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Chronic dietary exposure of zebrafish to PAH mixtures results in carcinogenic but not genotoxic effects

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Abstract :

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous contaminants that can be present at high levels as mixtures in polluted aquatic environments. Many PAHs are potent mutagens and several are well-known carcinogens. Despite numerous studies on individual compounds, little is known about the toxicity of PAHs mixtures that are encountered in environmental situations. In the present work, zebrafish were continuously fed from 5 days post-fertilisation to 14 months post-fertilisation (mpf) with a diet spiked with fractions of either pyrolytic (PY), petrogenic light oil (LO), or petrogenic heavy oil (HO) origin at three concentrations. A decrease in survival was identified after 3 mpf in fish fed with the highest concentration of HO or LO, but not for PY. All PAH fractions caused preneoplastic and neoplastic disorders in long-term-exposed animals. Target tissues were almost exclusively of epithelial origin, with the bile duct epithelium being the most susceptible to chronic exposure to all PAH fractions, and with germ cells being the second most responsive cells. Significantly higher incidences of neoplasms were observed with increasing PAH concentration and exposure duration. The most severe carcinogenic effects were induced by dietary exposure to HO compared to exposure to LO or PY (45, 30 and 7 %, respectively, after 9 to 10 months of exposure to an intermediate concentration of PAHs). In contrast, earliest carcinogenic effects were detected as soon as 3 mpf after exposure to LO, including the lowest concentration, or to PY. PAH bioactivation and genotoxicity in blood was assessed by ethoxyresorufin-O-deethylase activity quantification and comet and micronuclei assays, respectively, but none of these were positive. Chronic dietary exposure of zebrafish to PAH mixtures results in carcinogenotoxic events that impair survival and physiology of exposed fish.

Keywords : Polycyclic aromatic hydrocarbon, Zebrafish, Carcinogenesis, Genotoxicity, Neoplasia, Carcinoma, Toxicological pathology

Introduction

Persistent organic pollutants (POP) are contaminants of natural or anthropic origin including several chemical families such as dioxins, polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs) and polyaromatic hydrocarbons (PAHs). The diverse PAHs are commonly classified into low molecular weight (LMW) or high molecular weight (HMW) PAHs, based on their molecular structure and their number of rings. Differences in the structure and size of individual PAHs result in substantial variability in their physical, chemical and toxicological properties.

Because of increasing human activity, PAHs are emitted at an ever-growing level and their concentration in the environment has considerably increased over recent decades (Shen et al. 2013). They are widespread contaminants present in the environment as complex fractions of dozens of individual PAHs. They may be generated mainly by three processes: combustion of organic matter (pyrolytic origin), release of petroleum (petrogenic origin), or to a lesser extent diagenesis (degradation of the organic matter) (Neff 1979). Once formed, PAHs may enter aquatic environment through the spillage of petroleum, industrial discharges, atmospheric fallout, and urban runoff (Neff 1979). Pyrolytic PAHs mainly enter in the aquatic environment via the atmosphere, associated with particles that reduce their bioavailability. In contrast, petrogenic PAHs can be directly discharged in the aquatic environment. In polluted environments, bivalves accumulate high levels of PAHs via their filtering activity (Hylland 2006; Rocher et al. 2006). Fish are exposed through water, through contact with contaminated sediments and by feeding. In molluscs and fish, some HMW PAHs can be metabolized into reactive compounds with DNA-binding activity (Le Goff et al. 2006; Fernandez-Tajes et al. 2011). These metabolism processes involve activation of AhR pathway, in particular the cytochrome P450 1A (cyp1a)(Billiard et al. 2002; Otte et al. 2010).

Long-term exposures to sublethal concentrations of PAHs have been shown to dramatically impair survival and physiology of exposed fish and their progeny (White et al. 1999). Some PAHs are potent mutagens (Mersch-Sundermann et al. 1992), and many are well-known carcinogens both in laboratory and environmental conditions (Hawkins et al. 1990; Myers et al. 1991). Elevated prevalence of hepatic neoplasms has therefore frequently been reported in fishes from heavily PAH-contaminated environments (Baumann 1989). Additionally, their major role is suspected in the aetiology of human cancer following exposure to cigarette smoke, urban air pollution, coal combustion, and certain occupational situations (Mastrangelo et al. 1996; Warshawsky 1999).

Various small aquarium fish, like zebrafish, have been utilized as carcinogenesis models (Stanton 1965). In comparison to many rodent species (Ward 1981), advantages of zebrafish include heightened sensitivity to carcinogens with a very low background tumor rate (Reddy et al. 1999a). Due to their short generation time, studies can be conducted near the life span of the individuals in about 12 months (Hawkins et al. 2003), an invaluable advantage in long-term studies that involve environmentally realistic exposures. The potential of zebrafish as a model for cancer research has been emphasized by the demonstrated similarities of transcriptome profiles between zebrafish carcinogen-induced tumor and human cancer (Lam et al. 2006). Other advantages include small size of adults that is compatible with a complete histological examination of all organs and tissues in a few sections without bias of a selective tissue sampling.

Following the intensive analyses already made on individual prototypical compounds like dimethyl-benz[a]anthracene (DMBA) and benzo[a]pyrene (BaP), there is a need to study PAHs as fractions currently encountered in environmental situations and to minimize potentially confounding factors. Moreover, most previous laboratory studies in fish were conducted with dissolved PAHs (Jonsson et al. 2004) or PAH in sediments (Cachot et al. 2007). Dietetary exposure has been more rarely explored to date (Rice et al. 2000; Wessel et al. 2010), especially if considering carcinogenic effects. In this context, Spitsbergen *et al.*(Spitsbergen et al. 2000a) chose to explore the long-term carcinogenic effects of high concentrations of one model PAH, DMBA, regarded as one of the most potent known PAH with dibenzo[def,p]chrysene.

The aim of this study is to document carcinogenetic and genotoxic effects induced by environmentally relevant conditions of PAH exposure, using realistic fractions in both qualitative and quantitative points of view, and a long-term dietary exposure started at the first meal of the fish and maintained afterwards.

Materials and methods

PAH-contaminated diet preparation

Three aromatic fractions were used for exposures: i) a pyrolytic fraction (PY) extracted from sediments collected in the polluted site of Seine Estuary (Oissel, France) and ii) two petrogenic fractions obtained from Erika fuel (heavy oil; HO) and Arabian light crude oil (LO). PAH extractions were performed as previously described (Letellier et al. 1997; Cachot et al. 2007). Briefly contaminants were extracted from the sampled sediment with Accelerated Solvent Extraction (ASE300, Dionex). The two oils were dissolved in pentane to induce

asphaltene precipitation (Mazeas & Budzinski 2001, Mazeas & Budzinski 2002). The sediment extract and the two pentane extracts were then reconcentrated using a vacuum evaporation system (Rapidvap, Labconco, Kansas city, USA). All three extracts were then purified using alumina columns (preparative liquid chromatography) and eluted with dichloromethane (Acros Organics, Thermo Fisher Scientific, Geel, Belgium). After another re-concentration step, aliphatic fractions obtained after elution on silica columns with pentane were discarded and aromatic fractions were obtained using pentane/dichloromethane (65/35, v/v) as solvents (Mazeas & Budzinski 2001, Mazeas & Budzinski 2002). Finally, samples were re-concentrated and the final fractions kept in dichloromethane (stored at -20 °C) for spiking experiments. PAHs concentrations of the three fractions were reported by Vignet et al. (Vignet et al. In press) and are presented in supplementary table 1.

Diets were prepared using the commercial diet INICIO Plus 0.5 (Biomar, France). INICIO Plus 0.5 is made of fish oil and flour and some plants extracts. According to producer its composition is as follows: raw proteins 58 %, raw lipids 15 %, non-nitrogenous extract 10.6 %, raw cellulose 0.4 %, ashes 11 % and total phosphorous 1.6 %. Vitamin A (9000 U.I/kg), vitamin E (350 mg/kg) and vitamin C (1000 mg/kg) were added. Food pellets were ground to obtain age-adapted pellets and spiked with PAH fractions. Three concentrations, 0.3X, 1X and 3X, were used with the 1X concentration corresponding to 5 $\mu g.g^{-1}$ for the sum of concentration of 16 indicator PAHs selected by the US Environmental Protection Agency (US-EPA). This 1X concentration is representative of that found in molluscs in the Seine Estuary (Rocher et al. 2006). Diets are named after the origin of the fraction and its concentration: 1X pyrolytic fraction diet will be named: PY-1X.

Fish exposure and sampling

This study was conducted with the approval of the Animal Care Committee of France under the official licence of M.-L. Bégout (17-010). We used zebrafish wild type TU strain (ZFIN ID: 76 ZDB-GENO-990623-3), which was established in our platform (PEP – http://wwz.ifremer.fr/pep) 6 years ago as large batches of individuals originating from the Amagen platform (Gif-sur-Yvette, France) and the Pasteur Institute fish facility (Paris, France).

Detailed exposure procedures are described by Vignet et al. (Vignet et al. In press). Briefly, eggs were obtained by random pairwise mating of zebrafish placed together in spawning boxes the evening before collection (AquaSchwarz, Germany). Eggs were collected the next morning and the fertilisation rate assessed within two hours of collection: only spawns with

rate above 80 % were kept. To provide as homogeneous and similar incubation conditions as possible, a minimum of five spawns were mixed and 50 embryos randomly sampled and transferred to as many Petri dishes as required for all conditions. Exposures to PY, HO and LO fraction spiked-diet were performed independently and successively in triplicates. For each exposure, a fourth control condition was included corresponding to the plain food treated as spiked-food with dichloromethane, used as carrier solvent for PAHs spiking. Embryos and larvae were maintained at 28 °C in Petri dishes in clean medium in an incubator with the same photoperiod as for adults. From 5 days post-fertilisation (dpf), larvae from each replicate were reared in separated 1 L-tanks and transferred after 12 days in 10 L-tanks in a flow-through system with a water renewal increasing with age and reaching a daily rate of 40 % after 1 month. Starting at 5 dpf, fish were fed twice a day with size-adapted food and once a day with uncontaminated *Artemia nauplii*. The size of the food pellets provided was increased as the fish aged. The ration was *ad libitum* from 5 dpf until the first biometric measures (2 or 3 months post-fertilisation (mpf)); thereafter, the quantity of food provided was 2 % of the biomass in each tank, with the exception of the PY-spiked food, for which the ration was 5 % at 2 mpf and then reduced to 2 % at 3 mpf. Rations were then adapted after each biometry on a monthly basis.

In the case of samplings dedicated to comet, EROD and micronuclei assays, fish were starved overnight and day of sampling.

Survival monitoring

Survival was monitored daily from 5 to 30 dpf and then on a monthly basis as described by Vignet et al. (Vignet et al. In press). The present study focused on latter stages and survival was calculated as a percentage of the starting number of fish for each exposure.

Histopathological evaluation

Fish were sampled at 3 mpf and 9-10 mpf for all diets except for HO-3X for which the last sampling was 7 mpf. Additional samplings were performed at 7 mpf for HO-0.3X and HO-1X and at 14 mpf for PY diets. Control fish were systematically sampled in parallel. Fish were euthanized with a lethal dose of benzocaine (250 µg.L^{-1}; Sigma-Aldrich, Saint-Quentin Fallavier, France) and fixed in 4 % formalin after ventral incision of the abdomen for larger individuals (Daouk et al. 2011). A histopathological evaluation was performed on a total of 760 fish from 3 mpf to 14 mpf (mean sex ratio of 0.52) (Table 1). The principles of step sectioning adult zebrafish have already been published (Spitsbergen et al. 2000a). After 24 h

of fixation, fins, most scales and the caudal peduncle were carefully removed and samples were dehydrated in graded ethanol solutions 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 haematoxylin-eosin-saffron (HES). Additional sections were stained with Ziehl-Neelsen acid-fast stain for mycobacteriosis, Hall's bilirubin stain and Picrosirius red stain for collagen.

Immunochemistry assays were performed using i) a mouse anti-cytokeratin (CK) antibody (Dako, Glosdrup, Denmark; 1:500) for cells of epithelial origin or ii) a mouse Proliferating Cell Nuclear Antigen (PCNA) antibody (clone PC10, Abcam Inc., Cambridge, MA, USA; 1:3000) as a proliferation marker. Briefly, paraffin-embedded sections were deparaffinised and subjected to antigen retrieval respectively in Tris-EDTA pH 9.0 buffer (Zytomed Systems GmbH, Berlin, Germany) (98 °C, 40 min) and in boiling 10 mM citrate buffer (pH=6.0) for 20 min. Endogenous peroxidase activity was inhibited by incubating the samples in 3 % H2O2 in water for 10 min. The sections were then incubated for 30 min with 10 % normal goat serum (Dako). Primary antibody was incubated in 2 % BSA (Sigma, St. Louis, USA) for 1 hour at 37 °C and revealed using successively biotinylated secondary antibody (Dako, 1:300), streptavidin-peroxidase complex (Dako, 1:300) and 3,3'-diaminobenzidine tetrahydrochloride (DAB, Dako).

All lesions were reported by a skilled pathologist certified by the European College of Veterinary Pathology. A minimum of 4 tissue sections per fish was observed to ensure the observation of all tissues and organs. Due to the high occurrence of bile duct abnormalities, a semi-quantitative scoring was performed for all fish except those for which none bile ducts were visible on the all-4 sections. A score reflecting biliary pericanalar fibrosis (0, no lesion; 1, some collagen deposit of less than 5 µm thick; 2, collagen deposit of less than 10 µm thick; 3, severe continuous collagen deposit; 4, fibrodysplasia) and a score reflecting severity of the bile duct epithelium lesions (0, no lesion; 1, hyperplasia; 2, dysplasia; 3, epithelio-mesenchymal transition; 4, carcinoma) were attributed.

Comet assay

For measurement of DNA strand breaks by the alkaline comet assay, 2 mpf-fish were sampled in each control and assay tank (3 fish per tank) to assess the level of DNA damage in the erythrocytes. Due to the small size of the fish, whole fish head was sampled and placed directly into 500 µL of a freezing medium. The freezing medium made of RPMI 1640

supplemented with DMSO 20 % and SVF 25 % was then withdrawn in a 1 mL cryotube and stored in liquid nitrogen prior to analysis.

According to a previously described protocol (Akcha et al. 2003), two slides per fish were prepared with 10 µL of sample mixed with 75 µL of low melting point agarose (LMP). Once the last LMP agarose layer was deposited and polymerised, the slides were immersed in an ice-cold lysis buffer (2.5 M NaCl, 0.1 M $EDTANa_2$, 0.01 M Tris base, 1 % N-sarcosinate, 10 % DMSO, 1 % Triton X-100, pH=10) for 1 h at room temperature in the dark. DNA unwinding was then performed by pre-incubating the slides in electrophoresis buffer (0.3 M NaOH, 0.001 M EDTA, pH=13) for 20 min at room temperature in the dark. DNA migration was performed in the same buffer for 12 min at 23 V (390 mA, E=0.66 $V.cm^{-1}$). Slides were then washed, dehydrated and dried. Just before analysis, 75 µL of Gel Red (1:10,000) were spread on the slide for at least 1 h at 4 °C for coloration. Slides were then analysed using an optical fluorescence microscope (Olympus BX60, x40) fitted to a CDD camera (Luca-S, Andor Technology) and an image analysis system (Komet 6, Kinetic Imaging Ltd.). The percentage of DNA present in the comet tail was calculated for each observed nucleus (50 nuclei per slide).

Micronuclei assay

Micronuclei (MN) assay was performed on haemocytes from 8 to 9 month-old zebrafish. Twelve to fifteen fish (4 to 5 per replicate) from each control and assay tank were individually analysed. For HO, because of the high fish mortality observed with the highest concentration, only control, 0.3X, and 1X concentrations were examined.

Fish were anesthetised by immersion for 10 min in benzocaine solution (50 $mg.L^{-1}$). Caudal peduncle was cut using a clean razor blade and from 1 to 5 µl of blood was collected from the caudal vein with a clean and heparinised 10 µl-micropipette tip. Blood (1 µl) was then smeared on a clean slide, air dried and then fixed in absolute ethanol for 15 min. Just before reading, slides were stained with 40 µL acridine orange solution (0.003 % in PBS) and covered with a glass slip. Reading was processed using an epifluorescent microscope at x400 magnification (Olympus, Rungis, France). Micronucleated cells frequency was manually recorded from 1,000 cells per slide using blind review by a single observer. A cell was considered micronucleated if MN was round shaped, distinct from main nucleus, size inferior to a third of the main nucleus, and with a similar green staining to the main nucleus (Hayashi et al. 1998). Non-isolated, stacked, more than bi-nucleated or orange-red stained cells were not taken into account.

EROD activity

EROD activity was measured for 3 to 4 individual 2 mpf-fish per tank, according to a previously published protocol (Burgeot and Menard 2004). Briefly, whole fish were crushed in a Potter cylinder with 5 volumes of phosphate buffer. After centrifugation for 20 min at 9000 G, the kinetic of resorufin formation was measured on the supernatant fraction after addition of 7-ethoxyresorufin (2 µM) and NADPH (0.25 mM) in a 96-well micro-plate with a spectrofluorimeter (TECAN Safire). Proteins were measured by the Bradford method and results were expressed as fmol/min/mg protein.

Kinetics of cyp1a *and* ahr2 *genes expression*

In order to decipher the kinetics of *ahr2* and *cyp1a* expression in relation with feeding, a specific starvation/feeding assay was performed with PY-3X diet. Exposures were performed as previously described with the following modifications. At 13 dpf, larvae were transferred in clean water (without food) and a 24 h starvation period was carried out. At 15 dpf (30 min after the onset of light), one part of larvae was fed again and pools of 20 larvae were sampled after 1, 2, 4, 6, 8, 10 and 24 hours post-refeeding (hprf). Before this diet, 20 larvae were sampled to establish the basal level of *cyp1a* and *ahr2* at 0 hprf. The remaining part of larvae was kept starved and sampled after 10 and 24 h. As a control, an assay with the control diet was performed in parallel. Finally, larvae were euthanized, pooled, transferred in liquid nitrogen and stored at -80 °C until analysis.

Total RNA were extracted from larvae samples using Trizol® Reagent (Invitrogen, Carlsbad, CA, USA) with successive chloroform/ethanol purifications following manufacturer's instructions. Then, first-strand cDNA was synthetized with a standard reverse transcription procedure from total 1 µg of RNA using M-MLV Reverse Transcriptase (Promega, Madison, USA) according to manufacturer's instructions. Finally, real-time PCR reactions were performed in sterile 96-wells PCR plates with StepOnePlus™ instrument **(**Applied Biosystems®, Life Technologies, USA) using reaction fraction based on the Fast SYBR® Green Master Mix 5X (Applied Biosystems®), and primers at 600 nM. The thermal cycling conditions were following: 10 min at 95 °C (activation), 15 s at 95 °C (denaturation), 40 s at 60 °C (hybridization) and 30 s at 72 °C (annealing) for 40 cycles. Gene's expression levels of *cyp1a* and *ahr2* were quantified by a comparative method from threshold cycle (C_T) number and normalized from three housekeeping genes (*eef1*, *g6pd* and *β-actin*). Primer sequences of

target genes were designed with Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/) and reported in Table 2.

Statistical analysis

Unless otherwise mentioned, reported results are means ± SEM. All statistical analyses were carried out at a 95% level of significance. Differences in survival were assessed within each diet using Log-rank (Mantel-Cox) test using Prism (Graphpad) software. Statistical analyses of histopathological results were performed with Statistica 9.0 software (Statsoft, Tulsa, OK, USA).

A GLM model was applied with age or PAH fraction tested as fixed factors, replicate, tank and gender as random ones. Only results for fixed factors and for the interaction between them are presented in the text. Post-hoc tests were performed using Newman-Keuls in each case. No tank effect was detected and an individual effect was only detected on tumor incidence at 3 mpf and on presence of epithelium dysplasia in LO-exposed fish. Global incidences, i.e. proportion of fish presenting duct epithelium dysplasia, pericanalar fibrosis or duct carcinomas, were analysed using chi-square test.

Raw comet data were Ln transformed to reach normality and then analysed by a one way-ANOVA with Statistica Software. For micronuclei assay, comparisons of mean frequency were performed using one-way ANOVA after checking for normal distribution of data (Shapiro-Wilk's test on 1 % residues) and variance homogeneity (Levene's test).

Gene expression data were analysed with a Relative Expression Software Tool REST-2009© (Qiagen, http://www.REST.de.com). At 0 hprf, PY-3X data was given in relative expression ratio compared to the control condition. For other analysed times, relative expression of the fed larvae was compared to that of the starved ones.

Results

This work is part of a larger program that included analysis of other effects following exposure to the same diets. Chemical analysis of fractions, diets and metabolites are presented elsewhere (Vignet et al. In press). Briefly, PY fraction was characterized by a high proportion of heavy PAHs and almost no substituted derivatives. HO fraction contained moderate levels of heavy PAHs and alkylated derivatives. LO fraction contained low level of heavy PAHs and a high level of alkylated ones. Diets composition reflected the one of fractions (Supplemental table 2). The concentration for the 16 US-EPA PAHs in 1X diets were PY-1X: 4505 ± 1527, LO-1X: 2739 ± 231 and HO-1X: 1887 ± 130 ng.g^{-1}dry weight and the total concentration of

PAHs (included alkylated ones) was similar in the three 1X diets: 5816 ± 1433, 4663 ± 360 and 6726 ± 278 ng.g^{-1}for PY, HO and LO respectively. Quantification of hydroxylated metabolites in 15 dpf-larvae confirmed fish contamination and results were consistent with diet composition.

Mortality

Survival was assessed separately for each PAH fraction using Log-rank (Mantel-Cox) test (Fig. 1). No difference was observed between concentrations for PY fraction (Chi-square value=0.80; p=0.849). For HO and LO diets, survival curves were different between concentrations (respectively Chi-square value=36.30; p<0.0001 and Chi-square value=13.86; p=0.003). In the absence of post-hoc test, it is difficult to individualize differences for LO diet. In the case of HO, HO-3X curve is clearly under the other curves starting from 3 mpf. Due to the high mortality observed in fish fed with HO-3X fraction, we decided to anticipate the sampling of fish fed with HO to 7 mpf instead of the 9 mpf initially planned in order to avoid the risk of the complete loss of a treatment group. All HO-3X remaining fish and 25 fish from other HO concentrations were then sampled at 7 mpf to allow comparisons.

Global incidence of preneoplastic and neoplastic lesions

A total of 264, 258 and 238 fish fed during 3 to 14 months with a diet containing respectively PY, HO or LO at 4 concentrations (3 PAH concentrations and one control condition), were examined microscopically after sectioning and HES staining. Liver was the primary target organ in PAH contaminated zebrafish with recorded lesions in hepatocytes and bile duct epithelial cells. In addition to liver, neoplasia also occurred in testis, intestine and pancreas, although at a relatively low frequency (Table 1).

Some dissimilarity regarding tumor incidences were identified between PAH fractions (Fig. 2, left panel). Neoplasm incidences significantly increased with time of exposure to HO and LO (respectively F=11.92; p<0.001 and F=27.26; p<0.001) but not to PY (Fig. 2), and with concentration of PY (F=5.07; p=0.002), HO (F=11.34; p<0.001), and LO (F=18.57; p<0.001). There is an interaction between time and concentration in HO (F=3.09; p=0.010) and in LO (F=8.06; p<0.001). Considering in more details the evolution of carcinogenesis, at 3 mpf, we observed neoplasms in PY-3X, LO-0.3X, -1X and -3X(Table 1) with a significant effect of the concentration (F=6.45; p<0.001) but not of the fraction. At this time point, the number of tumor was significantly higher in PY-3X than in HO-3X (p=0.015). Due to the high mortality recorded all along the study in the HO-3X group, experiment was stopped in this group at 7

mpf, a time when neoplasm incidence reached already 33 % (8 out of 24). At 9 mpf, all concentrations considered, 17 % (5 out of 29) and 72 % (21 out of 29) of fish exposed to PY and LO respectively displayed neoplasm compared to 4 % (3 out of 82) in the control fish groups. An effect of the fraction ($F=9.13$; $p<0.001$), of the concentration ($F=18.08$; $p<0.001$) and an interaction between concentration and fraction ($F=6.18$; $p<0.001$) were identified at this time point. In the absence of data for HO-3X group at 9 mpf, we demonstrated a significant carcinogenic effect for HO-1X and LO-3X diets compared to control ($p=0.003$ and $p<0.001$) and a higher carcinogenic effect for LO-3X compared to PY-3X and for HO-1X compared to PY-1X (respectively $p<0.001$ and $p=0.002$). The carcinogenic effect of LO-1X was intermediate and could not be differentiated statistically from that of PY-1X or from HO-1X.

Nature of preneoplastic lesions

- Hepatocellular alteration foci

For consistency, only foci of cellular alteration (FCA) of more than 10 hepatocytes were considered and classified upon their staining with HES as eosinophilia, clear cell and basophilic foci (Fig 3). Respectively, 5.5 % (11 out of 199), 5.0 % (9 out of 180) and 2.8 % (5 out of 176) FCA were identified after PY, HO or LO exposure, all concentrations considered (Table 1). The global incidence in control groups was 2.2 % (4 out of 182). No significant effect of the gender, the age or the fraction could be identified on FCA incidences. Clear cell foci were globally more frequent (60 % of all observed FCA) and consisted of pale, finely vacuolated hepatocytes with granular cytoplasm. Eosinophilic foci (24 % of all FCA) appeared as round or irregular sharply delineated foci of hypereosinophilic slightly enlarged hepatocytes. Basophilic foci (16 % of all FCA) appeared round and consisted of clusters of smaller hepatocytes with hyperbasophilic or amphophilic cytoplasm.

- Biliary epithelium lesions

A systematic scoring of biliary epithelium lesions was performed. Bile duct epithelium appeared normally (score=0) as a monolayered cylindrical or cuboidal epithelium with a very low proliferation activity as assessed by PCNA labelling (Fig. 4, a-b). Bile duct hyperplasia (score=1) was a common finding in all zebrafish and consisted of an increased number of well-differentiated bile ducts with a typical epithelium displaying higher number of mitosis than in normal ducts (Fig. 4, d-e). The number of fish displaying hyperplastic bile ducts was stable between 3 and 9-10 months in control groups (34 % for both time points) while the number of fish with a higher score slightly increased from 3 to 18 %. In contaminated fish,

the number of fish with a score of 1 slightly decreased from 34 % to 26 % during this time period, with a parallel global increase from 24 % to 62 % in the number of fish with a higher score. Microscopically, dysplastic epithelium (score=2) appeared as a multi-layered and irregularly stratified epithelium and consisted of cells displaying pleomorphic, anisocytosis, anisokaryosis and high nucleo-cytoplasmic ratio (Fig.4, g). Number of mitotic cells was focally increased (Fig.4, h). In some samples displaying a dysplastic and highly proliferative duct epithelium, a few cells appeared elongated with loose intercellular bounds (Fig.4, j-k). They reached individually the pericanalar stroma through the basal membrane (Fig.4, l) that is highly evocative of epithelio-mesenchymal transition (score=3). A mean biliary epithelium score was calculated for each group (Fig. 2, right panel). No significant effect of the fraction could be detected at 3 mpf in fish exposed to PY and HO but score of fish exposed to LO-1X and LO-3X were increased compared to control. At 7 mpf, a significant effect of the exposure to HO was identified for all concentrations compared to control. From 9 mpf, the mean score ranged [0.1-1.2] in control animals and contrasted with the ones calculated in exposed animals, ranging [1.3-3.4] (Fig. 2). At 9-10 mpf, a significant effect of the fraction ($F=10.19$; $p<0.001$), and the concentration ($F=38.83$, $p<0.001$) was demonstrated with an interaction between both factors ($F=2.32$; $p=0.044$). The relative potency could be summarized as follow: LO-3X > PY-3X, HO-1X> PY-1X and LO-1X > PY-1X ($p<0.001$, $p=0.002$ and $p=0.002$ respectively) while potency of HO-1X and LO-1X could not be distinguished. At 14 mpf, an effect of PY was identified for all concentrations compared to control.

- Cholangiofibrosis

Bile duct epithelium lies normally on a basal membrane surrounded by scant connective tissue (Fig.4, c). In our study, the amount of connective tissue around bile ducts was frequently increased as evidenced in HES sections (Fig. 4, left panel) and this thickening was associated with the presence of several fibroblasts with a large euchromatic and nucleolated nucleus indicative of their active state. The collagenous nature of this densified tissue was confirmed with a specific Picrosirius red stain (Fig 4, right panel). The number of fish displaying mild to marked pericanalar fibrosis in control groups increased with time from 15 % at 3 mpf to 43 % at 9-10 mpf. Pericanalar fibrosis also increased following exposure to all contaminated diets from [11-55 %] to [37-100 %] at the same time points (Table 1). Significant effects of both the fraction, the concentration and an interaction between them were identified at 3 mpf ($F=3.52$, $p=0.031$; $F=3.81$, $p=0.011$; $F=2.24$, $p=0.041$ respectively) and 9-10 mpf ($F=14.28$, $p<0.001$; $F=25.47$, $p<0.001$; $F=2.87$, $p=0.015$). Pericanalar fibrosis was shown to be associated with bile duct epithelium dysplasia (Chi-squared test, $p<0.001$).

Neoplastic lesions

The nature of the 94 observed tumors is indicated in Table 1. The vast majority of tumors were of epithelial origin except one chondroma and one intestinal stromal tumor, both of mesenchymal origin and originating in these two cases from the intestinal wall (Table 1). Most tumors (91 out of 94) were malignant, with visible infiltration of peripheral tissues in 50 % of cases. These tumors are described below. Only 3 tumors were classified as benign, always consisted of a well-demarcated mass slightly compressing peripheral tissues and were identified as a chondroma, an adenoma of endocrine pancreas origin and a pneumatic duct adenoma.

- Duct carcinoma

Nodular proliferation of duct cells was observed in liver or more rarely in pancreas (Fig. 4 and 5). According to their common duct epithelium origin, we chose to indifferently call these two neoplasms “duct carcinoma” in this report, without considering their hepatic or pancreatic primary site. This name should reflected more properly their very similar appearance and their high propensity to infiltrate peripheral tissues that sometimes precluded identification of the tissue origin of the tumor. Duct cells proliferation and transformation most frequently occurred in the anterior area of the liver near the emergence of the common bile duct. The early very invasive behaviour and the abundance of atypias were indicative of their carcinomatous nature.

Duct carcinomas formed large tumors, without significant increase in the mean size of tumors from 3 to 14 mpf. Neoplasms were composed of disorganized clusters of atypical, variably sized, branching and tortuous ductules and ducts often admixed with abundant proliferating dense collagenous or myxoid stroma (Fig. 4, m and o and Fig. 5, a). Lining neoplastic epithelium was usually simple, but in some regular instance was irregularly stratified with occasional extension through the basement membrane. Neoplastic cells could either be cohesive and polygonal, reminiscent of their epithelial origin, or isolated and elongated with fine cytoplasmic processes. The proliferation activity was very high (Fig. 4, n). A spindle cell proliferation around ducts was frequently associated, sometimes making it difficult to distinguish between proliferating neoplastic ducts and a highly cellular stroma. Special staining and immunohistochemical analysis confirmed the presence of scattered epithelial cells in the stroma, isolated, in little clusters or forming small ductules with a collapsed lumen (Fig 5, c).

Neoplasm infiltration first occurred in the surrounding parenchyma and larger tumors in the

liver additionally extended through the hepatic capsule and invaded directly of adjacent tissues and organs, including intestine, pancreas and spleen. The only observed form of distant metastasis was transcoelomic after fragmentation of the primary tumor into the peritoneal cavity with occasional implantation and invasion into abdominal fat, pancreas and peritoneal wall. No vascular emboli or distant metastasis in tissues away from the pleuroperitoneal cavity could be observed.

Duct carcinomas were observed in control and exposed fish from 3 to 14 mpf. Global incidences ranged [0-3.2 %] in control fish compared to incidences of 7.4 %, 14.6 % and 23.4 % in fish exposed to PY, HO and LO respectively with a significant effect of the fraction (Chi-squared test, respectively $p=0.012$, $p<0.001$ and $p<0.001$).

- Seminoma

In the present study, seminomas were the second most commonly found tumor with 7 occurrences. These tumors were observed in fish exposed to PAHs at 1X and 3X concentrations all fractions considered. Fish age at the time of observation ranged from 3 to 9 mpf. Seminoma was not observed in non-exposed fish and no significant difference of incidence could be identified between the 3 PAH fractions. These neoplasms were often very large, always exhibiting a very aggressive behaviour, invading large parts of the body cavity and infiltrating abdominal tissues. The tumors were solid with only a few lobules containing spermatocytes and spermatids (Fig 6). A severe inflammatory infiltration was frequently associated.

Sporadic findings

Other lesions included developmental lesions of the heart, the eyes and the organs of reproduction. These results not directly related to the carcinogenic study will be reported elsewhere (Lucas et al. submitted; Vignet et al. In press). Some inflammatory lesions, predominantly macrophage aggregates and granulomas, were sporadically found in the body cavity or in the ovary. Embryonic nephrons that stained more basophilic than mature ones were infrequently and undifferentially observed in exposed and control fish. Hamartoma of pseudobranch was observed in 2 fish, near the normal pseudobranch. This was a well-differentiated redundant pseudobranchial tissue.

Genotoxic effects

Genotoxicity was evaluated at 2 mpf using comet assays to assess repairable DNA damages and at 9 mpf using MN assay to assess non-repairable DNA damages.

Due to the small size of the fish exposed to the HO and LO diets, blood was very difficult to collect and, as a consequence, the number of nuclei available on the slides was too small to be significant. By applying the comet assay in fish fed with the PY-contaminated diet, no difference in the extent of DNA damage was observed compared to the control group ($p=0.37$), whatever the concentration (Fig. 7).

Micronuclei analysis was performed on blood cells of adult zebrafish fed for 9 months with PY-, LO- or HO-contaminated diets (Fig. 8). MN frequency ranged from 0.07 to 0.33 ‰ with no significant difference (ANOVA, $p>0.05$) between fractions. A relatively high inter-individual variability was observed.

Metabolism activation and kinetics

EROD activity results are presented in table 3. No significant effect of the exposure to PAHs was detected (Tukey test) except between control group and fish fed with diet containing HO-0.3X ($p=0.020$) in which EROD activity was increased (fold-change=1.9).

To investigate the possible consequence of sampling procedure on comet and EROD assays, the kinetics of *cyp1a* and *ahr2* expression after feeding were analysed on PY-3X larvae. Expression of *cyp1a* was measured following a 24 h starvation period (0 hprf) of larvae exposed to PY-3X and of control larvae (Fig. 9). At 0 hprf, *cyp1a*expressionwas only slightly up-regulated in PY-3X fish (fold-change=1.3; $p<0.001$). This expression was highly up-regulated in refed PY-3X larvae compared to starved PY-3X larvae from 2 to 8 hprf (fold-change range [5.5-11.3]; $p<0.001$). No significant difference was observed in expression levels at 10 hprf ($p=0.177$). At 24 hprf, *cyp1a* expression was not different from the basal level (0 hprf) ($p=0.325$).

Regarding, the aryl hydrocarbon receptor 2 (*ahr2*), no significant expression change was revealed after a contaminated refeeding from 0 to 4 hprf ($p=0.346$, $p=0.325$, $p=0.825$ and $p=0.325$) and from 10 to 24 hprf ($p=0.830$ and $p=0.177$). However, *ahr2* expression was 0.9-fold repressed in refed PY-3X larvae at 6 and 8 hprf respectively ($p<0.001$).

Discussion

Despite numerous toxicological studies on individual PAHs, little is known about effects of long-term dietetary exposure to PAHs as mixtures containing dozens of molecules that are effectively encountered in environmental situations. This is in particular due to the fact that wild-caught fish could have been exposed to other carcinogenic compounds or to other

confounding factors. The aim of the present study is to document carcinogenic and genotoxic effects induced by exposure to PAHs from the first meal and maintained afterwards.

Neoplasm incidences

In order to exhaustively describe here all preneoplastic and neoplastic lesions and characterize the full range of carcinogen responsiveness of all tissues, serial step sections of the entire body of exposed and control zebrafish were observed microscopically. Descriptive studies of chemically induced tumors in zebrafish have frequently omitted in the past to report complete data concerning fish strain, fish age or spectrum of investigated tissue. Additionally, naturally occurring tumors in zebrafish have only been identified in a few published works (Spitsbergen et al. 1997b; Smolowitz et al. 2002; Spitsbergen et al. 2012). This information would be useful to compare results between carcinogenesis studies. Reported incidences of spontaneous neoplasia in zebrafish colonies must therefore be taken cautiously but always seems to be relatively low, evaluated to approximately 1 % (Spitsbergen et al. 1997a). An incidence of 4 % was found in our control fish, which is in agreement with this low basal level. The slightly higher rate observed in this study may be due to the genetic background. Indeed albeit being established as TU colony, some sporadic leopard or longfin phenotypes were observed suggesting that some *Tupfel* (TL) genetic background has been introduced at some time.

After chronic exposure of seven or more months to PAH fractions, incidences of neoplasia ranged from 17 % to 72 % with the lowest carcinogenic effect observed with the PY fraction and the highest with the HO one. A clear concentration-effect relationship was additionally demonstrated for all tested fractions. As a comparison with two of the most potent PAHs characterized to date, a maximal incidence of 23 % of zebrafish developing neoplasia was reported after dietary exposure to 1,000 ppm of DMBA for 12 weeks from 2 months of age (Spitsbergen et al. 2000a) and no increase of the incidence with time. In the present study, the carcinogen exposures started during the early highly sensitive stages in the life of zebrafish whereas previous dietary studies published by Spitsbergen et al. did not start until fish were juveniles 2 months of age. Medaka or even trout are believed to be much more sensible to carcinogenic effects in similar conditions retaining carcinogen sensitivity in dietary studies begun later in life (Reddy et al. 1999a).

Effect latency

Several times of exposure were documented here for the first time. The survival was decreased as soon as 3 mpf in the high concentration group of fish exposed to HO, confirming the already observed higher toxicity of this fraction (Le Bihanic et al. Submitted-a; Le Bihanic et al. Submitted-b; Vignet et al. In press). Effect on survival was only detected afterwards in fish exposed to LO and no effect on survival was identified in fish exposed to PY. Regarding tumors, neoplasms were observed as soon as 3 mpf only after exposure to the highest level of PY or LO.

Target tissues and cells

In the few published data, the most commonly reported spontaneous neoplasms in zebrafish are epithelial, including seminoma, adenoma of exocrine pancreas and hepatocellular adenoma and less frequently intestinal adenocarcinoma (Spitsbergen et al. 2000a; Smolowitz et al. 2002). In our study, a large majority of neoplasms were tumors of epithelial origin and corresponded mainly to duct carcinomas and to seminomas. Mesenchymal tumors were very few as previously reported (Spitsbergen et al. 2000a).

Our study identified liver as the main target organ after a chronic dietary exposure to PAHs. This is in accordance with other reports identifying liver tumors (including those derived from hepatocellular, bile duct, blood vessel and Kupffer cells) as predominant following exposure of various fish species to DMBA or BaP (Bailey et al. 1984; Hendricks et al. 1985; Fong et al. 1993; Bailey et al. 1996; Bunton 1996; Reddy et al. 1999b; Wills et al. 2010). Rare gastric tumors were additionally observed with lower incidence in the rainbow trout (Fong et al. 1993) and similarly, very few intestinal tumors were recorded here in the agastric zebrafish. In PAH-contaminated sites, a high prevalence of hepatic preneoplastic or neoplastic lesions was also reported in mummichog (*Fundulus heteroclitus*)(Vogelbein et al. 1990), North American winter flounders (*Pleuronectes americanus*) and European flounders (*Platichthys flesus*) (Murchelano and Wolke 1985, 1991; Cachot et al. 2013).

The large predominance of duct carcinomas of hepatic origin described here contrasts with the neoplasm incidences reported by Spitsbergen et al. in juvenile zebrafish (2 mpf) dietary exposed to DMBA for 4 months (500 to 1,000 ppm) (Spitsbergen et al. 2000a). In this last study, intestinal and gill neoplasms were identified with respective incidences of 7 and 4 %. Only 3 duct carcinomas, originating from the pancreas, and no liver neoplasm were observed. The main distinctive parameters with our study, that are age of onset of exposure and nature of the PAH, may explain the discrepancy in target tissues (Spitsbergen et al. 2000b). The influence of the genetic background of our fish colony and the aquaculture systems used in

our facility cannot be ruled out. Indeed as indicated previously, it is likely that some TL genetic background contaminated the TU background. TL line which has been shown to have a high rate of spontaneous bile duct hyperplasia as observed here (Kent et al. 2012; Spitsbergen et al. 2012) may have a higher sensitivity to biliary neoplasia upon exposure to carcinogens. In liver, in addition to bile duct tumors, FCA were sporadically seen in our study but their global low incidence precludes any correlation with the exposure to PAHs. None liver adenoma or hepatocarcinoma was observed although FCA is considered to represent a morphological appearance associated with the progression of hepatic neoplasia in rodents and fish (Hendricks et al. 1995; Boorman et al. 1997; Thoolen et al. 2010; Hobbie et al. 2012). In mummichog subjected to acute aqueous expositions to BaP, incidences of both FCA and hepatocellular carcinomas have been described to be increased (Wills et al. 2010).

The histologic appearance of tumors in zebrafish is similar to that of the human counterpart, including increased frequency of mitosis and atypical nuclei (Goessling et al. 2007). A comparison of the human and zebrafish genomes reveals the conservation of cancer-relevant oncogenes, tumor suppressor genes, and cell cycle genes (Amatruda et al. 2002). The histologic similarity and genetic conservation suggests that the genetic mechanisms underlying carcinogenesis in zebrafish may be highly similar to those in humans. A recent study compared the gene expression signatures in chemically induced liver tumors in zebrafish to human hepatocellular carcinoma (Lam et al. 2006). Another study analysed deregulation of genes involved in cell cycle, apoptosis, DNA repair, and metastasis in zebrafish liver tumors and revealed that the genetic changes in both species were significantly correlated (Goessling et al. 2007).

Carcinogenesis mechanisms

The carcinogenic mechanisms implicated in duct tumor progression were not elucidated here despite several investigations. In particular no evidences of repairable or irreversible DNA damages were observed. Unfortunately, genotoxic effects were only documented in haemocytes due to their higher accessibility for sampling using comet and micronucleus assays. We cannot rule out 1) the occurrence of DNA damages such as DNA-adducts and 2) that DNA damages may have occurred in other tissues, particularly in bile ducts whose cells displayed preneoplastic lesions correlated to the fraction and the dose of exposure and from which most neoplasms derived. Indeed, PAHs are known to be bioavailable and quickly metabolized by fish hepatic enzymes as confirmed here by the kinetic of *cyp1a* expression after feeding or, in other studies, by the rapid production of biliary PAH-metabolites after

exposure (Wessel et al. 2010; Le Du-Lacoste et al. 2013). Among these metabolites, 3-hydroxybenzo[a]pyrene is believed to be one of the most genotoxic (Aas et al. 2000; Ruddock et al. 2003; Brinkmann et al. 2010; Le Du-Lacoste et al. 2013). Several studies have also reported the genotoxicity of compounds such as nitrogen-, sulfur- and/or oxygen-containing PAHs or their alkyl substituted analogues (Burczynski and Penning 2000; Binet et al. 2002; Francioni et al. 2007; Ramos de Rainho et al. 2013).

All the three fractions tested here have genotoxic potential. In the case of the PY-contaminated diet, genotoxicity may be driven by high molecular weight PAHs such as BaP from which metabolites were quantified in larvae exposed to this diet (Vignet et al. In press). Regarding the LO-contaminated diet, genotoxicity should rather be due to the high level of alkylated PAHs as genotoxicity of these compounds and their metabolites have already been documented (Burczynski and Penning 2000; Francioni et al. 2007). As for HO-contaminated diet, it displayed high levels of both heavy PAHs, including BaP, and methylated ones. Additionally we cannot exclude the role of compounds that were not quantified here as some recent studies focused on the important role of uncharacterized polar contaminants such as nitro-PAHs, aromatic amines and sulfur compounds in the global mutagenicity of PAH fractions (Amat et al. 2004; Wahidulla and Rajamanickam 2010; Di Giorgio et al. 2011). Regarding other compounds, aromatic fractions were prepared with methods excluding the co-purification of heavy metals. PCBs and some other organic compounds are copurified with PAHs, but there were no PCBs in the petrogenic fractions. For the PY fraction extracted from Oissel sediment, previous reports indicated that PCBs were present at very low concentrations ($\sum$PCBs=70 ng.g^{-1} and dioxin-like congener CB118=8.4 ng.g^{-1} (Cachot et al. 2006)). We therefore concluded that the carcinogenic effects reported here were due to the PAHs present in the different fractions.

The chronic exposure of bile duct cells to some of these compounds may be responsible from their higher rate of proliferation depicted in the present study but also from the formation of DNA base oxidation or DNA adducts resulting in the induction of neoplasms originating from these specific cells. Noteworthy, genotoxicity assessed by the comet assay was demonstrated to be higher in trout larvae exposed for 10 days to HO fractions compared to larvae exposed to LO or PY fractions (Le Bihanic et al. Submitted-b). This result would be in accordance with the higher carcinogenic potency of the HO fraction highlighted in the present study.

Direct alteration to DNA is not the sole mechanism of chemical carcinogenesis and a large number of chemicals can produce cancer in animal models (as well as in humans) without any genotoxic effect. It is now assumed that the multistage carcinogenic process can result from

an increase in cellular proliferation (Winn et al. 2000; Cohen and Arnold 2011). Although some PAH-metabolites are well-known DNA-reactive carcinogens at high levels, our results at lower levels suggest that some PAHs might chronically increase the duct cell proliferation rate. In accordance to this hypothesis, it has been recently described in Medaka, the induction of TGF-β1 expression after dimethylnitrosamine exposure in some liver cells, including preductular epithelial cells. TGF-β1 expression results in collagenous matrix deposition, spindle cell proliferation and hepatocellular and biliary carcinomas (Hobbie et al. 2012). In accordance to this work, we found some collagenous deposits around bile ducts of fish exposed to PAHs eliciting a progressive thickening of the pericanalar space. Other mechanisms, like epigenetic alterations and DNA methylation alterations, may have also contributed to carcinogenesis without DNA-adduct formation by specific silencing of tumor suppressor gene expression or by aberrant activation of tumor-promoting genes (Oliveira et al. 2007; Pogribny and Beland 2012).

Taken together these results indicated that chronic dietary exposures of zebrafish to environmentally relevant doses and mixtures of PAHs elicited carcinogenic events with a high frequency and in a narrow range of tissues. Due to their invasive behaviour, tumor growth had detrimental effects on adult fish survival. Despite their very contrasted compositions, pyrolytic and petrogenic mixtures were similar regarding the array of induced preneoplastic and neoplastic lesions suggesting shared carcinogenic mechanisms.

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Figure legends

Table 1 Incidence of preneoplastic and neoplastic lesions in zebrafish dietary exposed to 4concentrations and 3 fractions of PAHs

Table 2 Specific primer sequences for target genes measured by real-time PCR

Table 3 Mean EROD activity in whole fish exposed to 4concentrations and 3 fractions of PAHs (N=3 to 4 in each group)

Figure 1 Survival of adults after exposure to PAH fractions. Kaplan-Meier survival plots are presented indicating survival rates for each fraction and concentration. Note that because of the high mortality occurring in the HO exposure, all the remaining HO-3X fish were sampled for histology analysis, at 6 mpf. This explains why the curve stops at 180 days. Log-rank (Mantel-Cox) test indicated significant differences for HO survival rates (Chi-square value=36.30; $p<0.0001$) and LO (Chi-square value=13.86; $p=0.003$) but not PY (Chi-square value=0.80; $p=0.849$)

Figure 2 Neoplasm incidence (left panel) and biliary epithelium mean score (right panel) of zebrafish exposed to various concentrations and fractions of PAHs. Mean scores are mean ± SD. Different letters indicate that values differ significantly ($p <0.05$)

Figure 3 Hepatic parenchyma of zebrafish exposed to various concentrations and fractions of PAHs. (a) Normal hepatic parenchyma of a control fish exposed to control. (b) Clear cell foci of cellular alteration (FCA) in a fish exposed to PY-0.3X, 14 mpf. (c) Eosinophilic FCA in a fish exposed to PY-3X, 9 mpf. (d) Basophilic FCA in a fish exposed to LO-3X, 3 mpf. (a-d) Haematoxylin-Eosin-Saffron. Bars = 100 µm (a, b, c) or 50 µm (d)

Figure 4 Bile duct of zebrafish exposed to various concentrations and fractions of PAHs. A semi-quantitative score (0 to 4) was attributed for each fish regarding bile duct epithelial lesions (left panel) and proliferation activity (middle panel). A second score (0 to 4) reflected the pericanalar amount of connective tissue (right panel). Contrasting with the normal appearance of bile ducts (a-c), hyperplastic ducts appeared with numerous mitotic cells (d, open arrowhead and e), sometimes surrounded with a thickened layer (*) of connective tissue (f). Dysplastic ducts were multi-layered with cellular atypias (g) and with a mild proliferation activity (h). During epithelio-mesenchymal transition, some epithelial cells appeared elongated, loosely attached to other epithelial cells (j-k, arrowhead) and disrupting basal membrane (l, arrowhead). Bile duct carcinomas (m) were composed of clusters and cords of neoplastic cells (arrowheads), sometimes with tubular differentiation. The proliferation activity was very high (n). Neoplastic cells were scattered in an abundant collagenic stroma (n, *). (left panel) Haematoxylin-Eosin-Saffron. (middle panel) Immunolabelling of the Proliferating Cell Nuclear Antigen. (right panel) Picrosirius red. (a-o) Bars = 10 µm

Figure 5 Duct carcinoma of zebrafish exposed to various concentrations and fractions of PAHs. (a) Bile duct carcinoma (black arrowhead), also called cholangiocarcinoma, in the liver (L) of a fish exposed to PY-3X, 14 mpf. The tumor breached parietal peritoneum (arrows) and infiltrated peripheral tissues. The neoplastic cells sometimes formed tubules (black arrowhead) in a loose connective stroma rich in inflammatrory cells. Note the normal adjacent bile duct (open arrowhead). (b) Pancreatic duct carcinoma (black arrowhead) in a fish exposed to LO-0.3X, 9 mpf. (c) Bile duct carcinoma in the liver of a fish exposed to HO-3X, 7 mpf. Cytoplasm of neoplastic cells was positive for cytokeratin, indicative of their epithelial origin. Some isolated neoplastic cells could be identified (arrow). (a, b) Haematoxylin-Eosin-Saffron. (c) Pancytokeratin immunohistolabelling. Bars = 100 µm (a, b) or 50 µm (c)

Figure 6 Spermatocytic seminoma in a zebrafish exposed to PY-1X, 9 mpf. Haematoxylin-Eosin-Saffron. Bar = 50 µm

Figure 7 Mean tail DNA value as an indicator of DNA fragmentation level in fish exposed to various concentrations of a pyrolytic fraction (N=12 to 15 in each group)

Figure 8 Micronuclei frequency (‰) in haemocytes of adult zebrafish fed with PAH-contaminated diet for 9 months. No significant differences were observed between treatment groups (ANOVA, $p>0.05$, N=3)

Figure 9 Expression kinetics of genes in 16 dpf larvae exposed to PY-3X spiked diet demonstrating an induction of *cyp1a* levels (a) and a repression of *ahr2* levels (b) following nutritional/starvation assay. Data are given in relative expression mean ± SEM. * denote significant differences as compared to control condition at t_0 and their respective starved condition for other analysis time ($p<0.001$). Stars placed at the top of markers indicate an up-regulation and below, a down-regulation

Supplementary Table 1 PAHs concentration in used aromatic fractions (µg.g $^{-1}$of fraction; mean ± SD)

Supplementary Table 2 Detailed concentration of individual PAHs in produced diets (ng.g $^{-1}$ food; mean ± SD; N=4-7)

Table1

Exposition			Fish		Preneoplastic lesions		Neoplastic lesions			
				Sex ratio	Pericanalar fibrosis					
PAH mixture	Dose	Duration	n	M/F %M	% mild to marked	FCA	Mesenchymal tumor	Adenoma	Duct carcinoma	Seminoma
PY	0	3	20	65%	13%	2/20			0/15	
	0	9	30	27%	21%	1/30			0/28	
	0	14	9	33%	33%	0/9			0/9	
	0.3	3	20	47%	23%	0/19			0/13	
	0.3	9	31	42%	37%	1/31			3/27	
	0.3	14	21	43%	58%	2/19	1/21 CHD		1/17	
	1	3	20	80%	15%	1/19			0/13	
	1	9	30	40%	39%	1/30	1/30 IST	1/30 EPA	0/28	
	1	14	20	35%	65%	1/20			0/20	
	3	3	20	95%	44%	0/20			2/18	2/20
	3	9	30	57%	85%	2/29			4/27	1/30
	3	14	13	38%	85%	0/12			3/13	
HO	0	3	20	85%	21%	0/20			0/19	
	0	7	25	48%	0%	0/23			0/21	
	0	10	22	91%	41%	0/22			2/22	
	0.3	3	20	63%	33%	0/19			0/18	
	0.3	7	25	56%	20%	2/25			0/25	
	0.3	10	27	56%	78%	4/27		1/27 PDA	2/27	
	1	3	20	89%	11%	0/19			0/19	
	1	7	25	52%	48%	0/24			3/23	2/25
	1	10	29	61%	86%	1/29			13/29	
	3	3	20	80%	8%	1/13			0/13	
	3	7	25	70%	29%	1/24			8/24	
LO	0	3	28	43%	11%	0/28			0/27	
	0	9	30	30%	63%	1/30			1/30	
	0.3	3	31	42%	29%	0/31			1/31	
	0.3	9	30	50%	63%	0/30			4/30	
	1	3	29	28%	55%	0/29			2/29	
	1	9	30	37%	90%	1/30			9/29	
	3	3	30	48%	52%	2/27			4/27	
	3	9	30	55%	100%	0/29			21/29	2/30
Total			**760**	**52%**						

CHD: chondroma; EPA: endocrine pancreas adenoma; FCA: foci of cellular alteration; IST: intestinal stromal tumor; PDA: pneumatic duct adenoma

Table2

-

Target gene	Accession Number	Primer (5' → 3')
eef1 (eukariotic elongation factor 1)	NM_131263.1	CAGCTGATCGTTGGAGTCAA[a]/TGTATGCGCTGACTTCCTTG[b]
g6pd (glucose-6-phosphate dehydrogenase)	BM_182602	GTCCCGAAAGGCTCCACTC[a]/CCTCCGCTTTCCTCTC[b]
β-actin (beta-actin)	NM_131031	CCCAGACATCAGGGAGTGAT[a]/CACAATACCGTGCTCAATGG[b]
cyp1A (cytochrome P4501A)	BC094977	GACAGGCGCTCCTAAAACAG[a]/CTGAACGCCAGACTCTTTCC[b]
ahr2 (aryl hydrocarbon receptor 2)	NM_131264	GCCTGGGATAAAGGAGGAAG[a]/CAGCTCCACCTGTCCAAAT[b]

a/b, Forward/Reverse primers

Table3

	Control	0.3X	1X	3X
PY	39.5 ± 8.8	94.8 ± 43.2	81.2 ± 33.0	78.8 ± 17.7
HO	27.6 ± 7.8	51.6 ± 5.9 *	37.9 ± 5.5	40.6 ± 10.9
LO	38.3 ± 8.5	53.6 ± 3.4	44.6 ± 4.9	58.9 ± 8.7

* $p<0.05$

Figure1

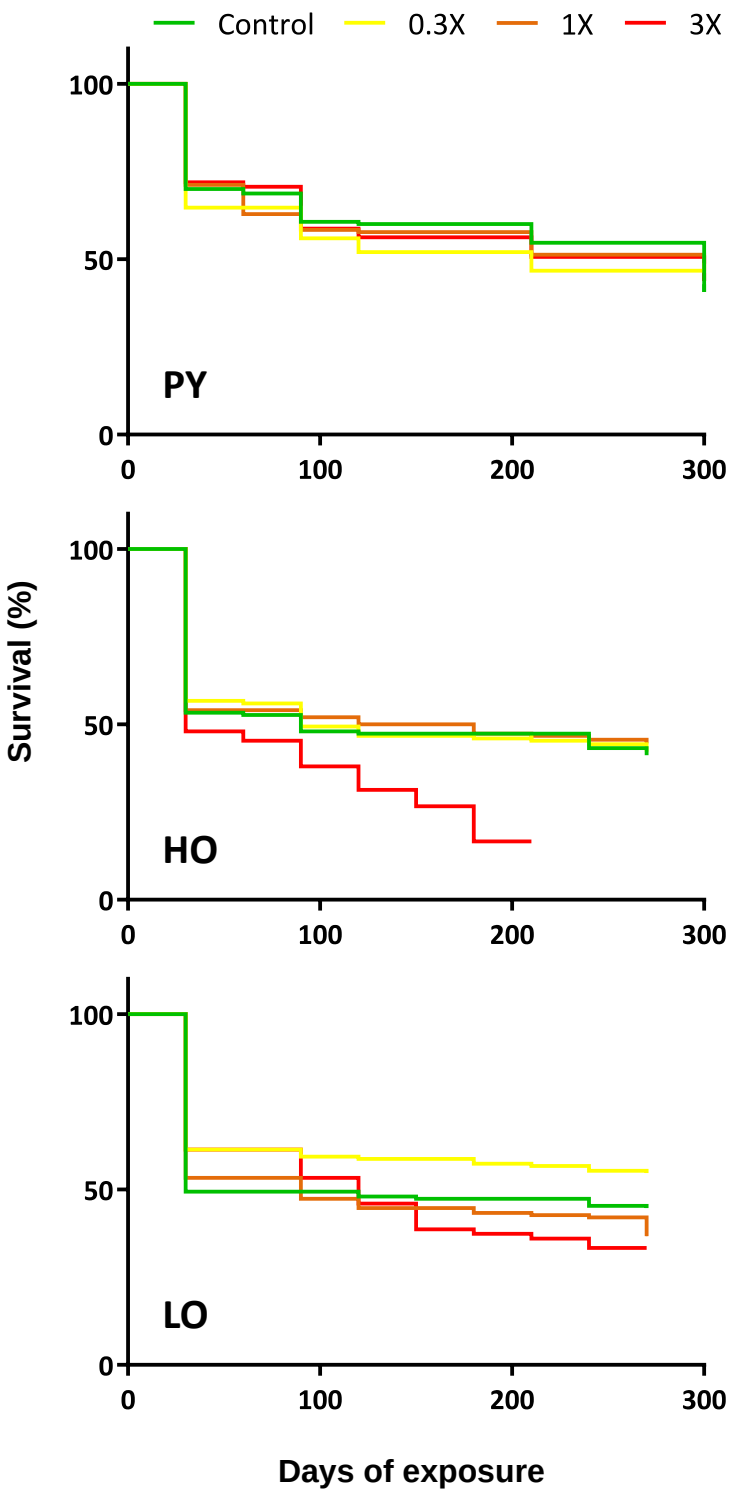

Control
0.3X
1X
3X
100
50
0
PY
HO
LO
0
100
200
300
Survival (%)
Days of exposure

Figure2

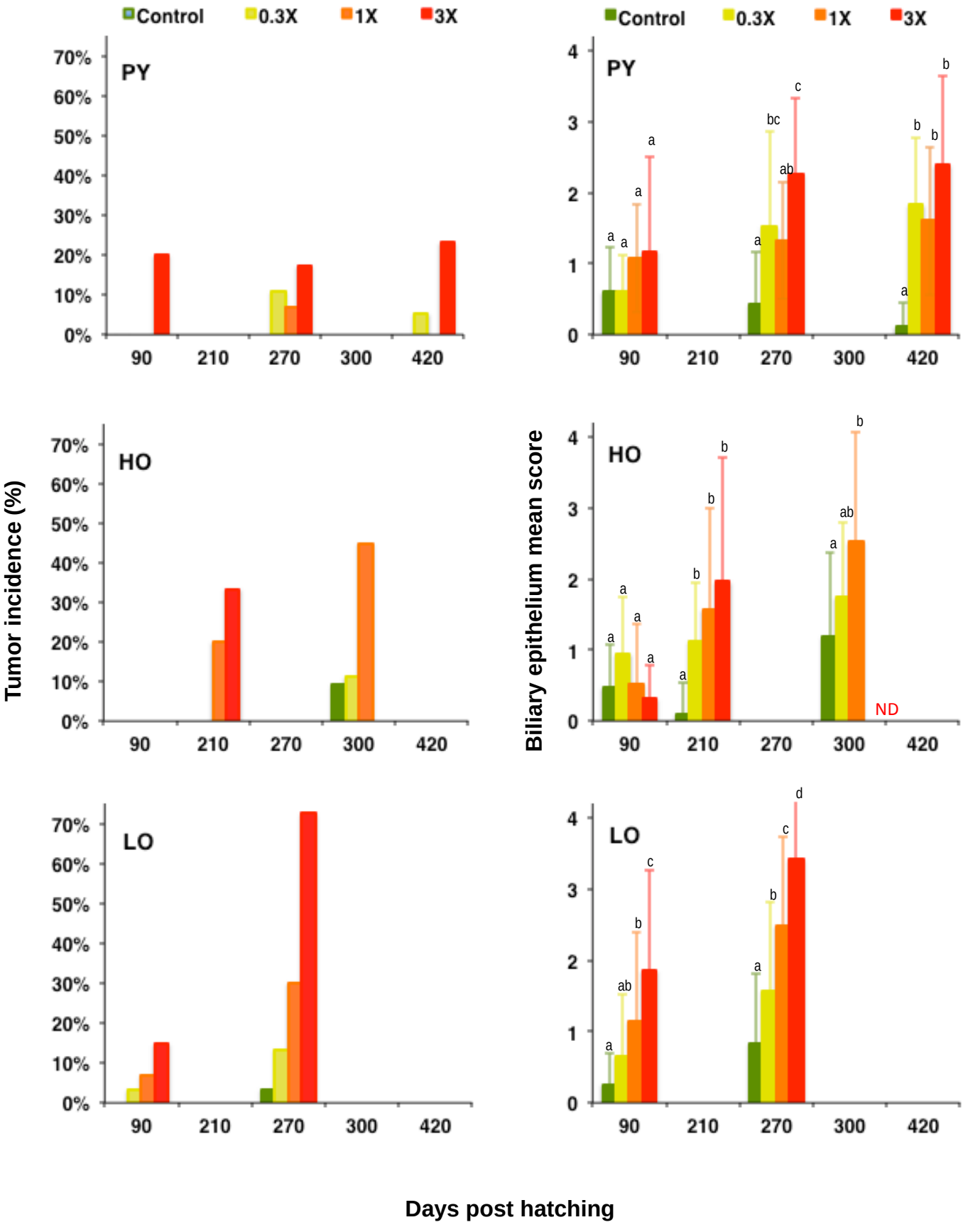

Control
0.3X
1X
3X
PY
HO
LO
Tumor incidence (%)
Biliary epithelium mean score
ND
Days post hatching
90
210
270
300
420

Figure3
Click here to download high resolution image

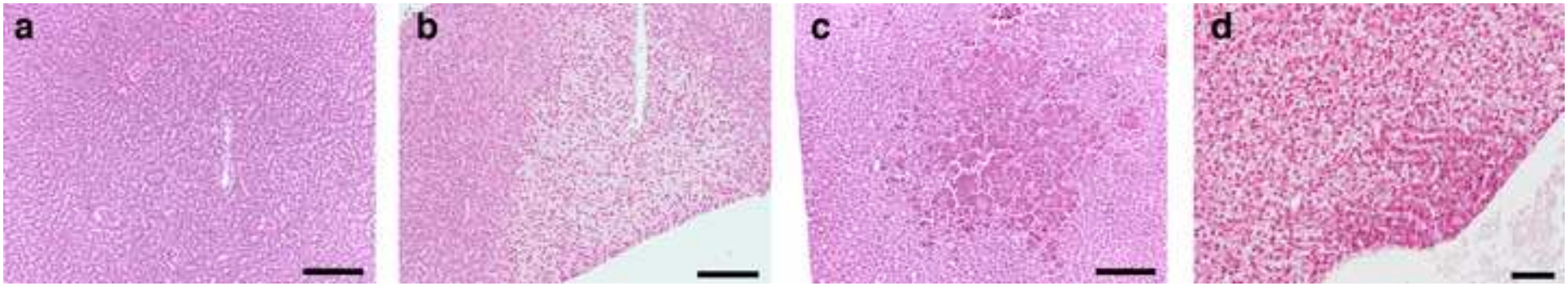


Figure4
Click here to download high resolution image

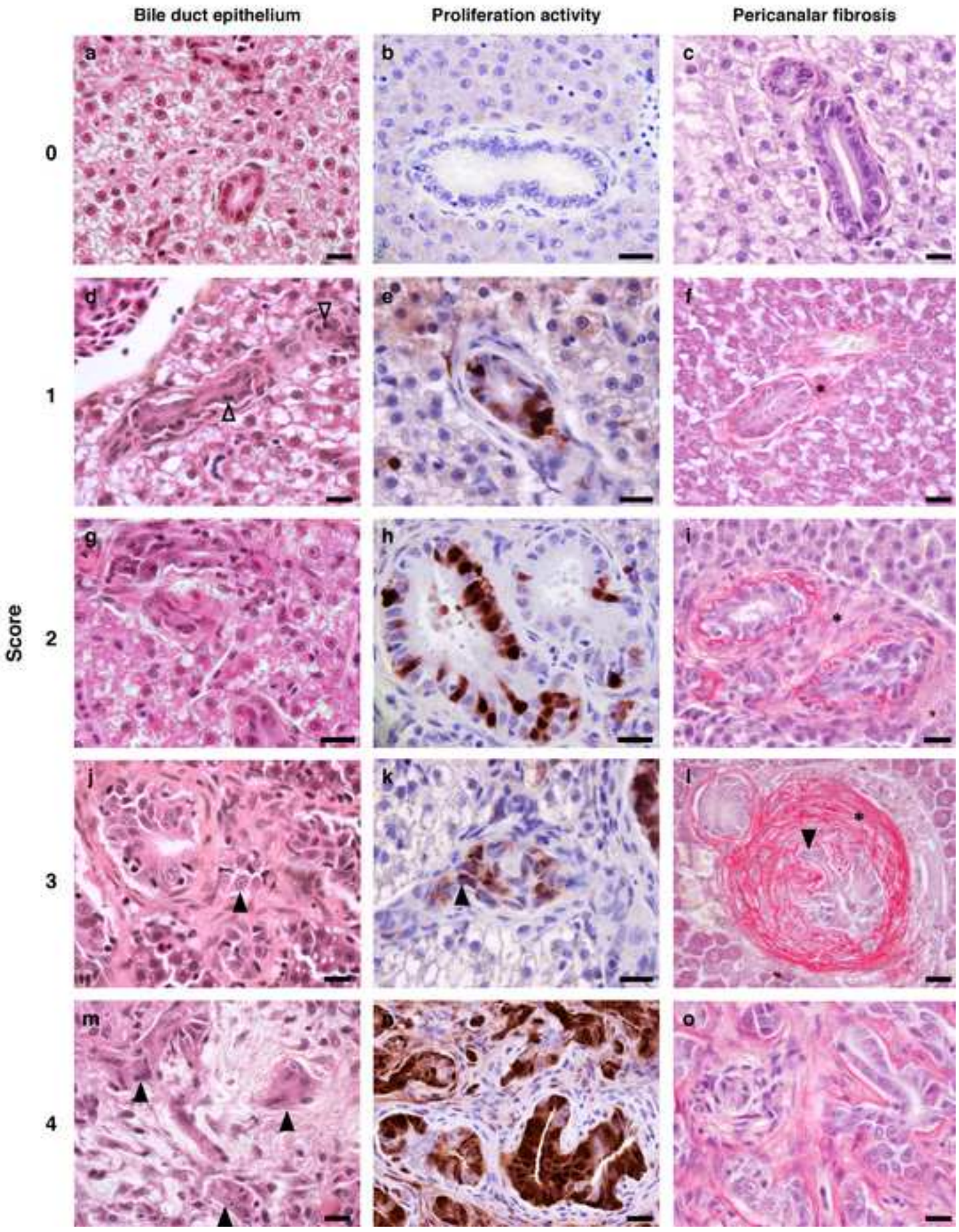


Figure5
Click here to download high resolution image

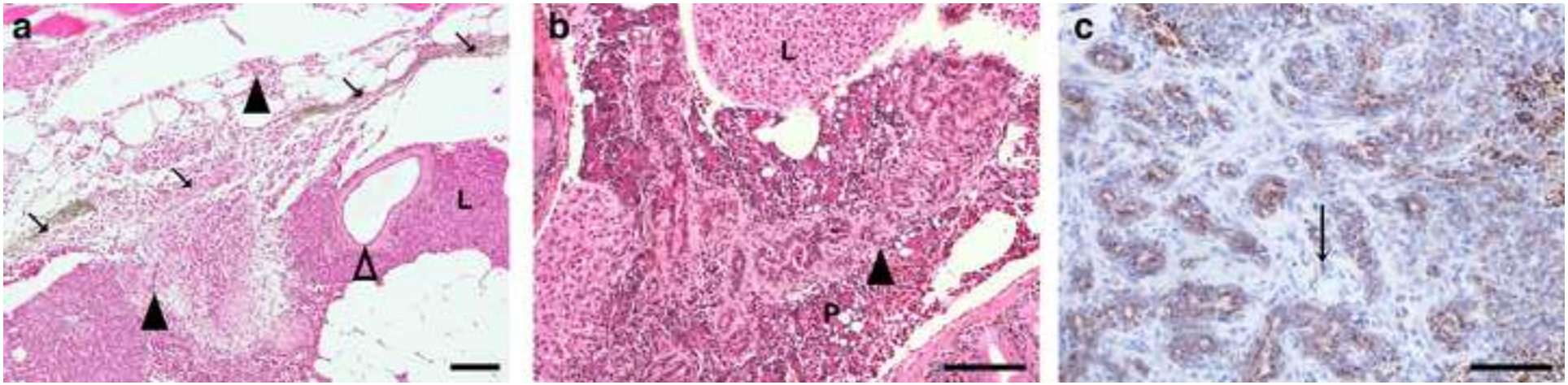


Figure6
Click here to download high resolution image

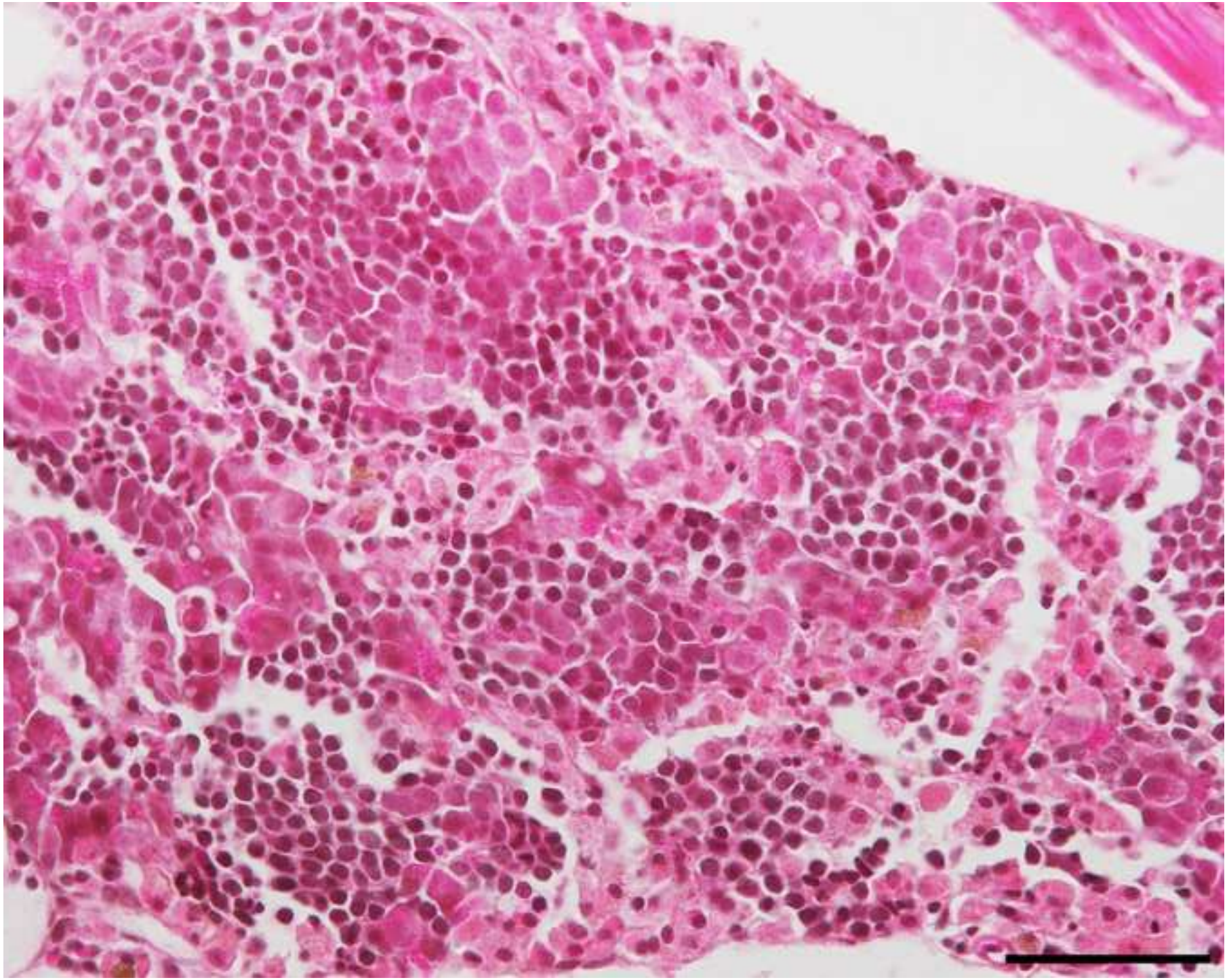


Figure7

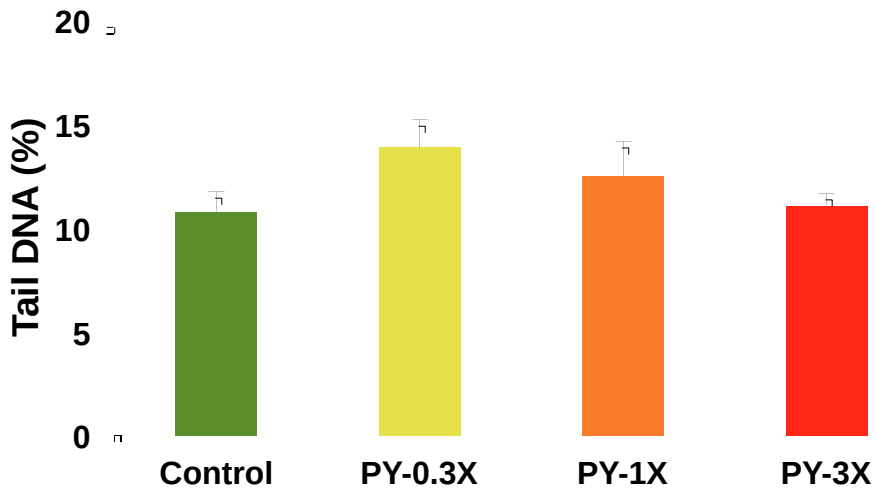


Figure8

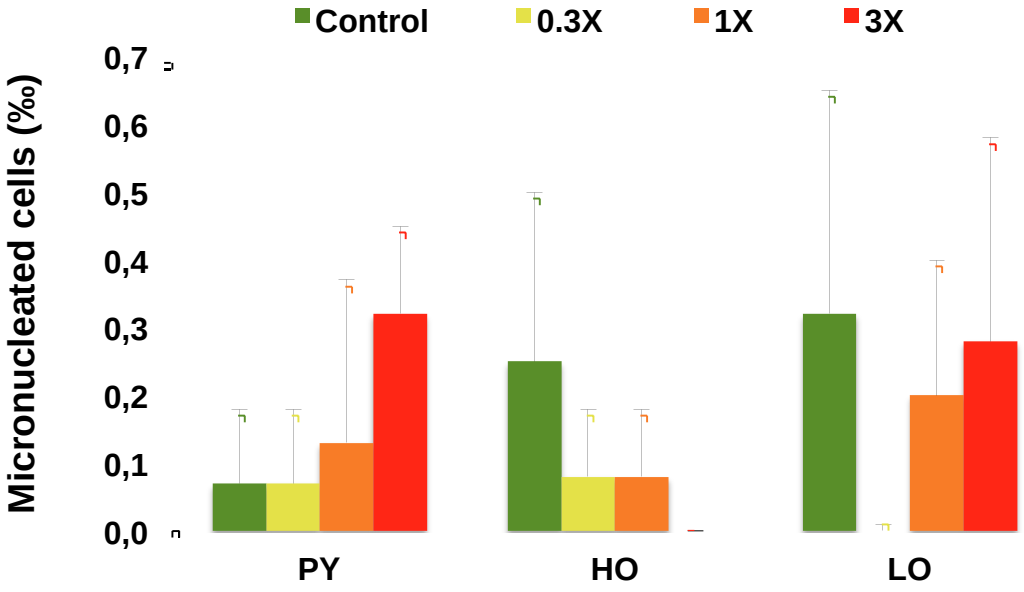
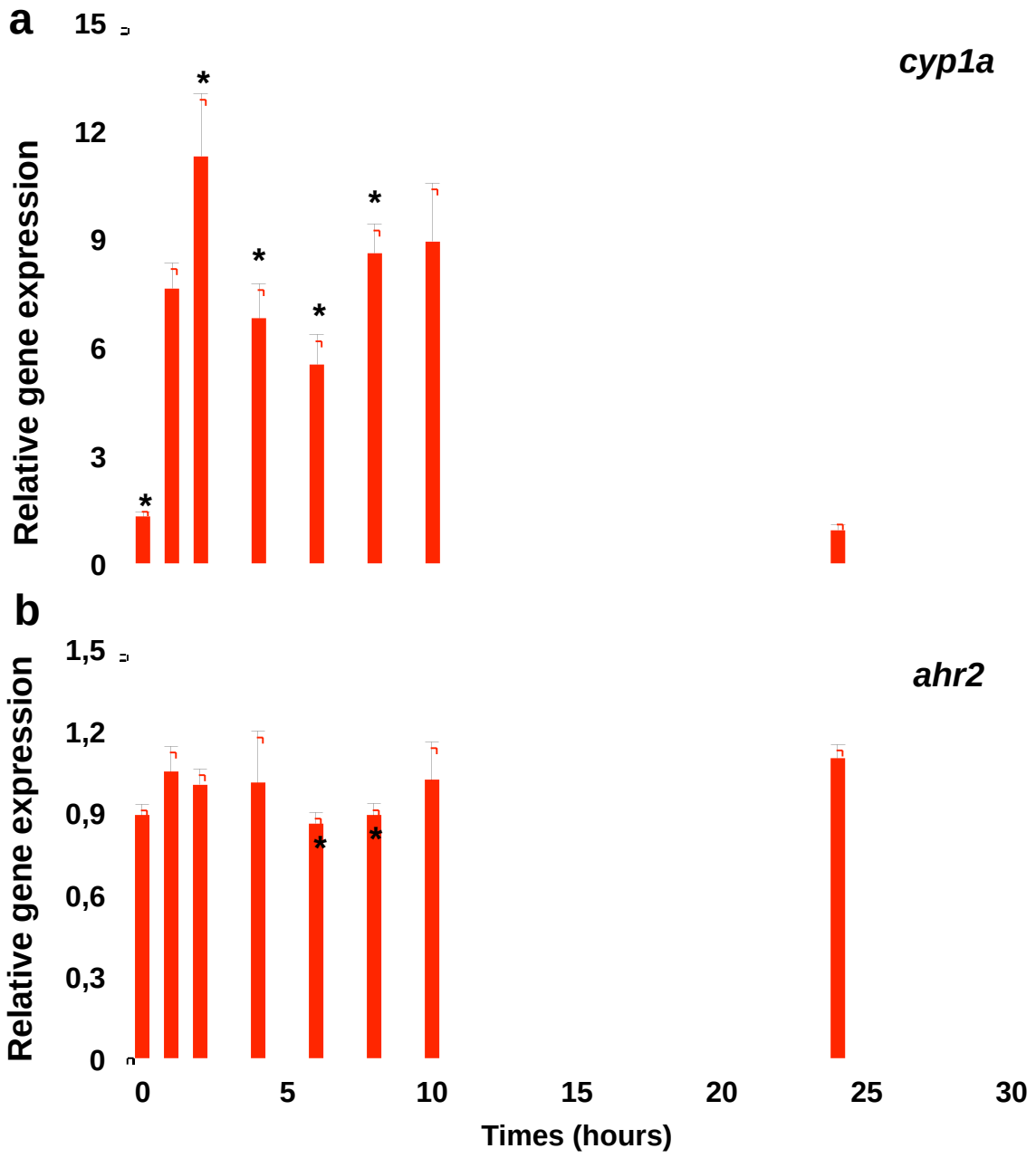

Control
0.3X
1X
3X
Micronucleated cells (‰)
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0,0
PY
HO
LO

Figure9


a
cyp1a
Relative gene expression
15
12
9
6
3
0
b
ahr2
Relative gene expression
1,5
1,2
0,9
0,6
0,3
0
0
5
10
15
20
25
30
Times (hours)

Supplemental Table 1: PAHs concentration in used aromatic fractions (µg.g $^{-1}$of fraction; mean ± SD)

PAHs	Ring #	PY	HO	LO
Naphthalene	2	7.8 ± 0.5	88.5	133.2 ± 4
acenaphthylene	2	4.8 ± 0.4	0.4	15.8 ± 1.9
Acenaphthene	2	2.8 ± 0.3	34.9	16 ± 7.3
Fluorene	2	4.7 ± 0.4	59.8	38.4 ± 1.1
Phenanthrene	3	26.4 ± 2.5	280.7	60.5 ± 4
Anthracene	3	14.5 ± 1.1	38.8	1.1 ± 0.5
Fluoranthene	3	55.7 ± 4.8	23.2	0.3 ± 0.2
Pyrene	4	49.5 ± 4.3	137.6	4.5 ± 0.4
benzo[a]anthracene	4	33.6 ± 2.8	112	2 ± 0.4
triphenylene + chrysene	4	39.9 ± 3.5	275.5	12 ± 0.6
benzo[b]naphto[2,1-d]thiophene	4	9.6 ± 0.6	154.4	21.6 ± 0.5
benzo[b]fluoranthene+benzo[k]fluoranthene+benzo[j]fluoranthene	4	76.8 ± 6.8	59.9	2 ± 0.2
benzo[e]pyrene	5	32.2 ± 2.9	92.2	4.5 ± 0.5
benzo[a]pyrene	5	33 ± 3.1	56.5	0.6 ± 0.2
Perylene	5	13.3 ± 1.3	30	1.1 ± 0.1
indeno(1,2,3-cd)pyrene	5	32 ± 2.4	5.3	ND
dibenz(ah)anthracene + dibenz(ac)anthracene	5	9.3 ± 0.7	17.1	ND
benzo[ghi]perylene	6	29.5 ± 2.4	93.1	1.2 ± 0.2
Sum PAHs		475.5 ± 40.7	1560	315 ± 10.2
2-methylnaphthalene	2	4.9 ± 0.5	311.9	247.1 ± 7.3
1-methylnaphthalene	2	2.7 ± 0.3	187.4	281.6 ± 14.2
Sum methylnaphthalenes		7.6 ± 0.8	499.3	528.7 ± 19.7
3-methylphenanthrene	3	4.9 ± 0.4	227.3	52.5 ± 7.3
2-methylphenanthrene	3	6.6 ± 0.5	291.5	55.1 ± 1.9
2-methylanthracene	3	2.9 ± 0.2	83.6	41 ± 10.8
9-methylphenanthrene + 1-methylanthracene	3	5.8 ± 0.6	190.7	100.5 ± 13.3
1-methylphenanthrene	3	3.5 ± 0.4	182.1	67.6 ± 15
Sum methylphenanthrenes		20.8 ± 1.9	975.2	316.7 ± 12.4
Total PAHs		504 ± 42.1	3034.5	1160.3 ± 35.1

ND: not detectable. Value without SD indicated the compound was quantified in only one sample.

Supplementary Table 2: Detailed concentrations of individual PAHs in produced diets (ng.g $^{-1}$ food; mean ± SD; n=4-7)

		PY				HO				LO			
	Ring #	Control	0.3X	1X	3X	Control	0.3X	1X	3X	Control	0.3X	1X	3X
naphthalene	2	6 ± 6	15 ± 7	56 ± 14	157 ± 74	4 ± 1	37 ± 6	120 ± 16	405 ± 73	3 ± 1	161 ± 21	315 ± 183	1110 ± 472
acenaphthylene	2	1 ± 0	11 ± 2	35 ± 6	114 ± 23	1 ± 0	3 ± 1	3 ± 1	13 ± 13	1 ± 0	15 ± 1	46 ± 2	136 ± 19
acenaphthene	2	2 ± 1	14 ± 12	29 ± 7	89 ± 24	27 ± 26	46 ± 23	74 ± 19	190 ± 17	23 ± 21	37 ± 26	67 ± 68	90 ± 30
fluorene	2	2 ± 1	14 ± 3	42 ± 8	137 ± 28	2 ± 1	34 ± 2	99 ± 8	312 ± 25	2 ± 1	79 ± 1	232 ± 2	677 ± 55
dibenzo[*b*,*d*]thiophene	2	2 ± 3	11 ± 1	34 ± 5	102 ± 26	0 ± 0	54 ± 3	166 ± 11	546 ± 54	0 ± 0	443 ± 8	1161 ± 18	3489 ± 208
phenanthrene	3	8 ± 4	95 ± 21	291 ± 60	895 ± 213	6 ± 3	152 ± 7	418 ± 32	1279 ± 51	6 ± 4	178 ± 7	492 ± 10	1438 ± 86
anthracene	3	1 ± 0	49 ± 13	159 ± 41	482 ± 165	0 ± 0	22 ± 1	70 ± 2	220 ± 10	0 ± 0	2 ± 1	4 ± 1	42 ± 54
fluoranthene	3	3 ± 3	130 ± 28	523 ± 182	1782 ± 353	2 ± 1	17 ± 1	44 ± 2	145 ± 18	2 ± 1	2 ± 2	1 ± 1	15 ± 17
pyrene	4	3 ± 3	112 ± 24	447 ± 160	1496 ± 311	1 ± 0	80 ± 3	227 ± 19	709 ± 64	2 ± 0	13 ± 2	18 ± 2	73 ± 28
benzo[a]anthracene	4	1 ± 0	171 ± 75	581 ± 221	1671 ± 763	0	57 ± 4	172 ± 11	543 ± 29	0	4 ± 1	14 ± 2	49 ± 28
triphenylene + chrysene	4	1 ± 0	215 ± 91	744 ± 290	2144 ± 1032	ND	108 ± 8	336 ± 21	1073 ± 79	ND	30 ± 1	98 ± 4	320 ± 62
benzo[*b*]naphto[2,1-*d*]thiophene	4	5 ± 3	52 ± 26	156 ± 70	472 ± 230	0	56 ± 4	186 ± 8	573 ± 36	0	66 ± 1	194 ± 9	588 ± 30
benzo[b]fluoranthene+benzo[k]fluoranthene+benzo[j]fluoranthene	4	2 ± 1	273 ± 72	868 ± 220	2740 ± 674	2.4	32 ± 2	110 ± 8	363 ± 18	2.4	6 ± 0	20 ± 1	66 ± 7
benzo[*e*]pyrene	5	1 ± 0	109 ± 29	346 ± 86	1084 ± 286	ND	56 ± 3	173 ± 11	536 ± 26	ND	17 ± 0	52 ± 1	160 ± 10
benzo[a]pyrene	5	1 ± 0	118 ± 33	373 ± 95	1168 ± 346	0 ± 0	ND	108 ± 6	342 ± 10	0 ± 0	ND	4 ± 1	17 ± 3
perylene	5	1 ± 0	37 ± 9	121 ± 27	390 ± 83	0 ± 0	ND	56 ± 4	172 ± 14	0 ± 0	ND	5 ± 1	13 ± 1
indeno(1,2,3-cd)pyrene	5	0 ± 0	123 ± 41	349 ± 89	1188 ± 265	ND	ND	ND	ND	ND	ND	ND	ND
dibenz(ah)anthracene + dibenz(ac)anthracene	5	2 ± 2	32 ± 11	108 ± 43	301 ± 106	0.3	ND	34 ± 2	113 ± 6	0.3	ND	3 ± 1	11 ± 1
benzo[ghi]perylene	6	0 ± 0	87 ± 27	268 ± 67	893 ± 191	0 ± 0	ND	146 ± 10	481 ± 12	0 ± 0	ND	12 ± 0	42 ± 12
Sum PAHs		**34 ± 6**	**1670 ± 448**	**5532 ± 1383**	**17305 ± 4798**	**39 ± 30**	**880 ± 28**	**2558 ± 169**	**8082 ± 305**	**33 ± 26**	**1053 ± 63**	**2739 ± 231**	**8335 ± 854**
2-methylnaphthalene	2	8 ± 9	15 ± 3	43 ± 8	116 ± 39	4 ± 1	137 ± 13	396 ± 32	1259 ± 118	4 ± 1	420 ± 19	1036 ± 96	2982 ± 725
1-methylnaphthalene	2	4 ± 6	8 ± 2	22 ± 5	62 ± 21	2 ± 0	86 ± 8	261 ± 20	854 ± 84	2 ± 0	458 ± 19	1147 ± 102	3300 ± 739
Sum methylnaphthalenes		**12 ± 15**	**23 ± 5**	**65 ± 13**	**178 ± 60**	**6 ± 2**	**223 ± 20**	**657 ± 52**	**2113 ± 201**	**5 ± 1**	**878 ± 38**	**2183 ± 197**	**6282 ± 1465**
3-methylphenanthrene	3	2 ± 1	17 ± 3	47 ± 11	149 ± 31	1 ± 0	126 ± 24	326 ± 47	934 ± 160	1 ± 0	114 ± 14	319 ± 38	850 ± 156
2-methylphenanthrene	3	2 ± 0	19 ± 5	60 ± 13	175 ± 41	1 ± 0	149 ± 11	400 ± 28	1172 ± 166	1 ± 0	121 ± 2	340 ± 16	915 ± 124
2-methylanthracene	3	0 ± 0	8 ± 2	25 ± 6	78 ± 18	ND	ND	ND	409 ± 32	ND	ND	ND	32 ± 8
9-methylphenanthrene + 1-methylanthracene	3	1 ± 0	14 ± 4	49 ± 12	165 ± 56	1 ± 0	137 ± 20	360 ± 85	990 ± 206	1 ± 0	283 ± 29	798 ± 42	2226 ± 394
1-methylphenanthrene	3	1 ± 0	12 ± 3	37 ± 11	100 ± 28	0 ± 0	73 ± 8	227 ± 15	617 ± 71	1 ± 0	122 ± 4	348 ± 14	956 ± 97
Sum methylphenanthrenes		**7 ± 3**	**70 ± 15**	**218 ± 41**	**668 ± 157**	**2 ± 2**	**530 ± 34**	**1448 ± 144**	**4122 ± 581**	**2 ± 2**	**640 ± 35**	**1804 ± 67**	**4957 ± 731**
Total PAHs		**55 ± 12**	**1763 ± 468**	**5816 ± 1433**	**18151 ± 4983**	**47 ± 33**	**1633 ± 71**	**4663 ± 360**	**14317 ± 813**	**40 ± 29**	**2572 ± 96**	**6726 ± 278**	**19574 ± 1945**

ND: not detectable