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Histopathological alterations, EROD activity, CYP1A protein and biliary metabolites in gilthead seabream *Sparus aurata* exposed to Benzo(a)pyrene

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Summary. This study compared for seabream, *Sparus aurata* exposed to benzo(a)pyrene-B(a)P-, the response of molecular cytochrome P450 1A (CYP1A) and cellular histopathology biomarkers. Male gilthead seabream, *Sparus aurata* specimens were exposed for 20 days via water to a series of high B(a)P concentrations. CYP1A was assessed by measuring enzymatic activity (EROD) and CYP1A protein content, and cellular responses were evaluated by routine histopathological methods. In addition, biliary metabolites were measured in order to verify that B(a)P was absorbed and metabolised. Histological lesions, both in liver and gills, increased in parallel to B(a)P concentrations, with the majority of changes representing rather non-specific alterations. Hepatic EROD and CYP1A proteins data showed a concentration-dependent induction, while in the gills, EROD activity but not CYP1A proteins showed a non-monotonous dose response, with a maximum induction level at 200 µg B(a)P.L^{-1} and decreasing levels thereafter. The findings provide evidence that short-term, high dose exposure of fish can result in significant uptake and metabolism of the lipophilic B(a)P, and in pronounced pathological damage of absorptive epithelia and internal organs.

Key words: B(a)P, Liver, Gills, Histopathology, CYP1A, Biliary metabolites, *Sparus aurata*

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Introduction

Strong indications of a relationship between water pollution and pathologies such as pre- and neoplastic tissue lesions, gill pathologies or skin lesions have been reported by several authors (Myers et al., 1994, Vethaak and Jol, 1996; Vethaak et al., 1996; Ortiz et al., 2003).

Polycyclic aromatic hydrocarbons (PAHs) are contaminants of the aquatic environment and originate mainly from anthropogenic sources. PAHs are components of crude oils and its refined products and they can be produced during the incomplete combustion of fossil fuels (Gelboin et al., 1990). In fish and other aquatic vertebrates, PAHs such as benzo(a)pyrene -B(a)P- are transformed by endogenous xenobiotic-metabolising enzymes. One of the key enzymes in B(a)P metabolism is cytochrome P4501A (CYP1A). The expression of the *cyp1a* gene is up-regulated by ligand binding of B(a)P to the arylhydrocarbon receptor (AhR) which activates cyp1a via responsive elements in the promoter region. B(a)P biotransformation leads to reactive electrophilic metabolites which are able to bind covalently to DNA to form adducts. In addition, B(a)P metabolism can induce DNA damage through generation of reactive oxyradicals (de Maagd and Vethaak, 1998). As a consequence, mutations can arise that may ultimately results in neoplastic changes and tumour formation (Bailey et al., 1996; Guengerich, 2000, Ostrander and Rotchell, 2005). In fact, a number of field studies on fish have provided epidemiological evidence for correlations between PAH exposure, CYP1A induction, DNA adducts and histopathological alterations including neoplastic changes (Krahn et al., 1986; Beyer et al., 1996; Myers et al., 1998; Reichert et al., 1998; Aas et al., 2000; Marty et al., 2003; Roy et al., 2003).

Several endpoints affected by PAH exposure have been used as biomarkers of exposure or effect of PAHs (Altenburger et al. 2003). CYP1A indicates exposure of fish to arylhydrocarbon receptor ligands, which include dioxins, furanes, polychlorinated biphenyls, as well as PAHs (i.e benzo(a)pyrene). CYP1A can be measured at the enzymatic catalytic level as 7-ethoxyresorufin-O-deethylase (EROD) activity (Whyte et al., 2000), at the protein level by means of Western blot or ELISA (Goksoyr et al., 1991), and at the mRNA level by means of Northern blot or RT-PCR (Cousinou et al., 2000). Since PAHs are rapidly metabolised in the fish (Varanasi et al., 1989), their tissue residues provide little information on exposure. Therefore, the determination of biliary PAH metabolites, has been suggested as an alternative estimate on actual PAH exposure (Melancon et al., 1992, Porte and Escartin, 1998; Gagnon and Holdway, 2000; Fuentes-Rios et al., 2005). For the determination of biliary PAH metabolites, fluorescence methods may be used such as the fixed wavelength fluorescence method (Aas et al., 2000) or the synchronous fluorescence spectrometry (Ariese et al., 1993). While both CYP1A and bile metabolites indicate exposure to metabolisable PAHs, histopathology, finally, diagnoses possible adverse effects of PAH exposure, e.g. (pre-)neoplastic tissue damages (Myers et al., 1991; Hinton et al., 2000, etc.).

The present study provides a combined analysis on biomarkers of exposure and effect (assessment of EROD and CYP1A protein, of biliary B(a)P metabolites and of histopathological alterations) in the seabream, *Sparus aurata*, exposed to the prototypic PAH -B(a)P-. *Sparus aurata* was selected as experimental species since on the one hand it is an economically important and intensively exploited species in the Atlantic and Mediterranean areas and, on the other hand, it has been suggested as a monitoring species for the coastal zones. As target organs, we choose liver and gills. Since the liver is the main organ of xenobiotic metabolism in fish (Lorenzana et al., 1989; Smolowitz et al., 1992; Hinton et al., 2000; Sarasquete et al., 2001), being most studies on cellular responses to PAH exposure focused on this organ. However, other organ systems also possess substantial biotransformation activities, for instance, the gut (James and Kleinow, 1994; van Veld et al., 1997), the brain (Ortiz-Delgado et al., 2002), as well as the gills (Carlson and Pärt, 2001). Whereas during chronic, low-dose exposure the digestive system probably is the main route for the lipophilic PAHs to enter the fish, the gills may be more important during acute, high dose exposures as they may occur during spills. Therefore, we intended to compare the CYP1A and histopathological response of liver and gills of seabream exposed over a relatively short period (20 days) to rather high concentrations of B(a)P.

Material and methods

Immature male specimens of seabream, *Sparus aurata* (average weight 250-300 g), from a commercial fish farm (CUPIMAR, SA, San Fernando, Cádiz, Spain) were acclimatised in tanks supplied with continuously flowing seawater at constant temperature (19±1°C) during two weeks.

Xenobiotic exposure

As in previous experimental assays (Ortiz-Delgado et al., 2002, 2005), after the acclimatisation, fish were randomly distributed to the experimental tanks for 20 days and submitted to the following treatments: (a) control (only vehicle added, toluene), (b) exposure to 100 $\mu g.L^{-1}$ B(a)P, (c) exposure to 200 $\mu g.L^{-1}$ B(a)P, (d) exposure to 300 $\mu g.L^{-1}$ B(a)P, (e) exposure to 500 $\mu g.L^{-1}$ B(a)P. Treatments were applied in triplicate, with 12 fish per each 120-L experimental tank. A stock solution of B(a)P was prepared in toluene and added to the water in suitable quantities to give nominal concentrations of B(a)P. The maximum solvent concentration in the water was 0.05 $\mu l.L^{-1}$ toluene.

The fish were exposed to the toxicant for 20 days under semi-static conditions. The water was renewed every 24 hours, followed by the addition of suitable quantities of B(a)P. Before the experiments were initiated, tanks were filled with water and B(a)P solutions were added and maintained during 24 h in order to guarantee complete adsorption of the compounds on the walls. Water temperature (18.8±0.2°C), pH (7.5±0.2), NO_2^- (<0.1 $mg.L^{-1}$), NO_3^- (<8$mg.L^{-1}$), NH^{4+} (<3 $mg.L^{-1}$), dissolved oxygen (8.3±0.3 $mg.L^{-1}$) and salinity (32‰) were measured daily during the experimental period. Throughout, no mortalities were recorded. Samples of liver and gills of control and exposed fish were taken at different days (5, 10, 15 and 20 days). Fish were fed with dried pellets during the experimental period but one group of fishes (100 $\mu g.L^{-1}$) was starved for a period of 10 days prior to sampling, in order to avoid bile evacuation from the gall bladder.

EROD measurements

EROD activity was measured as described by Scholz et al. (1997). Tissue samples corresponding to liver and gills, were homogenized in Tris-based homogenisation buffer and microsomes were prepared by means of ultracentrifugation (Scholz et al., 1997). The microsomes were suspended in 200 µl homogenisation buffer and EROD activities were determined in a microplate format assay using a fluorometer plate reader (Fluostar, SLT-Tecan). The assay was run with 47 µM NADPH and 0.4 µM ethoxyresorufin in phosphate-buffered saline. The concentrations of the reagents in the assay were optimised in preliminary experiments. The rate of resorufin formation in the assay was measured at an excitation wavelength of 344 nm and at an emission wavelength of 590 nm. A resorufin standard curve was used to convert the fluorescent readings into the amount of resorufin formed. Volume activity of EROD was normalized to microsomal protein.

CYP1A protein analysis

An indirect ELISA method was performed according to Goksøyr et al. (1991) and Scholz et al. (1997) in liver and gills of seabream specimens exposed during 20 days to B(a)P. The microsomal samples were adjusted to a protein content of 10 ng microsomal protein/ml. The primary antibody used for the ELISA was the C10-7 CYP1A monoclonal antibody directed against peptide 277-294 of rainbow trout (*Oncorhynchus mykiss*) CYP1A (Biosense AS, Bergen, Norway) diluted 1:500. As previously pointed out by Ortiz-Delgado et al., (2005), the C10-7 antibody presented cross reactivity with the CYP1A from *S. aurata*. The primary reaction was followed by a horseradish-peroxidase-conjugated goat IgG anti-mouse (Dako) as secondary antibody. Staining was performed using 3,3'-diaminobenzidine as substrate. The peroxidase reaction product was measured as optical density in a spectrophotometer (Pharmacia, Freiburg, Germany) at 405 nm; controls (non-specific binding, blank) were included.

Bile metabolites measurement

Bile metabolite analysis was performed by means of fixed wavelength fluorescence, according to Gagnon and Holdway (2000). The bile samples were diluted with distilled water from 1:1000 to 1:40000, so that the fluorescence reading was within the linear range of the standard. Fluorescent readings were performed for the B(a)P-type metabolites at 380/430 nm using 1-hydroxy pyrene (Sigma) as a reference standard. The analyses were made in a fluorescent plate reader (SLT Fluostar). B(a)P-type metabolites are reported as ng of 1-hydroxy pyrene units equivalent per ml bile fluid.

Histopathological studies

For light microscopy, small samples of liver and gills were fixed in Bouin's fluid and/or formaldehyde buffered with 0.1 M phosphate (pH 7.2). After dehydration in graded concentrations of ethanol, samples were embedded in paraffin wax. Sections 6-7 µm thick, were stained with either Haematoxylin and Eosin or Haematoxylin followed by Light Green-Orange G-Fuchsin trichromic stain (Gutiérrez, 1990; Sarasquete and Gutiérrez, 2005) for histomorphological studies. Histological abnormalities detected in liver and gills were recorded as present or absent and expressed as a percentage of fish affected (prevalence) per tank.

For transmission electron microscopy (TEM), small pieces of the organs were fixed for 2h in cold 2.5% glutaraldehyde-0.1 M cacodylate buffer (pH 7.2), rinsed several times in cacodylate buffer and postfixed with 1% OsO_4 in 0.1 M cacodylate buffer. Samples were dehydrated in a gradient series of acetone and embedded in Spur's medium. Ultrathin sections of 60-80 nm thickness (Reichert-Jung ultramicrotome) were stained with uranyl acetate and lead citrate prior to observation in transmission microscope (Zeiss EM 9S2).

Statistical analysis

Histological alterations quantified in liver and gills were analysed by means of a non-parametric ANOVA followed by the Tukey-Kramer test. The significance level adopted was $P<0.05$. Non-parametric Spearman rank correlation analysis was also used in order to investigate the relationship between EROD activities and histopathological disorders in liver and gills.

Results

CYP1A catalytic activity (7-ethoxyresorufin-O-deethylase, EROD)

In the liver, B(a)P treatment produced significant

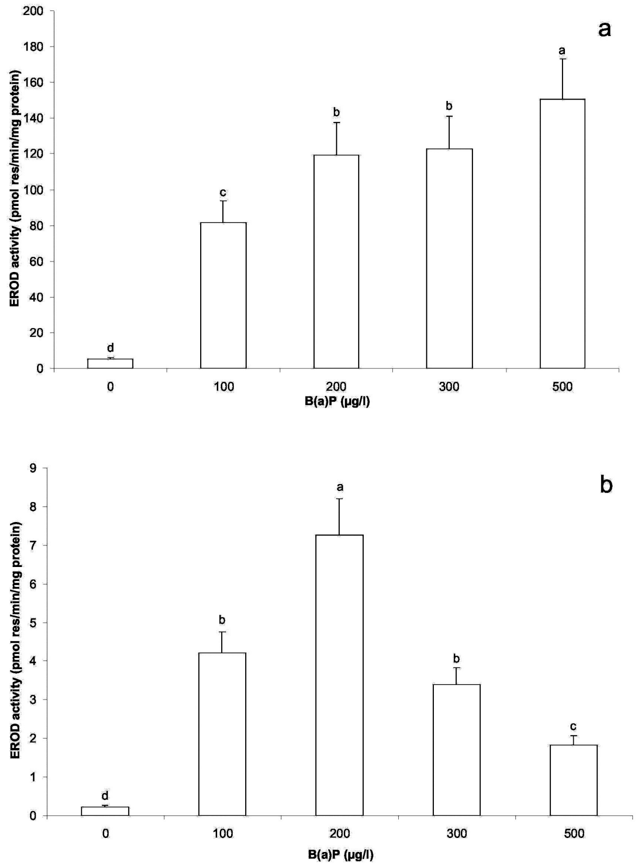

Fig. 1. a. Levels of EROD activity in hepatic microsomes of *S. aurata* following treatment at increased doses of B(a)P at 20 days of exposure. **b.** Levels of EROD activity in branchial microsomes of *S. aurata* B(a)P exposed specimens. Values are expressed as mean(±)SE. Different letters between groups of exposure indicate statistically significant differences (P<0.05).

EROD induction in 20 days of experiment. EROD activity gradually increased in a dose-dependent way, reaching a maximum of 28-fold of induction compared with control (Fig. 1a). For gills, exposure to B(a)P resulted in a gradual increase of EROD activity to a maximum level of 30-fold induction for fish exposed to 200 $\mu g.L^{-1}$ followed by a strong decrease of the induction response for fish exposed to the highest dose of B(a)P (8-fold induction compared to control for fish exposed to 500 $\mu g.L^{-1}$) (Fig. 1b).

CYP1A protein/ELISA

At 20 days of B(a)P treatment, induction of hepatic microsomal CYP1A protein followed a similar pattern as detected for EROD activity, resulting in a gradual dose-dependent increase of the protein content (Fig. 2a). CYP1A of the gills showed the same induction pattern as detected for liver with a maximal fold induction of 3.8 (500 µg.L-1) compared with control (Fig. 2b).

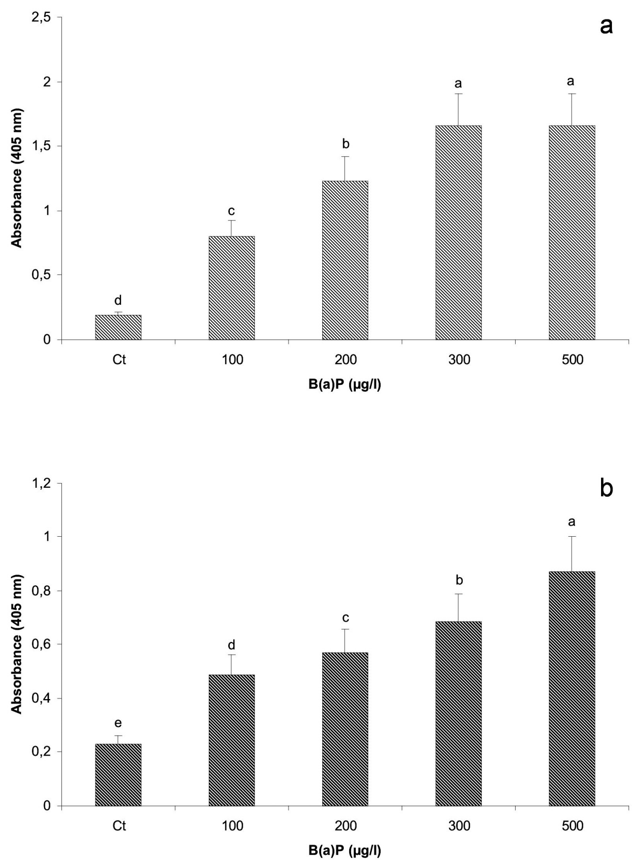

Fig. 2. ELISA measurements in liver **(a)** and gills **(b)** from *Sparus aurata* specimens exposed to B(a)P at 20 days of exposure (using the fish CYP1A antibody C10-7). Values are expressed as mean(±)SE. Different letters between groups of exposure indicate statistically significant differences (P<0.05).

Bile metabolites measurement

An increase in the biliary fluorescence was detected in exposed specimens, while the control showed partly negative values (below the detection limits). Exposed fish had bile metabolite levels significantly elevated over controls and they increased with increasing exposure time (Fig. 3). These findings suggesting that B(a)P was bioavailable to seabream and was rapidly absorbed and metabolised.

Histopathology

The liver of the seabream, *Sparus aurata*, showed the hepatocytes distributed in cordons concentrically placed around the sinusoids. In addition to hepatic tissue, the liver also contained a so-called "intrahepatic" exocrine pancreas (Fig. 4a,b). At ultrastructural level, the hepatocytes showed an ovoid nucleus containing a distinct nucleolus (Fig. 4c). Extensive stacks of rough endoplasmic reticulum (RER) cisternae, interspersed with few mitochondria were detected, although the smooth endoplasmic reticulum (SER) was restricted. The cytoplasm of hepatocytes contained lipid droplets without membrane (Fig. 4c) and glycogen granules.

Following exposure to B(a)P, the cellular architecture pattern of the hepatic parenchyma was altered, showing the hepatic parenchyma cellular disorganization of the hepatocytes as well as vascular dilation and blood stagnation (Fig. 5 a,b,d). Foci of cellular alteration, characterized by the presence of distinct focal areas within the hepatocytes presented different degree of staining compared to the surrounding parenchyma, was detected in exposed fish. Cellular disorganization, variation in nuclear size, pycnosis and hypertrophy with an increase in lipid droplets were also detected in exposed organisms (Fig. 5c) as well as blood infiltration of the hepatic parenchyma, as evident from

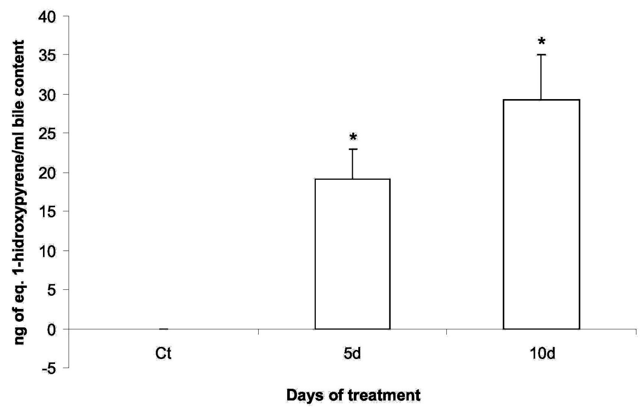

Fig. 3. B(a)P metabolites in bile from seabream specimens exposed during 10 days to 100 $\mu g.L^{-1}$ of B(a)P. Asterisk means significant differences with respect to the controls.

the presence of erythrocytes and leukocytes in the liver parenchyma. Furthermore, ultrastructural alterations, such as an increase in the number of lipid droplets (Fig. 6a), presence of pseudo-myelinic inclusions within the lipid droplets (Fig. 6b), glycogen augmentation (Fig. 6c), as well as mitochondrial disintegration (Fig. 6d) were detected. Moreover, seabream exposed for 20 days to the higher dose of B(a)P (500 $\mu g.L^{-1}$) showed severe disorganization of the stacks of RER with some of the cisternae surrounding the lipid droplets (Fig. 7 a, b and c). A rise in the number of secondary lysosomes (Fig. 7d) was also detected.

Seabream gills consist of hemibranchs containing a row of long thin filaments, the primary lamellae, with the surface area of these forming regular and parallel folds across its dorsal and ventral surface -the secondary

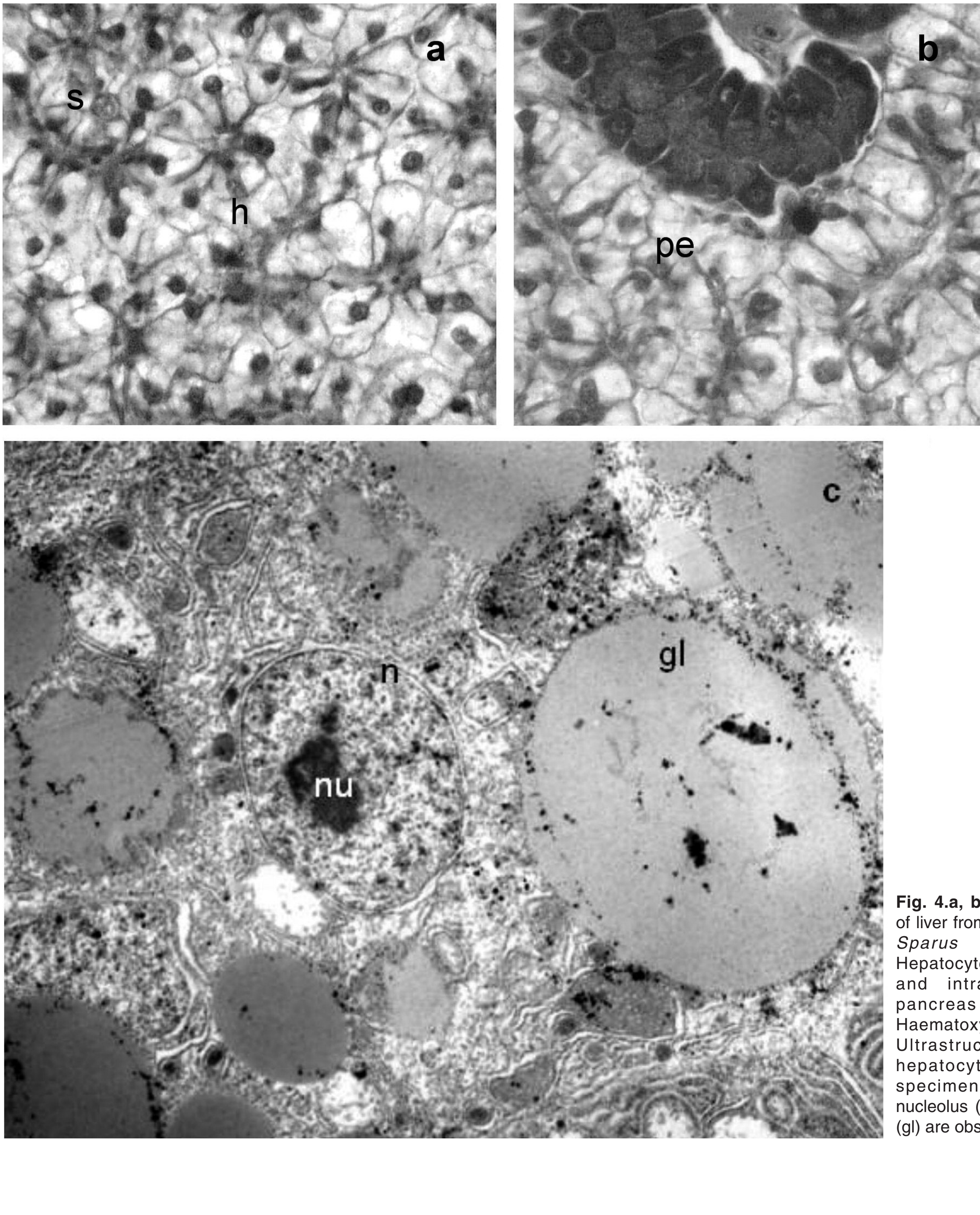

Fig. 4.a, b. Histological section of liver from a control seabream, *Sparus aurata* specimen. Hepatocytes (h), sinusoids (s) and intrahepatic exocrine pancreas (pe) are shown. Haematoxylin-Eosin, x 200. **c.** Ultrastructural section of hepatocytes of an untreated specimen liver. Nucleus (n), nucleolus (nu) and lipid droplets (gl) are observed. x 4,000

lamellae- (Figs. 8a,b). The respiratory lamellae were covered by an epithelial layer of respiratory epithelium (Fig. 8c) and internally, the lamellar blood sinuses were lined and spanned by pillar cells (endothelial cells) (Fig. 8d). In the filaments and through the interlamellar regions, chloride and goblet/mucous cells were easily observed (Figs. 8b,c).

The main alterations induced by B(a)P exposure in the gills of seabream specimens were dilation and rupture of blood capillaries, associated with blood extravasation into extracapillary location. The rupture of pillar cells and capillaries led to an accumulation of erythrocytes in the distal portion of the secondary lamellae (telangiectasis/aneurism) (Fig. 9a). These

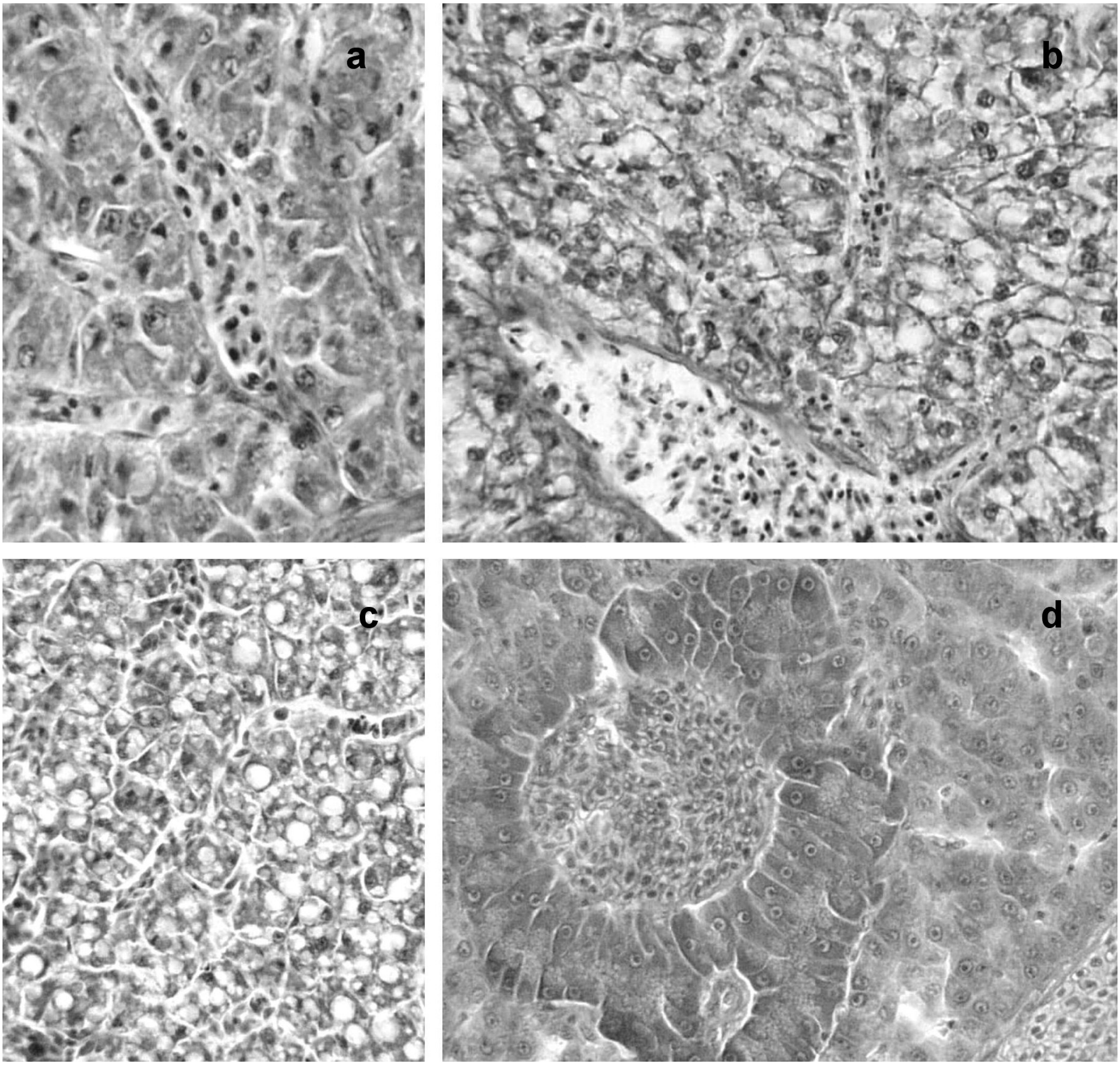

Fig. 5. Hepatic histological section of B(a)P-treated seabream specimen showing **(a)** capillary hyperemia. (15 days, 300 μg.L^{-1}, Haematoxylin and Eosin, x 400). **b.** Vascular dilation and disorganization of the normal parenchymal structure (20 days, 200 μg.L-1, Haematoxylin and Eosin x 400. **c.** Cellular disorganization, pycnosis and lipid droplets increases (20 days, 500 μg.L^{-1}, Haematoxylin and Eosin, x 100). **d.** Blood stagnation with vascular dilation, causing the compression of the adjacent hepatic parenchyma (20 days, 300 μg.L^{-1}, Haematoxylin and Eosin, x 250).

changes are indicative of a failure of the branchial circulatory system. Epithelial cells of the secondary lamellae increased in number with respect to control cells. Edema in the secondary lamella resulted in lifting of lamellar epithelium. The rise in the primary lamellar epithelium resulted in the obliteration of the inter-lamellar spaces between secondary lamellae (Fig. 9b,c). Finally, the mitochondria of the chloride cells, located in the interlamellar regions, appeared altered (Fig. 9d), while the tubular system showed a clearly ramified and

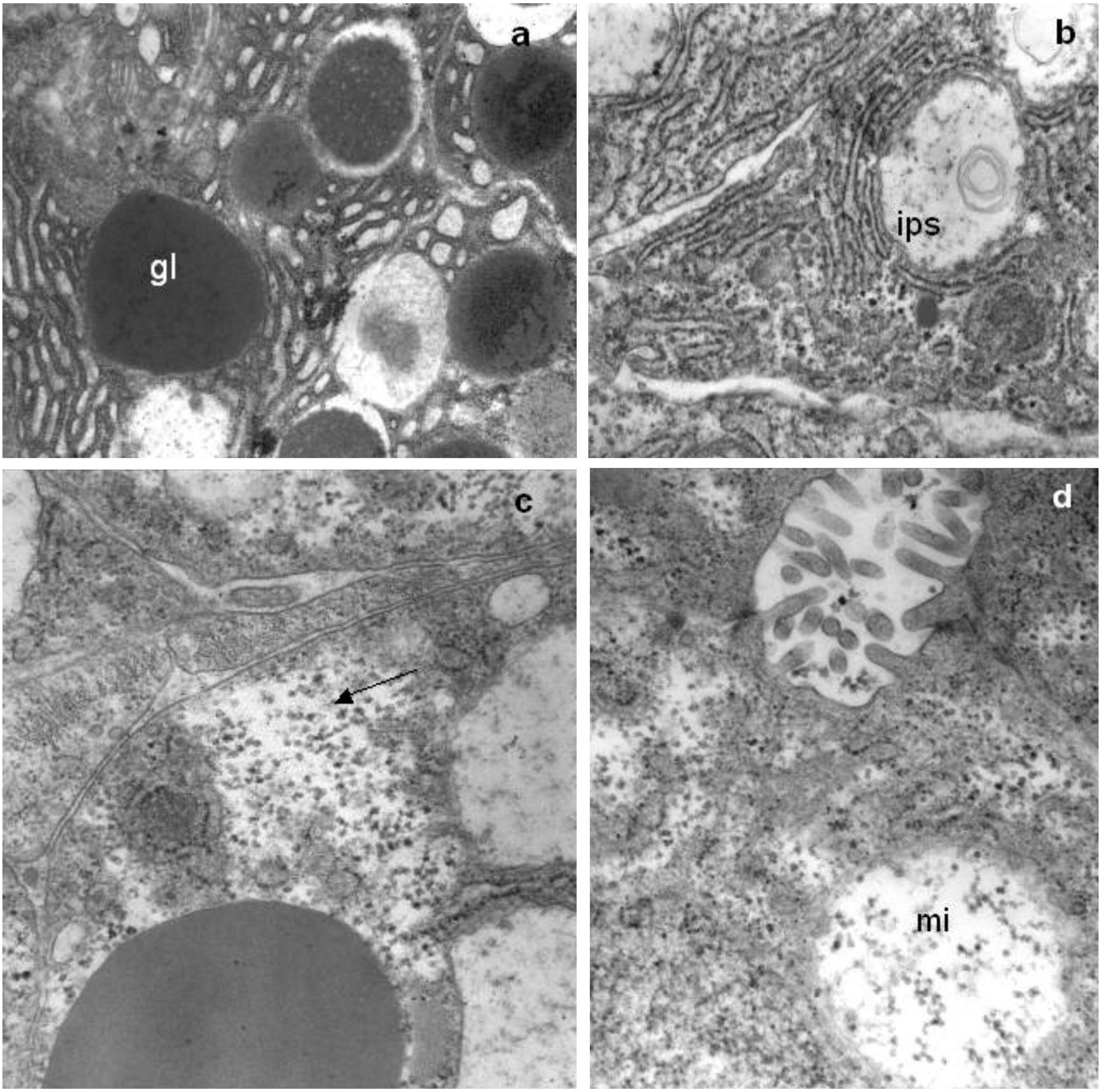

Fig. 6. Ultrastructural section of liver from B(a)P treated specimens showing **(a)** increase in the number of lipid droplets (gl) (20 days, 500 µg.L^{-1}, x 15,000), **(b)** pseudo-myelinic inclusions within lipid droplets) (20 days, 500 µg.L^{-1}, x 15,000. **c.** Increased hepatocellular glycogen cytoplasmic granules (arrow) (15 days, 300 µg.L^{-1}, x 25,000 and **(d)** altered mitochondria (20 days, 300 µg.L^{-1}, x 25,000).

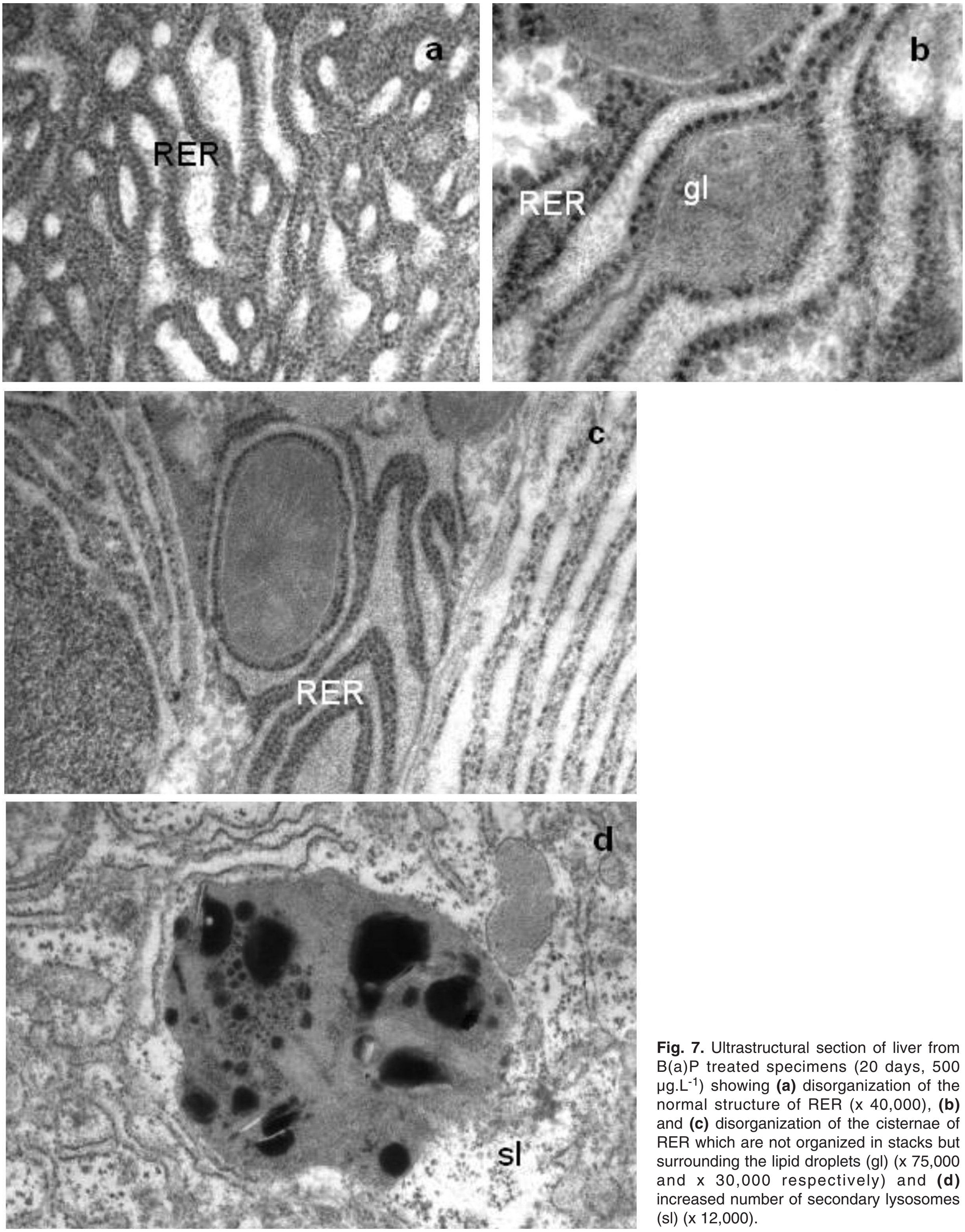

Fig. 7. Ultrastructural section of liver from B(a)P treated specimens (20 days, 500 $\mu g.L^{-1}$) showing **(a)** disorganization of the normal structure of RER (x 40,000), **(b)** and **(c)** disorganization of the cisternae of RER which are not organized in stacks but surrounding the lipid droplets (gl) (x 75,000 and x 30,000 respectively) and **(d)** increased number of secondary lysosomes (sl) (x 12,000).

elaborated structure, as observed in control sections.

Prevalence of histopathological damage

In general, the histological alterations detected in gills and liver from exposed seabream affected an important number of specimens at the end of the experimental period (Tables 1 and 2). Moreover, an increase in the percentage of affected specimens in those groups exposed to the higher B(a)P concentrations was

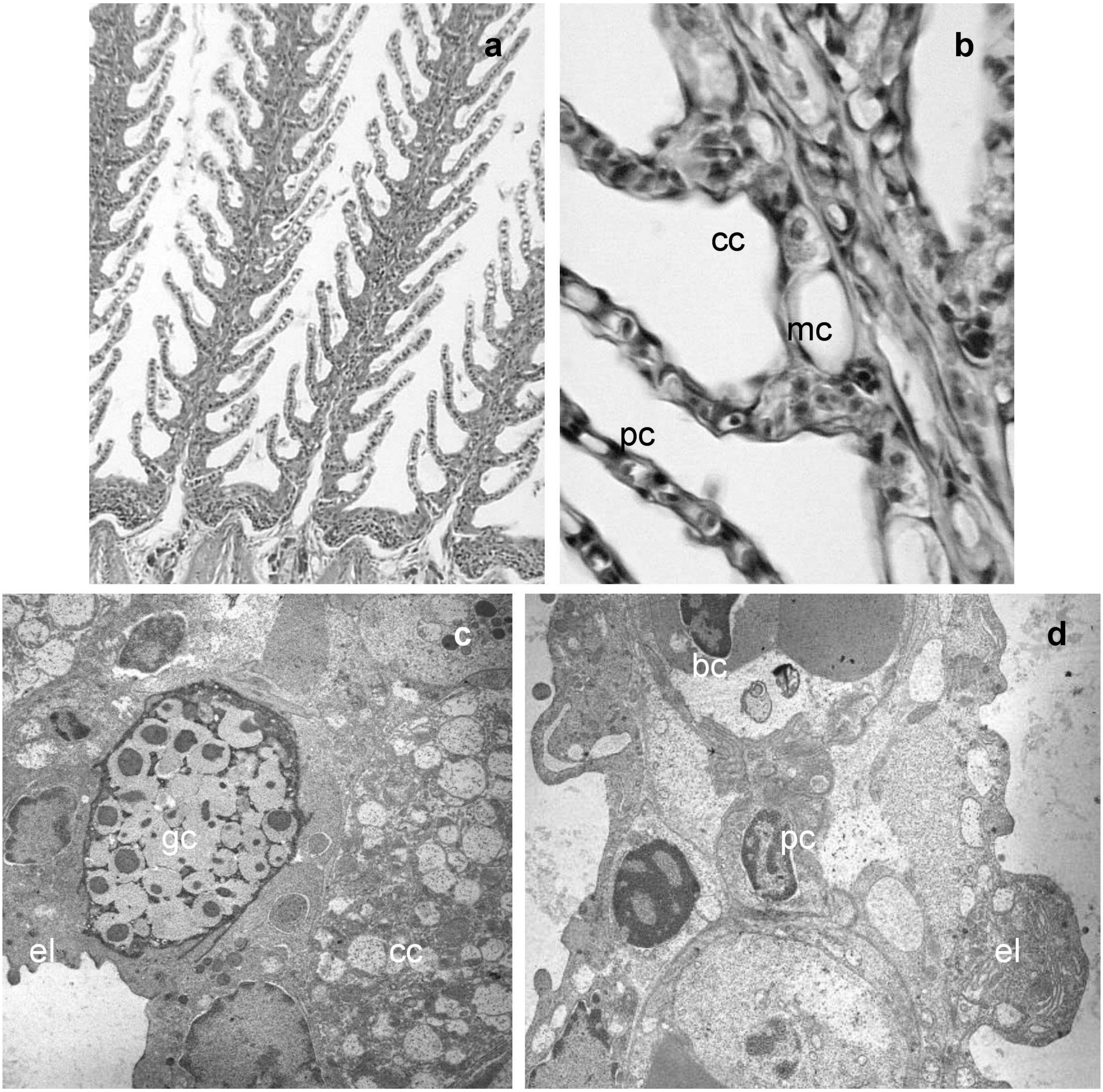

Fig. 8. Gills from control seabream specimens showing (**a and b**) basic features of the primary and secondary lamellae and the distinct cell types of its epithelium, chloride cells (cc), mucous cells (mc) and pillar cells (pc) (Haematoxylin-Eosin, x 100 and x 400, respectively). **c.** Ultrastructural section of a control specimen gill showing the primary lamella containing the chloride (cc) and goblet or mucous (gc) cells (x 3,000). **d.** Epithelial layer (el), blood cells (bc) and pillar/endothelial (pc) cells are detected in the secondary lamellae (x 4,000).

detected (Tables 1 and 2).

For the liver, blood stagnation was even present in control seabream specimens. Exposure to 500 µg.L^{-1} of B(a)P at the end of the exposure period (20 days) elicited the higher prevalence of capillary hyperhemia, nuclear pycnosis, and cellular necrosis (83,3 %), followed by foci of cellular alterations, inflammatory response and blood infiltration (Table 1). For gills, blood extravasation, followed by hyperplasia, edema and epithelial desquamation, were evident at the end of the exposure period (20 days) in fish exposed to all B(a)P concentrations. However, necrosis was only evident in fish exposed to the higher B(a)P concentrations (Table 2).

EROD, CYP1A protein and histopathological correlations

Spearman rank correlation analysis was performed to study the relationships between EROD, CYPIA protein content, and different types of histopathological damage observed in liver (Table 3) and gills (Table 4). Results of the analyses showed a significant correlation between EROD activities and semi-quantitative values of histopathological lesions in liver in all cases, except for blood stagnation. Moreover, this correlation was most marked for capillary hyperemia, inflamatory response, cellular necrosis and foci of cellular alteration (Table 3). However, there was no significant correlation between EROD activity and gill alterations. On the other

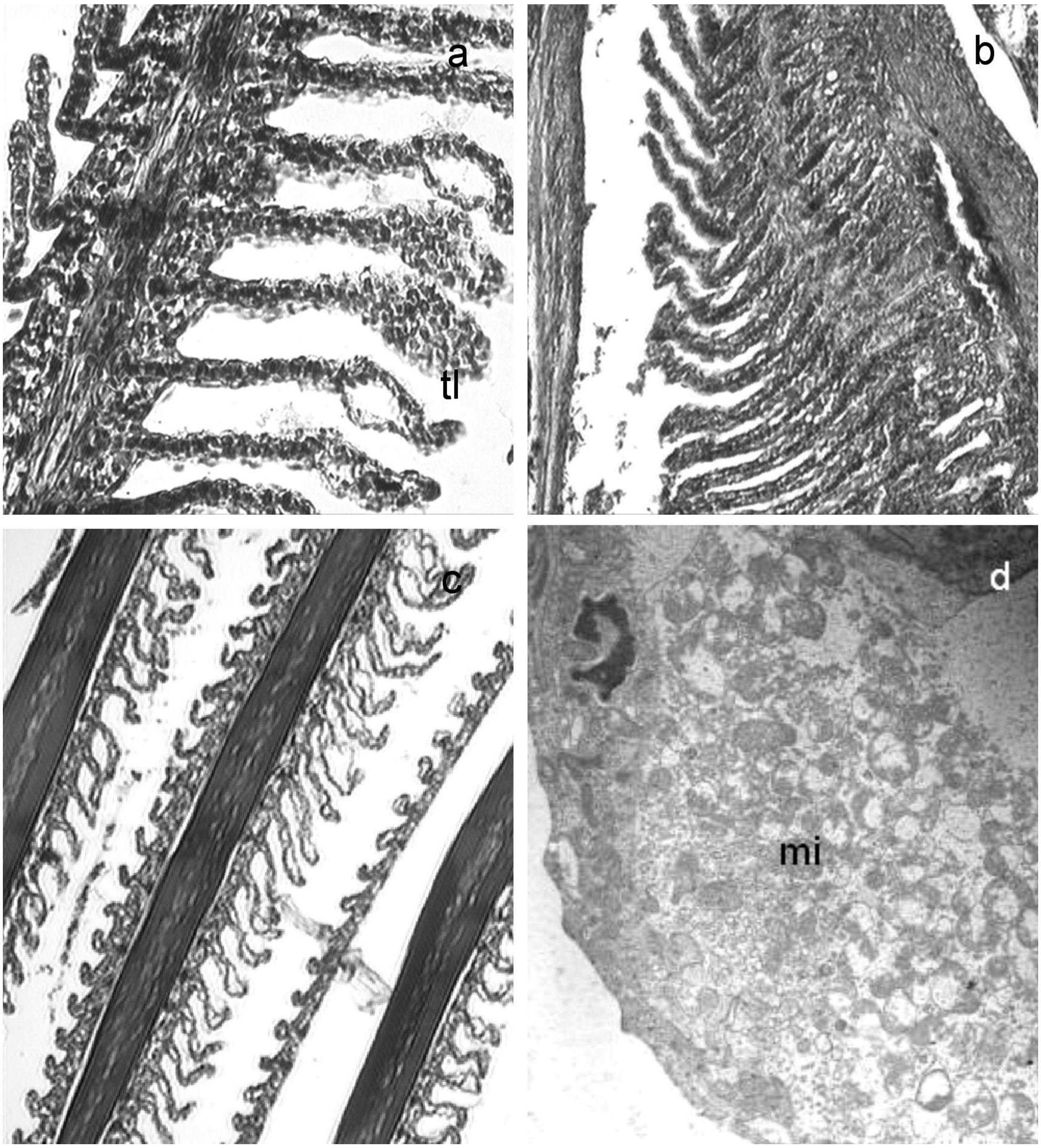

Fig. 9.a. Histological section of gills from treated specimens showing telangiectasis (tl) caused due to a rupture of pillar cells and accumulation of blood cells to the tips of the secondary lamellae (15 days, 300 µg.L^{-1}, Haematoxylin-VOF, x 100). **b.** Fusion of adjacent secondary lamellae with an increase in the cellular layers (20 days, 500 µg.L^{-1}, Haematoxylin-Eosin, x 400). **c.** edemas with epithelial lifting in the base and tips of the secondary lamellae (20 days, 200 µg.L^{-1}, Haematoxylin-VOF, x 40). **d.** Ultrastructural section of gills from treated specimens showing altered mitochondria (mi) while the tubular system is unaltered (20 days, 100 µg.L^{-1}, x 1,000).

Table 1. Prevalence of hepatic lesions in seabream specimens exposed to B(a)P.

Pathology	Exposure time (days)	Control	μg B(a)P/L			
			100	200	300	500
Capillary hyperemia	5d	8.3	8.3	16.7	16.7	25
	10d	0	25	25	41.7*	50*
	15d	8.3	25	33.3	50*	75*
	20d	8.3	33.3*	50*	75*	83.3*
Nuclear pycnosis	5d	8.3	8.3	16.7	33.3*	41.7*
	10d	0	33.3	33.3	41.7*	50*
	15d	8.3	66.7*	58.3*	66.7*	75*
	20d	8.3	75*	75*	83.3*	91.6*
Inflammatory response	5d	0	8.3	8.3	8.3	8.3
	10d	0	0	0	8.3	16.7
	15d	8.3	16.7	25*	25*	41.7*
	20d	0	33.3*	41.7*	50*	66.7*
Hepatocyte necrosis	5d	0	8.3	25	33.3*	33.3*
	10d	0	16.7	41.7*	50*	58.3*
	15d	0	25*	33.3*	66.7*	75*
	20d	8.3	41.7*	58.3*	75*	91.6*
Blood stagnation	5d	33.3	41.7	33.3	41.7	50
	10d	33.3	50	41.7	50	50
	15d	41.7	41.7	58.3	58.3	58.3
	20d	50	58.3	50	58.3	50
Blood infiltration	5d	8.3	0	0	8.3	25
	10d	8.3	16.7	16.7	16.7	33.3*
	15d	0	0	8.3	25	41.7*
	20d	0	0	0	41.7*	58.3*
Foci of cellular alteration	5d	0	0	16.7	16.7	33.3*
	10d	8.3	16.7	25	41.7*	41.7*
	15d	0	16.7	25*	50*	58.3*
	20d	0	0	33.3*	58.3*	75*

Data presented as percentages, means from three replicate tanks/ treatment (twelve fish/tank). *: Denotes percentage of fish exhibiting response significantly different than the control (ANOVA, $P<0.05$).

Table 2. Prevalence of branchial lesions in seabream specimens exposed to B(a)P.

Pathology	Exposure time (days)	Control	μg B(a)P/L			
			100	200	300	500
Edemas	5d	8.3	8.3	16.7	16.7	33.3
	10d	8.3	33.3	33.3	50*	41.7*
	15d	8.3	33.3	41.7*	58.3*	66.7*
	20d	0	41.7*	58.3*	83.3*	91.6*
Epithelial desquamation	5d	8.3	0	8.3	33.3	33.3
	10d	0	41.7*	41.7*	58.3*	41.7*
	15d	8.3	66.7*	58.3*	66.7*	50*
	20d	8.3	83.3*	75*	83.3*	91.6*
Hyperplasia	5d	0	0	16.7	33.3*	33.3*
	10d	0	16.7	41.7*	50*	58.3*
	15d	0	16.7	33.3*	50*	75*
	20d	8.3	41.7*	58.3*	75*	83.3*
Dilation of capillaries and blood extravasation	5d	8.3	41.7*	33.3	41.7*	41.7*
	10d	8.3	50*	41.7*	41.7*	58.3*
	15d	8.3	50*	58.3*	58.3*	75*
	20d	16.7	58.3*	58.3*	66.7*	83.3*
Necrosis	5d	0	0	0	0	16.7
	10d	0	8.3	8.3	16.7	33.3*
	15d	0	0	0	33.3*	41.7*
	20d	0	0	0	50*	58.3*

Data presented as percentages, means from three replicate tanks/ treatment (twelve fish/tank). *: Denotes percentage of fish exhibiting response significantly different than the control (ANOVA, $P<0.05$).

Table 3. Results of Spearman rank correlation (r) analysis on EROD activities and various histopathological parameters (n=15) of the liver.

	Histopathological alterations						
	CH	NP	IR	HN	BS	SI	FCA
EROD vs. Liver	r=0.85*	r=0.81	r=0.91**	r=0.94**	r=-0.17	r=0.81*	r=0.93**
ELISA vs. Liver	r=0.89*	r=0.90**	r=0.89*	r=0.87*	r=0.26	r=0.80*	r=0.85**

Significant correlations are indicated by asterisks *P<0.05, **P<0.01. CH: capillary hyperplasia; NC: nuclear pycnosis; IR: inflammatory response; HN: hepatocyte necrosis; BS; blood stagnation; SI: sanguineal infiltration; FCA: foci of cellular alteration.

Table 4. Results of Spearman rank correlation (r) analysis on EROD activities and various histopathological parameters (n=15) of the gills.

	Histopathological alterations				
	Ed	ED	Hyp	DC	Nec
EROD vs. Gills	r=0.09	r=0.062	r=0.084	r=0.058	r=-0.327
ELISA vs. Gills	r=0.97**	r=0.67*	r=0.96**	r=0.90**	r=0.87*

Significant correlations are indicated by asterisks *P<0.05, **P<0.01. Ed: edemas; ED: epithelial desquamation; Hyp: hyperplasia; DC: dilation of capillaries and blood extravasation; Nec: necrosis.

hand, the relationship between CYP1A protein content and histopathological alterations in liver and gills was significant for all types of histological damage (Tables 3 and 4) except for hepatic blood stagnation (r=0.267), as detected for hepatic EROD activity (Table 3).

Discussion

Cytological and histopathological changes are good indicators of toxic effects of a wide variety of pollutants (Au et al., 1999). In fish from contaminated areas, histopathological alterations of different organs and tissues show changes related to toxicant type, species sensitivity, age, sex, concentration and route of administration of contaminants, etc. Moreover, the degree of alterations in organs/tissues (liver, gills, digestive tract, vascular endothelium, etc.) may be related to different toxicants, concentration, exposure time and route of uptake (Livingstone, 1993).

In vertebrates, the liver is the most important site for the metabolism of xenobiotics. Histopathological alterations in fish liver have been regarded as good indicators of sublethal effects of environmental contaminant exposure (Braunbeck et al., 1990; Arnold et al., 1995, Hinton et al., 2000). Results of histological studies in wild fish have demonstrated a good correlation between chronic, low-dose exposure of fish to PAHs and the occurrence of liver tumours and other neoplasia-related liver lesions (Myers et al., 1987, 1988; Schiewe et al., 1991; Arcand and Metcalfe, 1995). The present study, however, used short-term and high dose exposure conditions. Accordingly, (pre-)neoplastic changes were not to be expected. Rather, the histopathological alterations of liver tissue as observed in this study appear to be of fairly unspecific nature: hepatocellular hypertrophy, necrosis, nuclear pycnosis, cytoplasmic vacuolisation, glycogen depletion, increase of lipid droplets, augmentation of lysosomal number, hemorrhages, widening of blood sinusoids. Similar hepatic alterations have been reported from short-term treatments of fish with a wide range of stressors, including acute pesticide exposure (Bhattacharya et al. 1975; Walsh and Ribelin 1975; Dutta et al., 1993), varying temperature (Braunbeck et al., 1987), inanition (Segner et al., 1994) or inadequate nutrition (Deplano et al., 1989; Braunbeck and Segner, 1992). Such unspecific changes may result partly from general stress-related alterations of circulation or metabolism (Wendelaar Bonga, 1997). However, also factors specific to B(a)P bioactivation may be involved, particularly the biotransformation-associated generation of reactive oxygen species which may lead to membrane damage, uncoupling of electrochemical gradients across membranes cytotoxicity, etc. (Winston and DiGiulio, 1991; Segner and Braunbeck, 1998; Altenburger et al., 2003). One histological parameter which showed no association with treatment, liver blood stagnation, appeared both in control and exposed fish suggesting that there is no association within treatment and the presence of such pathology, as indicated from Table 1 and Spearman rank correlation.

Also the histopathological changes in the gills of B(a)P exposed seabream are of rather unspecific nature, as they have been reported to be imposed on the gills by a wide range of stressors (Mallat, 1985): rupture of the gill epithelium, telangiectasis, epithelial lifting or lamellar fusion. Similar response of gill morphology were observed in bluegill, Lepomis macrochirus (Richmonds and Dutta, 1989) and seabream, *Sparus aurata* (Arellano et al., 2001) exposed to malathion and 2,3,7,8-TCDD, respectively. Since the gills are strongly vascularized, an important factor contributing to B(a)P-induced gill damage could be the induction of CYP1A in endothelial cells. Endothelial cells are rich in CYP1A (Sarasquete and Segner 2000; Ortiz-Delgado and Sarasquete, 2004) and it is well established that CYP1A induction can result in endothelial rupture and subsequent tissue damage.

A clear induction response of the CYP1A system measured as EROD activity was observed after B(a)P treatment in both liver and gills of the *S. aurata* specimens. Results of EROD analysis exhibited a good concentration-response-relationship with B(a)P exposure in liver from *S. aurata*. Congruent findings were reported by Au et al. (1999) in *Solea ovata* specimens after intraperitoneal injection of B(a)P. The strong CYP1A induction in the liver is consistent with the role of hepatocytes in the biotransformation of xenobiotics (Lorenzana et al., 1988; Grinwis et al., 2000; Sarasquete and Segner 2000; Sarasquete et al., 1999, 2001). Because of the relatively high CYP1A specific activities in fish liver compared to other tissues, the liver is considered to be proposed to be the major site of CYP1A-catalyzed biotransformation in fish (Lech and Bend, 1980; Binder et al., 1984).

Also in the present study, gill EROD activities were by an order of magnitude lower than liver activities. Despite the lower CYP1A catalytic rates in gills, its higher relative perfusion rates compared with the liver indicates that this organ may represent a significant biotransformation site (Barron et al., 1987, 1988). Thus the biotransformation in the gills may influence the toxicity of waterborne chemicals (Maren et al., 1968). Xenobiotics can be metabolised at significant rates during their passage through the branchial epithelium. Moreover, they may pass through the gills essentially as parent compounds and undergo metabolism in liver (van Veld et al., 1997). In this context, it is an interesting observation that although branchial EROD activity was decreasing at higher B(a)P concentrations, pathological gill damage was steadily increasing. This observation argues for a role of the parent compound as responsible agent, instead of B(a)P metabolites or oxygen radicals arising from B(a)P metabolism.

Hepatic CYP1A content (as ELISA measurement) presented the same induction pattern as detected for EROD activity, although the induction factor was lower (8.7 fold for CYP1A protein compared to 28-fold for EROD activity). Hepatic CYP1A protein showed a concentration-dependent increase with increasing B(a)P concentration. ELISA analysis offers the advantage than CYP1A protein can be measured in the presence of substances and chemicals which could inhibit catalytic activity or if the high concentration of the inducer became inhibitory for EROD activity (Achazi et al., 1994; Brüschweiler et al., 1996). An inhibitory effect of elevated B(a)P concentrations on EROD activity may explain the induction of branchial EROD activity, which showed a maximum level of 30-fold induction for fish exposed to 200 $\mu g.L^{-1}$, and a decrease at higher concentrations. The interpretation that this response pattern reflects substrate inhibition is supported by the ELISA results which show a monotonous increase of microsomal CYP1A protein in the gills at all tested concentrations.

A good relationship did exist between EROD and CYP1A protein responses on the one hand and histopathological responses on the other hand, except for the EROD/gill pathology relation (because of reduced branchial EROD activities at higher B(a)P concentrations – see above).

Several studies about the relationship of biochemical and morphological/ histological changes in fish have been described, showing that exposure to PAHs and PCBs provoked a clear relationship between cellular alterations and induction of mixed function oxygenase activities/MFO (Klauling et al., 1979, Kontir et al., 1986; Chui et al., 1985; Au et al., 1999). Furthermore, B(a)P has shown to stimulate RER (rough endoplasmic reticulum) and SER (smooth endoplasmic reticulum) proliferation in sea bass (Lemaire et al., 1992). In our study, the disorganization and proliferation of the cisternae of RER surrounding lipid droplets after B(a)P treatment were detected but not quantified.

In this paper we demonstrated that liver and gill histopathological changes are correlated with CYP1A induction (measured as EROD/protein) under conditions of short-term, high dose exposure to B(a)P - although the specificity of the two markers is different – the histopathological changes were indicative of a general stress response, while CYP1A induction is a specific response to the toxic agent. The biochemical-histopathological correlation was clearly expressed in the liver, while in the gills, the non-monotonic response curve of EROD obscured the relationship to some extent. Such organ-specific differences in responses, possibly due to toxicokinetic factors, have to be taken into account in exposure and effects assessment. Good correlations between biochemical and histological responses were reported from a number of field studies (e.g., Myers et al., 1998; Moore et al., 2003). By statistical analyses, Myers et al. (1998) could demonstrate that biochemical responses to contaminants were significant risk factors for prevalences of both neoplastic and non-neoplastic hepatic lesions, at least for the conditions of long-term, low dose exposure as it is usually the case under field conditions. However, our results indicate that also under high dose exposure, as it may occur in the case of spills, severe, unspecific damage of internal organs can take place.

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