

The Impact on Reproduction of an Orally Administered Mixture of Selected PCBs in Zebrafish (*Danio rerio*)

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Abstract. Zebrafish (*Danio rerio*) were orally exposed to a mixture of 20 PCBs in three different dose levels (0.008, 0.08, and 0.4 µg of each congener per gram of freeze-dried chironomids). Generally, the PCBs accumulated in a dose-related manner. After 13 weeks of exposure body, liver, and ovary weights, as well as the liver and ovary somatic index, were significantly lower in exposed groups. In addition, the PCB mixture was an effective inducer of hepatic EROD activity. The reproduction study performed with exposed females and unexposed males after 9 weeks revealed that median survival time for larvae was only 7.7 days in the high-dose group as compared with 14 days in controls. Furthermore, egg production was reduced in all three groups exposed. No differences in hatching frequency or median hatching time were recorded. Histologically, females in both the intermediate and high-dose groups contained a reduced number of mature oocytes. The present study demonstrates that the potency of the mixture of selected PCBs induces hepatic EROD activity and has a clearly negative effect on zebrafish reproduction.

Several studies indicate that PCBs (polychlorinated biphenyls) affect reproductive success in wild fish populations. Lake trout (*Salvelinus namaycush*) from the Great Lakes have been reported to suffer from decreased hatching success and high embryonic mortality (Mac *et al.* 1993). Hansen *et al.* (1985) reported decreased viable hatch in Baltic herring (*Clupea harengus*) and von Westernhagen *et al.* (1981) found impaired egg development and survival in Baltic flounder (*Platichthys flesus*). All of these reproductive failures have been associated with high PCB levels in ovaries or eggs. Under laboratory conditions, several studies have been conducted with commercial PCB mixtures or single PCB congeners to evaluate the effects on reproduction in fish. Bengtsson (1980) reported that adult minnows (*Phoxinus phoxinus*) exposed to Clophen A50 suffered from delayed spawning and offspring showed reduced hatching time and frequency. Later, Holm *et al.* (1993) reported

that three-spined stickleback (*Gasterosteus aculeatus*) exposed to Clophen A50 suffered from reduced spawning success. Furthermore, injection of a single PCB congener (3,3',4,4'-tetrachlorobiphenyl) in white perch (*Morone americana*) was shown to impair both maturation of adult females and survival of their offspring (Monosson *et al.* 1994). Other reproductive anomalies observed upon PCB exposure include inhibition of spermatogenesis and various testicular abnormalities (Sangalang *et al.* 1981; Freeman *et al.* 1982) as well as disruption of reproductive endocrine function (Khan and Thomas 1996).

However, only few long-term PCB exposure studies have been reported and to the best of our knowledge there is none performed on the zebrafish, which has become widely used in ecotoxicological test systems. Furthermore, most studies conducted to evaluate the effects of PCBs are, as described above, based on exposure of single PCB congeners, *e.g.* #126 and #169, or technical mixtures, such as Clophen A50 and Arochlor 1254. In order to cover the broad structural variation, tested PCBs must be carefully preselected. The PCBs consist of a large number of congeners, which represent a broad variation in physicochemical characteristics (Andersson *et al.* 1996). By using statistical design in combination with principal component analysis, 20 PCBs have been selected to represent the 154 tetra- to hepta-chlorinated congeners (Tysklind *et al.* 1995). A large spread in physicochemical characteristics of the included PCBs determined the selection. The present study employs these 20 PCBs, including congeners of each degree of chlorination, *i.e.*, tetra to hepta, as well as each number of chlorine atoms in *ortho* position.

The purpose of the present study was to evaluate reproduction effects in the zebrafish after long-term oral exposure to a mixture of 20 selected PCB congeners.

Materials and Methods

Food Preparation

The mixture of selected PCBs contained equal amounts of each congener and the purity, as controlled by HRGC/LRMS (MD800, Fisons, UK), was more than 99%. The PCBs was purchased from Ultra Scientific, North Kingstown, RI, USA (nos. 58, 78, 173, 188, and 190)

and from AccuStandard, New Haven, CT, USA (nos. 41, 51, 60, 68, 91, 99, 104, 112, 115, 126, 143, 153, 169, 184, and 193). Numbering of the PCBs throughout this paper is according to the IUPAC system (Ballschmiter and Zell 1980; Schulte and Malisch 1983). The PCB mixture was dissolved in 90 ml isooctane at different concentrations, each of which was mixed with 30 g of freeze-dried chironomids (Nutrafin[®]). The food was prepared in three dose levels of PCB, *i.e.* low (Ld), intermediate (Id), and high (Hd). The control food was treated with isooctane only. The solvent was evaporated at 50°C and 70 rpm using a rotary evaporator. The final concentrations in the low, intermediate, and high dose levels were 0.008, 0.08, and 0.4 µg of each congener/g food, respectively.

Experimental Design

Zebrafish, with a weight of 150–200 mg, were bought from a local pet shop. After 4 weeks of acclimatization, the experiment started. The exposure took place in 40-L aquariums, equipped with external filters (EHEIM 2211). Once a month, one-fourth of the water volume and the filter wadding was changed. The temperature was kept constant at 25°C, and the photoperiod was 10 h of light followed by 14 h of darkness. The zebrafish were divided into four experimental groups, each of 30 fish, *i.e.* one control group and one group for each dose level of PCB. The fish were fed chironomids daily, in amounts equivalent to about 2% of their body weight measured over the experimental period.

Sampling and Analysis

After 4 and 13 weeks of feeding, sampling took place. Fish were randomly sampled to the sum of 10 females from each of the four aquariums. At all samplings, body, liver, and gonadal weights were recorded. For each female, the liver somatic index (LSI, liver weight × 100/total body weight) and gonad somatic index (GSI, gonadal weight × 100/total body weight) were calculated. Ovaries were fixed in phosphate-buffered formalin, processed, and embedded in paraffin. Serial sections were cut, stained with eosin-hematoxylin and examined by light microscopy. In addition, female liver samples were used for measurement of the cytochrome P450-dependent ethoxyresorufin-*O*-deethylase (EROD) activity. The livers were weighed, immediately frozen in liquid nitrogen, and kept at –70°C until analysis. Homogenates were made from individual livers ultrasonicated for 5 s in 0.2 ml of 0.1 M sodium phosphate buffer (pH 7.4) containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.02 mM butylhydroxy toluene (BHT), 1 mM dithiothreitol (DTT), and 0.1 mM EDTA. EROD activity was measured immediately after ultrasonication as described by Andersson *et al.* (1985). Liver homogenate protein content was measured according to Lowry *et al.* (1951), using bovine serum albumin as standard.

Reproduction

After 9 weeks of exposure, the reproduction study was started. The temperature was raised and kept constant at 27°C and the photoperiod was 12:12 h of light:darkness. Rectangular spawning tanks were used for the study and water was aerated using Rena 301 air pumps. Five females from each experimental group were transferred to four spawning tanks (one tank for each dose group), each tank containing five net breeding traps, one per female, so that the females were separated from each other. Each female was placed with two unexposed males. Males were changed if spawning did not occur within a few days. The traps were examined every day, at a specific time, and the eggs were collected and transferred to 100-ml petri dishes. In cases where there were large numbers of eggs, each batch was divided into

several groups so that each petri dish contained no more than 30 eggs. Water was renewed every day in the dishes. The number of hatched and surviving eggs/larvae were recorded daily in order to evaluate median hatching time and median survival time. Parental fish were fed unpolluted chironomids and daphnia during the reproduction experiment whereas the larvae were not fed at all.

PCB Analysis

The female zebrafish from the two samplings, *i.e.* taken at 4 and 13 weeks, used for morphological studies and measurements of enzymatic activity, were also analyzed for contents of PCBs. Since livers and ovaries were dissected, the analysis of PCBs does not represent a whole-body analysis. Each sample contained 10 females, which were pooled at each dose level and time of exposure. The samples were homogenized with 5–10 g of Na₂SO₄ and transferred to a glass column for extraction of lipids. Before extraction PCB no. 50 and 189 were added as internal standards. Lipids were extracted with acetone:hexane (5:2) followed by hexane:diethylether (9:1). After gravimetric determination of lipids, a semipermeable membrane device (SPMD) was used for dialysis of lipids using cyclohexane as solvent. Further clean-up of the extracts included florisil-gel open chromatography and an acid silica column. As a keeper tetradecane was added and the extracts was evaporated to the final volume. Before analysis, PCB no. 199 was added as recovery standard. Analysis of the PCBs were carried out by HRGC/ECD (Hewlett Packard 6890). For identification and quantification of the PCBs in the mixture a standard was run. The tetra-chlorinated biphenyls were quantified versus PCB no. 52, the penta CBs vs. no. 101, the hexa CBs vs. no. 153, and the hepta CBs vs. no. 180.

Statistical Analysis

Statistical analyses of LSI, GSI, EROD, body weight, liver weight, and ovary weight were conducted using the nonparametrical Mann-Whitney U test, comparing the exposed groups to the control group. The significance level was set at 0.95 ($p \leq 0.05$). The symbols *, **, and *** represent p values of $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively. In the reproduction study, median hatching time and median survival time were calculated using a simple regression plot (Statview 4.0 for Macintosh) and tested for statistical significance using the Mann-Whitney U test, as described above.

Results and Discussion

In the present study, zebrafish were orally administered a mixture of selected PCBs for a total period of 13 weeks. Only few experimental long-term exposure studies are reported in the literature concerning effects of PCBs on fish. In the natural environment, fish are continuously exposed to a broad range of toxicants over their whole lifetime. Therefore it is important to focus on toxicological effects observed during long times of exposure. There are several reports concerning PCB residues in ovaries and the effects on reproduction in feral fish. Baltic flounder (*Platichthys flesus*) eggs with PCB concentration exceeding 0.12 µg/g (wet wt) showed a reduction in viable hatch (von Westernhagen *et al.* 1981). Furthermore, when the concentration was near or higher than 0.25 µg/g, viable hatch was lower than 15%. In whiting (*Merlangius merlangus*), von Westernhagen *et al.* (1989) reported the critical ovarian threshold level for PCB to be 0.2 µg/g. The PCBs in the present study consisted of

Table 1. Hepatic EROD activity (pmol resorufin · min⁻¹ · mg protein⁻¹) in female zebrafish after 4 and 13 weeks of PCB exposure^a

Dose Group	4 Weeks of PCB Exposure		13 Weeks of PCB Exposure	
	Mean	(n)	Mean	(n)
Control	14.4 ± 10	(n = 10)	5.20 ± 1.8	(n = 10)
Low	6.50 ± 3.4*	(n = 9)	9.26 ± 4.1*	(n = 8)
Intermediate	18.2 ± 16	(n = 9)	22.0 ± 11***	(n = 10)
High	170 ± 80***	(n = 10)	49.1 ± 61**	(n = 6)

^a Values are given as mean ± standard deviation (SD)

Table 2. Total mortality (n = 30) during the experiment and body weights recorded after 4 and 13 weeks of PCB exposure^a

Dose Group (n = 10)	Mortality (%)	4 Weeks of PCB Exposure		13 Weeks of PCB Exposure	
		Body Weight (mg)	Weight Index (%)	Body Weight (mg)	Weight Index (%)
Control	3	235 ± 47	+27	402 ± 110	+117 (+71)
Low	3	253 ± 59	+37	299 ± 70*	+62 (+18)
Intermediate	10	279 ± 87	+51	265 ± 46**	+43 (-5)
High	20	263 ± 98	+42	224 ± 71**	+21 (-15)

^a Weight indices are related to fish weight before exposure (185 ± 36 mg) and indices in parenthesis between samplings. Values are given as mean ± standard deviation (SD)

20 structurally diverse tetra- to hepta-chlorinated congeners. The chemical analysis of the PCBs, with recoveries of 68–90%, was conducted for pooled females sampled after 4 and 13 weeks, from the different dose groups. Generally, the concentrations of each congener increased by the dose given and with time of exposure. The levels found of each congener vary greatly depending on structure-specific characteristics. Congeners lacking chlorine atoms in vicinal *meta* and *para* position, such as PCB no. 104 and 143, as well as the non-*ortho* substituted PCBs, *e.g.* no. 126 and 169, are found in lower concentrations. These phenomena have recently been reported for three-spined sticklebacks (van Bavel *et al.* 1996). After 4 and 13 weeks of exposure, ΣPCB concentrations for control, Ld, Id, and Hd groups were 0.06, 0.11, 0.58, and 1.9, and 0.07, 0.14, 1.1, and 2.7 µg/g fish (wet wt), respectively. The tissue concentration of PCBs in the Id group increased twofold over the 4- to 13-week exposure period, whereas the Hd group increased only 1.4-fold over the same period. The lower increase of the Hd group might indicate a concentration near equilibrium of accumulation and clearance rate.

Monosson *et al.* (1994) reported that female white perch (*Morone americana*) accumulated PCB no. 77 in ovary > liver > skeletal muscle after a single intraperitoneal (IP) injection. Furthermore, the residues of PCB#77 were 10–15-fold higher in the ovaries and three–five-fold higher in livers, as compared with skeletal muscle. In a study similar to the present (Holm *et al.* 1993), sticklebacks were exposed orally to two different dose levels of Clophen A50. After 3.5 months of exposure, the whole-body PCB residues of the female sticklebacks were reported to be 102 and 289 µg/g (wet wt), respectively. These concentrations were 50–100-fold higher than those of the Hd group (13 weeks) in the present zebrafish

Table 3. Ovary weights and gonad somatic indices (GSI) in female zebrafish following 4 and 13 weeks of PCB exposure^a

Dose Group (n = 10)	4 Weeks of PCB Exposure		13 Weeks of PCB Exposure	
	Ovary Weight (mg)	GSI (%)	Ovary Weight (mg)	GSI (%)
Control	16.6 ± 11	6.84 ± 4.0	37.7 ± 25	8.90 ± 4.8
Low	12.4 ± 11	5.02 ± 4.8	21.1 ± 18	6.47 ± 5.6
Intermediate	19.4 ± 25	5.89 ± 5.7	7.51 ± 7.0**	2.92 ± 2.8**
High	23.6 ± 25	7.67 ± 5.9	3.62 ± 4.9***	1.36 ± 1.4***

^a Values are given as mean ± standard deviation (SD)

Table 4. Liver weights and liver somatic indices (LSI) in female zebrafish after 4 and 13 weeks of PCB exposure^a

Dose Group (n = 10)	4 Weeks of PCB Exposure		13 Weeks of PCB Exposure	
	Liver Weight (mg)	LSI (%)	Liver Weight (mg)	LSI (%)
Control	4.3 ± 2.1	1.84 ± 0.81	11 ± 4.5	2.74 ± 0.61
Low	4.8 ± 2.2	1.83 ± 0.56	6.3 ± 2.9*	2.00 ± 0.66*
Intermediate	5.2 ± 2.6	1.79 ± 0.67	2.9 ± 1.5***	1.13 ± 0.69***
High	5.1 ± 3.1	1.91 ± 0.91	2.8 ± 1.8***	1.17 ± 0.46***

^a Values are given as mean ± standard deviation (SD)

study (2.7 µg/g wet wt). However, in the present study the actual body burden was probably much higher than that recorded since livers and ovaries were dissected prior to analysis. Probably, these tissues contained higher levels of PCBs than that recorded in body concentration. In comparison, in minnows (*Phoxinus phoxinus*) exposed to Clophen A50, Bengtsson (1980) reported abnormally high egg mortality at ovary residue levels of 15 µg/g. Furthermore, Nebeker *et al.* (1974) reported a reduction in reproductive success when fathead minnow (*Pimephales promelas*) and flagfish (*Jordanella floridae*) reached a total body burden of 92 µg/g PCB.

Measurement of EROD (CYP1A) activity is a commonly used assay in the assessment of exposure of organisms to persistent organic pollutants, such as PCDDs, PCDFs, PCNs, and PCBs (Goksøyr and Förlin 1992). The present study shows that the PCB mixture used is an effective inducer of the hepatic cytochrome P450 system in zebrafish (Table 1). In a study by Buchmann *et al.* (1993), where zebrafish were exposed to TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin), the unexposed controls showed an EROD activity of 9.5 pmol · mg protein⁻¹ · min⁻¹. This is in accordance with the controls in the present study, where the first and second sampling showed 14.4 and 5.2 pmol · mg protein⁻¹ · min⁻¹ in mean hepatic EROD activity, respectively. After 4 weeks of exposure, a 12-fold elevated EROD activity was recorded in the Hd group compared with controls. The second sampling revealed a significant dose-dependent EROD induction (Table 1). However, in the Hd group a reduction in EROD was recorded, probably due to some adaptory response or intracellular damage of the hepatocytes.

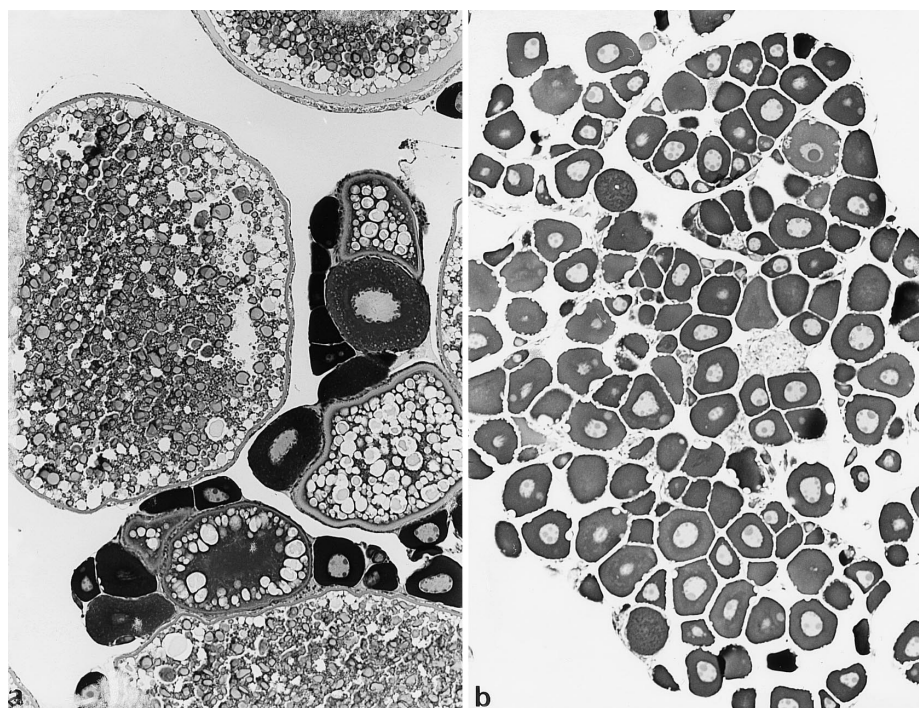


Fig. 1. Ovaries from (a) control zebrafish with oocytes in different stages of maturity and (b) from the high dose group containing only primary oocytes ($\times 110$)

Table 5. Spawning success, median hatching time, and median survival time in the reproduction study, which included unexposed males and female zebrafish exposed to three different dose levels of PCB for 9 weeks^a

Dose Group	Spawning			Median Hatching Time (days)	Median Survival Time (days)
	Spawning Females (%)	Mean # Eggs per Female	Early Mortality (%)		
Control	80	238	0	2.98 \pm 0.42 (n = 255)	13.8 \pm 1.2 (n = 178)
Low	40	220	12	2.81 \pm 0.57 (n = 114)	13.3 \pm 1.2 (n = 98)
Intermediate	60	167	9	2.84 \pm 0.63 (n = 70)	13.1 \pm 1.9 (n = 19)
High	60	96	5	2.63 \pm 0.43 (n = 123)	7.67 \pm 0.49*** (n = 92)

^a Values are given as mean \pm standard deviation (SD)

Inhibition of CYP1A1 by PCB congeners of commercial mixtures have recently been reported. In scup liver (*Stenotomus chrysops*), PCB no. 77 has been indicated to simultaneously cause catalytic inhibition, induction of CYP1A1 mRNA, and inhibition of CYP1A1 protein (White *et al.* 1997). In rainbow trout, IP injection with Clophen A50 altered the responsiveness toward PCB, measured as a reduction in CYP1A1 mRNA and CYP1A1 protein (Celander and Förlin 1995). The reason for this inhibition is not known, but it is suggested in both reports that it can be an effect directly on the CYP1A1 system by PCB.

During the exposure period, a low mortality in the control and Ld groups, with one fish (3%) dead in each group, was recorded. The numbers of deaths in the Id and Hd groups were three (10%) and six fish (20%), respectively (Table 2). No significant differences were recorded after 4 weeks of PCB exposure in body, liver, or ovary weights between controls and exposed groups. However, after 13 weeks of exposure, the mean body weight was almost half in the Hd group, 224 mg as compared with 402 mg in control (Table 2). The decreases in body weights in exposed groups are mostly due to decreases in

GSI (Table 3). Furthermore, there was also decreases in liver weights and LSIs in the Id and Hd groups (Table 4), which may be due to metabolic stress and toxic effects, leading to degeneration of the liver. In addition, ovary weights and GSI were found to be significantly lower in the Id and Hd groups (Table 3). The GSI in females in the Id and Hd groups were approximately one-third and one-sixth of that in the controls, respectively. This is in accordance with a study by Monosson *et al.* (1994), where adult white perch were given IP injections of PCB no. 77 prior to the spawning season. The results showed that fewer females matured in the group receiving the highest dose (3 IP injections of 5 μ g/kg) and those that did mature had a GSI approximately half that of control females. The study also showed that females from all groups were able to spawn, but there was a reduction in egg deposition in the groups exposed to PCB no. 77. This is in accordance with observations of reproduction disturbances within the present study. These results suggest that the PCB mixture, or certain PCB congeners, has a suppressive effect on oocyte maturation rather than affecting ovarian development. This is also supported by the

histological examination, which showed that after 13 weeks of exposure, both Id and Hd groups contained a reduced number of mature oocytes compared with the control group (Figure 1). Selman *et al.* (1993) divided oocyte development in zebrafish into five different stages (I–V). According to this classification, the oocytes, especially in the Hd group, seemed to be arrested in stage I (Figure 1), containing no cortical alveoli, no yolk bodies, and with few nucleoli in the nucleus. The exact mechanism by which PCBs negatively affect the reproduction system is unknown. Atlantic croaker (*Micropogonias undulatus*), fed the technical PCB mixture Aroclor 1254, were reported to have decreased plasma estradiol and vitellogenin concentrations, as well as decreased GSI (Thomas 1989). Furthermore, Thomas (1989) also observed a decreased secretion of gonadotropin from the pituitary when incubated *in vitro* after the *in vivo* exposure. PCBs might have a site in the hypothalamus-pituitary complex, and thereby decrease the secretion of gonadotropins (Thomas 1989). This would lead to decreased estradiol production and plasma vitellogenin concentration, which reduce oocyte maturation. Another factor that affect reproduction is the stress hormone cortisol. Cortisol has been shown to inhibit testosterone and estradiol production in rainbow trout ovarian follicles (Carragher and Sumpter 1990). Elevated plasma cortisol levels and EROD activity have recently been reported in rainbow trout exposed to PCB no. 77 (Vijayan *et al.* 1997). Therefore, the toxicological stress due to PCB exposure might have a suppressive effect on oocyte maturation.

When considering the spawning, there was a dose-related reduction of the number of eggs deposited per female (Table 5). This is in accordance with the histological evaluation showing a reduction of mature oocytes in the exposed groups. The control group showed the largest number of spawning females (80%), producing a mean of 238 eggs per female. The least number of eggs deposited was from the females in the Hd group, less than half of that in controls, followed by the Id and Ld groups, respectively (Table 5). The dose-related reduction of eggs laid in the exposed groups is in accordance with previous studies. Wannemacher *et al.* (1992) reported that, in female zebrafish, exposure to 5, 10, and 20 ng TCDD/fish led to a dose-related reduction of the number of eggs. Similar results were obtained in a study by Bengtsson (1980) where Clophen A50 was shown to reduce the number of spawning occasions in minnows. In the present study, the numbers of deaths occurring within the first three days (early mortality), were recorded to be 0% in controls, 12% in the Ld group, and 9 and 5% in the Id and Hd groups, respectively (Table 5). We have no explanation for the fact that the low-dose group produced more vulnerable embryos; however, it could be due to individual female variation in egg quality. No differences in hatching frequency were observed, with an almost 100% hatching success in all groups. Further, no statistical difference could be found in median hatching time (Table 5). However, the embryos of the Hd group tended to hatch earlier than embryos of the other groups. Although not statistically significant ($p = 0.14$), median hatching time of the Hd group was recorded as 2.63 days compared with 2.98 days in the control group.

When considering median survival time of the larvae, there was a dramatic decline in the Hd group. Larvae of the control

group showed a median survival time of 13.8 days, compared with only 7.67 days ($p = 0.0007$) in the Hd group (Table 5). Maternal exposure to PCB is well known to be associated with decreased larval survival (von Westernhagen *et al.* 1981; Black *et al.* 1988; Monosson *et al.* 1994). In the study by Monosson *et al.* (1994), a decline in survival near the end of the yolk-sac absorption was reported in larvae from female white perch exposed IP to 1.0 and 5.0 $\mu\text{g/g}$ of PCB no. 77. Walker *et al.* (1992) exposed eggs from rainbow trout (*Oncorhynchus mykiss*) and lake trout (*Salvelinus namaycush*) to TCDD, both by an egg-injection method and waterborne exposure, and the predominantly overall mortality occurred during the yolk-sac stage, not during the egg stage.

In conclusion, the selected PCBs accumulated in a dose-related manner. It is demonstrated that the levels of PCBs used, when exposed to zebrafish, affects reproduction negatively, with disturbances in oocyte maturation and offspring survival. Furthermore, the mixture of PCBs strongly induces hepatic EROD activity.

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