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Histological analysis of acute toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in zebrafish

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Abstract

Previous studies have demonstrated that acute exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) by injection leads to inhibition of caudal fin regeneration in zebrafish. Since the TCDD exposure in these studies is systemic, it is possible that pathology in organs other than the fin could result in inhibition of fin regeneration. Therefore, histopathology of adult zebrafish (*Danio rerio*) organs was characterized following abdominal cavity injection of a TCDD dose (70 ng/g). The most pronounced histopathologic changes 5 days post-injection included lipidosis and hypertrophy of liver hepatocytes and hypertrophy of gill lamellae. Effects of TCDD exposure on immunolocalization of the zebrafish aryl hydrocarbon receptor nuclear translocator (ARNT2), the heterodimer partner of the aryl hydrocarbon receptor (AHR2), and an AHR regulated gene cytochrome P450 1A (CYP1A) was also determined. ARNT2 was immunolocalized to the gastrointestinal tract, gill lamellae, kidney, ventricle of the heart, caudal fin, brain and liver of zebrafish. TCDD exposure had no measurable effect on ARNT2 abundance or localization. CYP1A was immunolocalized in TCDD exposed fish as a biomarker for cells with an activated AHR pathway. CYP1A was not detected in any tissue from vehicle exposed fish. Significant TCDD-dependent induction of CYP1A was detected in the proximal tubules of the kidney, in liver hepatocytes and in the gastrointestinal tract of TCDD exposed fish. Significant but lower TCDD-dependent CYP1A expression was evident in the gill, caudal fin and ventricle of the heart. Overall, TCDD exposure in adult zebrafish leads to histopathology similar to that reported in other fish species, and it appears unlikely that the histopathology in these organs completely explains the inhibition of fin regeneration.

Keywords: 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin, TCDD; Histopathology; Cytochrome P4501A, CYP1A; Zebrafish

1. Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is a potent developmental toxicant and a member of a group of environmental contaminants known as polychlorinated aromatic hydrocarbons, which include polychlorinated biphenyls (PCBs), dibenzofurans (PCDFs) and dibenzo-*p*-dioxins (PCDDs). These

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lipophilic compounds are persistent in the environment and known to bioaccumulate and biomagnify in the food chain. Signs of TCDD toxicity are shared among different species and are mediated by the aryl hydrocarbon receptor (AHR) pathway (Safe, 1986). In mammals, once AHR is bound by ligands such as TCDD, AHR translocates to the nucleus and dimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT). AHR/ARNT heterodimers interact with AHR responsive elements (AHREs) altering the transcription of a variety of genes including cytochrome P450 1A (CYP1A). TCDD-dependent induction of CYP1A is commonly used as an indicator of tissues possessing an activated AHR. TCDD-dependent induction of CYP1A has been demonstrated in zebrafish cell cultures, whole larvae and adult zebrafish (Collodi et al., 1994; Henry et al., 1997; Tanguay et al., 1999; Andreasen et al., 2002b). We have previously isolated and characterized two zebrafish aryl hydrocarbon receptors, AHR1 and AHR2 (Tanguay et al., 1999; Andreasen et al., 2002a). Four splice variants of ARNT2 have been characterized and include ARNT 2a, 2b and 2c and 2x (Tanguay et al., 2000; Wang et al., 2000; Hsu et al., 2001). Tissue-specific expression patterns and functional data suggest that AHR2, not AHR1 mediates TCDD toxicity in zebrafish development (Andreasen et al., 2002a).

Fish are among the most sensitive vertebrates to the toxicity of TCDD. Early life stages of fish are significantly more sensitive to TCDD than adults (Peterson et al., 1993; Walker and Peterson, 1994). The LCegg50s in seven different fish species ranged from 0.5 to 2.6 ng TCDD/g with fathead minnows (*Pimephales promelas*) five-fold more sensitive and lake trout (*Salvelinus namaycush*) 38-fold more sensitive than zebrafish (Walker et al., 1991; Elonen et al., 1998). The LC_{50} for zebrafish larvae is 2.5 ng TCDD/g egg (Henry et al., 1997). Among larval fish, common signs of TCDD toxicity include pericardial edema, yolk sac edema, craniofacial malformations, reduced blood flow, anemia, underdeveloped swim bladder, decreased growth and mortality (Wannemacher et al., 1992; Henry et al., 1997; Belair et al., 2001; Teraoka et al., 2002). Histopathologic examination of these TCDD exposed embryos revealed that they displayed a variety of epithelial lesions, including arrested gill development, ballooning degeneration and/or necrosis of the renal tubules, hepatocytes, pancreas and major brain regions. In addition, subcutaneous edema of the pericardium and skeletal muscle also occurred.

The LD_{50}s for TCDD in adult fish from six freshwater species range from 3 to 16 ng/g with a rank order of sensitivity of bluegill > largemouth bass > bullhead > carp = yellow perch (Kleeman et al., 1988; van der Weiden et al., 1994). The LD_{50} for TCDD in adult zebrafish has not been determined. Common histopathologic changes are observed in adult fish exposed to TCDD. Yellow perch (*Perca flavescens*) exposed to 25 or 125 ng TCDD/g fish display thymic involution, depletion of lymphoid tissue in the spleen, depletion of lymphoid and hematopoietic cells in the kidneys, hepatocyte hypertrophy, lipidosis and glycogen depletion in the liver, marked hyperplasia of the epithelium of the primary lamellae of the gill as well as epicardial myocyte necrosis and fibrinous pericarditis (Spitsbergen et al., 1988a). Mirror carp (*Cyprinus carpio*) exposed to 2.93 ng/g TCDD demonstrated lymphoid depletion, erythrocyte congestion and enlarged macrophages in the spleen. Rainbow trout (*Salmo gairdineri*) exposed to 10 ng TCDD/g fish displayed effects similar to yellow perch. These fish exhibited multifocal lymphoid necrosis in the thymus, lymphoid depletion in the kidney, thymus and spleen and glycogen depletion in the liver (Spitsbergen et al., 1988b). Trout treated with 25 or 125 ng/g TCDD exhibited occasional fusion of the secondary lamellae and increased mucous cells of the gill. Vacuolation and ballooning of hepatocyte cytoplasm with bile duct hyperplasia and biliary fibrosis were displayed in both rainbow trout and mirror carp, 34 and 42 days post TCDD exposure, respectively (Spitsbergen et al., 1988b; van der Weiden et al., 1994).

It is well established that CYP1A induction follows AHR ligand binding in many tissues, but the toxicologic significance of this induction remains uncertain (reviewed in (Tanguay et al., 2003)). Targeted gene repression of CYP1A by morpholinos in larvae zebrafish blocked the signs of TCDD developmental toxicity including pericardial edema and circulation deficiencies (Teraoka et al., 2003). Minimally, CYP1A expression can be used as a biomarker to follow activation of the AHR pathway. In scup (*Stenotomus chrysops*) exposed to 3.1 ng/g tetrachlorodibenzofuran, CYP1A expression is induced in liver, heart, gill and gastrointestinal tract (Smolowitz et al., 1991). CYP1A was also expressed in epithelial cells of kidney tubules and

the mucosal folds and glandular pits of the gastrointestinal tract. In some studies, CYP1A is expressed in organs without accompanying TCDD-dependent histopathology. European flounder (*Platichthys flesus*) exposed to 0.0125–500 ng TCDD/g displayed CYP1A expression in hepatocytes, spleen, digestive tract, mesonephros, gonads and gills however there was no observable exposure-related histopathology in any of these organs.

Dose-response studies involving TCDD injection in adult zebrafish indicate that caudal fin regeneration is completely inhibited by TCDD 70 ng/g (Zodrow and Tanguay, 2003). Fin regeneration was impacted at doses as low as 2.8 ng/g, however the 70 ng/g TCDD dose had the most significant impact. Importantly, the fish appeared healthy for up to 28 days with no change in weight, feeding or behavior. Fin regeneration is a complex process involving multiple stages, which include mesenchymal cell dedifferentiation as well as cellular differentiation and proliferation. Most of these stages occur in the first 4 days of fin regeneration. This TCDD-related inhibition of fin regeneration is also independent of the stage of regeneration, between 0 and 4 days post amputation. This suggests that multiple regeneration stages are impacted by TCDD.

TCDD was delivered by injection as opposed to dietary or waterborne routes of exposure to ensure a consistent delivered dose. This results in a systemic TCDD distribution and it is possible that damage to organs following exposure to TCDD could underlie the failure of the fin to regenerate in the presence of TCDD. For example, the cardiovascular system is a known target for TCDD toxicity in fish (Henry et al., 1997; Belair et al., 2001; Dong et al., 2002). If heart output or vascular perfusion were reduced, wound healing and regeneration would likely be impacted. When TCDD is administered at the day of amputation, fin regeneration is dramatically impacted and easily measured 5 days post amputation, therefore any pathologic changes affecting fin regeneration would be apparent at this post-injection time point. The goal of this study was to determine the histopathologic effects of an acute TCDD dose that is known to significantly decrease zebrafish caudal fin regeneration (Zodrow and Tanguay, 2002) and determine if these histopathological changes could partially explain the TCDD block of caudal fin regeneration. Histopathologic and immunohistochemical evaluation reveals that several organ systems are affected by TCDD exposure, and these tissues also express ARNT2 and are sites of induced CYP1A protein.

2. Materials and methods

2.1. Materials

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD, >99% pure) was purchased from Chemsyn (Lenexa, KS). Vectastain kits were obtained from Vector Laboratories Inc. (Burlingame, CA). Diaminobenzidine (DAB) was obtained from Sigma. Tissue distribution of CYP1A protein in zebrafish sections was determined using the monoclonal antibody MAb 1-12-3, which has previously been used to detect CYP1A in fish species (Park et al., 1986; Stegeman et al., 1989; Smolowitz et al., 1991; Guiney et al., 1997). Anti-ARNT2 (M-20) antibody was purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Anti-β-actin (AC-15) antibody was purchased from Sigma. Chicken egg yolk phosphatidylcholine (PC, >99% purity) in chloroform was obtained from Avanti Polar Lipids (Alabaster, AL).

2.2. Maintenance of zebrafish

Zebrafish (*Danio rerio*) were maintained in a recirculating tank rack system at 27 °C containing oxygenated reverse osmosis water supplemented with 0.3 g/l Instant Ocean Sea Salt and sodium bicarbonate (Marine Biotech, Beverly, MA). The water was filtered through 0.45 μm mesh, denitrified by bacterial filtration and finally disinfected by ultraviolet light exposure. Fish were fed twice daily, once with dry flake food (Tetra-min, Tetra, Melle, Germany) and once with live artemia (Great Salt Lake Artemia cysts, INVE, Grantsville, UT).

2.3. TCDD dosing of adult zebrafish

2,3,7,8-TCDD was added to phosphatidylcholine in chloroform (PC) as described previously (Walker and Peterson, 1991). Briefly, TCDD in 1,4-dioxane was added to PC, evaporated to dryness, rehydrated with 0.9% NaCl and sonicated to form liposomes with a final TCDD concentration of 20 ng/μl. Adult male

zebrafish were anesthetized with 0.16 g/l Tricaine (MS-222) before intraperitoneal injection with either 3.5 μl/g PC as vehicle (control) or 70 ng/g TCDD in PC liposomes. The fish were allowed to recover in 1 l tanks before transfer to 27 °C 10 gal tanks. Fish were fed twice daily for the duration of the experiment.

2.4. Histopathology

Five days post-injection, vehicle control and TCDD exposed zebrafish were euthanized in Tricaine (MS-222), fixed for 3 days in Dietrich's Fixative (30% methanol, 10% formalin and 2% acetic acid) and dehydrated in graded concentrations of ethanol before paraffin embedding. Serial 5 μm whole body sagital sections were prepared, mounted on slides and stained with hematoxylin and eosin (Colorado Histo-Prep, Ft. Collins, CO). Periodic acid Schiff (PAS) staining was performed on selected vehicle control and TCDD exposed sections to analyze glycogen content.

2.5. Histopathological Scoring

Three TCDD exposed and three vehicle control fish were analyzed for histopathology. Approximately 30 serial sections were produced from each fish for analysis. The slides were blinded to the observer and histopathologic scoring was performed on all serial sections. Previously, untreated zebrafish whole fish sections were stained with H and E to determine zebrafish histology. Control slides were determined based on similarity to these untreated zebrafish sections. Histopathology was determined based on severity of changes compared to control sections. Upon completion of the scoring, the slide key was revealed. Scores was based on the severity as well as the number of slides out of the total in which the histological changes were observed with $-$ = no histopathology in any field on the slides, $+$ = mild histopathology present in <25% of the fields on the slides, $++$ = moderate histopathology present in >75% of the fields on the slides; and $+++$ = all fields of the slides displayed severe histopathology.

2.6. Organ distribution of ARNT2 protein

Since ARNT2 is necessary for AHR signaling in zebrafish, ARNT2 organ distribution was determined. Six adult zebrafish were euthanized in Tricaine (MS-222) and the eye, brain, skeletal muscle, heart, fins and gills were immediately removed and individual organs were pooled from the six fish for total protein isolation. The organs were homogenized in extraction buffer (25 mM MOPS, pH 7.5, containing 1 mM EDTA, 5 mM EGTA, 10% (v/v) glycerol, 1 mM dithiothreitol (DTT), 5 μg/ml leupeptin, 1 μg/ml aprotonin and 5 μg/ml pepstatin A). The homogenate was transferred into a 1.5 ml centrifuge tube followed by sonication. The extract was centrifuged at 22,000 × g for 30 min and protein concentrations of the supernatant were determined by the Bradford protein assay using bovine serum albumin as a standard (Bradford, 1976). 40 μg of protein extract from each organ was resolved by 12% SDS PAGE, followed by transfer to nitrocellulose. The nitrocellulose was stained with Ponceau-S to confirm equal protein loading. The ARNT2 protein was detected using the goat anti-ARNT2 M-20 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:500 and ECL chemiluminescence. This ARNT2 antibody was designed against the carboxy terminal of the mouse ARNT2 and recognizes the C-terminus of the zebrafish ARNT2b/c protein but does not detect ARNT2a or ARNT2x (Andreasen et al., 2002a). The nitrocellulose was then reprobed using the anti-β-actin antibody at a dilution of 1:5000 (Sigma) and ECL chemiluminescence.

2.7. Adult in situ Immunohistochemistry

Five days post-injection, vehicle control and TCDD exposed zebrafish were euthanized in Tricaine (MS-222) and fixed for 3 days in Dietrich's Fixative followed by dehydration in graded concentrations of ethanol before paraffin embedding. Serial 5 μm whole body sagital sections were cut and mounted onto slides for immunohistochemical detection of ARNT2 and CYP1A. Slides were pretreated with 0.1 M citrate buffer at 100 °C for 5 min followed by several washes in PBS. The slides were processed using the Vectastain kit essentially as described by the manufacturer (Vector, Burlingame, CA). Briefly, following blocking in serum for 30 min, the slides were incubated with anti-ARNT2 M-20 polyclonal antibody or CYP1A MAb 1-12-3 (Stegeman et al., 1989) at 1:500 dilution for 14 h at 4 °C in a humid chamber.

Following several washes in PBS, the slides were incubated with horseradish peroxidase-conjugated secondary antibodies followed by diaminobenzidine detection. Adjacent sections were processed without the addition of primary antibody to serve as the control for these experiments.

To determine TCDD-induced CYP1A expression in regenerating caudal fin, zebrafish caudal fins were partially amputated immediately following TCDD exposure. Five days post-exposure, fish were euthanized and the caudal fins were removed for sections prepared as previously described.

3. Results

3.1. Histopathology

To evaluate the histopathology in adult zebrafish 5 days following a single TCDD exposure, control and TCDD exposed animals were anesthetized, sectioned and stained with hematoxylin and eosin. Table 1 summarizes the histopathologic observations for both control and TCDD treated zebrafish with representative images of the tissues displayed in Fig. 1. The most significant differences between control and TCDD exposed animals were observed in the liver. The livers of the TCDD exposed zebrafish displayed lipidosis, hepatocyte hypertrophy as well as an apparent decrease in the number of hepatocyte nuclei per field (Table 1, Fig. 1A and B).

The abundance of hepatic glycogen was determined using PAS staining in tissues from control and TCDD exposed fish (Table 1). While enhanced hepatocellular glycogen storage can result in a similar histological picture with increased non-eosinophilic cytoplasm in hepatocytes, PAS staining revealed that TCDD exposure resulted in significant glycogen depletion in the liver. TCDD exposed fish also displayed hypertrophy of the gill lamellae as well as apparent fusion of some of the secondary lamellae (Table 1, Fig. 1C and D).

The atrium and ventricle of the heart in TCDD exposed fish were indistinguishable from controls displaying no changes in blood cell or myocyte structure as well as no significant change in overall heart shape and size (Fig. 1E and F). In addition, the proximal and distal tubules of the kidney were similar in size and structure to controls and there were no significant alterations in hematopoietic tissue (Fig. 1G). Finally, the gastrointestinal tract of TCDD exposed zebrafish displayed no significant histologic differences in the structure of the mucosal folds or changes in epithelial or goblet cells from control fish (Fig. 1H).

3.2. Organ distribution of ARNT2 protein

Four ARNT2 splice variants have been characterized in the zebrafish, ARNT2a, ARNT2b ARNT2c

Table 1
Histopathologic analysis of vehicle control or TCDD exposed adult zebrafish

Organ	Pathology	Control	TCDD
Liver	Lipidosis	–	+++
	Hepatocyte Hypertrophy	–	+++
	Decreased number of hepatocyte nuclei	–	+++
	Glycogen depletion	–	+++
	Bile duct hyperplasia	–	–
Gill	Increased mucous cells in primary lamellae	–	–
	Hypertrophy of Gill lamellae	–	++
	Fusion of secondary lamellae	–	++
GI	Necrosis of epithelial cells in serous glands	–	–
	Hyperplasia of serous mucosal glands	–	–
	Atrophy of serous portion of glandular mucosa	–	–
Kidney	Depletion of lymphomyeloid elements	–	–
	Fewer blast cells	–	–
	More differentiated lymphomyeloid cells	–	–

(−) no histopathology; (+) histopathology in <25% of fields; (++) histopathology in >75% of fields; (+++) histopathology in all fields.

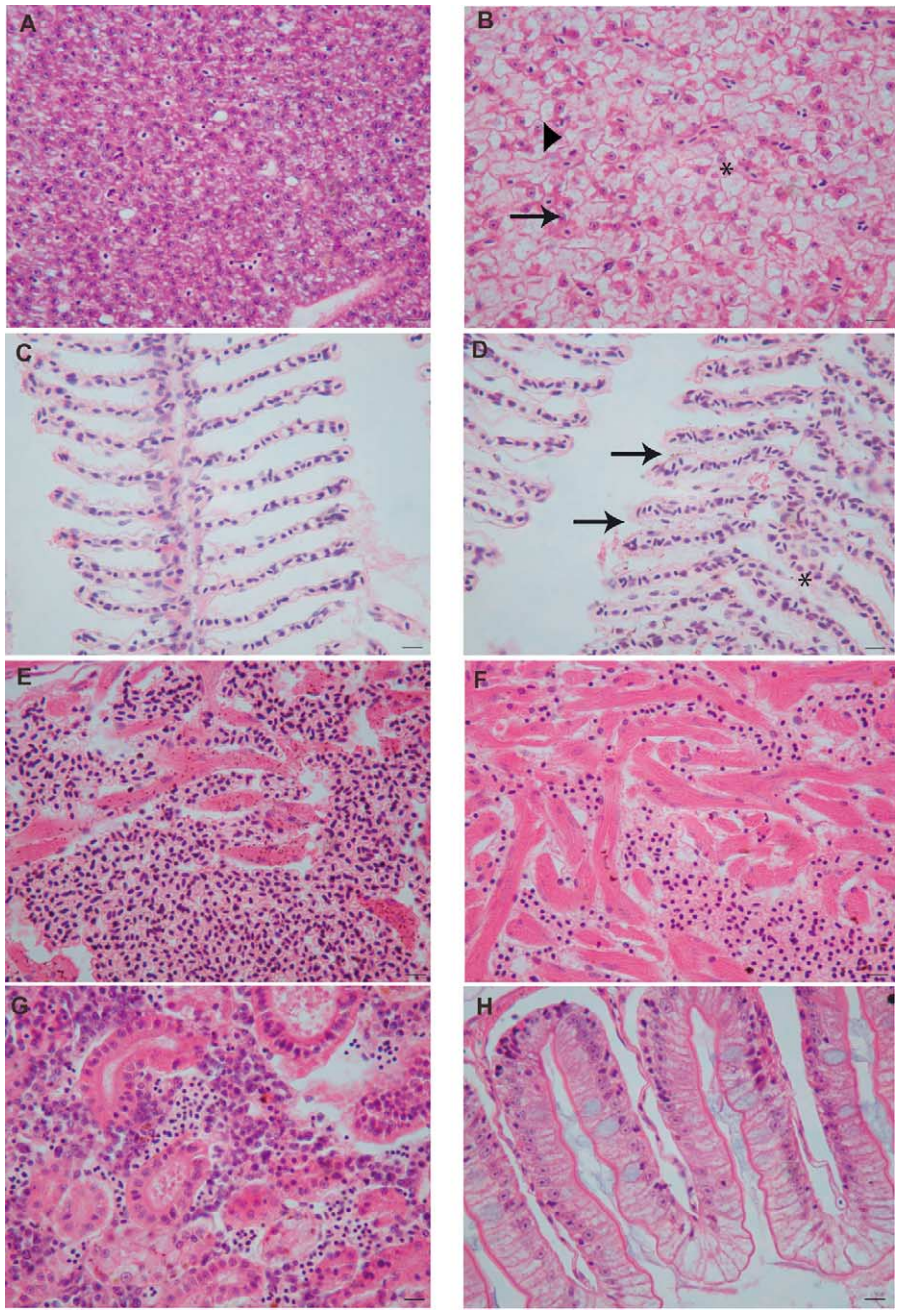

Fig. 1. Histopathology of TCDD in adult zebrafish exposed to 70 ng TCDD/g fish. Serial 5 μm whole body sagital sections were prepared and stained with hematoxylin and eosin. Images were digitally acquired using a 40× objective. (A) Liver from vehicle control zebrafish; (B) liver of TCDD exposed zebrafish displaying hepatocyte hypertrophy (arrowhead), lipidosis (asterisks) and decreased nuclei number (arrow) in liver sections from exposed zebrafish; (C) gill lamellae from control zebrafish; (D) gill tissue from TCDD exposed zebrafish displaying hypertrophy (asterisks) and fusion of secondary lamellae (arrows); (E–H) no significant changes from vehicle control zebrafish were detected in the atrium, ventricle, kidney and gastrointestinal tract from TCDD exposed zebrafish. Scale bar is 10 μm.

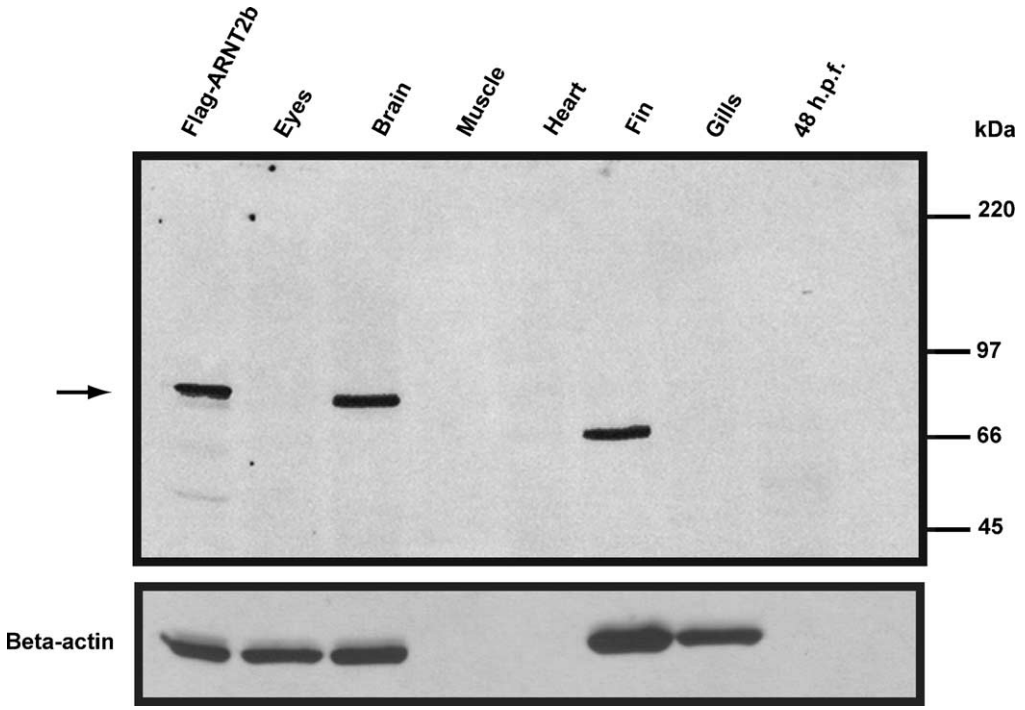

Fig. 2. Distribution of ARNT2 protein in adult zebrafish. Total protein extracts were prepared from the indicated adult zebrafish organs. 40 μg of each of the indicated organ extracts were resolved on a SDS PAGE gel and transferred to nitrocellulose. The membrane was incubated with goat anti-ARNT2 antibody and anti-goat HRP secondary antibody followed by chemiluminescent detection. The first lane contains 40 μg of COS-7 cell extracts expressing FLAG-ARNT2b. The arrow denotes the location of the ARNT2 protein.

and ARNT2X having theoretical molecular weights of 45, 81 and 79 and 44 kDa, respectively (Tanguay et al., 2000; Wang et al., 2000; Hsu et al., 2001). The ARNT2 antibody used in these studies recognizes the C-terminus of the ARNT2b/c proteins, and cannot detect ARNT2a or ARNT2x. Proteins from adult organs were analyzed by Western blotting. For reference, extracts from COS-7 cells transiently transfected to express FLAG-ARNT2b (Tanguay et al., 2000) were resolved on the same blot. The 83 kDa FLAG-ARNT2b protein is easily detected in the COS-7 extract (Fig. 2, lane 1) and ARNT2b/c is highly abundant in extracts isolated from the brain (Fig. 2, lane 3). Eye, muscle, heart, gill and 48 h post fertilization (hpf) extracts did not contain a significant amount of ARNT2b protein (Fig. 2, lanes 2, 4, 5, 7 and 8). This antibody however also detects a 65 kDa band in extracts isolated from the fin (Fig. 2, lane 6). The identity of this protein remains unknown, but may simply represent cross-reacting antigen, or perhaps is another splice variant of ARNT2. Upon longer exposure times, the ARNT2b/c band was detected in eye, heart, fin and gill and the 65 kDa protein was detected in eye, muscle, heart, and gill (data not shown). The β-actin antibody confirms equivalent loading in each lane (Fig. 2). β-actin expression was not detected in extracts from the heart, muscle or 48 hpf extracts as this antibody has been reported not to recognize cardiac or skeletal muscle actin (Gimona et al., 1994).

To detect ARNT2b/c in situ, immunohistochemistry was conducted using the anti-ARNT2b/c-specific antibody. ARNT2b/c is expressed in tissues including the epithelium of the intestinal tract (Fig. 3A) and the epithelial cells of the gill lamellae (Fig. 3B). ARNT2b/c is also detected throughout the kidney, with no preference to proximal versus distal tubules (Fig. 3C). There was minimal ARNT2b/c staining in the myocardial muscle of the ventricle, liver and brain (Fig. 3D–F). Importantly, ARNT2b/c abundance and localization was not affected by TCDD exposure.

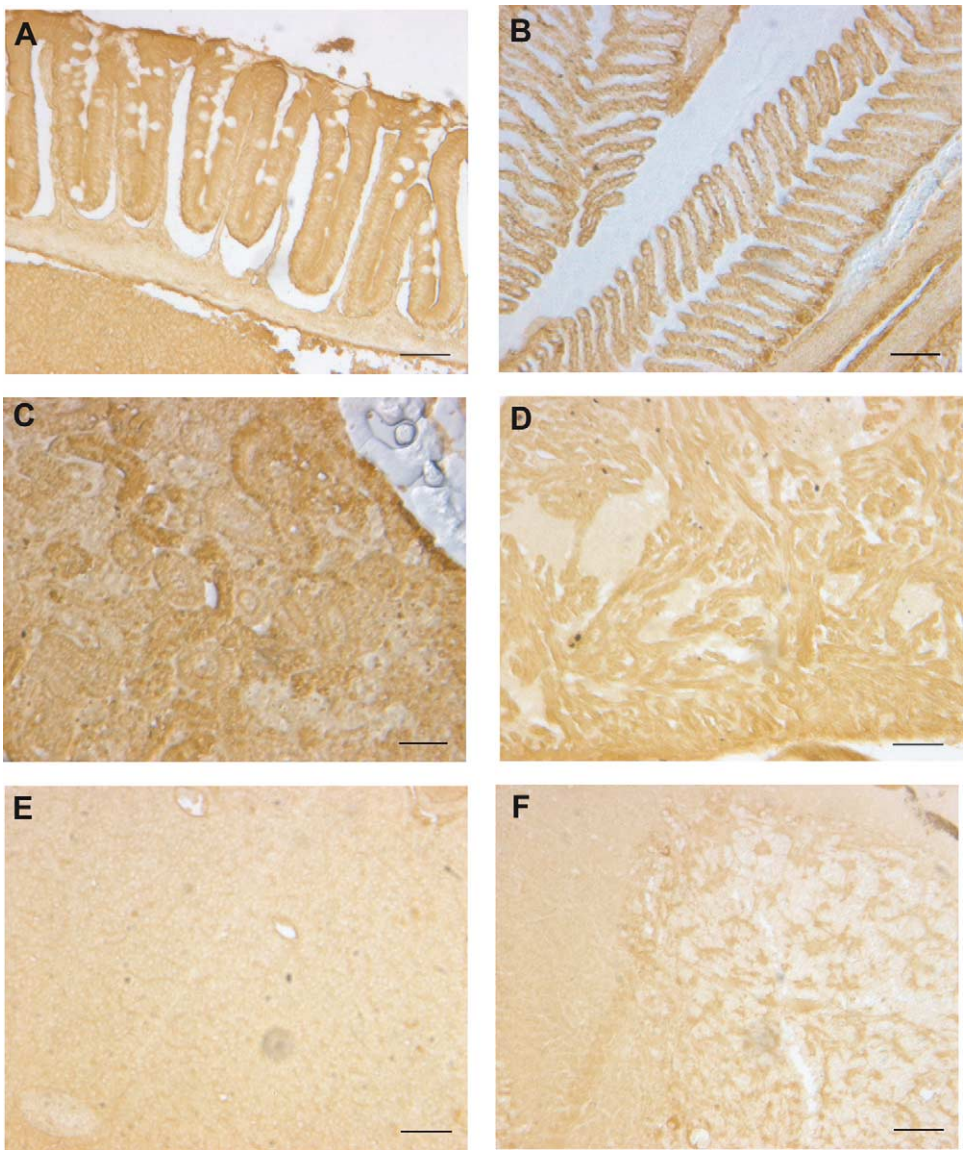

Fig. 3. Immunohistochemical localization of ARNT2b/c in adult zebrafish exposed to 70 ng TCDD/g fish. In situ immunohistochemical detection of ARNT2 in 5 μm whole body saggital sections. The bright field images were digitally acquired using a 20× objective. (A) Significant ARNT2b/c expression was observed in all layers of the intestinal tract; (B) the lamellae of the gills; (C) all layers of the kidney; (D) as well as in the cardiac muscles of the ventricle ARNT2b/c expression in the; (E) adult liver; (F) brain. Scale bar is 50 μM.

3.3. Immunohistochemical localization of CYP1A

TCDD-induced expression of CYP1A protein was used to monitor tissues with an activate AHR pathway. Serial sagital sections of adult zebrafish were prepared from control and TCDD exposed zebrafish for immunohistochemical detection of CYP1A. CYP1A protein was not detected in any adult tissue of the vehicle control animals (Table 1 and Fig. 4A). TCDD exposure-induced CYP1A protein expression in the proximal tubules of the kidney, the liver hepatocytes and the epithelium of the gastrointestinal tract. The

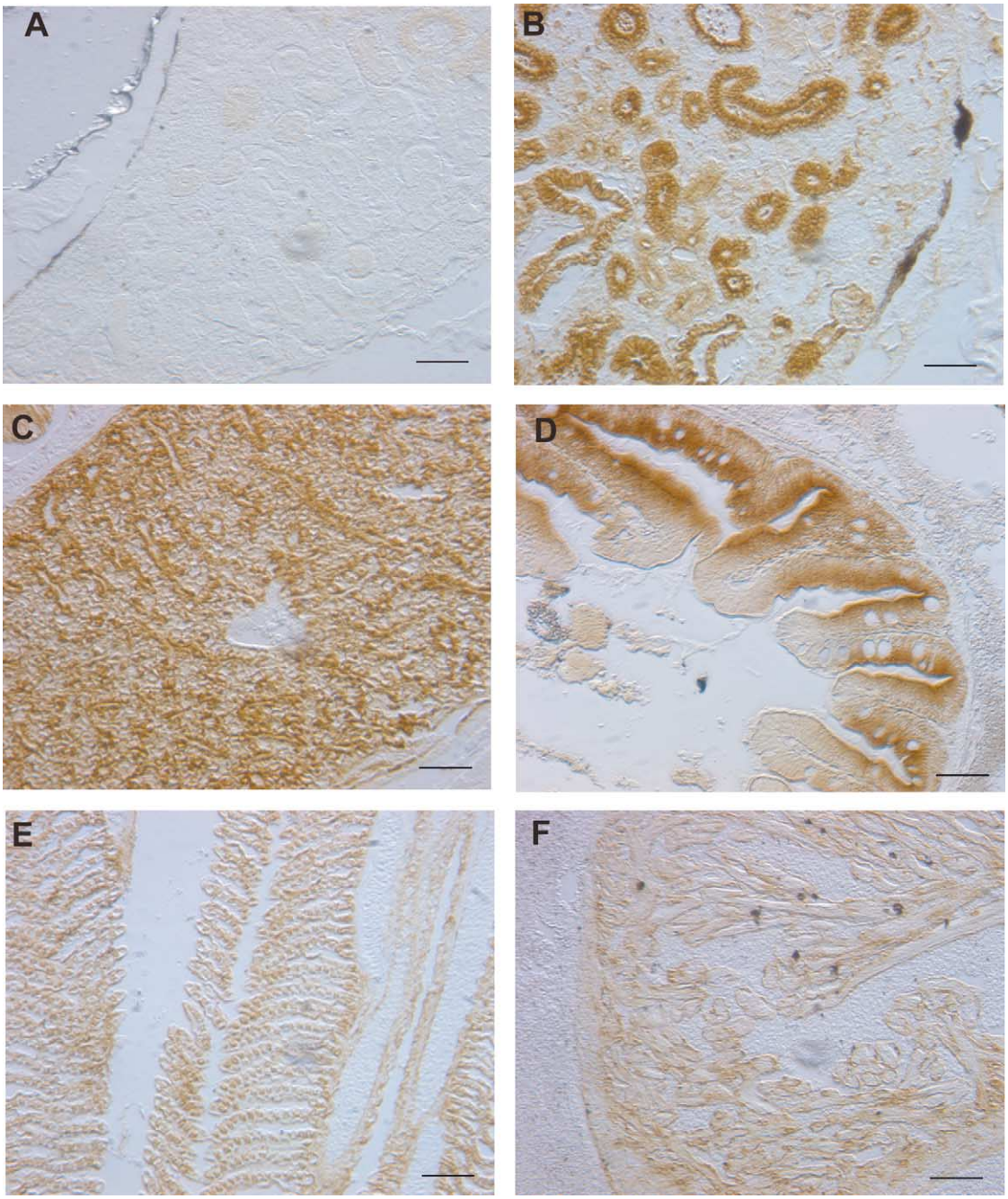

Fig. 4. Immunohistochemical localization of CYP1A in adult zebrafish exposed to 70 ng TCDD/g fish. In situ immunohistochemical detection of CYP1A in 5 μm whole body saggital sections. The bright field images were digitally acquired using a 20× objective. (A) Representative kidney tissue from vehicle control zebrafish displaying no CYP1A expression in control tissues; (B) significant CYP1A expression was present in the proximal tubules of the kidney; the (C) hepatocytes of the liver; (D) and the epithelium of the intestine from TCDD exposed zebrafish but not in control fish; (E) CYP1A is minimally expressed in the adult gill; (F) and cardiac muscle of the ventricle of TCDD exposed fish. Scale bar is 50 μM.

CYP1A staining in the gastrointestinal tract was consistently less intense at the mucosal folds (Fig. 4B–D). CYP1A expression was also detected in the gill epithelium and myocardium of the ventricle of TCDD exposed fish (Fig. 4E–F). In addition, CYP1A expression was detected in the intact and regenerating caudal fin of TCDD exposed fish (Fig. 5A) compared to controls (Fig. 5B) indicating that the AHR pathway is functioning in the regenerate (Table 2).

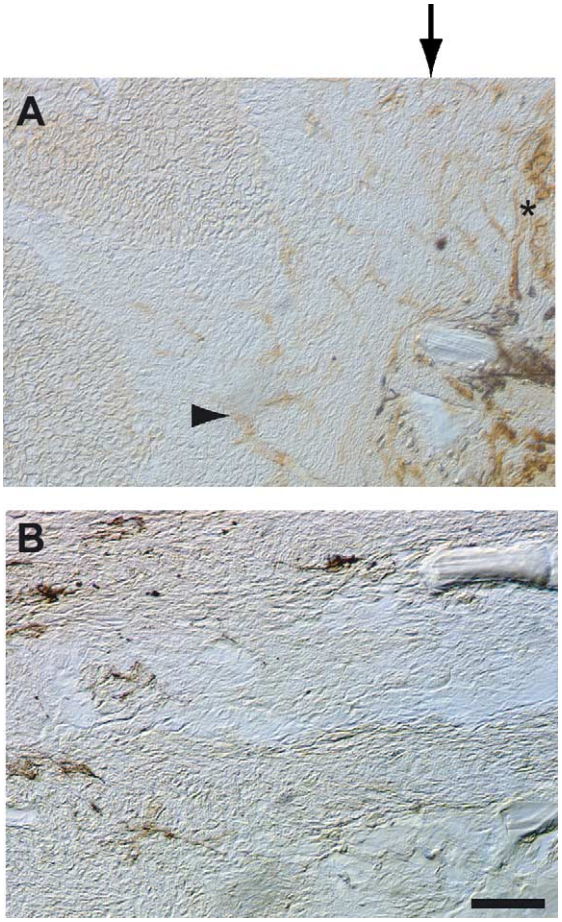

Fig. 5. Immunohistochemical detection of CYP1A in the regenerating fin tissue in adult zebrafish exposed to 70 ng TCDD/g fish. In situ immunohistochemical detection of CYP1A in 5 μm caudal fin sections. Images were digitally acquired using a 20× objective. (A) Fin section from TCDD exposed fish displaying CYP1A expression in regenerating tissue (arrowhead) in addition to intact fin tissue (asterisk); (B) caudal fin from TCDD exposed fish not expressing CYP1A without exposure to primary antibody. This is representative of the absence of CYP1A expression seen in the caudal fin of control zebrafish sections exposed to primary antibody.

Table 2
Summary of ARNT2 and CYP1A immunolocalization in vehicle control and TCDD exposed adult zebrafish

Organ	ARNT2		CYP1A	
	Control	TCDD	Control	TCDD
Liver	++	++	−	+++
Gill	+++	+++	−	++
Kidney	+++	+++	−	+++
Gastric mucosa	+++	+++	−	+++
Heart	++	++	−	++

(−) no immunoreactivity; (+) some immunoreactivity; (++) marked immunoreactivity; (+++) strong immunoreactivity.

4. Discussion

Previous studies in adult zebrafish indicate that caudal fin regeneration is significantly inhibited by TCDD (Zodrow and Tanguay, 2003). Due to the systemic nature of the exposure in these experiments it is possible that damage to organs following exposure to TCDD could result in decreased fin regeneration. The inhibition of fin regeneration is pronounced by 5 days post-exposure, therefore it is critical to evaluate the TCDD-dependent pathology at this specific time point. The LC_{50} for adult zebrafish was not determined in this study, however zebrafish exposed to 70 ng/g TCDD remained healthy for at least 28 days with no changes in feeding, behavior or weight indicating that zebrafish are relatively resistant to TCDD. For example, yellow perch receiving a single 25 μg/kg TCDD dose demonstrate 95% mortality within 28 days (Spitsbergen et al., 1988a). Recent molecular and genetic evidence may provide a partial explanation for the relative resistance of zebrafish to TCDD. Fish have at least two copies of many genes as a result of genome duplication events (Amores et al., 1998; Taylor et al., 2001). In many cases, the closely-related genes, or paralogs, remain biologically active. For example, two forms of AHR, AHR1 and AHR2 have been identified in both zebrafish and the Atlantic Killifish and functional studies indicate that both of the Killifish AHRs function as dioxin receptors. They bind TCDD, dimerize with ARNT, and transactivate genes containing

AHREs (Karchner et al., 1999). The zebrafish AHR2 is the receptor that mediates TCDD toxicity since it binds TCDD, dimerizes with ARNT2b and activates transcription. Surprisingly, the zebrafish AHR1 fails to bind ligands, only weakly associates with ARNT2b and fails to transactivate (Andreasen et al., 2002a). It is intriguing to speculate that zebrafish are less responsive to TCDD because of a reduced gene dosage. The identification and characterization of additional fish AHRs is necessary to investigate this hypothesis.

Here, we report that TCDD leads to hypertrophy of hepatocytes, glycogen depletion and lipidosis of the liver as well as hypertrophy and fusion of secondary lamellae. These results are similar to those obtained in juvenile yellow perch and rainbow trout exposed to 25 or 125 ng TCDD/g fish (Spitsbergen et al., 1988a,b). However, studies in yellow perch, mirror carp and trout also reported a variety of pathologic effects that we did not observe at this specific time point. Specifically, in those studies there was depletion of lymphomyeloid elements, fewer blast cells and more differentiated lymphomyeloid cells in the kidney while necrosis of epithelial cells in the serous glands, hyperplasia of serous mucosal glands and atrophy of the serous portion of the glandular mucosa were observed in the gastrointestinal tract. In addition, the heart displayed necrosis of myocytes near the ventricle and hypertrophy and hyperplasia of the pericardium (Spitsbergen et al., 1988a,b). Hyperplasia and fibrosis of liver bile ducts results from single TCDD exposures in both rainbow trout and mirror carp (Spitsbergen et al., 1988b; van der Weiden et al., 1994). Notably, with yellow perch rainbow trout and mirror carp, exposed fish were examined for histopathology between 35 and 42 days post-injection. The differences in time to examination and interspecies differences in sensitivity to TCDD may partially explain the differences between our results with zebrafish and those in yellow perch and rainbow trout. The histopathologic effects displayed in other fish species may appear in zebrafish upon longer exposure times, however this study was designed to specifically evaluate histopathology at 5 days post-injection. Future time course studies following injection are required to compare TCDD responsiveness in zebrafish to other species.

It is largely accepted that TCDD toxicity is mediated by activation of the AHR pathway and this activation is often correlated with increased CYP1A expression (DeVito et al., 1996). Furthermore, organ-specific TCDD toxicity often correlates with CYP1A expression. Previous studies reveal that larval zebrafish exposed to a waterborne concentration of TCDD at 0.5 μg/l expressed AHR2 mRNA in the caudal fin at 24 h post fertilization, trunk kidney at 36 hpf, the head kidney and vasculature of the heart at 48 hpf, and gill and intestinal mucosa at 120 hpf (Andreasen et al., 2002b). AHR2 and mRNA expression was also observed in the liver at 120 hpf. ARNT2a/b/c mRNA was detected in the vasculature of the heart and trunk kidney at 48 hpf, caudal fin, intestinal mucosa, gill and liver at 120 hpf with no measurable ARNT2a/b/c expression in the head kidney up to 120 hpf. CYP1A mRNA was expressed in the gill arches at 48 hpf and heart, intestinal mucosa and liver at 120 hpf. TCDD exposure in the zebrafish larvae resulted in increased expression of CYP1A relative to vehicle control fish, however, AHR2 and ARNT2a/b/c expression were not altered significantly. In addition, AHR2, ARNT2 and TCDD-induced CYP1A mRNAs colocalized in tissues affected by TCDD exposure (Andreasen et al., 2002b).

The pattern of ARNT2a/b/c mRNA expression in larval zebrafish is similar to expression in adults. ARNT2b/c mRNA is expressed in the adult brain, eye, gill, skin, muscle and fin with little expression in the liver, heart and kidney (Tanguay et al., 2000). Adult male zebrafish have high expression of AhR2 mRNA in the heart, muscle, liver and gill with lower expression in brain, kidney and fin (Andreasen et al., 2002a). Exposure to 10 ng TCDD/g fish resulted in 100–500-fold induction of CYP1A mRNA in brain, heart, muscle, swimbladder and liver with a lower fold induction in the gill, kidney and fin. However, AHR2 mRNA expression was not altered significantly from vehicle control fish, except in the heart (Andreasen et al., 2002a).

In the present study, immunoblot experiments localized ARNT2b/c protein primarily to the brain. There was a smaller potential splice variant in the fin, however, exposing the immunoblot to film for longer time revealed expression of a band the correct size for ARNT2b/c and also the potential splice variant, in eye, heart, fin and gill (data not shown). This pattern is similar to adult zebrafish ARNT2b/c mRNA expression and abundance (Tanguay et al., 2000). ARNT2b/c was immunolocalized to the

gastrointestinal tract, gill and kidney with lower levels of expression present in the ventricle of the heart, liver, and brain (Fig. 3). CYP1A was highly expressed in the proximal tubules of the kidney, liver hepatocytes and intestinal epithelium with lower levels of expression displayed in the gill and cardiac muscle of the ventricle (Fig. 4). In scup (*S. chrysops*) exposed to 3.1 ng/g tetrachlorodibenzofuran, CYP1A expression is induced in all cell types of the liver, endothelial cells of the endocardium of the heart, pillar (endothelial) cells of the gill and vascular endothelium of the gastrointestinal tract (Smolowitz et al., 1991). CYP1A was also expressed in epithelial cells of kidney tubules and the mucosal folds and glandular pits of the gastrointestinal tract. In adult Atlantic cod (*Gadus morhua*) exposed to 50 mg/kg of the AHR agonist β-napthoflavone (βNF), CYP1A is induced in liver hepatocytes and endothelial cells, in atrial and ventricular endothelial cells and endocardium, in the epithelial cells of the proximal kidney tubule, and in the endothelial cells of the spleen, intestine and gills (Husoy et al., 1994). In some studies, CYP1A expression has been detected in organs displaying no TCDD-dependent histopathology. European flounder (*P. flesus*) exposed to 0.0125–500 ng TCDD/g displayed no exposure-related histopathology of liver, thyroid gland, stomach, gut, spleen, mesonephros, gonads or gill (Grinwis et al., 2000). However, an exposure-related increase of CYP1A immunodetection was observed in hepatocytes, endothelium of spleen, digestive tract, mesonephros, gonads and gills.

ARNT2 and TCDD-dependent CYP1A were colocalized in liver hepatocytes and the gill. Hepatocytes and gills were also the most adversely affected by TCDD. Upon exposure to TCDD, ARNT2b/c and CYP1A expression were observed in the gastrointestinal tract and tubules of the kidney, however these organs displayed no significant histopathology 5 days post-exposure. The ventricle of the heart displayed lower expression of both ARNT2 and CYP1A compared to other tissues, but no observable signs of TCDD-dependent histopathology were detected in the ventricle or atrium of the heart. ARNT2 and CYP1A were also coexpressed in the intact caudal fin, and TCDD exposure significantly impairs caudal fin regeneration (Zodrow and Tanguay, 2003). To determine if the AHR pathway plays a role in the inhibition of fin regeneration, immunohistochemical expression of CYP1A was determined in regenerating fins of control and TCDD exposed fish. TCDD-induced CYP1A expression was detected in the regenerating caudal fin of exposed fish compared to controls. mRNA expression studies also confirmed the presence of AHR2, ARNT2b/c and CYP1A induction in regenerating caudal fin of TCDD exposed zebrafish (Zodrow and Tanguay, 2003). In extracts prepared from fin tissue, a putative 65 kDa ARNT2 is detected and in this tissue CYP1A is induced. This would suggest that this putative ARNT2 isoform is an additional functional ARNT2, which requires further study.

In conclusion, adult zebrafish are less sensitive to TCDD than other fish, yet display many of the same signs of TCDD organ-specific toxicity. ARNT2b/c and CYP1A colocalize in liver and gill, organs significantly affected by TCDD exposure. Importantly, the pathology observed in this study cannot completely account for the inability of caudal fin to regenerate in the presence of TCDD and suggests that localized mechanism(s) at the regeneration site remain to be elucidated.

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