Int. J. Dev. Biol. 41: 411-423 (1997)

The aquatic vertebrate embryo as a sentinel for toxins: zebrafish embryo dechorionation and perivitelline space microinjection

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ABSTRACT **Pollution of aquatic ecosystems poses a serious threat to aquatic organisms and ultimately the entire ecosystem. Understanding how a toxin affects embryonic development is key to determining the risk a pollutant represents to the environment. Extraembryonic membranes, such as the chorion of fish eggs, provide a protective barrier between the embryo and the environment. Although the fish chorion excludes many chemical pollutants, some noxious agents can still gain access to the aquatic embryo. Therefore a monitoring system that tests the effects directly upon the embryo must be established. Although exposure to a single toxin in the laboratory can determine the concentration at which a pollutant becomes a health or environmental hazard, embryos and adults in nature are not merely affected by a single chemical, but are exposed to mixtures of different pollutants. Zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*) embryos were employed for the rapid observation of the effects of single chemicals and chemical mixtures on development. Using dechorionation and a perivitelline space microinjection system, the embryos were effective sentinels for low concentrations of aquatic pollutants. The developmental effects of small quantities of toxins were observed. Embryos treated during the late gastrula stage of development with hexachlorobenzene (HCB); 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD); toluene; benzene; or mixtures of these chemicals developed cardiovascular abnormalities. The zebrafish dechorionation exposure technique, Micro Intrachorionic Zebrafish Embryo Live Laboratory test, was especially effective in testing the pollutant mixtures. Combinations of both TCDD and benzene (as well as the toluene and benzene combinations) were tested and the mixtures acted synergistically; the combinations were more toxic than either chemical by itself. Hexachlorobenzene- and TCDD-treated embryos tested positively for expression of cytochrome P450 1A indicating that the cytochrome metabolic pathways were already functional in these early embryos, and suggested that a product of the cytochrome system may be involved in HCB and TCDD pollution associated cardiovascular defects.**

KEY WORDS: *zebrafish, medaka, heart defects, sentinels for aquatic pollution, AhR agonist/non agonist*

Introduction

Vertebrate embryos are exquisitely sensitive to pollutants, carcinogens, viruses and other noxious agents. The evolution of a variety of extra-embryonic membranes and organs have provided the vertebrate embryo with various degrees of protection from these agents. Our laboratory has used a variety of vertebrate embryos as sensitive test systems; however we have focused on (1) the frog embryo *(Rana pipiens)*, to microinject and test the carcinogenic activity of a herpes-type virus: The Lucké Tumor Herpes Virus (LTHV) (Mizell *et al.*, 1969; Mizell, 1969, 1972, 1985), (2) the opossum embryo *(Didelphys virginiana)* to test *in vitro* fetal culture (New and Mizell, 1972; New *et al.*, 1977); to microtransplant fetal opossum nervous tissue into embryonic opossum limbs to induce mammalian limb regeneration (Mizell, 1968) and to determine the origin of fetal opossum immunoglobulins (Hindes and Mizell, 1976); and (3) fish embryos the Japanese medaka, *Oryzias latipes*, and the zebrafish, *Danio rerio* to test the developmental and carcinogenic potential of aquatic pollutants (Mizell *et al.*, 1995, 1996a,b,c).

Abbreviations used in this paper: TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; HCB, hexachlorobenzene; PAH, polyaromatic hydrocarbon; Ah, aromatic hydrocarbon; AhR, aryl hydrocarbon receptor; CYP, cytochrome P450; bpm, beats per minute.

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0214-6282/97/$05.00

TABLE 1

SURVIVAL OF MEDAKA EMBRYOS INJECTED WITH HCB[a]

Time after injection / Concentration (ppm)	N	24 h	48 h	Heart defects*	72 h	96 h	Hatched (%)
1000 ppm	24	16 (66%)	16 (66%)	3/16	8 (33%)	8 (33%)	8 (33%)
100 ppm	24	21 (88%)	21 (88%)	3/21	21 (88%)	21 (88%)	12 (50%)
10 ppm	24	23 (96%)	23 (96%)	3/23	23 (96%)	23 (96%)	13 (54%)
1 ppm	24	20 (83%)	20 (83%)	3/20	20 (83%)	20 (83%)	14 (58%)
0.01 ppm	24	20 (83%)	20 (83%)	2/20	20 (83%)	20 (83%)	14 (58%)
0.0001 ppm	24	23 (96%)	22 (92%)	1/22	22 (92%)	22 (92%)	14 (58%)
Control, oil injected	60	53 (88%)	52 (87%)	0	51 (85%)	51 (85%)	49 (82%)
Control, uninjected	60	55 (92%)	55 (92%)	0	55 (92%)	55 (92%)	52 (87%)

[a]Medaka embryos hatch in approximately 14 days. *Heart defects in 48 hour survivors.

TABLE 2

SURVIVAL OF MEDAKA EMBRYOS INJECTED WITH TCDD

Time after injection / Concentration (ppm)	N	24 h	48 h	Heart defects*	72 h	96 h	Hatched (%)
10 ppm	9	1 (11%)	0	-	-	-	0
1 ppm	32	18 (56%)	13 (41%)	2/13	13 (41%)	13 (41%)	11 (34%)
0.01 ppm	24	23 (96%)	23 (96%)	1/23	23 (96%)	23 (96%)	22 (92%)
0.0001 ppm	34	34 (100%)	32 (94%)	1/32	32 (94%)	32 (94%)	30 (88%)
Control, uninjected	12	11 (92%)	11 (92%)	0	11 (92%)	11 (92%)	11 (92%)

*Heart defects in 48 hour survivors.

Embryos of aquatic species have encountered increasing problems of water-borne pollution. Amphibians, such as the common North American leopard frog, *Rana pipiens*, are becoming less common. In fact, the worldwide frog population has undergone severe depletion during the last few decades. Pollution of the world's waterways are suspect as the major reason for the plummeting frog populations.

Fish populations are also in decline throughout the world and pollution is also suspect as one of the major causes. Our laboratory has been investigating the Mississippi River Basin, the largest water drainage system on the North American continent, and the effects of pollution in this system of fresh water. Hexachlorobenzene (HCB) was found in high concentration [280 mg/Kg (ppm)] in the sediment of the Mississippi River Basin near Baton Rouge, Louisiana (Mizell *et al.*, 1995). HCB therefore was chosen as one of the toxins we employed in embryo microinjection outlined below. Since HCB is known to bind to the aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor (Whitlock *et al.*, 1996) we decided to also test 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). The effects of the highly toxic TCDD, which also binds to the AhR, have been studied in several fish species; pericardial edema and other cardiovascular defects were prominent features in these previous studies (Peterson *et al.*, 1993; Walker and Peterson, 1994). The molecular mechanisms of these events are not known, nor is known the function of the Ah receptor during *normal* embryogenesis. In conjunction with testing these embryos with two aryl hydrocarbon receptor agonists, we also employed common pollutants which cause similar cardiovascular defects: toluene and benzene (Non AhR agonists).

Zebrafish and Japanese medaka embryos are excellent models for monitoring aquatic pollution and its toxic effects. Their rapid development and transparent chorions allow embryogenesis to be easily followed. Both traits make these fish ideal for observing the effects of pollutants on early development. In the past the method of exposure to a toxin was a limitation to the use of fish embryos (Abel, 1989). Ambient exposure to a chemical was unreliable because the chorion of the zebrafish and medaka acted as a barrier to toxins, and only at very high concentrations did deleterious effects occur (Jones and Huffman, 1975). This made an accurate comparison between the effects of toxins in the environment and the effects of ambient exposure difficult (however the residue analysis could be conducted on samples of exposed embryos to determine chemical dose). Many investigators did not attempt to quantify the amount of toxin which penetrated the medaka chorion during ambient exposure (Ishikawa *et al.*, 1975). Moreover, experiments which involved submersion of embryos and fry in toxins generated large quantities of contaminated waste water creating a disposal problem. In one typical experiment ambient exposure to water-borne toxins generated approximately 18 liters of polluted waste water (Koenig and Chasar, 1984).

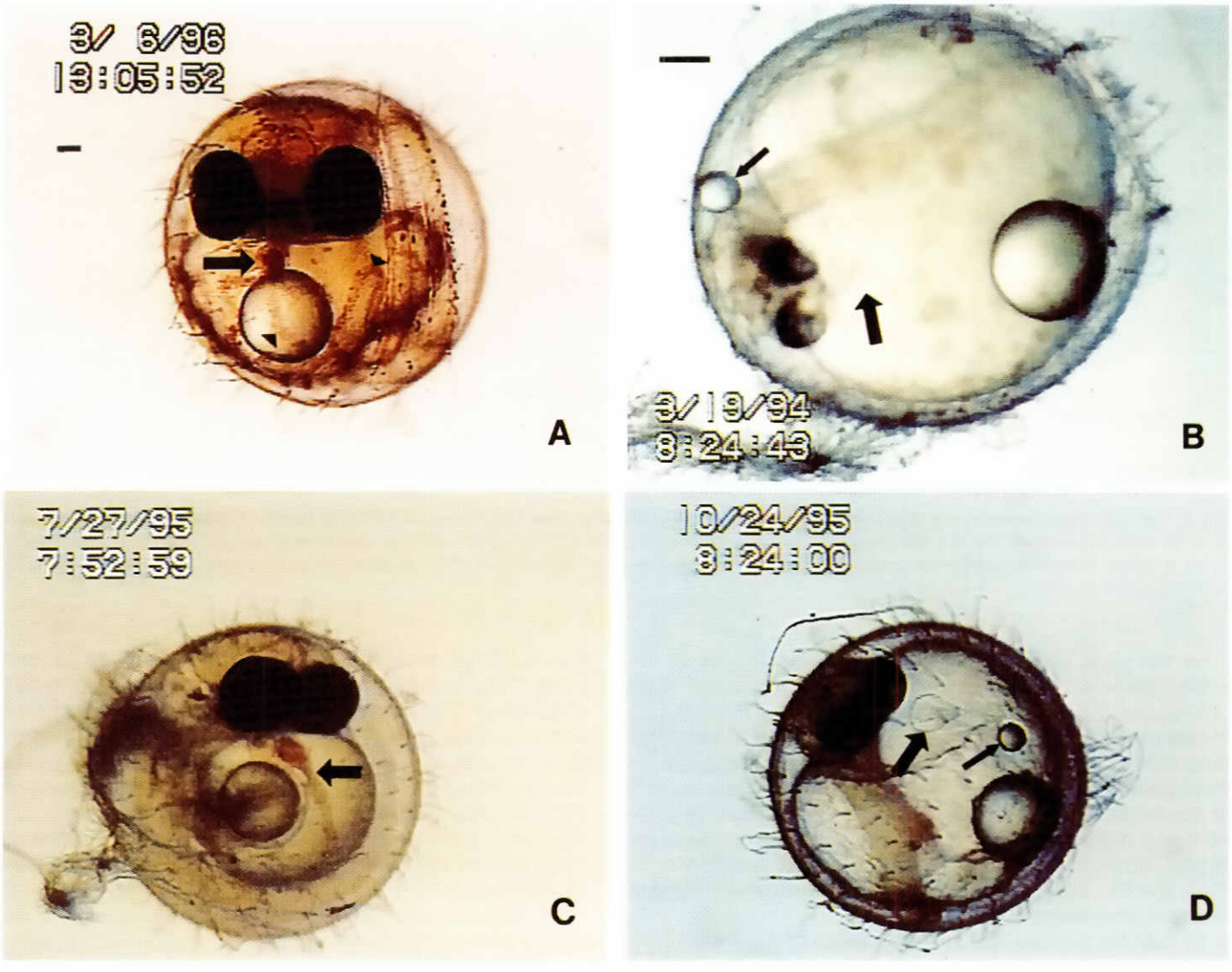

Fig. 1. Video prints comparing medaka heart formation after treatment with various chemicals injected at the late gastrula stage. *Embryos shown 120 h after fertilization (96 h after injection).* **(A)** *Uninjected medaka control. Normal heart (large arrow) and yolk sac vasculature(arrowhead points) are visible. Two pigmented eyes are prominent and the tail is wound around the yolk sac. A single native oil droplet is visible. Bar, 0.1 mm.* **(B)** *Hexachlorobenzene injected medaka with droplet still positioned in the perivitelline space and in contact with the yolk sac. Droplet at 9 o'clock (small arrow). Thin-walled straight tube heart shown (large arrow). Bar, 0.1 mm.* **(C)** *Medaka injected with benzene. Non-circulating blood stasis present in heart tube (large arrow).* **(D)** *Retinoic acid injected medaka. Droplet (small arrow) seen on yolk sac. Single chambered heart (large arrow) has differentiated at some distance from the body.*

The limitations of medaka ambient exposure may be overcome by microinjection, and previous investigators utilized this approach in chemical carcinogenesis studies of rainbow trout and salmon embryos (Black *et al.*, 1985). They injected the carcinogen under study through the chorion and directly into the yolk sac. Microinjection of a toxin through the chorion results in effects at far more realistic concentrations, approximating those found in the environment. Until recently, however, microinjection of fish embryos was limited in its usefulness. Medaka and other killifish embryos have exceptionally tough chorions which are difficult to puncture. In past experiments microinjection through the chorion had resulted in such high mortality that it was difficult to distinguish between the effects of toxins which were injected and the damage caused by the injection procedure (Black *et al.*, 1985; Metcalfe and Sonstegard, 1985; Grizzle *et al.*, 1988). Either direct embryo injection or yolk sac penetration and injection, led to high mortality because of injuries incurred by needle penetration of vital tissue. By using a *perivitelline microinjection method* recently developed in our laboratory, these limitations have been overcome. After penetrating the chorion, instead of mechanically damaging the yolk sac or the embryonic tissue by needle penetration of the embryo's cells, the injected material was delivered to the small perivitelline space of the gastrula-stage medaka. Thus precise quantities of a toxin were placed within the perivitelline space of an intact developing embryo with minimal mortality. The toxin-containing droplet was positioned to be absorbed via the adjacent developing yolk sac vasculature.

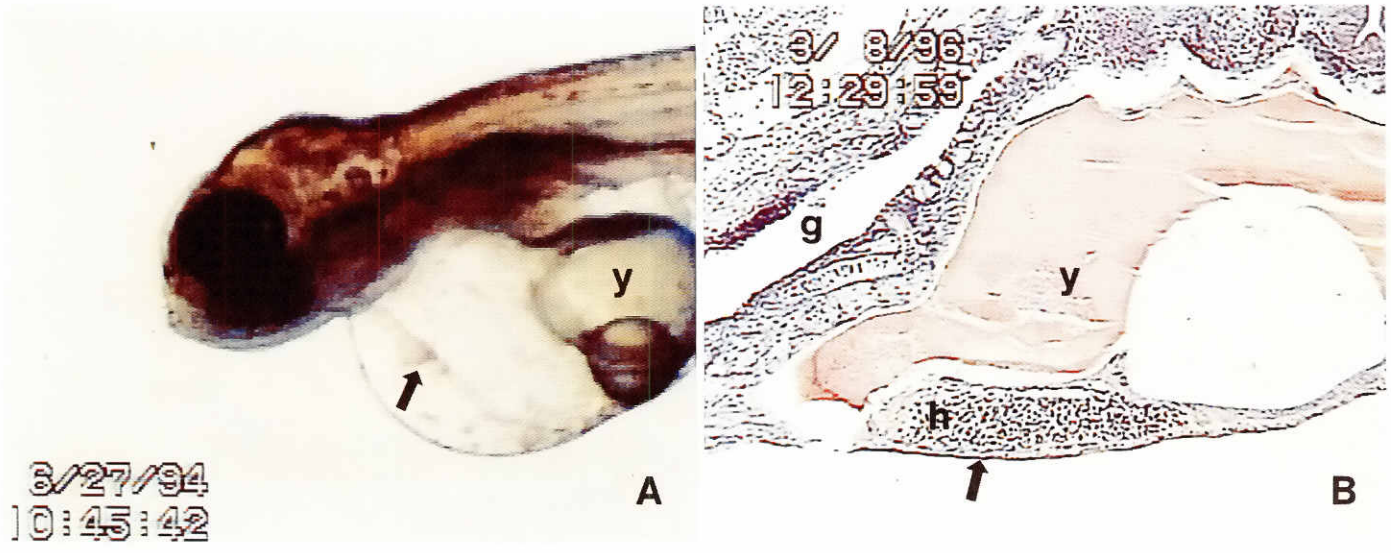

Fig. 2. Typical hexachlorobenzene cardiovascular malformation induced in a medaka embryo injected at the late gastrula stage. *A 40 nl droplet of HCB was administered. Severely affected animal removed from the chorion at 8 days of age.* **(A)** *The heart (arrow) consists of a thin tube surrounded by an enlarged, edematous pericardial cavity.* **(B)** *Photomicrograph mid-sagittal section of animal in* **A**. *y, yolk; g, gut; h, heart. Nucleated red blood cells fill the tube-shaped heart. H & E stain. x120.*

In the other series of experiments, *zebrafish gastrula dechorionation and static exposure*, zebrafish embryos were dechorionated and exposed for 30 min to a variety of toxins. Like medaka perivitelline space microinjection, static 30 minute exposure allowed zebrafish gastrulae direct access to pollutants. An important feature of these experiments was the rapid development of the zebrafish: they reach swim-up stage (begin feeding) within 96 h post fertilization (hpf). On the other hand, medaka embryos require two weeks to hatch and do not survive early dechorionation. Thus medaka embryos were microinjected, whereas zebrafish embryos were tested by dechorionation.

In both the medaka and zebrafish series of experiments the developing embryos were tested for the early presence of the cytochrome P450 1A system and their ability to metabolize planar aromatic hydrocarbons. Using these protocols zebrafish and medaka embryos were exposed to either TCDD, HCB, benzene, toluene, or mixtures of these chemicals. Preliminary results of these microinjection studies were presented at the biannual Zebrafish Meeting: Zebrafish Development and Genetics, Cold Spring Harbor, New York (Mizell *et al.*, 1996a); and the preliminary results of zebrafish dechorionation studies were presented at the General Scientific Meetings of the Marine Biological Laboratory, Woods Hole, Massachusetts (Mizell *et al.*, 1996b,c).

Results

Perivitelline space microinjection

Utilizing our microinjection technique, we have been able to successfully microinject into the perivitelline space of both zebrafish and medaka gastrula stage embryos.

The injection of HCB, TCDD, benzene, or retinoic acid (RA) into medaka late gastrula stage embryos induced abnormal heart development. Medaka embryos injected with vegetable oil and uninjected controls developed normally (Fig. 1A). Exposure to HCB or TCDD resulted in a high percentage of similar lethal medaka heart malformations (Tables 1,2). Affected embryos lacked visible extra- or intraembryonic circulation (Fig. 1B). The heart of such an embryo consisted of a thin, beating tube stretched between the yolk sac and embryo (Weis and Weis, 1974). Pooling (non-circulating) blood was observed in the yolk sac vasculature and in the hearts of many of the affected medaka embryos (Fig. 1C). Pericardial edema was a constant occurrence in these severely affected embryos. Heart contraction rate was also noticeably decreased.

When retinoic acid was injected into developing medaka embryos, a different though equally severe heart defect occurred. Our results were similar to zebrafish cell lineage studies of dechorionated embryos in RA (Stainier and Fishman, 1992). In our medaka studies the affected embryos were injected at late gastrula stage, and they developed hearts which lacked anterior chambers (Fig. 1D). These hearts differentiated in distant, almost ectopic sites. These embryos also lacked visible circulation; however, their heart contraction rate was unaffected.

Heart rate was important in determining the severity of the heart defects observed in these embryos. In severely affected medaka embryos injected with HCB or benzene the average heart rate of the embryos was 34 beats per minute (bpm). Affected medaka embryos injected with RA had an average heart rate of 132 bpm. Uninjected medaka embryos and oil-injected controls had an average heart rate of 124 bpm. During routine daily observation of the injected embryos, heart development was carefully monitored and video recorded. In medaka the presence or absence of these severe heart defects could accurately be determined two or three days after injection; at this time the medaka embryo had reached *stage 30* (102 hpf). By this stage, controls had developed extensive yolk sac vasculature and red blood was seen circulating through the chambers of the heart which had differentiated. The absence of blood circulation and the shape of the heart were used as

determining factors in scoring these as animals with severe heart defects. In embryos injected with HCB, TCDD, or benzene, heart rate was also an early indicator of developmental heart abnormalities. Many animals lacked severe heart malformation, but their reduced heart rate indicated they were affected by the chemical injection. These moderately affected animals would continue to develop and would hatch. However, hatching was usually delayed in these animals. The control medaka would hatch in 14 days; whereas, many moderately affected medaka hatched in 20 or more days. The former, severely affected medaka would not hatch and would die a few days after exhibiting gross abnormalities. However, in a few cases the severely affected medaka would continue to develop at a slow rate, but would die before hatching. The medaka in Figure 2 is an example of such an animal: it was manually removed from its chorion after 8 days and video taped before fixation (Fig. 2A). Its heart rate was reduced to 60 bpm. Histological examination of these embryos revealed that the heart was little more than a tube separated from the rest of the embryo by edema. The medaka heart lacked chambers and lacked appreciable cardiac muscle (Fig. 2B). On the other hand, uninjected controls fixed at a similar age possessed an atrium and ventricle which were clearly visible and had well differentiated cardiac muscle.

To understand how these chemicals affect early cardiovascular development some of the medaka embryos were prepared for cytochrome P450 gene activation testing. The living, moderately-affected, embryos exposed to HCB, TCDD, benzene, or RA were examined upon hatching for the ability to respond with P450 gene expression. Activation of the P450 monooxygenase system represents an initial detoxification step leading to the eventual elimination of toxic compounds. Polyaromatic hydrocarbons (PAH's) are ubiquitous environmental pollutants, and exposure to these compounds results in the induction of P450 IA. All vertebrates respond in this manner to a variety of planar aromatic hydrocarbons. Although such a response can be demonstrated in most adult vertebrates it has not been ascertained when the P450 monooxygenase system develops in the vertebrate embryo. In our medaka studies, differentiation of the cardiovascular system proceeded in the less severely affected HCB-injected animals, and the cytochrome P450 system had apparently developed by the time of hatching (Fig. 3). In the adult, CYP IA expression has been shown to occur in response to polyaromatic hydrocarbons (PAH's) such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) binding to the aromatic hydrocarbon receptor (AhR) (Hahn *et al.*, 1988; Whitlock, 1993; Hahn and Karchner, 1995). HCB injected embryos showed a positive response when stained for CYP IA. The endothelium of the heart stained positively for CYP IA gene activity(Fig. 3A). The endothelium of peripheral blood vessels of the brain and capillaries in the gills also stained (Fig. 3B). Benzene and retinoic acid exposed fish and uninjected controls were *negative* for CYP IA expression.

At hatching many other medaka organ systems are well developed. Gut derivatives such as the liver and pancreas are well established and differentiation of the islet cells of the pancreas into alpha and delta cells, with their characteristic granules, can be readily demonstrated at this time (Fig. 4).

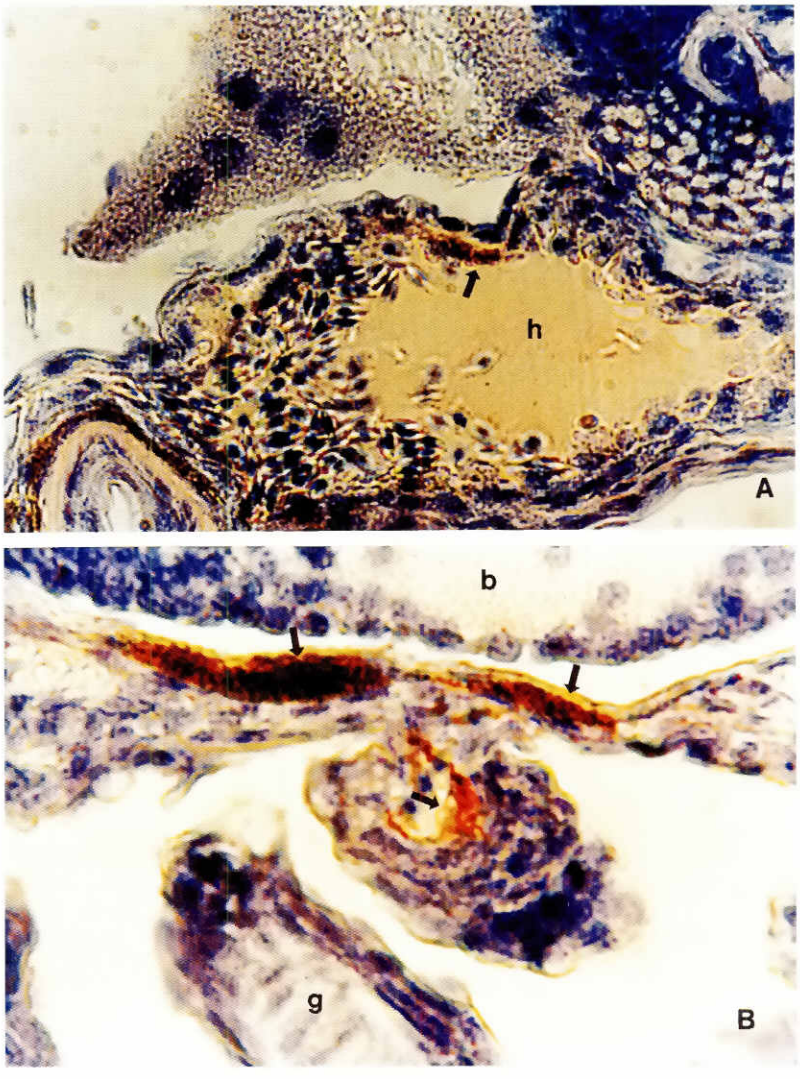

Fig. 3. Photomicrograph of the heart of another HCB-injected medaka embryo fixed at hatching. (A) *Immunohistochemically stained for CYP IA1 activation. Note endocardium staining (arrow). h, heart. Lumen filled with nucleated red blood cells, x450.* **(B)** *Photomicrograph of HCB injected medaka embryo fixed at hatching. Sagittal section immunohistochemically stained for CYP IA1 expression. Antibody staining (arrows) visible in the endothelial cells of blood vessels. b, brain; g, gill. See text for details. (x450).*

Zebrafish dechorionation and static exposure

Dechorionated zebrafish embryos exposed to TCDD for 30 minutes during the gastrula stage also developed severe cardiovascular defects (Fig. 5A,B; Table 3). These embryos had heart defects similar to those seen in medaka embryos microinjected with TCDD (Table 2). In those zebrafish embryos with cardiovascular defects, the heart remained tube-like with little musculature and a greatly reduced rate of contraction. As in medaka the effects on zebrafish heart development were dose-dependent (Table 3). Abnormal development was not, however, limited to the heart.

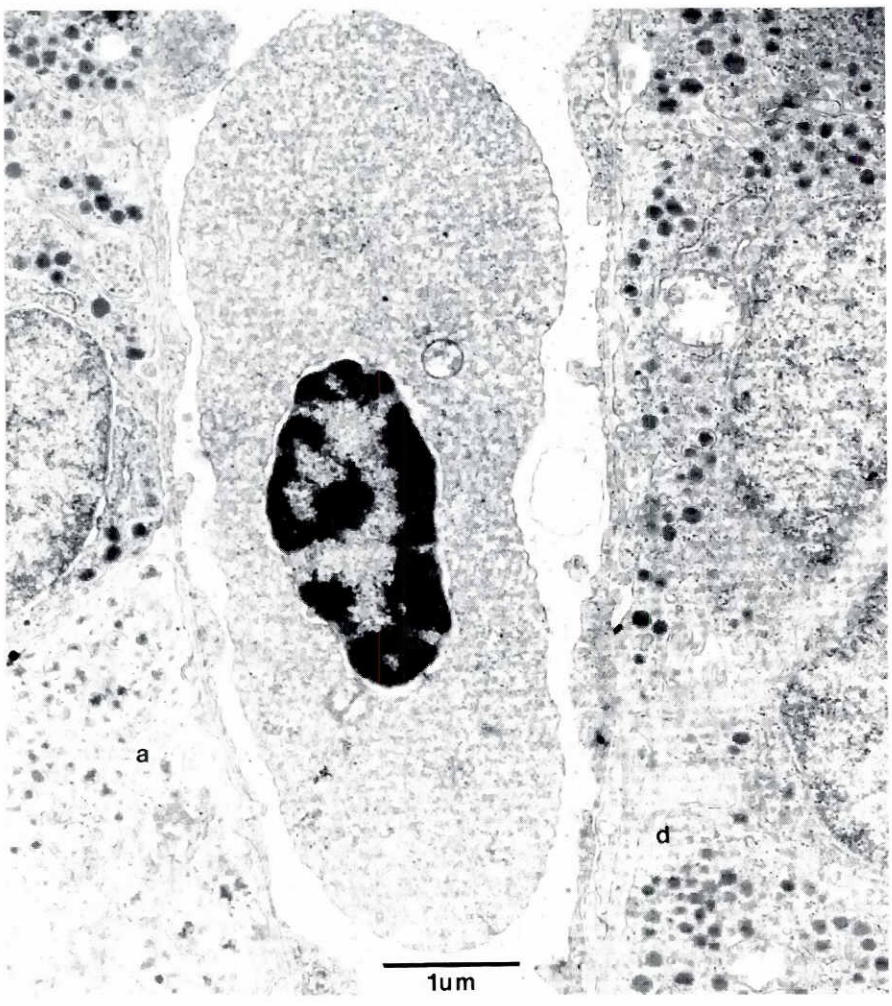

Fig. 4. Transmission electron micrograph of pancreatic islet cells surrounding a capillary of a newly hatched control medaka. *Differentiated alpha- and delta-cells are visible. A single nucleated red blood cell fills the lumen of the capillary. a, alpha cell; d, delta cell.*

Many of the heart affected embryos exposed to TCDD displayed other deformities. These included retarded craniofacial development, a lack of eye pigment, retarded kidney development, and several skeletal malformation. Furthermore, many of the TCDD-exposed embryos failed to survive to swim-up stage despite the appearance of normal development.

Because TCDD is a strong AhR agonist, zebrafish embryos exposed to this pollutant were tested for CYP IA expression. Immunohistochemical analysis of TCDD-exposed zebrafish embryos revealed early activation of this cytochrome pathway. Gut and liver stained positively for CYP IA activity (Fig. 5c). Positive staining was present in the animals' vascular endothelium which indicated that a by-product of TCDD metabolism might be involved in causing the cardiovascular abnormalities.

Cardiovascular defects, similar to those caused by HCB and TCDD, were also observed in zebrafish embryos exposed to toluene or benzene, two AhR non-agonists. A tube-like heart, absence of circulating blood, and pericardial edema characterized the toluene or benzene cardiovascular defects (Fig. 6). Like TCDD, toluene or benzene affected cardiovascular development in a dose-dependent manner (Tables 4,5). Additional deformities associated with exposure to toluene or benzene included anterior-posterior axis truncation, lack of cranial structures, and poor kidney differentiation. Survival of these embryos was also dose-dependent. Many embryos lacked gross abnormalities and appeared unaffected; however, they failed to survive to swim-up stage (96 hpf).

Mixtures

Embryos exposed to a combination of TCDD and benzene developed cardiovascular and other defects similar to those abnormalities seen in embryos exposed to the individual compounds (Fig. 5B). However, the mixture of TCDD and benzene (Table 6) was more toxic that either chemical by itself (compare Tables 3,5,6). The LC_{50} of the TCDD/benzene mixture was approximately 0.01 ppm compared with TCDD's LC_{50} of 0.1 ppm or benzene's LC_{50} of 0.05 ppm.

Similarly the combination of toluene and benzene acted synergistically. Those embryos exposed to a toluene and benzene mixture developed cardiovascular abnormalities and other defects similar to those abnormalities seen in embryos exposed to the individual compounds (Table 7). However, the mixture of toluene and benzene was more toxic to the embryos than the individual chemicals (compare Tables 4,5,7). The LC_{50} of the toluene and benzene mixture was approximately 0.005 ppm while toluene's LC_{50} was 0.1 ppm and benzene's LC_{50} was 0.05 ppm. Thus benzene interacted synergistically to appreciably augment the lethality of Toluene as it did with the TCDD/benzene mixture.

Discussion

Our experiments have shown that microinjection into the perivitelline space of developing medaka and zebrafish embryos provided a useful method for observing the effects of chemicals on early development. When compared to the results of injections into the yolk sac (Black *et al.*, 1985) or directly into the developing embryo (Metcalfe and Sonstegard, 1985), our injected embryos had a far higher survival rate. In medaka embryos microinjected with HCB during the late gastrula stage of development, control injections of vegetable oil resulted in low mortality (Tables 1,2). Walker *et al.* developed a yolk sac injection technique whereby they employed Superglue^{R} to seal the injection site. Although the trout, *Onchorhynchus mykiss*, egg retained 92-97% of the injected TCDD dose, the controls' mortality was 73% (Walker *et al.*, 1992). Thus, yolk sac injection resulted in 30-70% mortality in controls and as many as 50% of the fish died when injecting directly into the embryo (Black *et al.*, 1985; Metcalfe and Sonstegard, 1985). Observation of our microinjection effects was possible because of the transparency of both the chorion and tissues of the medaka and

TABLE 3

SURVIVAL OF DECHORIONATED ZEBRAFISH AFTER 30 min STATIC EXPOSURE TO 2,3,7,8-TETRACHLORO-DIBENZO-P-DIOXIN (TCDD)

	N	24 h	48 h	Heart defects*	72 h	96 h (Swim up)
1 ppm TCDD	20	12 (60%)	11 (55%)	2/11	10 (50%)	9 (45%)
0.1 ppm TCDD	20	14 (70%)	12 (60%)	1/12	11 (55%)	11 (55%)
0.01 ppm TCDD	16	12 (75%)	11 (69%)	0	11 (69%)	11 (69%)
0.001 ppm TCDD	16	12 (75%)	12 (75%)	0	12 (75%)	12 (75%)
0.0001 ppm TCDD	16	14 (88%)	14 (88%)	0	13 (81%)	13 (81%)

Zebrafish dechorionated and exposed at the gastrula stage of development to different concentrations of TCDD. *Heart defects in 48 hour survivors.

zebrafish embryos. Placement of a droplet of the injectate adjacent to the developing yolk sac vasculature provided an efficient means of delivering a test chemical to an embryo. Such direct contact is impossible in ambient embryo exposure to toxins because of the resistance of the chorion. The results of ambient exposure, long used for determining dose response to a toxin, may now be "fine tuned" through this microinjection technique. Most dose/response curves to toxins are plotted with relatively high concentrations of toxin and then the low portion of the curve is extrapolated down through the "zero point". By employing this method of microinjection the effects of very low doses of a toxin may be directly observed.

Medaka embryos exposed at late gastrula stage (stage 16) developed cardiovascular defects. Normal embryo heart beat began at stage 23. However, if embryos were injected during either earlier development, e.g. blastula stage (stage 10), or at a later stage, liver rudiment formation (stage 32), then heart defects did not occur. This indicated that there was a window of opportunity in which these chemicals may affect heart differentiation. It has been further noted that medaka embryos injected with HCB or TCDD developed these cardiovascular defects in a dose-dependent manner(Tables 1,2). More work must be done to determine the stage(s) at which HCB and benzene affect heart development, and how they affect heart differentiation.

HCB and benzene have been shown through these assays to cause circulatory defects common to several aromatic compounds: toluene, carbaryl, parathion, tolbutamide, dinitrophenol, and 2,4,5-trichlorophenoxyacetic acid (Smithberg, 1962; Wilde and Crawford, 1966; Weis and Weis, 1974; Schreiweis and Murray, 1976). Formation of a thin tube-like heart without visible circulation and with the presence of pericardial edema were common results of exposure to each of these chemicals. These aromatic compounds may form a family of toxins which all affect heart development via a common mechanism(s).

By employing the above microinjection technique we have shown that HCB and benzene produced characteristic heart defects (Mizell *et al.*, 1995). Exposure to retinoic acid was useful for

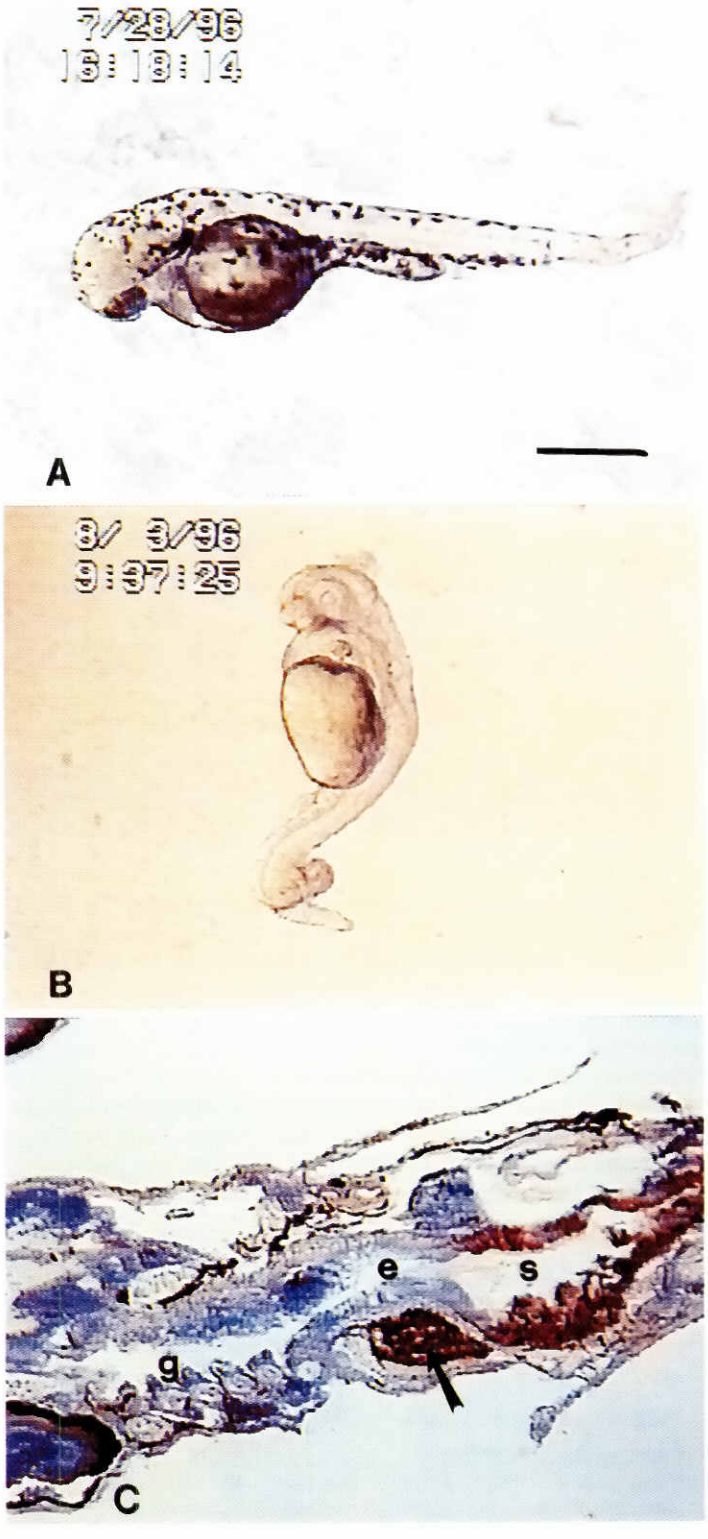

Fig. 5. Video prints of TCDD-treated zebrafish. Embryos were exposed for 30 min at gastrula stage (5.25 hpf), and shown at 48 hpf. (A) *Embryo exposed to 1 ppm TCDD. Note reduced head structures, pericardial edema, and retarded development. This embryo lacked blood circulation and the heart rate was reduced. Bar, 0.5 mm.* **(B)** *Embryo exposed to a mixture of TCDD and benzene at a concentration of 1 ppm. Note retarded cranial development, lack of pigment, malformation of distal structures, and pericardial edema.* **(C)** *Video print of parasagittal histological section of zebrafish exposed to 100 ppm of TCDD. Stained for Cytochrome P450(CYP) IA activation. Cells lining the stomach (s) stain positively, whereas the cells lining the gills (g) and esophagus (e) did not stain. The liver (arrow) also demonstrated CYP IA activation. x120.*

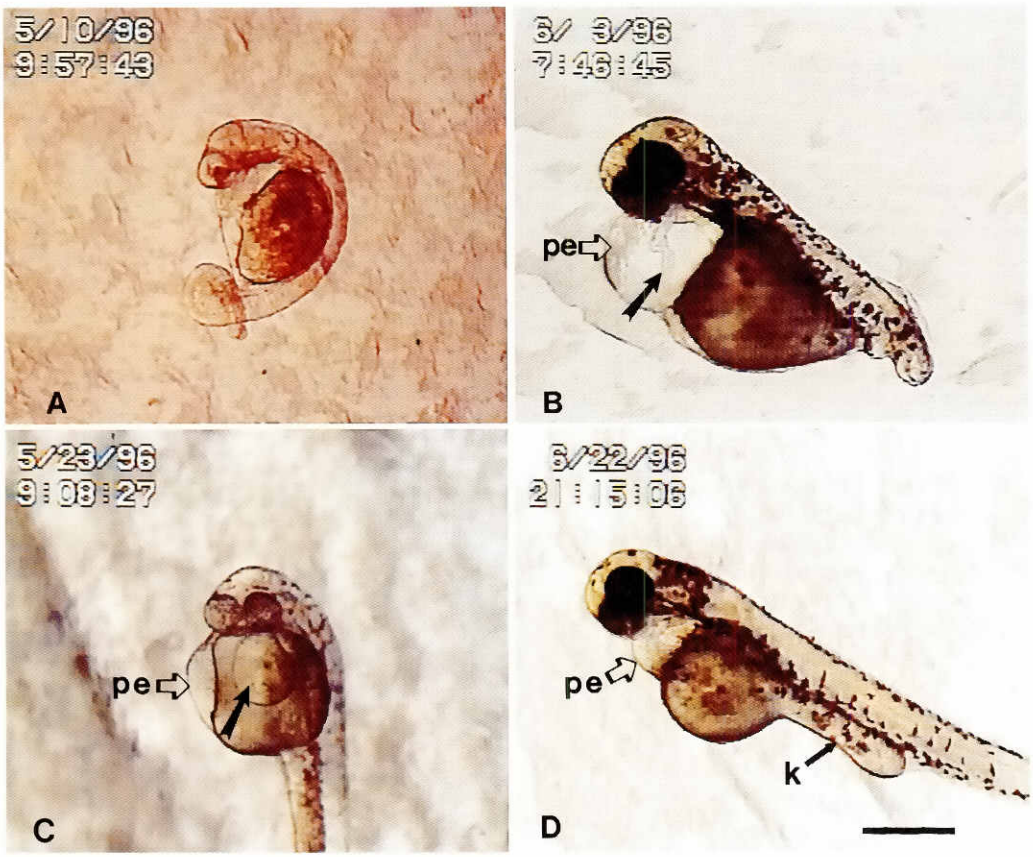

Fig. 6. Video prints of dechorionated benzene and toluene-treated zebrafish shown at 48 hpf. Embryos were exposed for 30 min at gastrula stage (5.25 hpf). (A) *Embryo exposed to 100 ppm toluene. Note retarded development, extreme microcephally, lack of cranial structures, lack of pigmentation, pericardial edema, minimal kidney differentiation, and malformation of distal structures.* **(B)** *Embryo exposed to 1 ppm toluene. Note truncated anterior/posterior axis, extreme pericardial edema (pe, open arrow) enclosing a poorly differentiated tube-heart (large arrow) also note lack of kidney differentiation.* **(C)** *Embryo exposed to 1 ppm benzene. Note enlarged heart chamber (large arrow), pericardial edema (PE, arrow) minimal pigmentation, and generally poor development.* **(D)** *Embryo exposed to a 0.001 ppm mixture of toluene and benzene. The embryos has reduced cranial differentiation, and edema, especially pericardial edema (pe, open arrow) enclosing a tube-heart with a reduced heart rate and lacking circulation. Kidney (k) Bar, 0.5 mm.*

comparing heart defects. Investigators had shown that heart defects caused by retinoic acid were the result of a dose-dependent deletion of cardiac tissue (Brockes, 1989; Stainier and Fishman, 1992). The defects caused by RA were visibly distinct from those caused by HCB or benzene. Embryos affected by RA lacked pericardial edema, had a normal heart rate and experienced truncation and deletion of portions of the heart: first the bulbis cordis, then the ventricle and so on. However, in embryos exposed to HCB or benzene the entire heart was affected simultaneously. The most severe effect resulting in a thin tube-like heart surrounded by pericardial edema. These differences along with HCB's and benzene's reduction of heart rate indicated that retinoic acid and aromatic compounds probably affect heart differentiation by different mechanism(s).

Medaka embryos required two weeks development to hatch and did not survive gastrula dechorionation. The medaka embryo has a small perivitelline space and the 40 nl microinjected droplet was not diluted appreciably by the perivitelline fluid (see Fig. 1B). However, the relatively large perivitelline space of the zebrafish embryo diluted the 40 nl droplet. Thus, while microinjection of 1 ppm TCDD into the medaka gastrula resulted in only 34% survival (Table 2), a similar microinjected dose of TCDD into the zebrafish

TABLE 4

SURVIVAL OF DECHORIONATED ZEBRAFISH AFTER 30 min STATIC EXPOSURE TO TOLUENE

	N	24 h	48 h	Heart defects*	72 h	96 h (Swim up)
100 ppm toluene	10	2 (20%)	2 (20%)	2/2	2 (20%)	0
10 ppm toluene	11	5 (45%)	5 (45%)	3/5	5 (45%)	2 (18%)
1 ppm toluene	36	25 (69%)	21 (58%)	1/21	20 (56%)	17 (47%)
0.1 ppm toluene	20	12 (60%)	11 (55%)	1/11	11 (55%)	10 (50%)
0.01 ppm toluene	20	15 (75%)	15 (75%)	1/15	14 (70%)	14 (70%)
0.001 ppm toluene	16	14 (88%)	12 (75%)	0	12 (75%)	12 (75%)
0.0001 ppm toluene	16	13 (81%)	13 (81%)	0	13 (81%)	13 (81%)

Zebrafish dechorionated and exposed at the gastrula stage of development to different concentrations of toluene. *Heart defects of 48 hour survivors.

TABLE 5

SURVIVAL OF DECHORIONATED ZEBRAFISH AFTER 30 min STATIC EXPOSURE TO BENZENE

	N	24 h	48 h	Heart defects*	72 h	96 h (Swim up)
10 ppm benzene	12	4 (33%)	3 (25%)	3/3	2 (17%)	0
1 ppm benzene	36	17 (47%)	11 (31%)	1/11	10 (28%)	9 (25%)
0.1 ppm benzene	20	9 (45%)	9 (45%)	1/9	9 (45%)	8 (40%)
0.01 ppm benzene	20	12 (60%)	12 (60%)	0	12 (60%)	12 (60%)
0.001 ppm benzene	16	12 (75%)	11 (69%)	0	11 (69%)	11 (69%)
0.0001 ppm benzene	16	15 (94 %)	14 (88%)	0	12 (75%)	12 (75%)
Control	20	16 (80%)	16 (80%)	0	16 (80%)	16 (80%)

Zebrafish dechorionated and exposed at the gastrula stage of development to different concentrations of benzene. *Heart defects of 48 hour survivors.

TABLE 6

SURVIVAL OF DECHORIONATED ZEBRAFISH AFTER 30 min STATIC EXPOSURE TO A MIXTURE OF TCDD AND BENZENE

	N	24 h	48 h	Heart defects*	72 h	96 h (Swim up)
1 ppm TCDD/benz.	16	6 (38%)	4 (25%)	2/4	4 (25%)	2 (13%)
0.1 ppm TCDD/benz.	16	10 (63%)	8 (50%)	1/8	6 (38%)	5 (31%)
0.01 ppm TCDD/benz.	16	16 (100%)	16 (100%)	0	11 (69%)	8 (50%)
0.001 ppm TCDD/benz.	16	13 (81%)	10 (63%)	0	10 (63%)	10 (63%)
0.0001 ppm TCDD/benz.	16	14 (88%)	12 (75%)	0	12 (75%)	12 (75%)

Zebrafish dechorionated and exposed at the gastrula stage of development to different concentrations of a 50-50 mixture of TCDD and Benzene. *Heart defects in 48 hour survivors.

TABLE 7

SURVIVAL OF DECHORIONATED ZEBRAFISH AFTER STATIC 30 min EXPOSURE TO A MIXTURE OF TOLUENE AND BENZENE

	N	24 h	48 h	Heart defects*	72 h	96 h (Swim up)
10 ppm	12	3 (25%)	3 (25%)	2/3	3 (25%)	1 (8%)
1 ppm	12	3 (25%)	3 (25%)	0/3	2 (17%)	2 (17%)
0.1 ppm	22	8 (36%)	8 (36%)	2/8	8 (36%)	6 (27%)
0.01 ppm	22	16 (73%)	14 (64%)	4/14	14 (64%)	10 (45%)
0.001 ppm	22	16 (73%)	15 (68%)	1/15	15 (68%)	14 (64%)
0.0001 ppm	22	18 (82%)	17 (77%)	1/17	16 (73%)	16 (73%)
Control	9	8 (89%)	8 (89%)	0	8 (89%)	8 (89%)

Zebrafish embryos dechorionated and exposed at the gastrula stage of development to various concentration of 50/50 mixtures of benzene and toluene. *Heart defects of 48 hour survivors.

gastrula had no effect on survival. Furthermore, a zebrafish dose-response relationship could not be demonstrated with low concentrations of other *microinjected* pollutants. However, zebrafish embryos developed very rapidly and only required 2-3 days to hatch. Moreover, zebrafish gastrulae were readily dechorionated and continued normal development. Mortality of dechorionated zebrafish embryos exposed for 30 min to low concentrations of TCDD was similar to the mortality of microinjected medaka and resulted in a dose-response relationship (Table 3).

Thus medaka embryos were microinjected with low concentrations of pollutants, whereas zebrafish embryos were effectively tested by dechorionation. In both procedures the embryos were directly tested without the intervention of the chorion

Like the medaka embryo, dechorionated zebrafish embryos were exposed to TCDD, toluene, benzene, or a mixture of these chemicals at the gastrula stage and developed severe cardiovascular abnormalities. The embryos had tube-like hearts, reduced heart rates, pericardial edema, and lacked blood circulation just as medaka embryos which were exposed to HCB or TCDD. This may mean that these pollutants affected embryonic heart development via the same mechanism(s) as other aromatics (Smithberg, 1962; Wilde and Crawford, 1966; Weis and Weis, 1974; Schreiweis and Murray, 1976).

In an effort to uncover the mechanism(s) of heart malformation caused by aromatic compounds, the activation of CYP IA in HCB- and TCDD-exposed embryos were tested. Using a sensitive fish monoclonal antibody to cytochrome P450, the endothelium of blood vessels and gut cells stained positively for CYP IA expression, in embryos exposed to HCB or TCDD. Activation of CYP IA in response to these pollutants may or may not be the first step toward causing heart malformation. However, CYP IA expression in these newly hatched fish exposed to HCB or TCDD indicated that the cytochrome metabolic pathways were already functional during medaka and zebrafish early embryonic development and may be involved in generation of the heart defect.

Mixtures

In our experiments benzene was the most lethal of the individual pollutants tested on embryonic development. Contamination of aquatic environments usually does not occur from single chemical pollutants, but from mixtures. To simulate environmental conditions found in the wild, zebrafish embryos were exposed to simple combinations of two pollutants. Embryos were exposed to mixtures

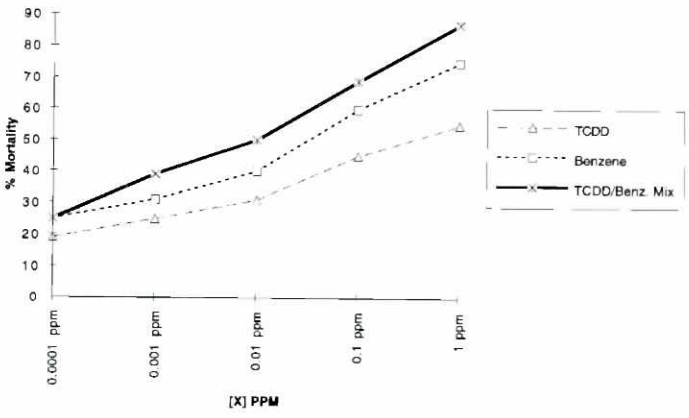

Fig. 7. Dose response of dechorionated zebrafish exposed to TCDD, benzene, and mixture of both.

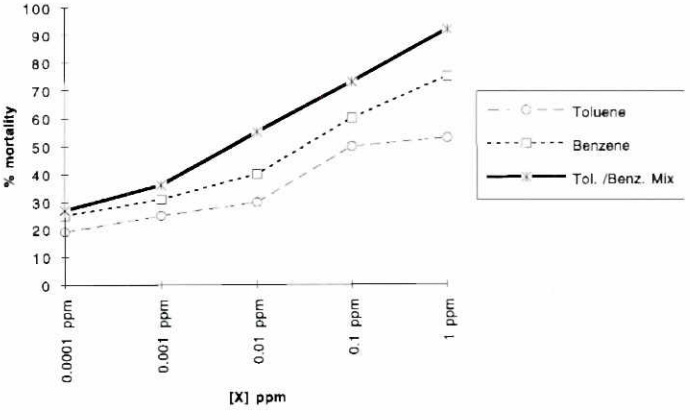

Fig. 8. Dose response of dechorionated zebrafish exposed to toluene, benzene, and a mixture of both.

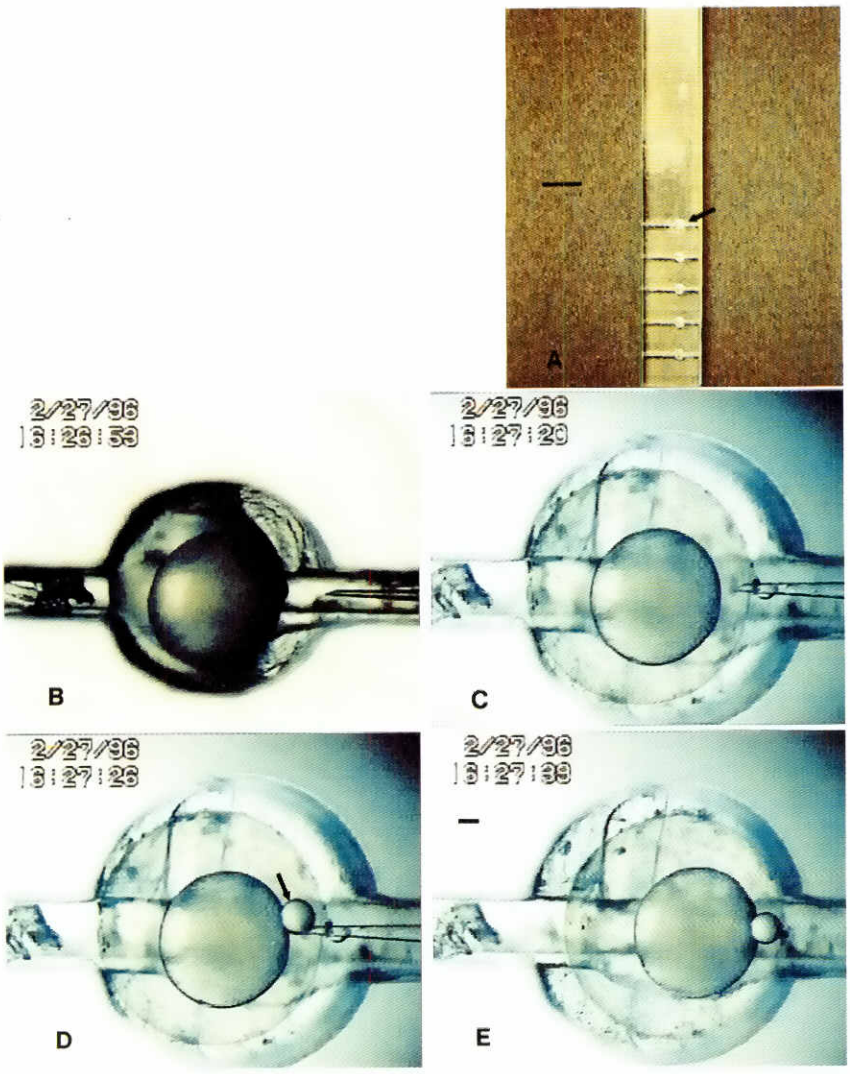

Fig. 9. Video prints of zebrafish microinjection sequence, late gastrula stage. (A) *Plastic injection slide. Five wells (arrow) hold the eggs in place and the channels provide a path for the micropipette. Bar, 1.0 cm.* **(B)** *Needle positioned in channel for penetrating the chorion. Both incident and transmitted light used to position the needle.* **(C)** *Needle immediately after penetration of chorion. Illuminated only by transmitted light (incident light extinguished). Tip of needle positioned in perivitelline space.* **(D)** *Droplet (arrow) of 40 nl of toxin in oil delivered to the perivitelline space. The droplet was positioned where it would be in contact with the developing yolk sac vasculature.* **(E)** *Micropipette withdrawn from the chorion and removed from the field of view. Time in the upper left corner of each figure recorded in seconds. Bar, 0.1 mm.*

of TCDD and benzene (AhR agonist/non-agonist), or toluene and benzene (both AhR non-agonists). Benzene also reacted synergistically with TCDD or toluene to produce a mixture consistently far more lethal than the individual pollutants (Figs. 7,8).

The chorion of the medaka and zebrafish embryo acts as a protective barrier against pollutants, bacteria, and other pathogens. By directly exposing these embryos to toxins, either by perivitelline space microinjection or by dechorionation, the effects of low doses of a pollutant on embryonic development were observed.

The results of a decade of zebrafish saturation mutagenesis research have recently been published in the journal *Development* (Vol. 123, 1996). The genetic screens for mutations affecting early zebrafish embryogenesis have already uncovered many of the fundamental genes of vertebrate development and differentiation, and have the promise of answering questions that *Drosophila* studies could not answer. The ability to perform genetic analysis combined with the rapidly increasing number of developmental genes that are being characterized make the zebrafish system especially favorable as a pollution biomarker. Therefore the zebrafish dechorionation exposure technique, Micro Intrachorionic Zebrafish Embryo Live Laboratory test, could eventually be used to uncover the genetic and molecular basis for the defects produced by toxins examined in this investigation.

These methods of exposure have relevance in mammalian as well as aquatic health. The placenta, like the chorion, is a barrier to many pathogens. However, aromatic compounds like HCB, TCDD, toluene, and benzene are capable of crossing the placental barrier and affecting fetal development (Roper, 1990). Direct exposure of fish embryos to low doses of toxins can provide realistic insight to the risks these pollutants pose to mammalian embryos. Thus the medaka or zebrafish embryos can be useful as sentinels for detecting small amounts of toxins, like a canary in a coal mine.

Materials and Methods

Medaka and zebrafish embryos were collected from our breeding colonies daily. The breeding fish were maintained at 26°C, with a 14 h light/ 10 h dark cycle. Eggs were laid within 1 h after the light period began. Zebrafish females dropped their eggs to the marble-covered bottom of the tank; embryos were then siphoned from under the marbles (see Westerfield, 1995 for natural spawning procedures and husbandry).

Medaka females observed to have produced eggs were separated from the main population into a medium sized bowl. When a medaka female lays her eggs, they remain attached via filaments and the eggs typically adhere to the anal fin. Eggs were removed from the anal fin with a Pasteur pipette. All eggs were collected within 2 h of the beginning of the light cycle (approximately 1 h after fertilization). Unfertilized eggs were discarded and fertilized eggs were transferred to a Petri dish containing embryo rearing solution (7.50 g NaCl, 0.20 g KCl, 0.20 g $CaCl_2$, 0.1 M $NaHCO_3$, in 1000 ml distilled H_2O). Embryos developed under constant fluorescent light at 26°C (see Kirchen and West, 1969 for further details).

Perivitelline space microinjection

The usual mammalian egg "holding pipette" could not be used with the rigid medaka chorion: ordinary negative pressure did not hold the egg for needle penetration; and additional negative pressure would deform and injure the medaka egg. Medaka or zebrafish gastrula stage embryos were placed in the plastic injection slide specially machined for fish embryo microinjection (Fig. 9A). The slide has five wells (arrow) each 1.3 mm in diameter and 1.2 mm deep. Each well is bisected by a channel cut across the entire face of the slide. An egg was placed in each well and the

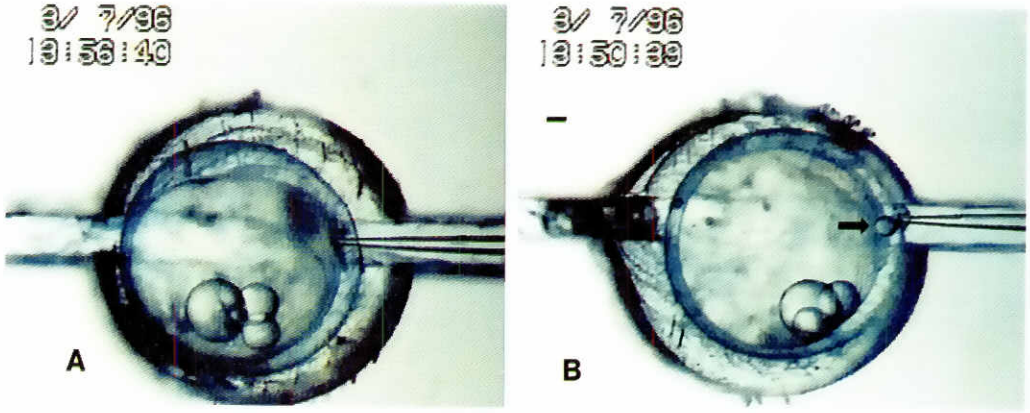

Fig. 10. Video prints of medaka injection sequence, late gastrula stage. (A) *Needle enters medaka chorion. The left side of the well steadies the embryo allowing penetration.* **(B)** *Microinjection of droplet (arrow). Now egg moves to opposite wall of the well which permits smooth retraction of the needle. Native oil droplets in the yolk sac are seen at 6 o'clock. Bar, 0.1 mm.*

previously prepared micropipette was brought into the channel by focusing on it and the surface of the slide. The micropipette was lowered to mid channel, and thus the needle approached the egg on a level plane. This method facilitated proper alignment of the needle and the chorion of the embryo. If the needle was not properly aligned, it would not strike the chorion squarely and might even tear the chorion, killing the embryo. If it deviated too much from the eggs' equator, it would "roll the egg".

Zebrafish microinjection

After the embryo was positioned in the well, the micropipette was brought into the channel. Excess water was removed from the well. During this time the embryo in the dried well was illuminated with incident and transmitted light; this allowed positioning of the needle against the chorion (Fig. 9B). The needle was then advanced through the chorion and into the perivitelline space by the micromanipulator fine adjustment. Balance pressure within the needle was then increased to bring the injectate to the tip of the needle. Once the needle had penetrated the chorion, the egg was covered with water and the incident light was extinguished; this provided the best visualization through the transparent embryo and chorion (Fig. 9C). After penetrating the chorion the balance pressure was increased so that any perivitelline fluid which had entered the needle would be returned to the perivitelline space (during penetration a positive pressure was exerted by the perivitelline fluid). The droplet (arrow) was then injected and positioned in the large perivitelline space of the zebrafish gastrula (Fig. 9D). As the needle was removed from the chorion, the balance pressure was reduced so that neither excess injectate would enter the egg nor would the contents of the micropipette be expelled after removal from the chorion (Fig. 9E).

Medaka microinjection

By using the plastic injection slide the egg was held securely during needle penetration and removal. As the needle pushed against the chorion, the egg was pressed against the far side of the well allowing the needle to puncture the chorion (Fig. 10A). Forty nanoliters were delivered to the small perivitelline space of the medaka gastrula. As the needle was removed after the droplet (arrow) had been placed in the perivitelline space, the egg was pulled against the near wall of the well permitting smooth extraction of the needle from the chorion (Fig. 10B).

Each injection was set to deliver a droplet volume of 40 nanoliters (nl) to the perivitelline space of the embryo. HCB, TCDD, toluene, and benzene were injected at a wide range of concentrations. Retinoic acid was also injected as a positive control for affecting heart development. Vegetable oil lacking HCB, benzene, or RA was used as a negative control. Over three thousand embryos were injected for these experiments.

Medaka and zebrafish embryos were injected upon reaching late gastrula stage. The plastic microinjection slide (specially machined for these experiments by machinist Frank Sylvia of the Marine Biological Laboratory, Woods Hole, MA, USA) held embryos in position for injection. Micropipette needles were fabricated according to techniques described by Brown and Flaming (1986). A standard borosilicate glass micropipette with a tip diameter of 2.5 μm was used for injection. The needle was "pulled" on a horizontal microforge from a micropipette with an outside diameter of 1.0 mm and an inside diameter of 0.5 mm (Sutter, Cat.# BF100-50-10). To pull the needle we used a P-97 Flaming/Brown micropipette puller (Sutter).

Microinjection was carried out on a Leica Wild M8 stereo microscope fitted with a 360° rotating, polarizing stage. Injection was performed with a Narishige IM 300 microinjector in conjunction with a Leica M micromanipulator. High purity nitrogen (Air Products) was used to deliver a droplet of 40 nl to the perivitelline space of each embryo. The range of injected concentrations varied from 1000 parts per million (ppm) to 1 part per billion (ppb) of hexachlorobenzene (Sigma) dissolved in vegetable oil. Benzene (Sigma) was injected at a range of concentrations which varied from 500 ppm to 6 ppm dissolved in vegetable oil. TCDD (Ultra Scientific) was injected at a range of concentrations which varied from 10 ppm to 0.0001 ppm dissolved in vegetable oil. Retinoic acid (all trans Sigma, Cat# R2625) was injected at 3000 ppm dissolved in oil.

After injection each embryo was placed in a separate chamber in a twenty-four well (3.25 ml vol./well) plastic cell culture plate (Costar). Approximately 2 ml of embryo rearing solution was added to each chamber. The transparent embryo enabled rapid daily monitoring in the chamber. Deviations from normal development were video-recorded. Upon hatching fry were sacrificed and prepared for histopathological examination: after fixation in 10% N-buffered formalin and dehydration in a graded alcohol series, these fish were embedded in paraffin, and sagittally sectioned. The slides were either stained with hematoxylin and eosin or prepared for immunohistochemistry.

Zebrafish dechorionation and static exposure

Zebrafish embryos were collected each morning, sorted, and staged. At gastrulation [5.25 h post fertilization (hpf)] the embryos were placed for 20 sec in a 1.6 ug/ml solution of trypsin (Sigma), made up in embryo rearing solution [(ERS), Carolina Biological]. The embryos were then rinsed three times in ERS. This treatment softened the chorion sufficiently that it could be removed with a pair of forceps (see Fig. 11). The dechorionated embryos were placed in a Petri dish lined with Parafilm (to prevent sticking). A 250 ul droplet of the various chemical pollutants was placed in a Petri dish (100 mm) lined with Parafilm, and a single embryo was placed in each droplet. After 30 min of exposure, the embryos were removed from the droplets, rinsed 3 times in ERS, and transferred into Parafilm-lined Petri dishes half-filled with ERS. The embryos were examined immediately after removal from the pollutant, and every 24 h until they reached the swim-up stage (96 hpf). Deviations from normal development were recorded on video tape. When the swim-up stage was reached, a final video recording was made and the embryos were killed, fixed and stained with hematoxylin and eosin for histological examination or immunohistochemistry.

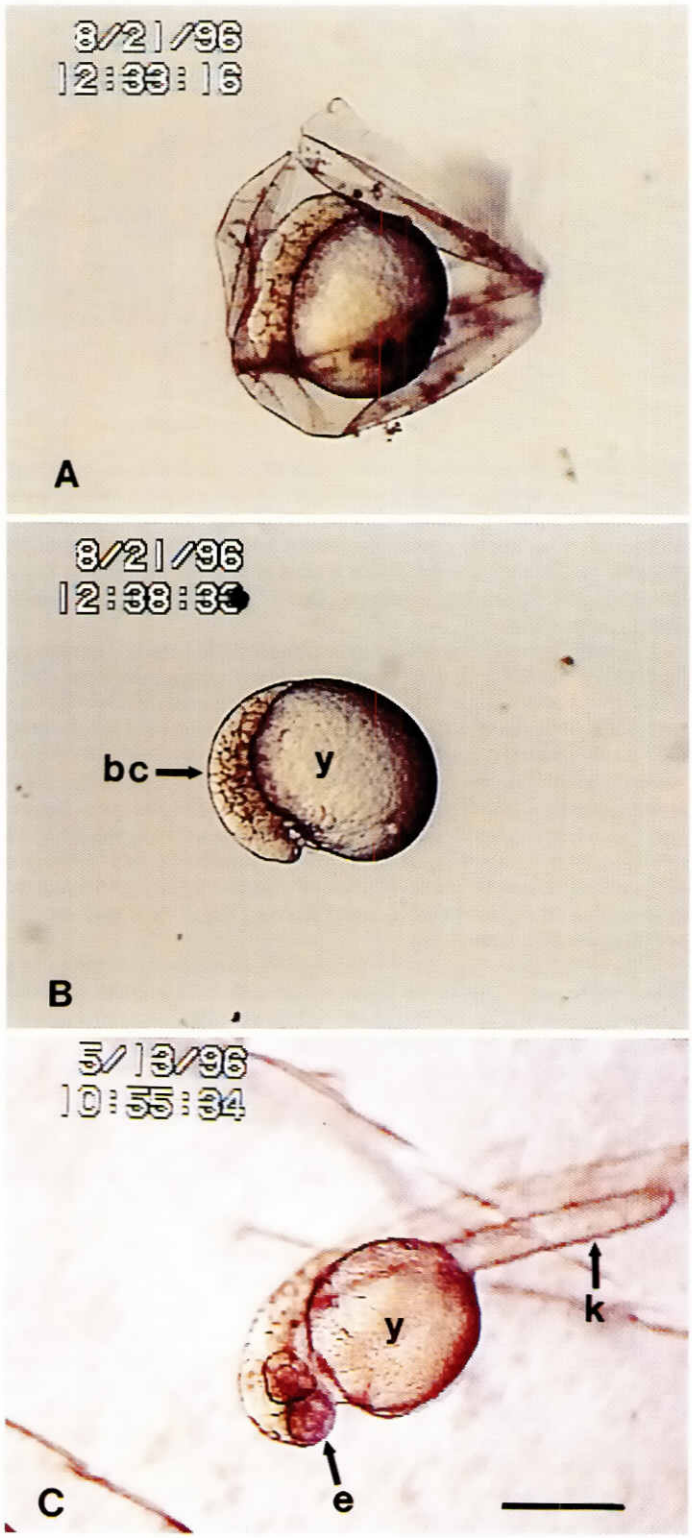

Fig. 11. Video prints of zebrafish embryo dechorionation and subsequent development (controls). (A) *Zebrafish blastula with chorion partially removed.* **(B)** *Same embryo completely removed from chorion; blastodisc cells (bc) on the surface of the yolk(y).* **(C)** *Control embryo 42 h post-fertilization (hpf), dechorionated at 5.25 hpf. e, eye; k, kidney; y, yolk. Bar, 0.5 mm.*

We followed this procedure in exposing embryos to the following chemicals: benzene, toluene, and a mixture of the two; TCDD and a mixture of TCDD and benzene. The concentrations of the benzene and toluene in the ERS ranged from 1 part per million (ppm) to 0.0001 ppm; mixtures of benzene and toluene concentrations were in a similar range; as were TCDD, and TCDD/benzene. e.g. Equal volumes of equal concentrations of benzene and toluene were combined to provide a series of 50-50 mixtures. Dechorionated gastrula-stage embryos were immersed in the different pollutant concentrations for 30 min, and the lethal concentration that killed 50% of the animals (LC^{50}) was determined.

Immunohistochemistry

Immunohistochemistry testing for expression of CYPIA consisted of using an indirect peroxidase labeling method and a monoclonal antibody prepared against the fish, *Stenotomus chrysops* (scup), P450IA (Mab 1-12-3) (Smolowitz, *et al.*, 1991). The small size of these newly hatched fish allowed the entire animal to be mounted on two slides. The antibody staining procedure was carried out in Shandon-Lipshaw special slide staining racks (Cat.# 7331017). Specific binding of the antibody to its substrate was then observed by using an avidin-biotin complex (ABC) technique to provide sensitive staining. We used the Signet immunohistochemical staining kit (Level 2 Ultra Streptavidin Detection System (Signet Cat.# 2248)), and the modified procedure employed by Smolowitz *et al.* (1991). These slides were then read and graded for stain intensity.

Acknowledgments

We thank Rajesh Katayani for excellent technical assistance and diligent care of our medaka and zebrafish colonies; and Roxanna Smolowitz for her guidance in utilizing Mab 1-12-3 in immunohistochemistry. We also thank John Stegeman for providing the monoclonal antibody, Mab 1-12-3 and for the productive discussions and suggestions concerning cytochrome P450 and the Ah receptor. We are pleased to acknowledge the assistance of others in our laboratory: Danielle Dienert, Charles Dugal, Donovan Fuqua; Karl Linderman, Melissa Mortenson, Brian Moss, and Kim Schussler. Kim Schussler provided Figure 4. We also gratefully acknowledge the superb assistance of the library and library staff of the MBL, Woods Hole, MA, USA. These studies were supported by Department of Energy Grant: DoE EMIFG0193E; Department of Defense/CBR Grant: CBR 93DNA-2; and United States Environmental Protection Agency Grant: CR822766.

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