was associated with a two-amino acid deletion outside of the receptor's ligand binding domain at the site of interaction with the chaperone protein, AIP/ARA9/XAP2, which stabilizes AHR enhancing its ability to bind ligand and activate transcription (Wirgin et al., 2011). Accordingly it is possible that the two-amino acid deletion altered the conformation of AHR2-1 so dioxin-like AHR agonists had reduced access to the ligand binding domain and/or stability of AHR2-1 may have been altered. Either effect could contribute to the resistant phenotype. Thus, from the killifish and tomcod examples above, it appears that multiple mechanisms of resistance to dioxin-like compounds exist in fish, both AHR-dependent and AHR-independent. Further work is needed to identify key molecular targets, in addition to AHR2, that mediate the multiple mechanisms of resistance to AHR agonist toxicity in fish (Whitehead et al., 2010; 2011).

TCDD Effects on the Zebrafish Transcriptome

As appreciation of the biological complexity of dioxin toxicity has grown one is forced to acknowledge the futility of trying to understand the AHR signaling pathway as a linear process. In truth, the AHR is just one node in a vast, interconnected and self-supporting network whose interactions at any given time define the state of the cell. How AHR activation by dioxin-like compounds perturbs this state is dependent upon the initial conditions at the time of exposure, which is to say that AHR signaling and toxicity can vary depending upon developmental time and the specific cell or tissue being examined. Fortunately, technology is allowing us to delve more broadly and deeply into this complexity. Much of the recent knowledge that has been discovered in zebrafish linking endpoints of TCDD toxicity to specific gene targets has been obtained using a transcriptomic approach, which provides a snapshot of the transcriptional changes following TCDD exposure. The first use of this approach to explore TCDD inducible target genes in zebrafish was by Stegeman and coworkers (Handley-Goldstone et al., 2005). At the time, methods to extract embryonic zebrafish hearts (Burns et al., 2005) were not available. To circumvent this limitation and still probe for cardiac specific effects, arrays were synthesized using a heart specific cDNA library. This method identified several categories of transcripts disrupted by TCDD consistent with the cardiac phenotype, providing targets for further exploration that would not have been achievable otherwise. Since then, a number of labs have utilized the transcriptomics approach to interrogate multiple tissues at different times to explore various endpoints of TCDD toxicity in zebrafish including heart malformations (Carney et al., 2006), jaw defects (Planchart et al., 2010; Xiong et al., 2008) and regeneration defects (Andreasen et al., 2006). The work on the TCDD inducible jaw and regeneration defects identified gene targets that can be directly manipulated to rescue their respective toxic endpoints (Mathew et al., 2008; Xiong et al., 2008).

The commercial availability of zebrafish microarrays have made the technique more accessible and practical for labs to perform; however the approach has been hampered by differences and limitations in coverage of the zebrafish genome, which means that important AHR gene targets have likely been missed along the way. With the advent of deep sequencing technology, isolated RNA can be converted into cDNA and directly sequenced then mapped to the genome to provide a transcriptional readout in a process known as RNA-Seq (Wang et al., 2009). This approach does away with the need for specific probe based arrays and has already been applied to zebrafish embryos (Zheng et al., 2011). Work in other systems have applied the technique to single cells (Tang et al., 2009), which may prove

useful in dissecting cell specific interactions and mechanisms of TCDD toxicity in complex multi-cellular tissues, or homogenous tissues exposed to morphogenetic gradients.

Given the inherent complexity of developmental processes within particular organs and across the organism as a whole, some endpoints of dioxin toxicity may not be traceable to a specific gene and may instead involve the interplay of several factors. (Carney et al., 2006; Waits and Nebert, 2011). However, as transcriptomic approaches have matured so have the tools for data analysis. These tools can give added dimension to transcriptomic data and place the results in context with gene and protein interaction networks, which may provide insight into potential epistatic effects. In addition, these interactome maps might provide insight into similarities and differences in endpoints of TCDD toxicity between tissues and across species. Several of these tools were utilized in a recent analysis of transcriptomic data following dioxin exposure in zebrafish with interesting results that demonstrate the power of this approach (Alexeyenko et al., 2010).

Relevance of TCDD Toxicity in Zebrafish to Human Health

Several reviews highlight the usefulness of zebrafish as a model for developmental biology and toxicology research (Ankley and Johnson, 2004; Carney et al., 2006b; Carvan et al., 2005; Hill et al., 2005; McGonnell and Fowkes, 2006; Spitsbergen and Kent, 2003; Teraoka et al., 2003). The zebrafish has also been used as a model for human diseases such as DiGeorge syndrome (Piotrowski et al., 2003), hepatoerythropoietic porphyria (Wang et al., 1998), erythropoietic protoporphyria (Childs et al., 2000), cardiovascular disease (Chan and Mably, 2011) and diabetes (Kinkel and Prince, 2009) and has emerged as the premier model for screening chemical libraries to identify compounds that suppress a particular disease phenotype associated with a known human disease (Burns et al., 2005; Margolis and Plowman, 2004; Peterson et al., 2000).

By using zebrafish, great strides have been made in understanding TCDD reproductive and developmental toxicity (Tanguay et al., 2003; Carney et al., 2006b; Hill et al., 2005). Some of the endpoints of TCDD toxicity such as alterations in heart and craniofacial development also have been observed in mammals (Allen and Leamy, 2001; Ikeda et al., 2000; Ivnitski et al., 2001; Keller et al., 2007; Thackaberry et al., 2005a, 2005b;) and may have implications for human health. The adverse effects of TCDD and PCB 126 on zebrafish larval heart development resemble hypoplastic left heart failure in human newborns (Grimes et al., 2008). Humans living near inland waters contaminated with HAHs and other contaminants exhibit a greater risk for heart related defects such as valvular stenosis along with other cardiac malformations (Goldberg et al., 1990). More recently a study identified a region of Baltimore where an increased frequency of hypoplastic left heart syndrome is correlated with the industrial release of solvents, dioxins and PCBs (Kuehl and Loffredo, 2006). Further analysis of TCDD-induced cardiotoxicity in zebrafish is certain to yield more insight into the genes and signaling pathways underlying this developmental defect. And, while further research is required, the association of downregulation of *Sox9b* with jaw malformation in TCDD-exposed zebrafish may also prove useful in understanding the skeletal abnormalities associated with campomelic dysplasia in humans with *sox9* deficiency (Foster et al., 1994; Wagner et al., 1994).

Conclusion

Although there is still much to be learned, our knowledge of dioxin toxicity and AHR signaling pathways has been enriched by the examination of small fish species, particularly zebrafish. In addition to addressing questions concerning the toxic effects of TCDD, the zebrafish model promises to offer insights into the endogenous function of the AHR protein

that are relevant to feral fish and human health. Demonstrating and understanding mechanisms of AHR-mediated toxicity that are common between humans and fish and wildlife are necessary if we are to integrate findings from laboratory and ecotoxicology studies with human health risk assessment (Carvan et al., 2008; Cook et al., 2003; Hinton et al., 2005; Tillitt et al., 2008).

By understanding the mode of action of dioxin-like chemicals in greater depth, we may be able to develop biomarkers for specific adverse effects that can be used for both ecotoxicological and human health risk assessment because of the high degree of evolutionary conservation among vertebrates. Fortunately, transcriptome analysis and interactome mapping (Alexeyenko et al., 2010) along with new methodologies in zebrafish such as ChIP-chip (Wardle et al., 2006), more rapid transgenic construction techniques (Kwan et al., 2007), RNA-Seq (Wang et al., 2009), genome-wide quantitative trait loci (QTL) analysis (Waits and Nebert, 2011) and advances in morpholino technology (Shestopalov et al., 2007) can be expected to further accelerate knowledge of AHR biology, including the identification of new transcriptional targets and mechanisms underlying the diverse endpoints of TCDD toxicity (Alexeyenko et al., 2010; Waits and Nebert, 2011; Yoshioka et al., 2011).

Highlights

- TCDD causes “blue sac” toxicity in fish larvae that culminates in death
- “Blue sac” consists of yolk sac edema, jaw and heart malformation and heart failure
- TCDD toxicity is mediated by AHR signaling-induced changes in gene expression
- Downregulation of *Sox9b* in the jaw is required for TCDD-induced jaw malformation
- Developmental exposure causes reproductive toxicity in adults and the next generation

Abbreviations

HAHs	halogenated aromatic hydrocarbons
PCBs	polychlorinated biphenyls
PCDDs	polychlorinated dibenzo-*p*-dioxins
PCDFs	polychlorinated dibenzofurans
DDE	1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethylene
AHR	aryl hydrocarbon receptor
ARNT	aryl hydrocarbon receptor nuclear translocator
TCDD or dioxin	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
CYP1A1	cytochrome P450 1A1
PAS	Per-Arnt-Sim
HSP90	heat shock protein 90
AIP, ARA9 or XAP2	AHR interacting protein

AHRE	AHR response element
XRE	xenobiotic response element
DRE	dioxin response element
AHRR	AHR repressor
CCGC	cell cycle gene cluster
EDC	endocrine disrupting compound
AV	atrio-ventricular
BV	bulbo-ventricular
hpf	hours post fertilization
TEC	TCDD toxicity equivalence concentration
TEF	toxicity equivalence factor
BSAF	biota-sediment accumulation factor
COX-2	cyclooxygenase 2
PCB 126	3,3’,4,4’,5-pentachlorobiphenyl
NOEC	no observable effect concentration

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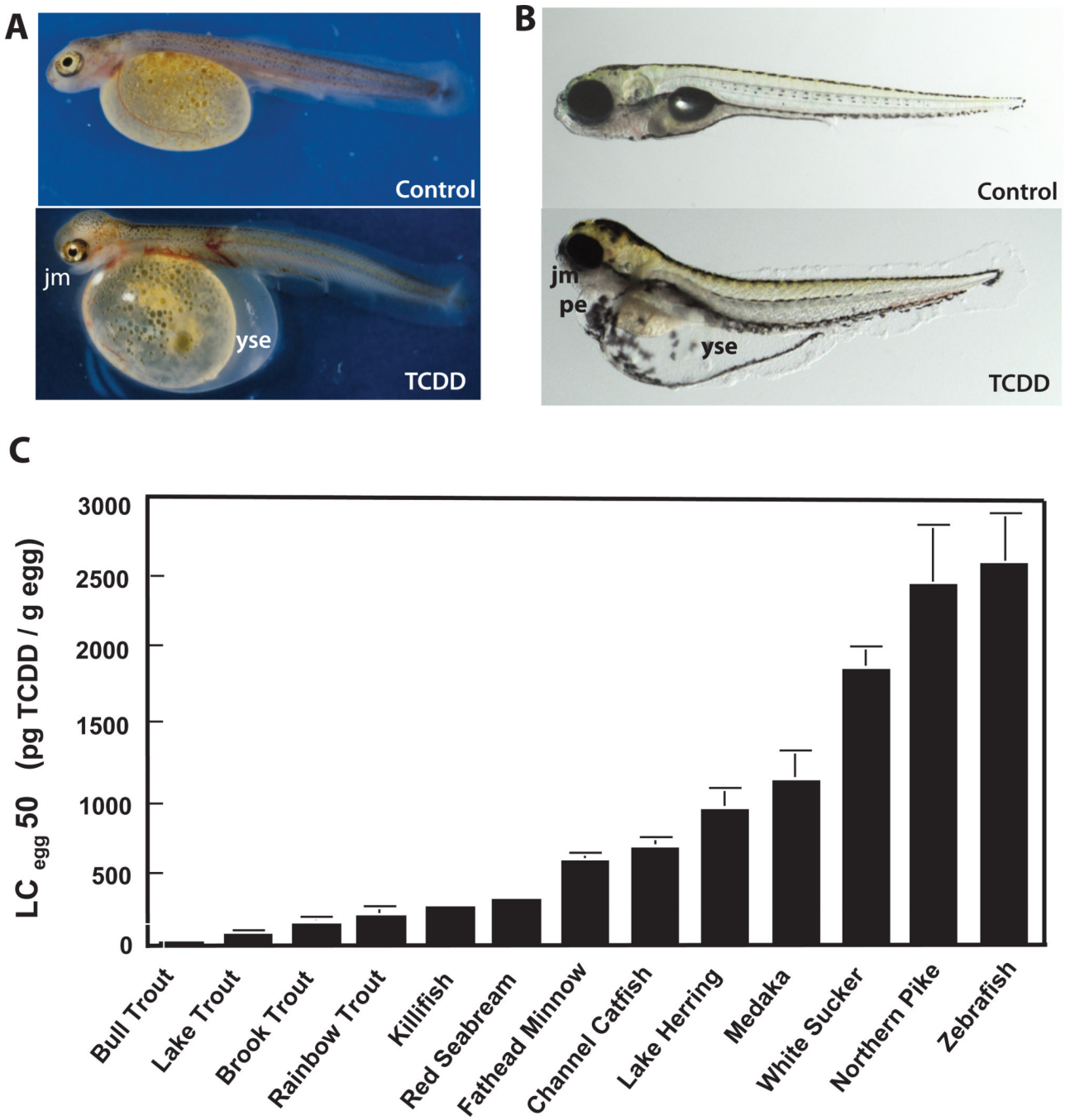


Figure 1. TCDD potency in causing early life stage mortality by "blue sac" syndrome varies 120 fold across different fish species

Bull trout and lake trout larvae are the most sensitive to TCDD-induced mortality and zebrafish larvae, the most resistant (C). However, provided a high enough dose of TCDD is administered to cause fish larval mortality, essentially the same signs of TCDD toxicity are seen prior to death in lake trout larvae (A), zebrafish larvae (B) and larvae of all other fish species studied (C). This common toxicity syndrome, that precedes mortality, is referred to as "blue sac." The hallmark signs of "blue sac" in TCDD-treated lake trout larvae (A) and zebrafish larvae (B) include: yolk sac edema (yse), pericardial edema (pe), jaw malformation (jm), uninflated swim bladder and hyperpigmentation. In lake trout larvae,

hemorrhage is also observed, but this is not seen in zebrafish larvae treated with TCDD. These endpoints of TCDD developmental toxicity are accompanied by a smaller heart in both trout and zebrafish larvae (not shown). Heart malformation in TCDD-exposed zebrafish larvae culminates in heart failure and death prior to swim-up. Figure 1A is from Cook et al. (2003) with permission (results were taken from Walker et al., 1991). $LC_{egg}50$ values in Figure 1C were taken from Elonen et al., 1998; Guiney et al., 1996, 1997; Helder, 1981; Henry et al., 1997; Johnson, et al., 1998; Spitsbergen et al., 1991; Toomey et al., 2001; Walker and Peterson 1994b; Walker et al., 1991; Wright and Tillitt, 2006; Yamauchi et al., 2006; Zabel et al., 1995b).

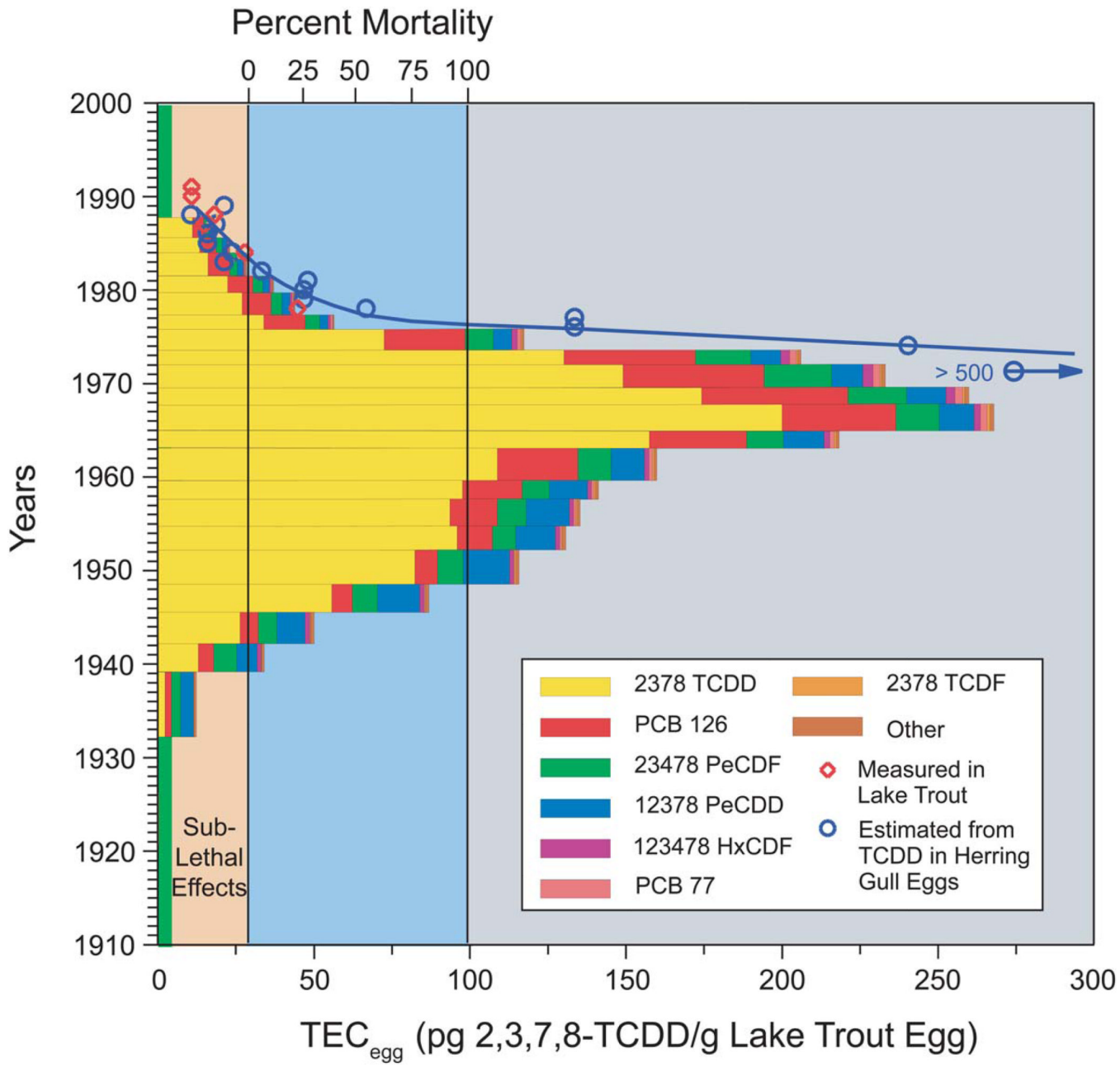


Figure 2. Retrospective prediction of early life stage mortality in Lake Ontario lake trout exposed as eggs to TCDD and related compounds

Estimates of percent mortality predict a 40 year period, 1940 to 1980, when early life stage mortality of lake trout caused by the exposure of lake trout eggs to dioxin-like chemicals would adversely impact the wild lake trout population in Lake Ontario, regardless of effects caused by other chemical or nonchemical stressors. The vertical, light blue area goes from 0% mortality, at a lake trout egg TCDD toxicity equivalence concentration (TEC) of 29 pg TCDD equivalence/g lake trout egg to 100% mortality, at a TEC of 100 pg TCDD equivalence/g lake trout egg. The vertical, light brown area shows the TEC range in lake trout eggs predicted to be associated with sublethal effects. The vertical, gray area represents 100% mortality. The length of each horizontal bar indicates the total TEC contributed by the various dioxin-like chemicals (box in figure) in lake trout eggs, determined from sediment

core analysis for the year that the sediment was deposited on the bottom of the lake. More specifically, the TECs contributed by individual dioxin-like chemicals in lake trout eggs for a particular year were determined from the analysis of these dioxin-like chemicals in radionuclide-dated 1-cm sections of a sediment core (LO87-20) taken from the bottom of eastern Lake Ontario. The concentration of each dioxin-like chemical in each 1-cm sediment section was multiplied by the appropriate biota-sediment accumulation factor (BSAF) for dioxin-like chemicals in lake trout eggs, BSAFs were adjusted for the effect of temporal changes in the chemical distributions between water and sediment, and multiplied by the toxicity equivalence factor (TEF) of that particular dioxin-like chemical based on trout early life stage mortality (Walker and Peterson, 1991; Zabel et al., 1995b) to predict the percent mortality of lake trout sac fry in the year the sediment was deposited on the bottom surface of Lake Ontario (Cook et al., 2003). A red open diamond indicates the TEC in lake trout eggs for a particular year estimated from measurements of dioxin-like chemicals in lake trout tissues that were collected in that year. A blue open circle indicates the TEC in lake trout eggs for a particular year estimated from measurements of the TCDD concentration in herring gull eggs in the same year. Figure 2 is modified from Cook et al. (2003) with permission.

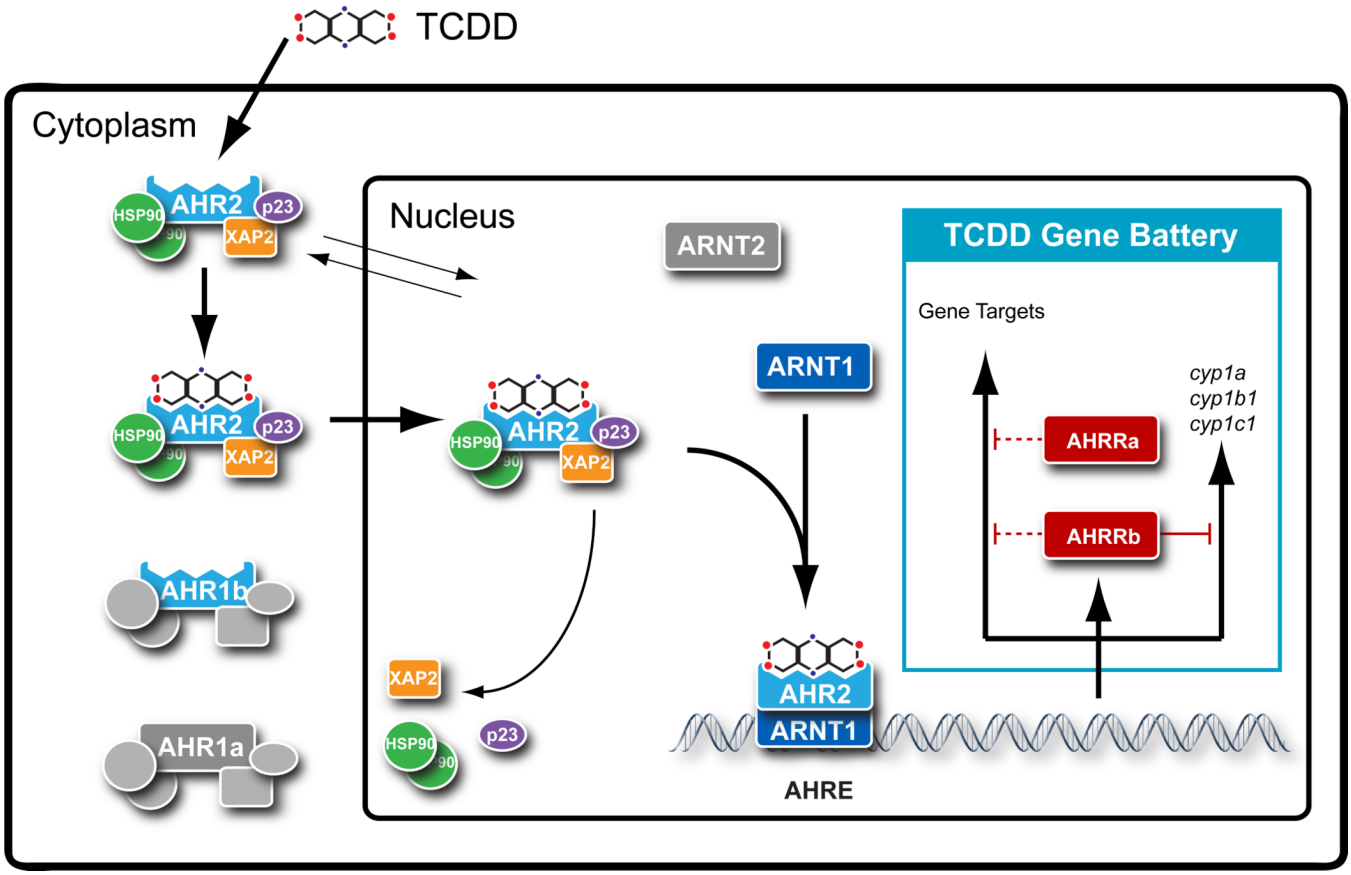
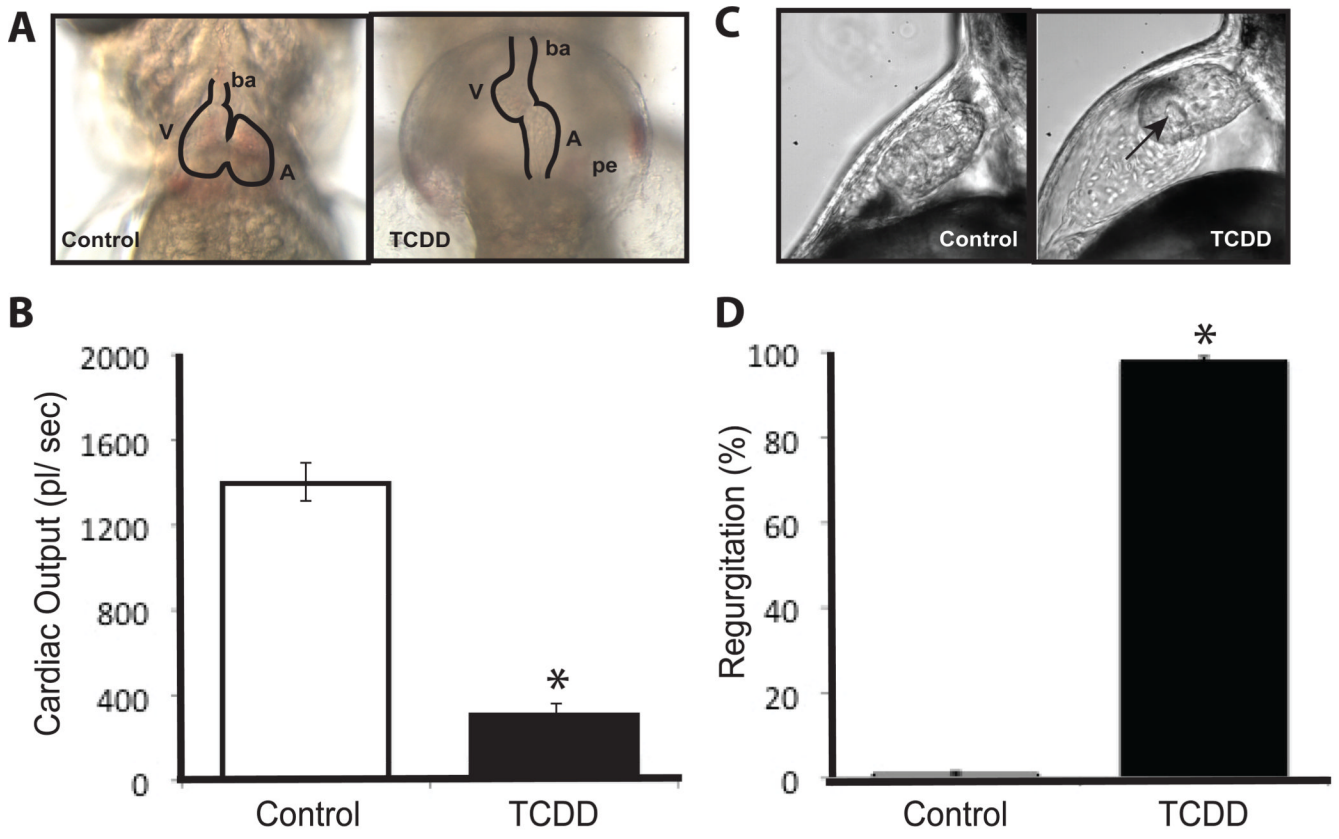


Figure 3. The aryl hydrocarbon receptor 2 (AHR2) signaling pathway mediates TCDD toxicity in zebrafish

As with many fish species, zebrafish have multiple copies of the key proteins, AHR and ARNT that mediate TCDD toxicity. Studies in zebrafish larvae have shown that AHR2 and ARNT1 dimers mediate gene transcription, following activation of AHR2 by TCDD, to cause developmental toxicity (see text for references).



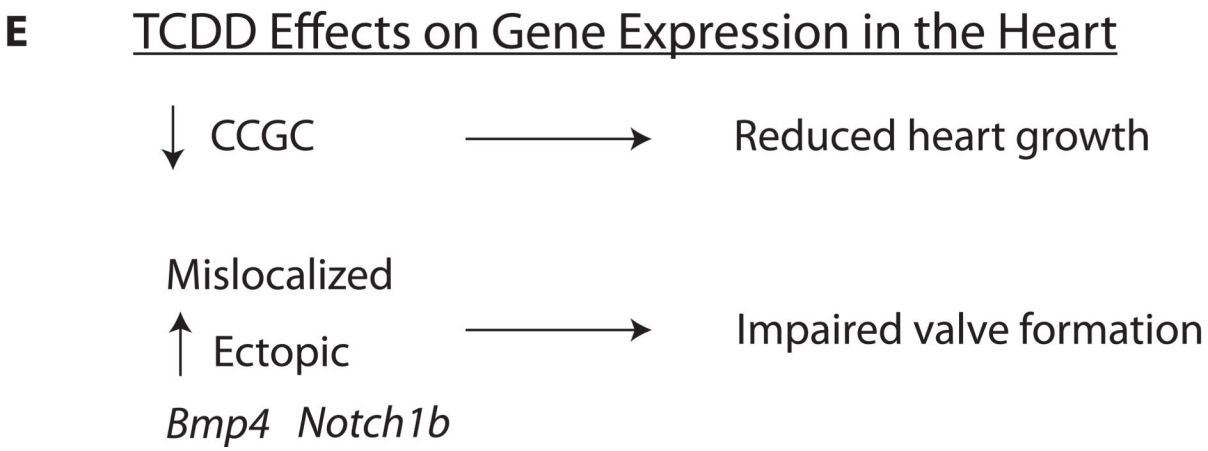


Figure 4. TCDD-induced heart toxicity in zebrafish larvae

Representative photograph of a gross heart malformation in a TCDD-treated zebrafish larva at 96 hpf (A). Abbreviations are: V = ventricle, A = atrium, ba = bulbus arteriosis and pe = pericardial edema. Reduced cardiac output caused by TCDD exposure (B). Representative photograph is shown of an impaired AV valve closure during systole in a TCDD-treated zebrafish larva heart (C). Arrow points to the open AV valve during systole which is abnormal. Increased percent of TCDD-treated larvae with regurgitation of blood at the AV valve (D). Effects of TCDD on gene expression in the heart (E) includes downregulation of a cell cycle gene cluster (CCGC) that leads to a reduced number of cardiomyocytes and increased and mislocalized ectopic expression of *Bmp4* and *Notch 1b* which impairs heart

valve formation. * Denotes significant differences ($p<0.05$). (A) and (B) are modified from King-Heiden et al. (2009) with permission; (C) and (D) are modified from Mehta et al. (2008) with permission.

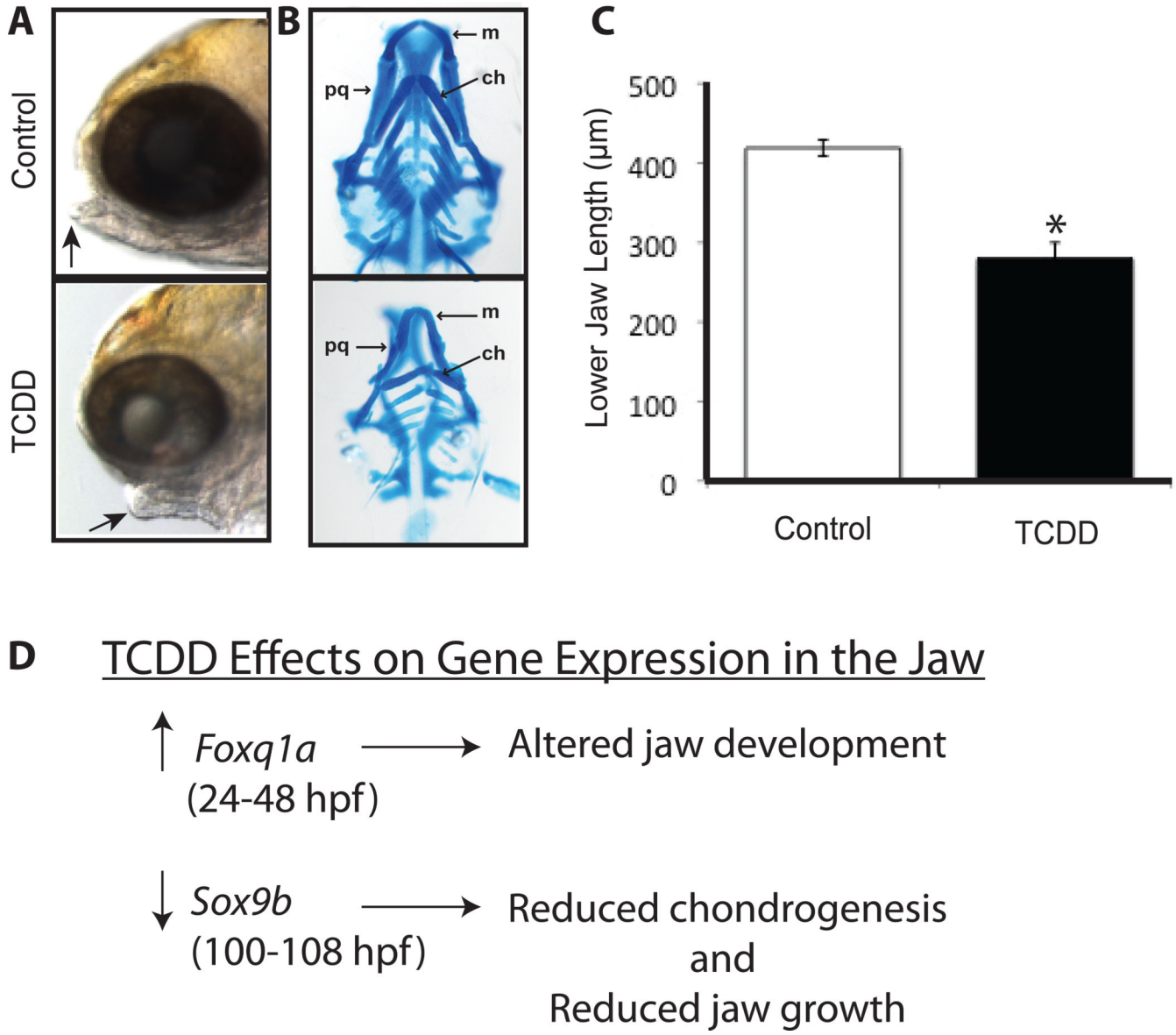


Figure 5. TCDD-induced jaw malformation in zebrafish

Representative photograph of impaired jaw formation in a TCDD-treated zebrafish larva at 120 hpf (A). The arrow points to the end of a shorter lower jaw in a representative TCDD-exposed larva (A). A representative photograph shows reduced size and altered shape of Alcian blue stained jaw cartilages in a TCDD-exposed larva (B). Decreased lower jaw length is shown for the TCDD treatment group at 120 hpf (C). Effects of TCDD on gene expression in the zebrafish larva jaw (D) reveals early upregulation of *Foxq1b* and later downregulation of *Sox9b* mRNAs. Jaw cartilage abbreviations are: m = Meckel's, pq = palatoquadrate, and ch = ceratohyal. * Denotes significant differences ($p<0.05$). Photographs in (B) were taken from Xiong et al. (2008) with permission.

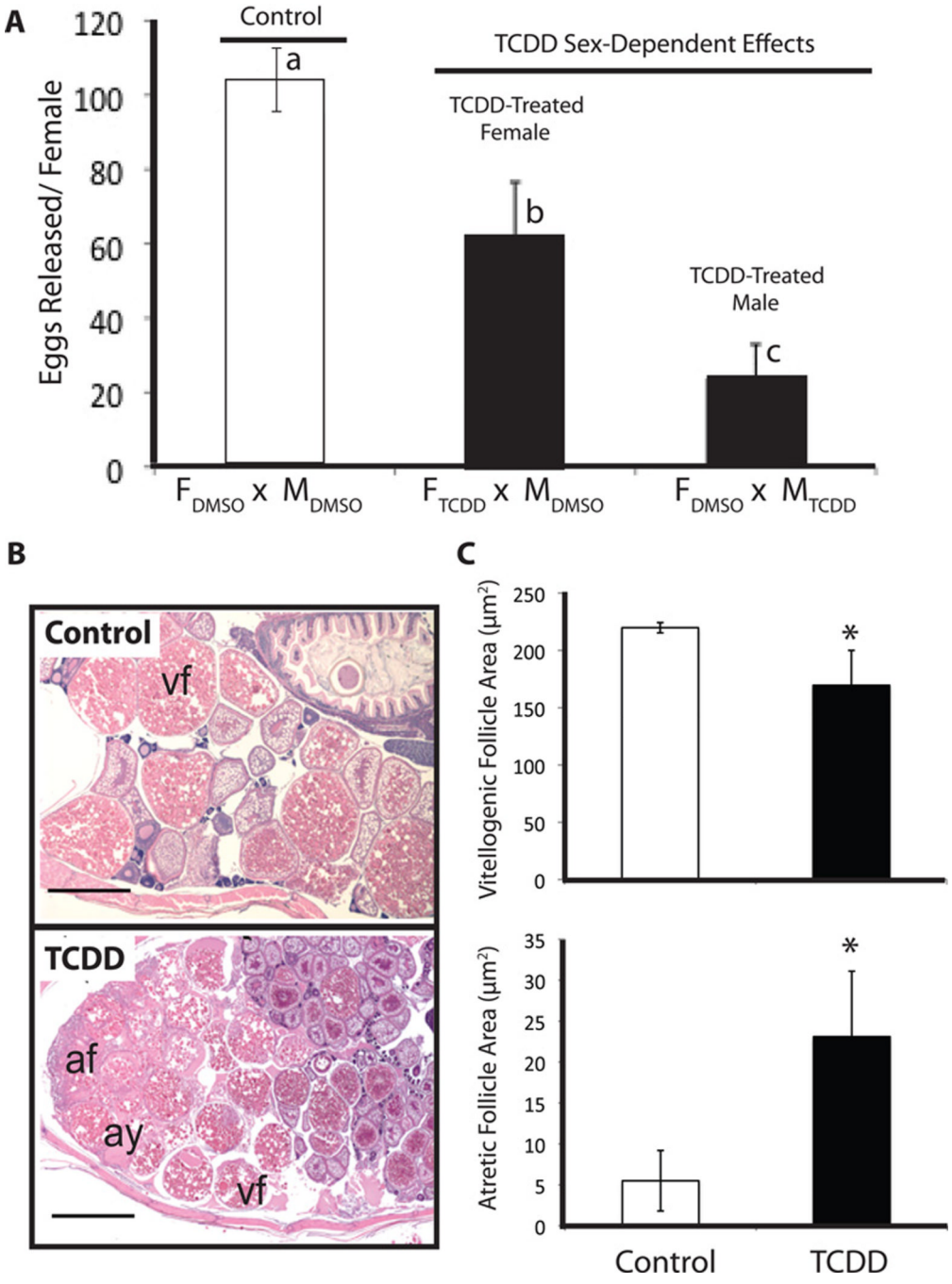


Figure 6. Reproductive toxicity in adult zebrafish following TCDD exposure from embryonic development through gonad differentiation

TCDD exposure reduces the number of eggs released by a female zebrafish during spawning (A) with TCDD-exposed males contributing more to the effect than TCDD-exposed females. Abbreviations: F_{DMSO} = control females; F_{TCDD} = TCDD-exposed females; M_{DMSO} = control males; M_{TCDD} = TCDD-exposed males. Representative photomicrographs demonstrating ovarian lesions in TCDD-exposed females (B) where vf = vitellogenic follicle, af = atretic follicle, and ay = amorphous yolk material. TCDD exposure alters follicular development (C). In female zebrafish exposed to TCDD, vitellogenic follicles were about 23% smaller and area of the ovary (normalized to area observed)

containing atretic follicles and amorphous yolk material was increased (C). Either a lowercase letter or an * denote a significant difference ($p<0.05$). Length of the horizontal line at the bottom of photomicrographs in panel B = 1000 um. Results in (A) and (B) are modified from King-Heiden et al. (2009) with permission.