

Long-term food-exposure of zebrafish to PCB mixtures mimicking some environmental situations induces ovary pathology and impairs reproduction ability

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Abstract:

Although the use of polychlorinated biphenyls (PCBs) has been banned for several decades, they are still present in the environment and are occasionally mechanically released from sediment or transferred through the trophic chain. Field analyses have established correlations between exposure to PCBs and alterations in fish physiology including reproductive function. Experimental exposures have been mainly performed using dioxin-like PCBs or other congeners at very high concentrations. However, these studies are often difficult to relate to real-life conditions. In the present study, we performed a life-cycle exposure using zebrafish model and mixtures representative of some environmental situations in terms of doses, composition and containing mainly non dioxin-like congeners. Exposure was performed through diet which is the main contamination route in the field. We demonstrated a bioaccumulation of PCBs in males and females as well as a maternal transfer to the eggs. Survival, growth and organ size were similar for all conditions. Several reproductive traits were altered after exposure to a PCB-contaminated diet, including a reduction in the number of fertilized eggs per spawn as well as an increase of the number of poorly fertilized spawns. This latter observation was found irrespective of the sex of contaminated fish. This is related to modifications of ovary histology revealing a decrease of maturing follicles and an increase of atretic follicles in the ovaries of females exposed to PCBs. These results indicate that exposure to PCBs mixtures mimicking some environmental situations, including mainly non dioxin-like congeners, can lead to a dramatic reduction in the number of offspring produced by a female over a lifetime. This is of great concern for wild species living under natural conditions.

Highlights

► Zebrafish lifecycle exposure to PCBs mixture with low content in dioxin-like congeners. ► PCBs bioaccumulation in adults and transfer to eggs. ► Reduction of fertilization rate due to an increase in poorly fertilized spawn number. ► Fertilization rate decreases is observed regardless of the sex of the contaminated fish. ► Reduction of the proportion of maturing follicles and increase of atresia.

Keywords: Zebrafish ; PCBs ; Chronic exposure ; Physiology ; Reproduction ; Atresia

1. Introduction

Polychlorinated biphenyls (PCBs) are synthetic chlorinated aromatic hydrocarbons that are non-flammable, hydrophobic, and chemically stable. In the 80's, when PCBs were banned, global production was estimated to be over 1.5 million tons (Breivik et al., 2002). Because of their extensive industrial use and chemical stability, PCBs have accumulated in the environment and biota. Their high lipophilicity and environmental stability has allowed them to persist in the environment for long periods resulting in a wide geographical distribution. In the environment, PCBs are buried in sediments and particles can be released by, for example, trawling, dredging or floods. This explains why biota can still be contaminated. The long biological half-life and high liposolubility of PCBs can lead to their bioaccumulation and biomagnification along food chains involving a wide range of trophic levels, with a potential risk for high trophic level predators (Borga et al., 2001 ; Fisk et al., 2001 ; Nfon and Cousins, 2006). For human and fish populations, dietary intake, especially the consumption of marine organisms, is considered as one of the most important sources of exposure to PCBs (Johansen et al., 1996 ; Muir et al., 2003 ; Nyman et al., 2002 ; Pompa et al., 2003)

Data collected from fish in the field suggests that PCB could have physiological effects although the consequences of PCB exposure are difficult to ascertain. Some of these effects have been confirmed by laboratory experiments and include progressive weight loss ('wasting syndrome'), alteration of lipid metabolism, hepatotoxicity, immunotoxicity, alteration of endocrine system function and reproduction, teratogenicity and developmental toxicity, and tumor promotion (Foekema et al., 2008 ; Monosson, 1999/2000 ; Ross, 2004 ; Safe, 2005 ; Schell and Gallo, 2010 ; van Ginneken et al., 2009)

The use of fish models to address ecotoxicology issues has become more prevalent in recent years. Small fish models have allowed more detailed investigations of the mechanisms that underlie the adverse effects of pollutant exposure (Hill et al., 2005 ; Hinton et al., 2005 ; Spitsbergen and Kent, 2003). Many publications have reported the effects of exposing zebrafish or medaka to pollutants, particularly PCBs. These studies have investigated pollutant exposure *via* different routes and at different stages of development from embryo to adult. Alteration of several physiological processes including development, reproduction, and behavior have been demonstrated Billsson et al., 1998 ; Grimes et al., 2008 ; Lyche et al., 2010 ; Na et al., 2009 ; Nakayama et al., 2004 ; Nakayama et al., 2005a ; Nakayama et al., 2005b ; Nourizadeh-Lillabadi et al., 2009 ; Örn et al., 1998 ; Westerlund et al., 2000). However in most of these studies the exposures were performed using individual congeners and or high doses.

In the present study, we have tried to use exposure conditions that resemble as closely as possible those occurring in the wild. For this purpose, juvenile and adult zebrafish were exposed to a mixture of PCBs which were environmentally relevant in terms of dose and composition. To further mimic naturally occurring situations, long-term exposure was performed through diet. The aim of the present study was to evaluate the physiological consequences of lifetime exposure of fish to environmentally relevant PCB mixtures in their diet. The mixtures we used included three mono-ortho substituted congeners which are less coplanar than non-ortho dioxin-like ones and belong to priority group 1B (McFarland and Clarke, 1998) and ten non dioxin-like congeners (priority groups 2 and 3). We monitored the bioaccumulation of PCBs in fish over time and as well as transfer to eggs. In particular, we assessed several key issues for reproduction including spawn size and number, fertilization rate, and gonad histology.

2. Material and methods

2.1. Fish, exposures, and sampling

Wild type zebrafish (*Danio rerio*) were bought at 6 weeks of age from a provider dedicated to breeding zebrafish especially for ecotoxicology experiments (Elevage de la grande rivière, Calluire, France). Exposure began 2 weeks later. These fish were maintained and exposed to a PCBs mixture in Ifremer research station L'Houmeau France, under standard conditions: water and room temperature were kept between 26-28°C and the photoperiod was 10 h dark and 14 h light. Water was obtained after a mix of reverse osmosis treated water and tap water, both being filtered beforehand with dust and charcoal filters, to obtain a pH of 7.5 ± 0.5 and a conductivity of $300 \pm 50 \mu\text{S}/\text{cm}$. Ammonia, nitrites, and nitrates were monitored daily for 2 months then weekly and remained within recommended ranges (Lawrence, 2007). The fish were raised in a flow-through system made of 20 identical 10L tanks in a rack. Water was automatically changed every hour leading to an exchange rate of 4L/day/tank. Contaminated water was collected and treated with activated charcoal before being discharged in a sewer. This study was conducted under the approval of the Animal Care Committee of France under the official licence of M.L. Bégout (17-010).

Commercial food (Neo supra, pellet-size 1400 μm), obtained from Le Gouessant (France), consisted of a formulated feed containing 58% crude protein and 13% crude fat. Spiked food was prepared by batch to avoid deterioration and by slowly adding a solution in iso-octane of known amounts of thirteen congeners, (AccuStandard Inc., New Haven, USA, purity above 98%) to food pellets in order to prepare diets. PCBs mixtures are composed of the seven indicator PCB congeners (CB28, 52, 101, 118, 138, 153, 180), and a few others to describe a larger range of chlorination from 3 to 8 chlorine atoms (CB105, 132, 149, 156, 170, 194). The latter compounds were chosen because they can highlight any underlying mechanisms acting on the distribution of organic contaminants in biota *i.e.* bioaccumulation or biotransformation.

CB153 is not metabolized and is used as a representative of all thirteen congeners and therefore only its concentration is reported here. Two diets were prepared: PCB-Medium and PCB-High for which targeted concentrations of individual congeners are shown in Table 1. These concentrations correspond to the one monitored in respectively medium and highly contaminated sites (respectively Loire and Seine estuaries; data from French coastal chemical contamination survey network – ROCCH). Batches of spiked food were stored in amber containers in a cool, dark place throughout their use (*circa* 1.5 months). Individual PCB congener concentrations were determined in each batch of spiked food (Table 1). Non-spiked food was also analysed for PCB levels, and concentrations were between $<0.05 \text{ ng/g}$ dry weight (dw) and 3.0 ng/g dw (mean value calculated on 5 replicates) depending on the congener. In addition to congeners included to spike the contaminated food, CB 31 (not detected; nd), CB 77 (nd), CB110 (0.75 ng/g dw), CB126 (nd), CB128 (nd), CB187 (nd) non-added congeners and p,p'DDE (3.50 ng/g dw) were measured with the exception of other molecules. The control food exposed only to solvent is further referred as Solvent whereas the unmodified food is referred to as Plain. We used the Plain food to exemplify potential deviation from untreated situations that could be due to solvent or spiking procedures.

After 2 weeks of acclimation, 30 fish were randomly distributed in each tank respecting an equivalent biomass of $214.1 \pm 2.9 \text{ mg}$ (mean \pm SEM) within range [188.3-239.1] corresponding to a coefficient of variation of 5.9%. Diets were then assigned to each tank with all diets represented in each rack shelf with 3 tanks for Plain, 3 tanks for Solvent control, 7 tanks for PCB-Medium, and 7 tanks for PCB-High diets. Fish were fed twice (morning and afternoon) on week days and once with a double dose on weekends. During all of the

experiments, fish were fed 2 % of their wet weight per day. This ration was updated after each biometry. Contamination lasted for 300 days.

Samples were made during biometries for PCB dosage; one or more fish from each sex and condition were euthanized with a lethal dose of benzocaïne (Sigma) and then stored directly at -20°C. In some cases, newly spawned eggs were also conserved at -20°C without any water and these samples were dosed for PCB.

2.2. Chemical analysis

PCB analysis was performed according to the protocol described by Bodiguel *et al.* (2009). Briefly, entire sampled fish were extracted in a hot Soxhlet extraction apparatus (Soxtec) over 1.5 h with a hexane:acetone mixture (80:20). The amount of extracted fat was determined by gravimetric analysis. Then, two successive clean-ups were performed on the extractable material. First, lipids and co-extractible material were destroyed by adding concentrated sulphuric acid. The cleaned extract was then purified by adsorption chromatography on a Florisil column (16% MgO + 84% SiO₂, activated for 1.5 h at 500°C, deactivated with 3% of demineralised water) and eluted with pentane. Finally, PCB congeners were analyzed by gas chromatography with an electron capture detector, on a HP 5890 series II equipped with a CP-Sil19 capillary column (60 m length, internal diameter 0.25 mm, and 0.15 µm phase film thickness) following optimized conditions described by (Jaouen-Madoulet *et al.*, 2000). The system was calibrated within quite a large range using a six-point calibration curve to define the linearity range of our detector (ECD) for all contaminants. The relative precision of the method was checked for this type of sample by analyzing five aliquots of a homogeneous-tissue preparation of fish muscle. The results showed standard deviations of less than 10% for all congeners, indicating that the method had a satisfactory reproducibility. During analysis of the real samples, analytical blanks were systematically measured for every ten samples. The blank concentrations were always less than the concentrations of the lowest standards of all congeners. Finally, 5 replicates of a reference material, BCR-CRM349 (Cod Liver Oil) were analyzed to determine the accuracy and precision of the method. PCB recoveries varied between 74 and 125%. Furthermore, the RSD values ranged from 4 to 19 %, with a mean of 10% for all PCBs. All of these results were in agreement with certified reference values and published data (Schantz *et al.*, 1993). The laboratory regularly takes part in Quality Assurance of Information for Marine Environmental Monitoring in Europe (QUASIMEME) intercomparison exercises for PCBs in biota and our Z-scores are satisfactory, *i.e.*, between -2 and +2.

2.3. Phenotypic analysis

Survival was monitored each day throughout the entire duration of the experiment.

To track fish growth, total body mass and standard lengths were measured approximately every two weeks and sex was recorded when possible. Fish were anaesthetized for 5 min in benzocaïne (50mg/l) during biometric analyses then released into their tank of origin. Specific growth rates (SGR, %) were also calculated from growth data according to the equation:

$$\frac{(\ln [\text{weight date 2}] - \ln [\text{weight date 1}])}{(\text{time between date 1 and date 2 in days})} * 100$$

At the end of the experiment, 9 females of each condition were weighed as well as their liver and gonads after dissection to calculate the Liver Somatic Index (LSI) and the Gonad Somatic Index (GSI) as a percentage of the organ weight relative to the total body weight.

Onset of spawning was determined by placing a spawning box (AquaSchwartz, Germany) within each tank from day 48 until a spawn was found in all of them (*i.e.* day 141). Fish were then regularly set into pairs in spawning boxes. Reproduction couples were obtained by setting up couples on the evening before spawning using fish coming from the same tank. In some cases, fish from Solvent and PCB 500 tanks were mixed to assess the dependence of fertilization rate on sex. Generally, spawning occurs progressively after a few minutes of light illumination. One hour later, eggs were collected, cleaned, sorted (into fertilized or none fertilized), and counted. From each spawn 100 fertilized eggs were set aside into a 10 cm diameter Petri dish filled with 30 ml of E3 solution (5mM NaCl, 0.17mM KCl, 0.33mM CaCl₂, 0.33mM MgSO₄) at 28° C. Embryo deaths were recorded each day to calculate the embryo-survival percentage. Embryo survival percentage was calculated in all conditions on starving larvae till their death (12-14 days) according to (Örn et al., 1998).

2.4. Histopathology evaluation

A histopathological evaluation was performed on 109 fish (69 females and 40 males). The principles of step sectioning adult zebrafish have already been published (Spitsbergen et al., 2000). Briefly, whole fish carcasses were fixed in 4% formalin after ventral incision of the abdomen for larger individuals. 24 h later, scales and fins were carefully removed and samples were dehydrated and embedded in paraffin. Serial sagittal step sections were cut from the left side of the fish. Four step sections from each adult fish were mounted on glass slides, 1 from eye anterior chamber level, 1 from eye posterior chamber level, 1 just medial to the eye, and 1 at the midline. Sections were routinely stained with hematoxylin-eosin-safran (HES). Ovarian and testicular tissue features were quantitatively assessed in a blind manner using a digital camera (Nikon DXM 1200, Champigny, France) linked to image-analysis software (Nikon Imaging Software). For ovaries, all oocytes present on mid-level section and on sections medial to the eye were counted along their morphological stage using middle magnification (Koç et al., 2008). Atretic oocytes, as assessed by their irregular shape and yolk-envelope disruption, were also counted. Individual frequencies of each type of oocyte and indexes of oocytic apoptosis were calculated using ratio of the total ovarian area. For testis, the total area occupied by differentiated E3 spermatids was measured (Schulz et al., 2010) and a ratio was calculated with total observed testis area on two middle magnification fields.

2.5. Statistical analysis

Because data do not follow a normal distribution, continuous data, *i.e.* weight, eggs numbers, fertilization rates, atretic oocytes numbers were analyzed using the Kruskal–Wallis test with Dunn's *post hoc* test when appropriate. Proportions, *i.e.* fertilization rate categorization and follicle-stage proportions were analyzed using chi-square test. In other cases, statistical tests are indicated. Statistical analysis was performed with Statistica 9.0 (StatSoft) or Prism 5.0 (GraphPad). Unless otherwise stated, values are indicated as mean ± SEM. Significance was set at $p < 0.05$.

3. Results

3.1. Fish contamination

The mixture designed to mimic environmental conditions with the concentration of each congener is presented in Table 1. In Solvent and Plain fish, CB153 concentration ranged from 12 ng/g dw at the beginning of the experiment to 40 ng/g dw after 230 days of

exposure. Since concentrations in both control groups showed no significant difference (Paired Student's t-test; $p > 0.05$ $n=4$), they were all considered together. All congeners administered in spiked food were detected in exposed fish at higher concentrations than in control fish. As shown in Figure 1, linear PCB accumulation kinetics were observed in fish during the first three months of exposure and in a similar manner for males and females. From this time point, CB153 concentration in females became highly variable in the [351-1644] ng/g range for PCB-Medium exposed females and in the [990-9305] ng/g range for PCB-High exposed females. In most cases, females with low CB153 concentrations were sampled immediately after having spawned (open circle in Fig. 1), which suggests that females may expel PCBs through spawning. This hypothesis was confirmed as CB153 concentration in control eggs is less than 0.1 ng/g fresh weight (fw). For eggs produced by females exposed to PCB-Medium diet, CB153 concentration was 16.0 ± 0.8 ng/g fw and 47.6 ± 7.8 ng/g fw for the PCB-High diet (see Figure 2 for the increases observed in PCB-Medium and PCB-High eggs *versus* control eggs).

3.2. Physiological indexes

Over the 300 days of contamination, we noticed no changes in survival rate between groups (Table 2). During the first 52 days of contamination (3 biometries), fish of both sexes were considered together since sex could not be unequivocally determined for every fish. Afterwards, when all fish were sexed, size and weight were calculated for each sex. Whatever the diet, we observed no differences in growth between groups as ascertained by comparison of final weight, specific growth rate (SGR) or condition index (not shown), which were all unchanged (Table 2). Final weight and SGR displayed a very large inter individual variability.

We determined the LSI and GSI for females in each group and observed no significant differences in any of these variables compared with Solvent fish (Table 3).

3.3. Reproduction

Although onset of spawning appears delayed after exposure to PCBs, there were no significant differences between the different conditions ($p=0.769$). On average, Solvent fish began to spawn at day 73.7 ± 25.7 , whereas contaminated fish began to spawn at day 93.2 ± 17.5 for PCB-Medium and day 109.8 ± 14.5 for PCB-High (Table 4).

Embryonic development was assessed throughout the duration of this study. We observed neither modification of embryonic development nor changes in the rate of development. We observed no differences in embryo survival, which was assessed until death of all larvae in absence of feeding (data not shown).

Although there was a trend in reduction of spawn size for PCB-High couples, there is no significant difference in the number of eggs per spawn (Table 4). However, if we consider the numbers of fertilized eggs, we observe a trend for PCB-Medium and a significant reduction for PCB-High with approximately one third less fertilized eggs per spawn compared with control spawn. Surprisingly, the fertilization rate is similar for PCB-Medium and PCB-High around 63%, *versus* 85% for Solvent control.

Since we obtained highly fertilized spawn from PCB-High couples, we analyzed in more detail the distribution of fertilization rate for each spawn (Fig. 3). In control fish, most of the spawns were highly fertilized with around 80% of spawns with a fertilization rate over 80% and less than 10% of spawns with a fertilization rate under 40%. Spawns from PCB-Medium or PCB-High couples were mainly characterized by a reduction of highly fertilized spawns (less than 50%) and an increase of poorly fertilized spawns (around 30%).

Crosses performed using Solvent and PCB-High fish showed that the fertilization rate was impaired regardless of the sex of the contaminated fish (Fig. 4A) with the same modification of fertilization rate distribution (Fig. 4B). This indicated that the lowering of fertilization rate following PCB exposure was not due to a particular sex. Surprisingly, no additive effect was observed when two contaminated fish were crossed.

3.4. Gonad histology and follicle atresia

Irrespective of the diet, examination of whole male gonads revealed the presence of testis with typical architecture, and normal spermatogenesis as evaluated by histology. A quantitative evaluation of the testicular areas occupied by spermatids revealed no differences between control and exposed fish. Ovaries of one-year old females displayed different features depending on diet. The main tissue alterations were then quantified. Relative frequencies of all four stages of follicles including, pre-vitellogenic stages 1 and 2, vitellogenic and post-vitellogenic, were first assessed. Both PCB-Medium and PCB-High female ovaries were characterized by a trend towards a higher proportion of pre-vitellogenic stage-1 oocytes, albeit not significant. In PCB-High females, there was a highly significant lower proportion of pre-vitellogenic stage 2, vitellogenic and post-vitellogenic stages (Fig. 5). Major alterations of oocytic maturation were observed in 4 out of 10 PCB-High fish with a complete absence of vitellogenic and post-vitellogenic stages. In these cases, ovarian tissue was only composed of pre-vitellogenic stages and interstitial tissue was markedly infiltrated by inflammatory cells, mainly macrophages and lymphocytes (Fig. 6).

Ovaries from PCBs females were also characterized by an increase in the number of atretic follicles. In control fish, the number of atretic follicles per microscopic field (mean \pm SEM) is 1.18 ± 0.28 in Nature females and 0.76 ± 0.23 in Solvent females. These numbers were tripled in PCB-exposed females to 3.19 ± 0.54 for PCB-Medium and 3.70 ± 0.45 for PCB-High (only ovaries with all four stages of follicles could be considered; $p < 0.01$). The presence of atretic follicles was often associated with a peripheral influx of some macrophages phagocytizing oocytic debris.

4. Discussion

In this study, we exposed zebrafish to mixtures of PCBs mimicking some environmental situations (as Loire or Seine estuary) through the diet over 300 days. This long period represents almost half the lifespan of zebrafish under laboratory conditions and includes approximately 200 days of reproduction.

PCBs are very persistent pollutants because of their high lipophilicity, very limited biotransformation, and slow depuration. Our findings are in agreement with these elements with a time- and dose-dependent increase in PCBs concentration in fish bodies (Anderson et al., 2001)

No steady-state was achieved for any concentration during the exposure time. The uptake curves observed in this experiment were typical of those observed by other authors during bioaccumulation studies of hydrophobic contaminants in fish (Fisk et al., 1998 ; Tomy et al., 2004). Concentration in fish is directly related to the concentration in diets. During the first 90 days of contamination, CB153 concentration in male and female follows a similar kinetic. After this time point, there was a clear differentiation in contamination levels between males and females. For females with the lowest CB153 concentration (identified immediately after spawning), this level was similar to that of males whereas some females had CB153 concentrations four- to fivefold higher than in males. Differences between females may be

related to the expulsion of PCBs during spawning as we found some PCBs in eggs at a concentration that correlated with the level in females. However, the quantity of PCBs contained in a spawn cannot account for the difference between heavily contaminated females and females which have spawned. It should nevertheless be noted that in a tank, competition exists between females and dominance can greatly reduce the number of spawning females (Gerlach, 2006). It is thus possible that heavily contaminated females are dominated females with a low number of spawns and thus reduced expulsion of PCBs.

Several studies have already been performed to establish the physiological consequences of exposure of zebrafish to PCB among other fish. However, in many cases only one congener is studied while PCBs always occur as mixtures in the wild, in addition doses and/or exposure route may not be relevant. All of these data are very useful to understand the mechanisms underlying possible effects of exposure to PCBs but they may not reflect what happens in an environment contaminated with PCBs. The composition of the mixtures that we have used has been designed to closely mimic some environmental situations in a biological matrix respecting relative proportions of each congener (Antunes et al., 2007 ; Bodiguel et al., 2009 ; Muir et al., 2003) and concentrations have been chosen to reflect the actual concentration in medium and heavily contaminated areas (respectively Loire and Seine estuaries).

Recruitment is the ability of an embryo or a fish to participate in the production of next generation. This includes the ability of an embryo to reach adulthood, and for the adult, the ability to produce gametes suitable for reproduction in terms of quantity and quality. In the present study, we investigated several endpoints related to recruitment including survival of larvae produced by adults exposed to PCBs, egg production, and fertilization rates. For these reasons, we have monitored endpoints which may potentially account for the ability of fish to participate in recruitment using a model species.

There were no differences in the global physiological parameters that we measured including, survival, growth, and organ weight. These results clearly differ from those reported by Örn and colleagues (1998) who found an increase in mortality and a reduction of body weight, LSI, and GSI in their intermediate- and high-dose groups. As mentioned above, the authors used an equivalent dose of the twenty chosen congeners with 80ng/g food for the intermediate dose and 400ng/g of highly toxic co-planar congeners 126 and 169 (Örn et al., 1998), which are absent from the mixture that we used. It has also been reported that survival is reduced following direct aqueous exposure of summer flounder larvae to CB126 congeners (Soffientino et al., 2010). Co-planar effects are thought to be mediated by AhR and therefore may elicit a dioxin wasting syndrome which includes weight loss and sometimes death (Walker and Peterson, 1994). However, in some cases, exposure to PCB promote growth; this has been observed after long-term exposure to food dosed with Clophen A50 (Bengtsson, 1980). Increase of growth has also been described in zebrafish exposed to mixtures of POP including DDT and PBDEs in addition to PCBs (Lyche et al., 2010 ; Nourizadeh-Lillabadi et al., 2009). Our results nevertheless agree with those demonstrating an absence of correlation between fish growth and the load of PCBs in wild fish (Bodiguel et al., 2009 ; Rypel and Bayne, 2010) or experimental models (Loizeau et al., in preparation). Mortality and growth alteration thus appear to be induced in fish after exposure to co-planar PCBs congeners, in particular CB126.

PCBs have been reported to disrupt endocrine activity in fish and other vertebrates including humans (Crain et al., 2008 ; Mendola et al., 2008 ; Mills and Chichester, 2005). This has been demonstrated at the molecular level (e.g. hormone or genes expression levels), the cellular level, in organs (e.g. gametes properties or GSI), and individuals (e.g. spawning events, embryo development). To gain insights into the mechanisms underlying alterations in

reproduction after exposure to PCBs, and to evaluate the functional consequences of this exposure, several endpoints were monitored.

No embryonic deformities or changes in larval survival rates were observed after exposure of parents to PCBs. Again, it appears that the effects depend upon the congeners studied. Several studies report alterations of fish embryonic development or survival after exposure to PCBs. This is the case of exposure to Aroclor 1254 or CB126 which cause general embryotoxicity (McCarthy et al., 2003), alterations in embryonic growth (Na et al., 2009), and changes in heart development (Grimes et al., 2008). Exposure to individual congeners, such as CB126 or CB77, can reduce fish-embryo survival (Foekema et al., 2008 ; Grimes et al., 2008 ; Monosson et al., 1994 ; Soffientino et al., 2010). This has also been demonstrated after exposures to complex mixtures (Örn et al., 1998). However, as mentioned above, CB126 and CB169 in this study were present at high levels. In contrast, exposure to PCB-48 technical mixture, which among other PCBs contains high levels of CB77, did not alter larval survival rate at 10 days post-hatching (Nakayama et al., 2005b). Alterations in survival have been reported with other congeners or mixtures for zebrafish embryos after intraperitoneal injection of females with 1µmol kg⁻¹ CB60, 104, and 190 (Olsson et al., 1999) and for minnow embryos after exposure to Clophen A50 (Bengtsson, 1980).

We conclude that the PCBs mixture that was used in the present study has no effect on egg development and viability either through egg quality or embryonic development. The deleterious effects reported in other studies are mainly due to the use of coplanar dioxin-like congeners.

We observed alterations in ovary structure in both contaminated groups with a dose effect. Indeed, there was a decrease in the proportion of stage 2 to 4 follicles in PCB-Medium female ovaries and in a six out of ten PCB-High female ovaries. This phenotype gets worse for the remaining PCB-High females with the absence of maturing stage 3 and 4 oocytes. These phenotypes are associated with an increase of atretic follicles in females fed both contaminated diets. These observations agree with previous reports of increased in atretic follicles after exposure to PCBs (Cross and Jo Ellen, 1988) and a bias towards primary follicles (Örn et al., 1998). It is to note that exposures presented here began after sex determination but overlap with differentiation of both sex gonads (Orban et al., 2009). However, the present study is the first report of such ovary anomalies after exposure to a mixture of PCBs mimicking some environmental situations. Our approach is thus suitable to investigate mechanisms underlying the alterations in ovary function that occur in polluted environments.

At the functional level, we observed a significant reduction of the overall fertilization rate. This was not due to a reduction of fertilization of each spawn but instead to an increase in the number of poorly fertilized spawns at the expense of highly fertilized ones. To our knowledge, this is the first report that a mixture of PCBs mimicking some environmental situations can decrease the fertilization rate of fish spawns. Our study revealed that, irrespective of the sex of the contaminated fish, a strong decrease of fertilization rate is observed with a non-additive effect when both males and females are contaminated. Alteration of fertility has already been reported for several vertebrate species (Letcher et al., 2010 ; Pocar et al., 2006). Reduction of fertility has also been described for oocytes directly incubated in PCBs and then submitted to *in vitro* fertilization (Campagna et al., 2001 ; Kholkute et al., 1994). Several mechanisms could underlie this reduction. There may be alterations in oocyte competence (Krogenaes et al., 1998), or sperm quality or release. We did not observe gross alterations of testes structure, but sperm defects appear to be more related to sperm properties (Hauser et al., 2003 ; Njiwa et al., 2004 ; Richthoff et al., 2003). Fertilization and/or sperm release defects can also be due to alterations in male reproductive behavior (Njiwa et al., 2004). The absence of additive effects of female and male alterations

on the fertilization rate is puzzling. However, several reports have shown that PCBs can act as both oestrogenic and anti-oestrogenic depending on their chlorination level (Pliskova et al., 2005).

In summary, our study shows that PCBs mixtures representatives of some environmental situations may lead to changes in the reproduction abilities of fish that may impair recruitment and population stability. It is possible that much of these observations are due to non dioxin-like congeners contained in the diet. Additional experiments should be performed to clarify this point. Several other questions remain unanswered, in particular concerning the timing of structural damage to the ovaries and the mechanisms underlying alteration in fertilization rate for both females and males.

5. Conclusions

Our data indicate that exposing zebrafish to mixtures of PCBs through diet leads to contamination of fish with a clear bioaccumulation and a transfer from adult females to eggs.

In terms of physiology, we observed alterations of ovary structure with an increase of atresia which can be related to a decrease in the number of eggs per spawn and fertilization. This can be combined with a decrease in the number of spawn per female. These findings indicate that, despite starting breeding under ideal conditions for reproduction, an exposure to mixtures of PCBs mimicking some environmental situations including mainly non dioxin-like congeners can lead to a dramatic reduction in the number of offspring produced by a female over a lifetime. This is of great concern for wild species living in natural conditions. More studies are needed to identify the mechanisms underlying these observations and a new series of exposures is currently underway.

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Tables

Table 1. Diets composition. List of PCBs congeners used in this work along with the chlorine number. Target and measured PCBs concentrations in PCB-medium and PCB-high diets are indicated (mean \pm SD). Reference congener CB153 is indicated in bold.

Congeners	Chlorine number	PCB-medium diet		PCB-high diet	
		Targeted concentration (ng/g)	Measured concentration (ng/g)	Targeted concentration (ng/g)	Measured concentration (ng/g)
CB28	3	10	4.8 \pm 0.6	50	16 \pm 3.3
CB52	4	25	20 \pm 0.8	125	82 \pm 11.5
CB101	5	50	45 \pm 2.3	250	191 \pm 9.5
CB149	6	50	47 \pm 4.9	250	217 \pm 11.6
CB118	5	50	54 \pm 4.4	250	207 \pm 7.9
CB153	6	100	106 \pm 5.5	500	420 \pm 15
CB132	6	25	18 \pm 0.9	125	104 \pm 5
CB105	5	25	26 \pm 5.3	125	133 \pm 19.5
CB138	6	100	99 \pm 4.9	500	431 \pm 16.4
CB156	6	25	17 \pm 1.5	125	113 \pm 4
CB180	7	50	43 \pm 2.1	250	215 \pm 4.5
CB170	7	25	26 \pm 1.6	125	126 \pm 4.5
CB194	8	10	9 \pm 1.1	50	47 \pm 1.7

Table 2. Biometry and survival among different groups. Initial and final weight and mean SGR over the period for male and female and survival rate for both sex exposed to control feeds or PCB diets, measured after 247 days of exposure (mean \pm SD). There is no significant difference between groups.

Dose group	Initial weight	Final weight male (mg)	SGR male	Final weight female (mg)	SGR female	Survival rate (%)
Plain	196 \pm 81	641 \pm 70	0.33 \pm 0.5	929 \pm 219	0.62 \pm 0.45	69.6 \pm 21.9
Solvent	224 \pm 75	594 \pm 94	0.42 \pm 0.2	1008 \pm 200	0.54 \pm 0.64	56.9 \pm 13.7
PCB-medium	215 \pm 83	623 \pm 117	0.35 \pm 0.44	1036 \pm 251	0.67 \pm 0.74	78.9 \pm 13.9
PCB-high	217 \pm 82	598 \pm 136	0.42 \pm 0.6	1030 \pm 287	0.63 \pm 0.71	64.8 \pm 12.3

Table 3. Organs biometry. Mean liver somatic index (LSI) and gonad somatic index (GSI) measured after 247 days of exposure for female (\pm SEM). There is no significant difference between groups.

Dose group	Fish (<i>n</i>)	LSI	GSI
Plain	9	2.29 \pm 0.22	14.22 \pm 1.17
Solvent	8	3.16 \pm 0.27	15.39 \pm 2.24
PCB-medium	10	3.09 \pm 0.29	17.75 \pm 1.84
PCB-high	11	2.56 \pm 0.28	14.00 \pm 1.42

Table 4. Spawn characteristics. Mean of spawning onset, total size of spawn, number of fertilized eggs per spawns and calculated mean fertilization rate obtained from adults exposed to control or PCB diets. The number in parenthesis after the spawning onset value indicates the number of tanks considered (mean \pm SEM; **p* < 0.05).

Dose group	Spawning onset (days)	Spawns (<i>n</i>)	Number of eggs	Number of fertilized eggs	Fertilization rate (%)
Plain	126.7 \pm 1.2 (3)	23	408.4 \pm 43.1	317.3 \pm 38.6	80.7 \pm 6.3
Solvent	73.7 \pm 25.7 (3)	48	422.6 \pm 36.9	378.4 \pm 37.5	84.8 \pm 3.9
PCB-medium	93.2 \pm 17.5 (6)	48	434.1 \pm 32.6	277.9 \pm 31.9	62.9 \pm 5.3 *
PCB-high	109.8 \pm 14.5 (6)	41	384.6 \pm 38.5	236.2 \pm 34.3 *	62.6 \pm 6.0 *

Figures

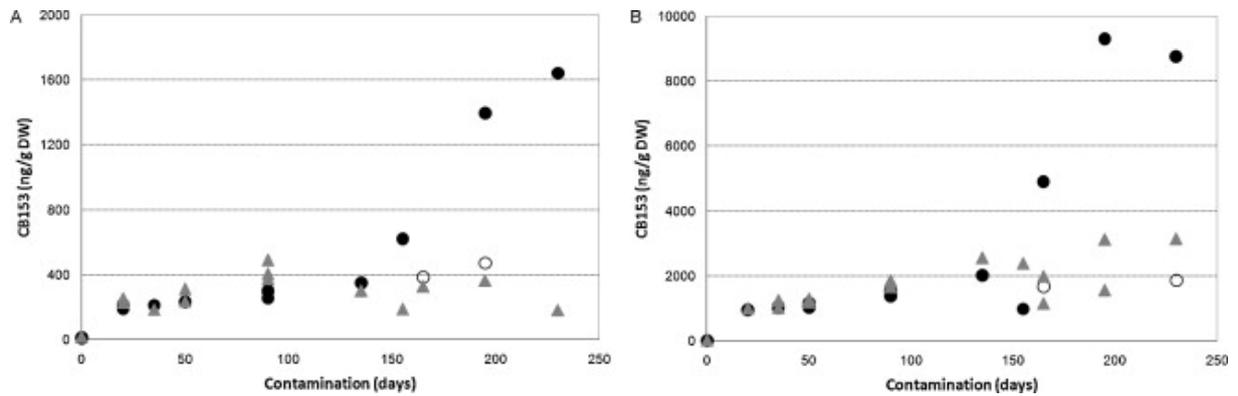


Fig. 1. PCBs concentration in fish over-time. Concentration of CB153 as a representative of all congeners has been determined for males (triangles) and females (filled circles) after exposure to PCB-medium (A), or PCB-high (B) diets. In both cases, concentration of PCBs has also been determined in females just after spawning (empty circles).

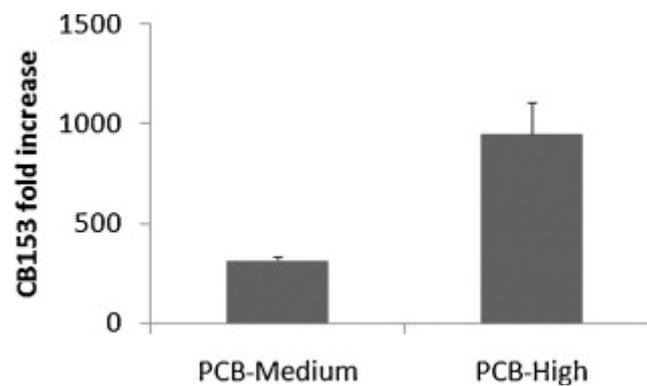


Fig. 2. PCB concentration in eggs. Fold-increase of CB 153 concentration in egg spawn by females exposed to PCB-medium and PCB-high diets compared with Solvent diet.

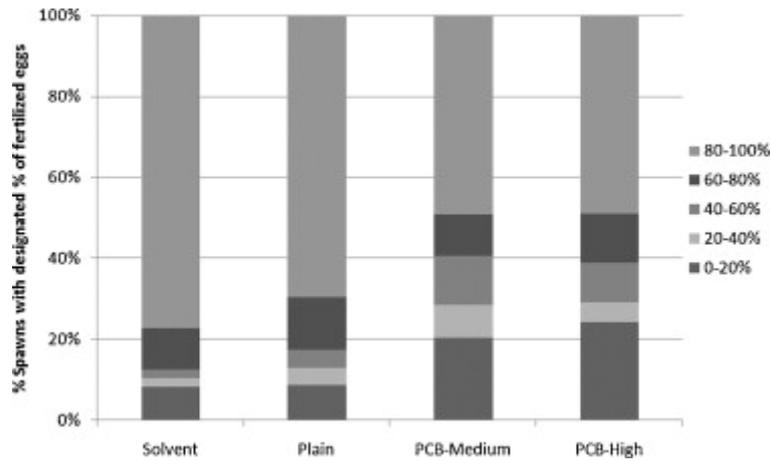


Fig. 3. Spawn quality distribution. Distribution of fertilization rate frequencies obtained from adults exposed to control or PCB diets. Chi-square test indicates that the distributions are different ($p = 0.0002$).

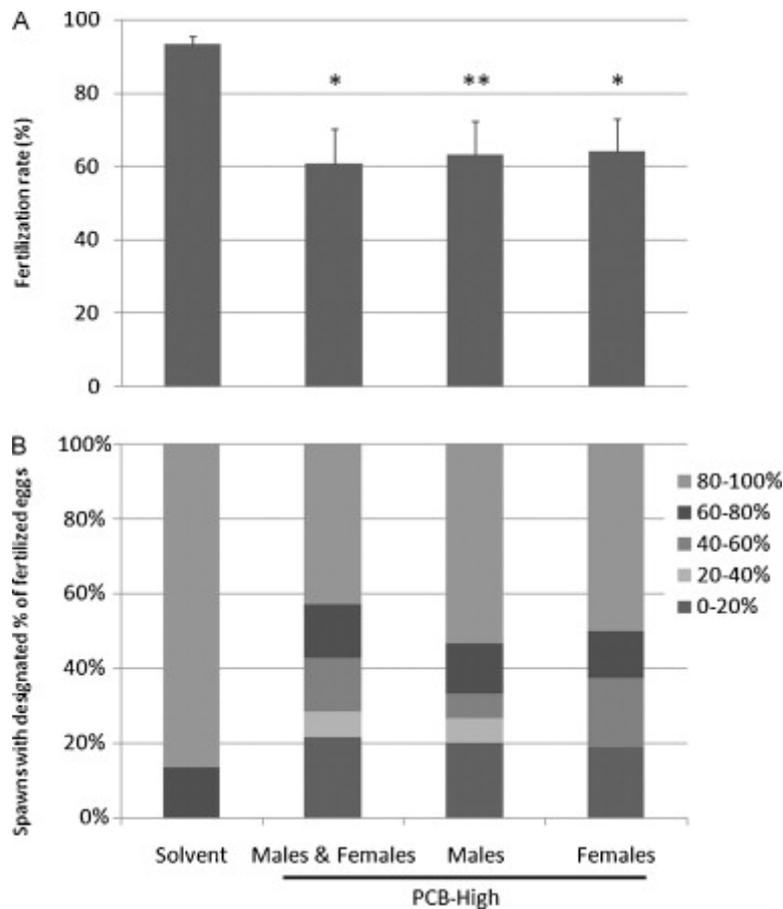


Fig. 4. Spawn quality distribution according to sex of the contaminated fish. (A) Fertilization rate obtained from adults exposed to control or PCB diets. In Solvent group both fish are from the Solvent group ($n = 15$ spawns) while other 3 conditions the sex of fish fed PCB-high is indicated at the bottom of the figure. In the case only one fish has been fed PCB-high diet, the other fish is from Solvent group. Number of spawns are $n = 14$ for males and females, $n = 15$ for males and $n = 16$ for females. (mean \pm SEM; * $p < 0.05$; ** $p < 0.01$). (B) Percentage of

spawns with the designated fertilization rate for spawns described above. Chi-square test indicates that the distributions are different ($p < 0.0001$).

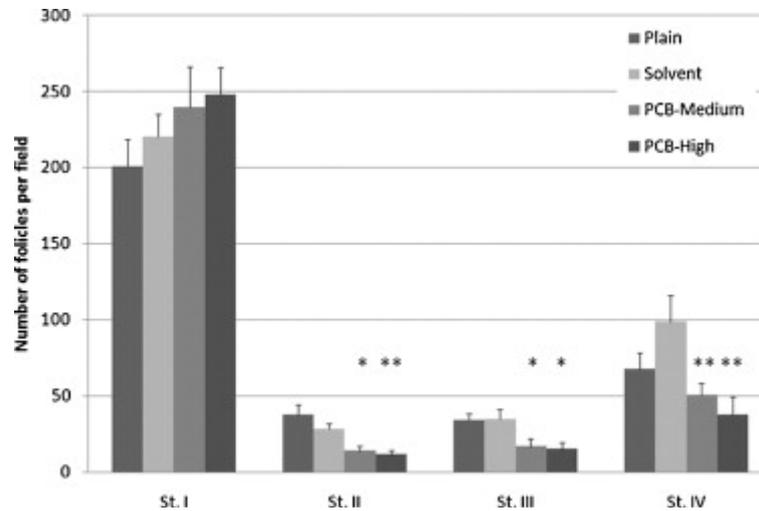


Fig. 5. Follicle stages. Number of different follicles in ovaries of females exposed to control or PCB diets according to their stage. The statistical significance of the differences was calculated for each stage between the different groups using an ANOVA and a PLSD Fisher test ($*p < 0.05$; $**p < 0.01$).

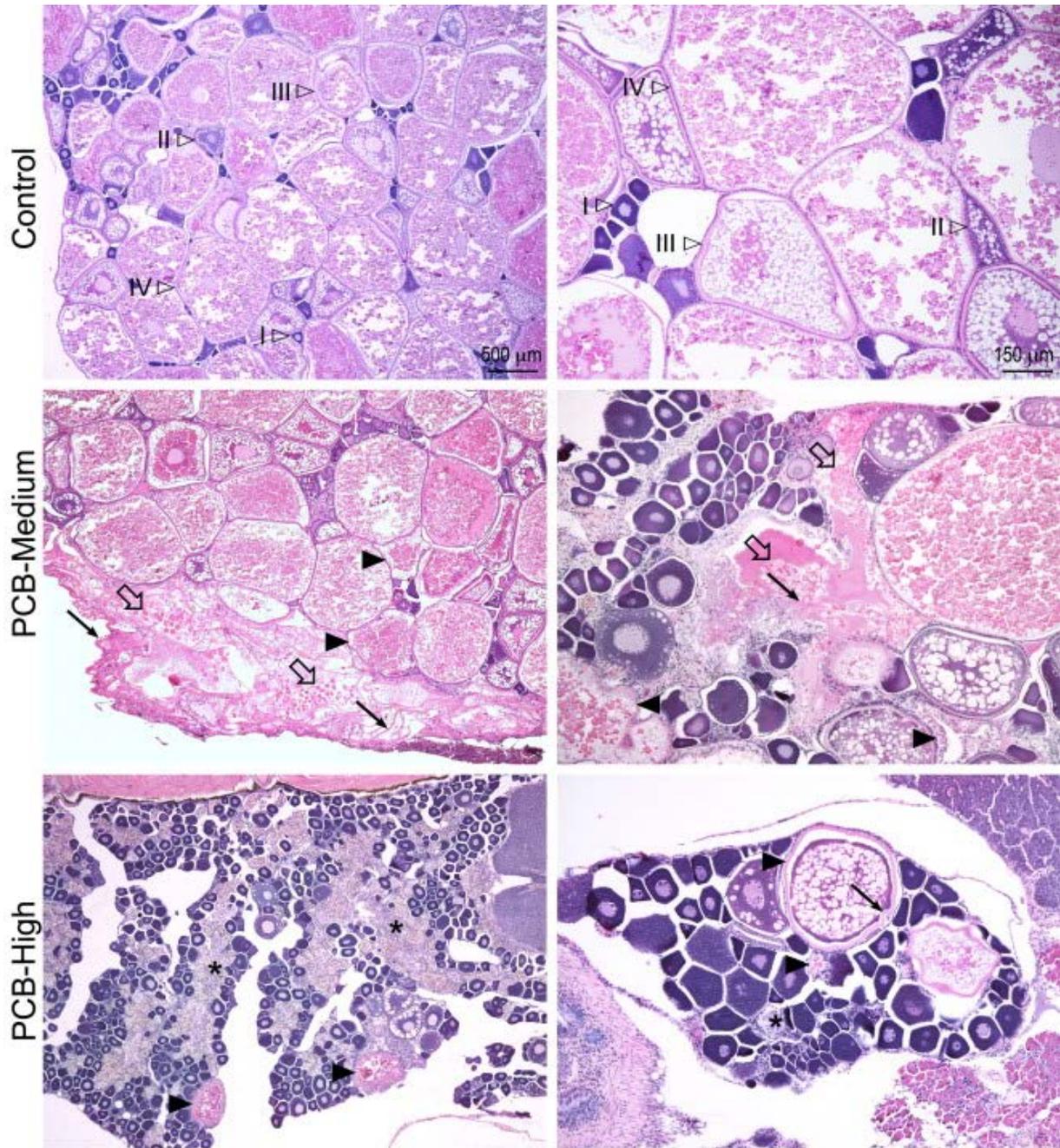


Fig. 6. Histological structure of ovaries. Zebrafish female ovaries stained with HES were photographed at low (left) and medium (right) magnification. The different stages of maturation of ovarian follicles (I–IV) can be identified in the ovary of a control fish (Solvent and Plain). The ovary from fish exposed to PCB-medium diet (middle panel) presents a number of atretic follicles (arrow head) as well as oocyte clusters in which there are fragments of vitelline membrane (full arrow) and yolk granules (open arrow). The number of follicles at stage III (vitellogenic) and stage IV (mature oocyte) is slightly decreased compared with the control. The ovary of fish exposed to PCB-high diet (lower panel) presents an almost complete absence of follicular stages III and IV. A few rare follicles at stage IV are atretic (arrow head) with a vitelline membrane forming characteristic folds (full arrow). Follicular depletion leaves large coalescent areas, corresponding to a granulomatous inflammatory reaction (*).