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Immunocytochemical distribution of cytochrome P4501A (CYP1A) in developing gilthead seabream, *Sparus aurata*

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Summary. CYP1A is a major inducible enzyme in the metabolism of xenobiotic substrates. In this paper we investigate by means of immunohistochemistry, the tissue distribution of constitutive cytochrome P4501A (CYP1A) during the period of endogenous nutrition (from hatching until day 4) in developing gilthead seabream, *Sparus aurata* larvae. For this purpose, a polyclonal antiserum (BN-1, Biosense Laboratories) directed against conserved piscine CYP1A sequences was used on paraffin-embedded sections from seabream larvae. From hatching onward, CYP1A immunoreactivity was observed in the following tissues and cells: syncytial, oil-globule envelopes and matrix of the yolk-sac, kidney (epithelia of renal tubules), cardiac muscle cells, skin epidermal cells, troncal musculature, enterocytes of different intestinal regions, goblet cells of the bucco-pharyngeal region, gill epithelial cells and the endothelia of the vascular system of various tissues (especially from liver and brain). Moreover, eye (retina), olfactory epithelium and some positive nerve fibers located in the proximity of the olfactory bulbs and running ventrally toward the posterior brain were strongly CYP1A immunoreactive. In general, the intensity of immunostaining increased with larval development.

Key words: Immunohistochemistry, CYP1A, Tissues, Larvae, Endogenous period, *Sparus aurata*

Introduction

The cytochrome P450 monooxygenase system comprises a family of structurally and functionally related heme proteins. In this family, multiple subfamilies are recognized that are active in the oxidative metabolism of diverse substrates, including drugs and environmental chemicals, as well as endogenous compounds such as steroids, neurohormones, fatty acids and prostaglandins (Nelson et al., 1996).

The cytochrome P4501A (CYP1A) subfamily has attracted particular attention due to its role in the biotransformation of many foreign compounds including dioxins, furanes, polychlorinated biphenyls (PCBs) and polyaromatic hydrocarbons (PAHs) (e.g., Nebert, 1989; Stegeman and Hahn, 1994; Parkinson, 1995). Exposure of organisms to these compounds leads to an induction of CYP1A via the cytosolic Ah (arylhydrocarbon) receptor (Hankinson, 1995). The induced CYP1A protein catalytically converts the lipophilic xenobiotics to more water-soluble compounds what is a first step towards excretion and detoxification. However, some of the metabolites are highly reactive and may ultimately result in enhanced toxicity and carcinogenicity (Parkinson, 1995).

In mammals, the CYP1A subfamily consists of two isoenzymes, CYP1A1 and CYP1A2. In fish, originally only the presence of a single CYP1A enzyme has been demonstrated (Nebert and Gonzalez, 1987; Heilmann et al., 1988), and, therefore, only the term CYP1A instead of CYP1A1 or CYP1A2 was used. However, recent studies presented evidence for the existence of two distinct CYP1A genes in fish although the functional significance of this finding remains unknown (Berndtson and Chen, 1994; Gooneratne et al., 1997).

Fish early life stages, i.e. embryos and larvae, appear to be particularly sensitive to the toxicity of CYP1A-inducing chemicals (Spitsbergen et al., 1991; Walker et al., 1994; Zabel et al., 1995; Henry et al., 1997). The presence of inducible CYP1A in whole body homogenates of fish embryos and larvae has been demonstrated repeatedly (Binder and Stegemann, 1980, 1983, 1984; Binder et al., 1985; Goksoyr and Solberg, 1987; Wisk and Cooper, 1992; Peters and Livingstone, 1995; Monod et al., 1996), however, organ distribution of CYP1A in early life stages of fish has been rarely studied (Guiney et al., 1997; Reinecke and Segner, 1998;

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Segner et al., 1998). In juvenile and adult fishes, major site of CYP1A expression is the liver, but CYP1A is also expressed in a number of extrahepatic organs such as gills, heart, kidney and intestine (Miller et al., 1988; Smolowitz et al., 1991; Buchmann et al., 1993; Husoy et al., 1994; Stegeman and Hahn, 1994; van Veld et al., 1997; Jaksic et al., 1998). Although specific CYP1A activities in extrahepatic organs usually are lower than in the liver, their possible importance for chemical toxicity should be not overlooked.

The aim of the present study is to investigate by means of immunohistochemistry, the tissular localization of constitutive CYP1A during the phase of endogenous nutrition (yolk period) of the marine gilthead seabream, *Sparus aurata* larvae.

Materials and methods

Sparus aurata larvae from hatching until day 4 were reared according to the procedures described by Dinis et al. (1998). The larvae were offered food -rotifers, *Brachionus plicatilis*- for the first time at 3 days posthatching. For histomorphological and immunohistochemical studies, larvae were fixed in buffered-formaldehyde 0.1M pH 7.2 for 24 hours. Then they were dehydrated through graded alcohols, cleared in xylene and embedded in paraffin. Serial sections were cut in coronal and sagittal plane and mounted on gelatinized slides. Sections were rehydrated in distilled water. Haematoxylin-eosin and Haematoxylin-VOF (light green-orange G-acid fuchsin) morphological techniques were performed according to Gutierrez (1990) and Sarasquete et al. (1995).

Immunocytochemical staining was performed using a streptavidin-biotin-peroxidase complex method. Endogenous peroxidase activity was blocked with 1% hydrogen peroxide in Coons buffer (0.01M Veronal, 0.15M NaCl) with 0.1% Triton X-100 (CBT) during 30 minutes at room temperature. Before immunostaining, sections were transferred for 5 minutes to CBT and saturated in CBT with 0.5% casein for 30 min. Sections were incubated overnight in a moist chamber at room temperature with a rabbit polyclonal antibody (BN-1, Biosense Laboratories, Bergen, Norway) raised against cod cytochrome-CYP1A diluted 1:250 in CBT with 0.5% casein. After several washes in CBT, sections were incubated during 1 hour at room temperature with biotinylated anti rabbit-IgG dilutes 1:1000 in CBT-0.5% casein. After washing in CBT, sections were incubated 1 hour at room temperature with streptavidin-peroxidase complex diluted 1:1000 in CBT. Finally, sections were washed with CBT followed by Tris-HCl (0.05M, pH 7.4) and peroxidase activity was visualized in Tris-HCl 0.05M, pH 7.6 containing 0.04% 4-chloro-1-naphtol (Sigma, St Louis, MO) and 0.005% hydrogen peroxide. To demonstrate the specificity of the CYP1A antibody, some sections were incubated with normal fish serum instead of the primary antibody.

Results

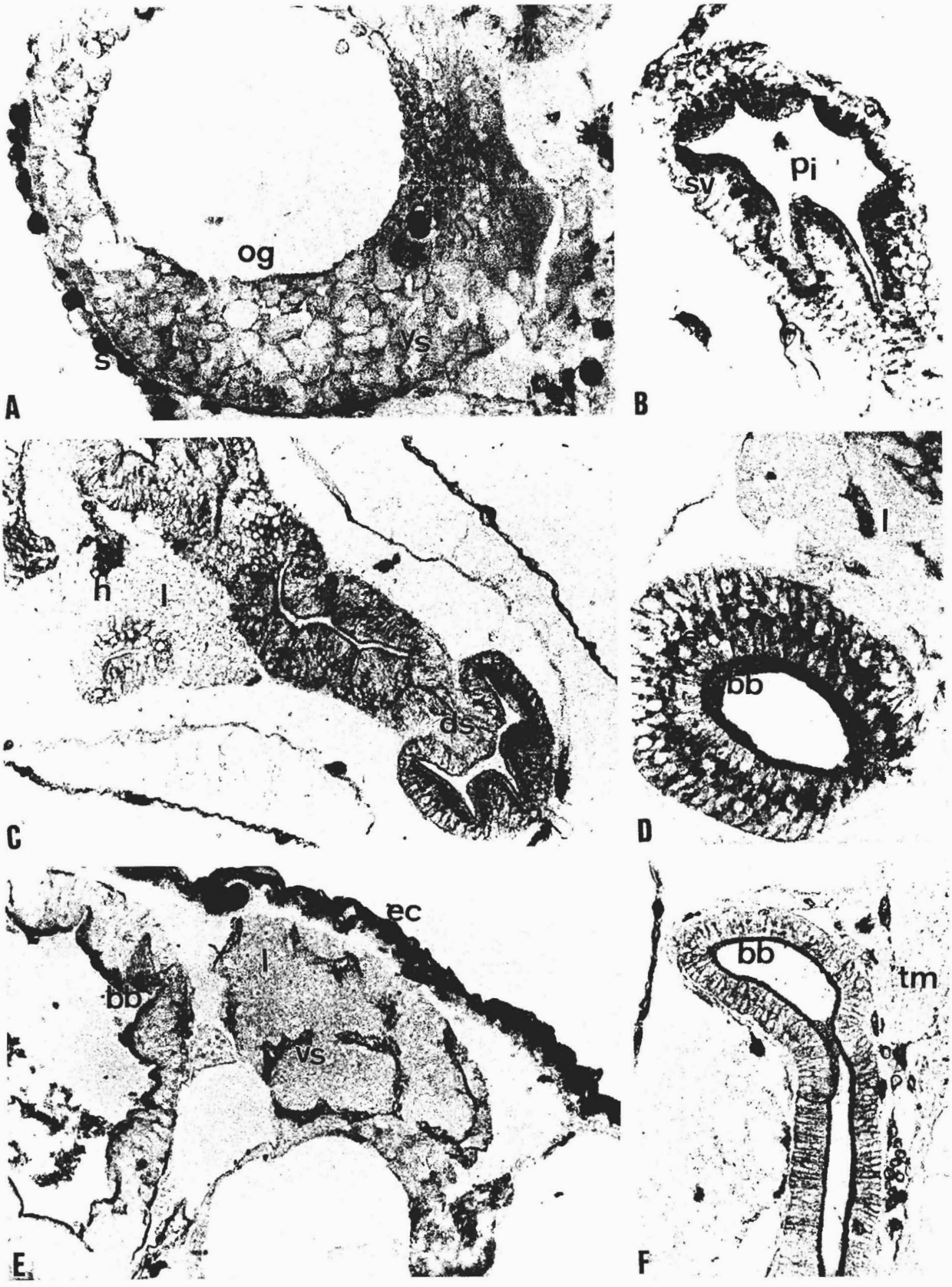
During the first three to four days after hatching, seabream, *Sparus aurata* passes through a period of endogenous nutrition when the organism relies on the yolk material as nutritional source. Between days three and five, exogenous feeding starts although the animal still partly relies on the endogenous yolk reserves.

From hatching until day four, seabream larvae showed CYP1A immunoreactivity in different organs and tissues (Figs. 1-3). A moderate CYP1A immunoreactivity was observed in the syncytium and in the oil globule envelope of the yolk-sac, while a weak immunostaining was found in the yolk-sac matrix (Figs. 1A,D, 2A,B). Control sections did not show positive immunostaining of these sites. In the organs of the digestive tract, both the liver and the alimentary canal were strongly positive (Fig. 1B-F), whereas no immunoreactivity was seen in the exocrine pancreas. Among the liver cells, the endothelia of the vascular system (blood vessels and sinusoids) as well as the biliary epithelia were distinctly immunostained, whereas the hepatocytes showed only weakly positive response (Fig. 1C-E). CYP1A immunostaining of the liver was more evident in 4 day-old larvae than in larvae at hatch. In the alimentary canal, CYP1A immunoreactivity was observed in the epithelium of the buccal cavity, the pharyngeal region (Fig. 2B) with epithelial and mucous cells stained and in the intestinal mucosa. In the anterior part of the intestine (midgut or intestine I), CYP1A immunostaining was evident in the cytoplasm, as well as along the brush border of the enterocytes (Fig. 1C-F); in the rear part of the intestine (hindgut or intestine II), immunoreactivity was located at the supranuclear vesicles in the upper half of the enterocytes, and in brush border (Fig. 1B).

During the period of endogenous nutrition, seabream gills do not possess secondary lamella but only gill filaments are developed. The epithelia of the filaments showed (Fig. 2A) positive CYP1A immunostaining

Further CYP1A positive tissues included the kidney,

Fig. 1. A. CYP1A immunoreactivity in yolk-sac of *Sparus aurata* larvae on day 3 post-.haching. x 400. **B.** Strong immunoreactivity in the posterior intestine (brush border and supranuclear vesicles) in 4-day-old larvae. x 400. **C.** Histological section of a larva 4 post-haching, showing a CYP1A immunoreactivity in the digestive system and liver. x 200. **D.** Larvae on day 3 post-hach; the incipient digestive system (cytoplasm of enterocytes and brush border), the yolk-sac, and the liver (vascular system) are stained. x 400. **E.** Larva at 4 days after haching showing an evident CYP1A staining in some epidermal cells of the skin, in the mucosa of the digestive system (specially in the brush border of enterocytes) and in vascular system of liver. x 400. **F.** CYP1A immunoreactivity in the brush border of the enterocytes of the intestinal epithelium, as well as in troncal musculature. x 200. bb: brush border; e: enterocytes; ec: epidermal cells; h: hepactocytes; l: liver; og: oil-globule; pi: posterior intestine; s: skin; sv: supranuclear vesicles; tm: troncal musculature; vs: vascular system; ys: yolk-sac.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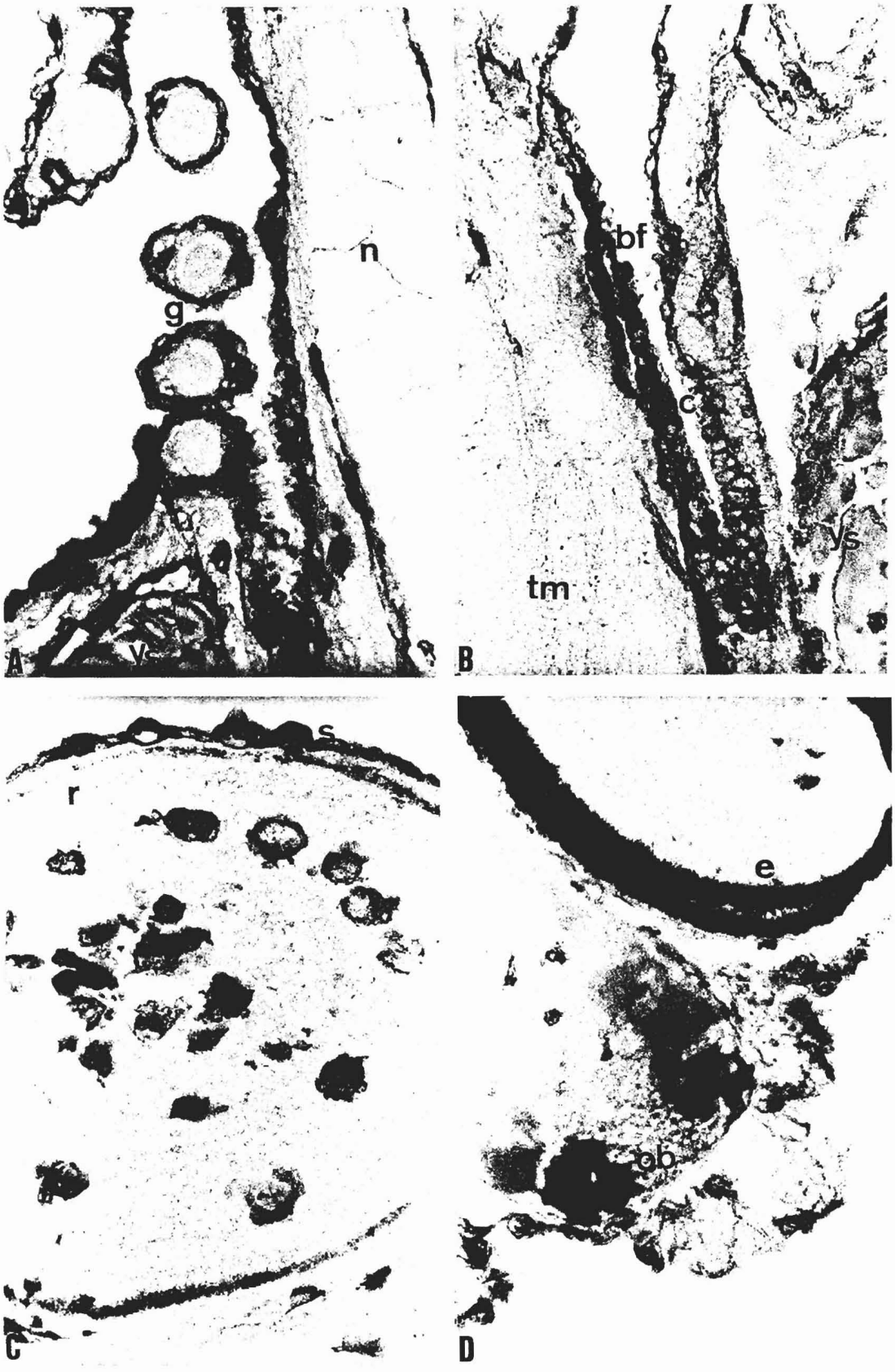

Fig. 2. A. Strong CYP1A immunoreactivity in different organs/tissues of *Sparus aurata* larvae at 3 and 4 days post-haching. Larva on day 3 post-hach showing a CYP1A staining in the gill filaments. x 400. **B.** Bucco-pharyngeal epithelium of a larva on day 3 post-hach. x 200. **C and D.** Strong CYP1A staining in eye - retina- (C), as well as in olfactory bulbs located in the rostral part of larvae brain at 4 days post-hach (D). x 400. bf: bucco-faryngeal epithelium; e: eye; g: gills; mc: mucous cells; n: notochord; ob: olfactory bulbs; r: retina; tm: troncal musculature; ys: yolk-sac.

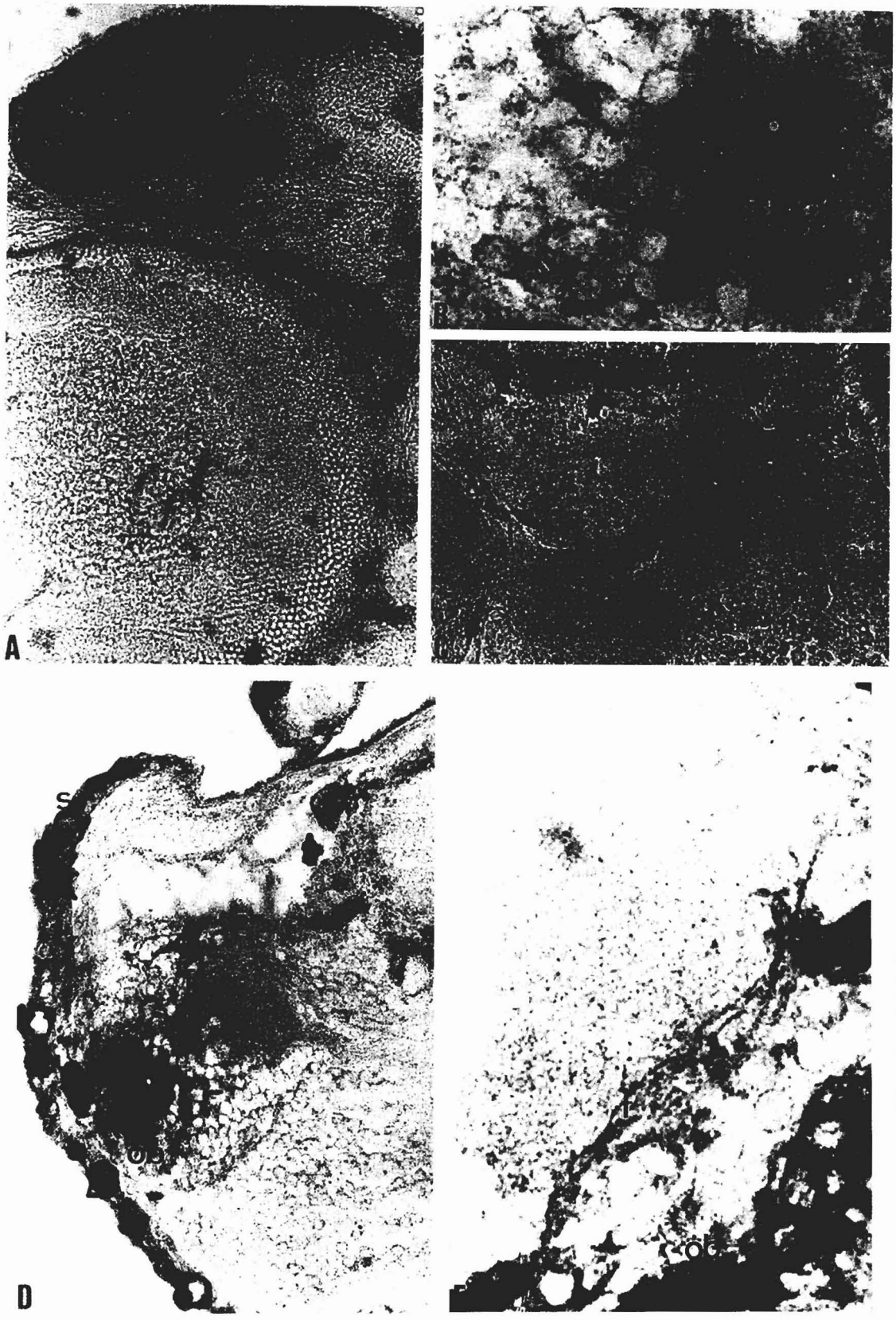

Fig. 3. A and B. CYP1A immunoreactivity in different cerebral areas of *Sparus aurata* larvae at day 4 posthaching. Strong immunoreactivity in placodeal olfactory epithelium (A), as well as in "putative" glial cells (B). x 400. **C-D.** Presence in seabream larvae of CYP1A immunoreactivity in nerve fibers (D) located in the proximity of the olfactory bulbs and runnig ventrally toward the posterior brain (C). The olfactory bulbs which are located in the most rostral part of the seabream larval brain (D), as well as the neuronal fibers **(D and E)** showed a strong CYP1A immnostaining. x 250. e: eye; f: neuronal fibers; gc: glial cells; ob: olfactory bulbs; po: placodeal olfactory epithelium; s: skin.

where the epithelia of the tubuli were stained, as well as epidermal cells of the skin (Fig. 1A,E). A moderate CYP1A immunoreactivity occurred in the muscle cells of the heart. On the contrary, in the trunk musculature CYP1A (Fig 1A,F) staining was restricted to the endothelia of the vascular system.

Certain sense organs and brain areas of the seabream responded positive in CYP1A immunostaining, i.e. the olfactory epithelium and the retina of the eye (Figs. 2C, 3A). In the brain, the vascular endothelia were clearly immunostained. CYP1A immunostaining was also evident in cells of the olfactory bulbs and in the ventral forebrain (Figs. 2D, 3). Some positive nerve fibers were observed in the proximity of the olfactory bulbs and running ventrally toward the posterior brain (Fig. 3D,F) CYP1A immunoreactivity seems to be present in neurons (Fig. 3C,D) although the presence of immunostained glial cells (Fig. 3B) can not be excluded.

Discussion

In this paper, we describe the distribution of the xenobiotic-metabolizing enzyme CYP1A in non-exposed embryos and early larvae of the gilthead seabream, *Sparus aurata*. The period of investigation covered the phase from hatching until start of exogenous feeding (4 days posthatching). During this period, the young fish has to prepare the transition from endogenous to exogenous feeding, what implicates profound anatomical and physiological alterations (Cousin and Baudin-Laurencin, 1985; Boulhic and Gabaudan, 1992; Sarasquete et al., 1993a,b, 1995; Segner and Verreth, 1995; Segner et al., 1993, 1994, Ribeiro et al., 1999). Major changes during early development include resorption of the lipid-rich yolk and oil-globule, opening of mouth and anus, functional differentiation of alimentary canal, liver and pancreas (Sarasquete et al., 1995), as well as of sensory organs. Whereas these processes are mainly completed until start of exogenous feeding, additional physiological changes occur during the larval period until metamorphosis in a juvenile fish: reorganization of trunk muscles (differentiation in aerobic red and anaerobic white fibres), differentiation of gills, and development of a functional stomach. The end of the larval period ends can be assignated to the appearance of the gastric glands indicative of a concomitent change in digestive capacity and prey selection (Stroband and Dabrowski, 1979; Segner et al., 1994; Ribeiro et al., 1999).

The ontogenetic appearance of (inducible) cytochrome P450 monooxygenase is important for the ability of fish embryos and larvae to metabolize, eliminate and eventually detoxify xenobiotics (Binder and Stegeman, 1980; Goksoyr et al., 1991; Peters et al., 1994, 1996). Xenobiotics may bioaccumulate particularly well in fish early life stages due to their high lipid content.

In gilthead seabream, CYP1A immunoreactivity is expressed before onset of exogenous feeding. This agrees with literature data on quantitative measurements of the CYP1A-associated enzymatic activities EROD and AHH in sac fry of rainbow trout, *Oncorhynchus mykiss*, and killifish, *Fundulus heteroclitus* (Binder and Stegeman, 1980; Vigano et al., 1993, 1995). As observed in this study and reported in the literature as well, enzymatic activities generally seem to increase with developmental stage (Binder and Lech, 1984; Stahl and Kocan, 1986; Goksoyr et al., 1991; Peters and Livingstone, 1995; Vigano et al., 1995; Segner et al., 1998). In some species, a transitory peak of CYP1A activity seem to occur around hatching (Binder and Stegeman, 1984).

CYP1A expression in the gut of larval seabream could be related with the presence of natural or anthropogenic inducers in the diet, because usually CYP1A expression in the gut is low (van Veld et al., 1987, 1997). However, in *Sparus aurata* a CYP1A immunoreactivity was observed in the incipient digestive tract from larvae at hatch, an observation which would argue against a contamination via diet.

The olfactory bulbs which are located in the most rostral part of the seabream larval brain showed a strong CYP1A immunostaining. This immunoreactivity seems to be present in neurons, although the presence of immunostained glial cells can not be excluded. At least in rats, the presence of cytochrome P-450 dependent monooxygenases has been evidenced in both neuronal and glial cells (Dhawman et al., 1990). Further double immunocytochemical studies, using anti-CYP1A and anti-GFAP antibodies, could clarify this aspect. As in mammalian brain (Das et al., 1982; Iscan et al., 1990), the olfactory bulbs also contain higher CYP-dependent activities than other brain areas in control rainbow trout (Andersson and Goksoyr, 1994).

It should also be noted the presence in seabream larvae of CYP1A immunoreactivity in nerve fibers observed in the proximity of the olfactory bulbs and runnig ventrally toward the posterior brain. In mammals, there is evidence of the presence of cytochrome P-450 in the synaptic mitochondria of the nerve fibers, suggesting that xenobiotic oxidation and detoxification can occur inside nerve endings (Walther et al., 1986, 1987).

The olfactory epithelium of seabream larvae strongly stained for CYP1A-immunoreactivity. In fish, olfaction is a crucial function for food detection, social behaviour and reproduction (Hara, 1993). A CYP1A immunoreactivity was observed in the olfactory organ of topminnows, *Poeciliopsis* sp. (Smolowitz et al., 1992). In rainbow trout, *Oncorhynchus mykiss*, strong P4501A1-immunoreactivity was detected in various cellular types forming the non-sensory olfactory epithelium including goblet cells, microridge cells and ciliated non sensory cells of olfactory organ. However, olfactory neurons and olfactory receptor cells were unreactive with anti-P450A1 antibody (Monod et al., 1994). According to these authors, the olfactory organ is involved in the detoxication/toxication of pollutants, as well as in the olfactory function, because induction of EROD by ß-naphthoflavone, as well as specific α-hydroxylation of testosterone was observed in olfactory organ of rainbow trout. Cytochrome P450A induction by

xenobiotics was also reported for topminnows exposed for 2 days to benzo(a)pyrene.

For the liver of seabream early life stages, we observed a stronger CYP1A immunoreactivity in endothelia than in hepatic parenchymal cells. In early developmental stages of the lake trout (prehatch embryos and posthach sac fry), Guiney et al. (1997) did not found evidence for constituive expression of CYP1A but CYP1A was detected only after exposure of prehatch embryos and posthach sac fry to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). CYP1A staining in endothelium appeared at a lower dosis of TCDD (22 pg/g egg) than in hepatocytes and the response was stronger than in other parenchymal cells. At the higher dosis (176 pg/g TCDD egg), liver cells types consistenly showing the strongest CYP1A induction were vascular endothelial cells and sinusoideal endothelial cells while the hepatocytes were fairly weakly stained.

The presence of constitutive and inductive CYP1A in endothelia of vascular system is a common feature in different organ/tissues of larvae and adult fish. A role for cytochrome P-450 monooxygenases in the vascular metabolism of the endogenous substrate, arachidonic acid, to vasoactive compounds has been suggested in vertebrates (Pinto et al., 1986; McGiff, 1991). Interaction of CYP1A with such endogenous substrates could alter the barrier function of the endothelium.

According to the observations from this study, no major changes in CYP1A tissue distribution occur from the early yolk-sac phase until the onset of exogenous feeding. Obviously, the qualitative pattern of tissue-specific expression of CYP1A in *Sparus aurata* is accomplished essentially already during the phase of endogenous nutrition. Congruent findings were reported for turbot (Reinecke and Segner, 1998). Alterations of CYP1A after start of exogenous feeding may involve mainly quantitative changes (Wisk and Cooper, 1992; Peters et al., 1996, Reinecke and Segner, 1998).

In conclusion, early developmental stages of seabream, *Sparus aurata*, express constitutive CYP1A in essentially the same organs and tissues which are known to be CYP1A-positive in older life stages. Fish larvae are highly sensitive to the toxicity of CYP1A-inducing compounds such as dioxins, PCBs, etc., and exposure to these lipophilic contaminants seems to provoque severe histopathological changes in endothelia. Further studies will have to elucidate the effects of exposure of seabream (during endogenous and exogenous periods) to CYP1A inducing chemicals in order to reveal the possible role of CYP1A induction for histopathological alterations of the vasculature and the related toxic effects such as edema and mortality.

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