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Phthalate esters immunolocalized in the gastrointestinal tract of shi drum *Umbrina cirrosa* (L.) and rainbow trout, *Oncorhynchus mykiss* (W.)

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Summary. The occurrence of phthalate esters in freshwater and marine aquacultural species like rainbow trout *Oncorhynchus mykiss* and shi drum *Umbrina cirrosa*, respectively, were determined by immunohistochemical approach. The results showed a similar distribution in the gastrointestinal tract of both species. In particular, intense immunoreactivity was found at gastric gland level. In the intestinal tract, goblet cells failed to stain, whereas enterocytes showed the highest binding of phthalates restricted to the apical cytoplasm. This distribution of phthalate esters at gastric gland and enterocyte level may have implications for the physiology of the digestive process and intestinal biotransformation. Phthalates are confirmed to be widely diffused contaminants, absorbed via the alimentary canal; thus a multidisciplinary approach could be useful to examine sea and freshwater environments.

Key words: Phthalates, Immunohistochemistry, Confocal microscopy, *Oncorhynchus mykiss*, *Umbrina cirrosa*

Introduction

Several natural and synthetic compounds, with different chemical properties, act as endocrine disruptors (EDs) even if they are often present at low concentration *in vivo*. Some EDs are widely present in the environment and accumulate in organ tissues and secretions rich in lipid compounds. In mammals, the major risk involves growing organisms during development. Indeed, in embryos some maternal hormones promote and regulate the development and the activity of the central nervous, immune and reproductive system in a harmonic and very complex functional interconnection; considering their potential risks, EDs like phthalates are continuously under investigation by the European Commission (EC) (Scowen, 1996).

Phthalate esters, or phthalates, are lipoid fat-soluble organic molecules widely used as plasticizers in many industries; they are also used as additives in polyvinyl chloride (PVC) giving mechanical resistance and hampering its light deterioration. Since phthalates are even employed as toy softeners, studies carried out on baby saliva showed the presence of phthalates from PVC belonging to several toy specimens (Sugita et al., 2003). Phthalates could also be present in oil lubricant, perfumes, cosmetics and others. They are also used in alimentary plastic packaging films or in aluminium foil-paper laminate and many of these compounds were suspected of migrating onto food depending probably on fat concentration and temperature (Page and Lacroix, 1995). Due to interaction with plastics, phthalates could also be released and found in water and soil partially adhered to suspension particles (Furtmann, 1994). In fact, phthalates are emitted into the environment during the production, use and disposal of plastic products; thus phthalates are now considered ubiquitary contaminants and environmental xenoestrogens and/or antiandrogens (Sultan et al., 2001; Iguchi et al., 2002; Sweeney, 2002).

Particularly, the reproductive endocrine system of fish is under investigation considering the growing interest about phthalate environmental contamination (Metcalfe et al., 2001). In rainbow trout, it has been shown that a variety of estrogen mimics interact with the plasma sex steroid-binding protein (rtSBP) and in a comparative study these receptors were found to bind both native steroids and estrogen mimics (Tollefsen, 2002; Tollefsen et al., 2002).

In the area of endocrine disruption, there are still many gaps in the knowledge about the routes of exposure of aquatic organisms to EDs and where these chemicals accumulate within the tissues. Occurrence of

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phthalate esters has been demonstrated in the alimentary canal of fish and amphibians (Menghi et al., 2002, 2003), but so far there is no clear-cut information about the gastrointestinal tract of other teleosts. Thus, using immunodetection with an antibody that selectively recognizes o-phthalate esters, the present work is aimed at localizing phthalate esters in the gastrointestinal tract of two aquacultural species such as the freshwater rainbow trout *Oncorhynchus mykiss* (W.) and the marine shi drum *Umbrina cirrosa* (L.). Rainbow trout has been extensively used as a sensitive experimental model for environmental toxicology research in aquatic systems (Darnerud et al., 1989; Jarboe et al., 1993; Brack and Schirmer, 2003). Shi drum is a relatively new species, interesting for Mediterranean aquaculture (Cardellini et al., 1998).

Materials and methods

Tissue collection

Ten adult rainbow trout (*Oncorhynchus mykiss*), of both sexes, (five males, five females) weighing 250-350 g, obtained directly from a local hatchery and without any additional treatment, were killed by decapitation after brief anaesthetization on ice to avoid possible interferences with phthalate esters. The nomenclature of the sampling sites used along the gastrointestinal tract follows that of Lee et al. (2001).

Stomach (cardiac and pyloric region), intestinal cecae, proximal and distal intestine were removed intact, stretched out and subsequently cut. Samples were rinsed and then fixed at room temperature in Bouin's fluid for 8 h or for 6 h in 6% mercuric chloride in 1% sodium acetate solution containing 0.1% glutaraldehyde.

Samples of stomach, proximal, medium and distal intestine from eight adult shi drum, (*Umbrina cirrosa*), of both sexes, (four males, four females), weighing 700-1000 g, were rinsed and then fixed for 6 h at room temperature in 6% mercuric chloride in 1% sodium acetate solution containing 0.1% glutaraldehyde, which was kindly provided by Prof. P. Ceccarelli (Faculty of Veterinary Sciences-Unicam-Italy).

All tissues were dehydrated in ethanol gradient, cleared in xylene and embedded in paraffin wax. In order to remove mercury deposits, sections were treated with Lugol's solution (Sigma-Aldrich, Milano, Italy) prior to staining.

Paraffin blocks were stored at 4°C prior to sectioning by routine standard histological procedures. Samples were serially cut (5 μm thickness) and sections were collected on SuperFrost/Plus slides (BioOptica, Milano, Italy).

Immunohistochemistry

Adjacent sections were de-paraffinized, re-hydrated and pre-treated with 0.1 M phosphate buffered saline (PBS), pH 7.4, containing 0.5% bovine serum albumin (BSA) and 0.03% Triton X-100, for 20 min at room temperature. Samples were then incubated with the primary antibody which selectively recognizes o-phthalate esters (Ius et al., 1993) and diluted 1:50 in PBS for 2 h at room temperature. After rinsing in 0.1M PBS, sections were incubated with the secondary antibody goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC) (Sigma Chemical Co., St. Louis, MO, USA) diluted 1:75 in PBS for 2 h at room temperature. Experimental controls were performed by exchanging the primary antibody with non-immune rabbit immunoglobulin G (Sigma-Aldrich, Milano, Italy). After three rinses, coverslips were mounted on slides with PBS-glycerol (1:1). The optimisation of the procedure in situ was performed by testing different concentrations of antibodies. Some de-paraffinized and re-hydrated sections were immersed in PBS-BSA-Triton X-100 for 2 h at room temperature to reveal the background aspecific staining.

Confocal analysis and image acquisition

Immunofluorescence patterns were analyzed under a Bio-Rad Ar/Kr MRC-600 Confocal Laser Scanning Microscopy (Bio-Rad, Herdfordshire, U.K.) attached to a Nikon Diaphot-TMD-EF inverted microscope equipped with a Plan Apo, oil immersion, objective (x60, NA=1.4) to obtain optical sectioning of samples (Shotton, 1989; Pawley, 1995). The standard BHS block (excitor filter 488 DF) was used for FITC.

High resolution images for a qualitative evaluation of the immunofluorescence patterns were captured and analyzed with Confocal Assistant 4.02 (Bio-Rad) and finally transferred to PhotoShop v7.0 (Adobe) for generation of graphics, then printed with Epson Stylus photo 890 on Epson glossy photo paper (Menghi et al., 1999).

Results

Appreciable inter-sectional as well as inter-individual differences between sexes were not observed, indicating that immunohistochemical differences did not occur. Also fixative mixtures did not appreciably affect the binding.

Oncorhynchus mykiss

The epithelial lining of the cardiac region reacted with the antibody directed against o-phthalate esters; in particular, the intercellular boundaries and cytoplasm, except for secretory granules, were strongly labelled for o-phthalate esters. Labelling was also observed at tubular gastric gland level, whereas nuclei were never stained. The connective tissue of plicae and surrounding glands showed intense labelling (Fig. 1). The pyloric region of the stomach showed the most intense immunoreactivity at the connective axis of longitudinal plicae (Fig. 2). Control performed by exchanging the

primary antibody with non-immune rabbit IgG showed lack of appreciable immunofluorescence (inset Fig. 2).

Cecae, as well as proximal and distal intestinal mucosae are lined by enterocytes with interspersed goblet cells. Intestinal cecae (Fig. 3) as well as proximal intestine (Fig. 4) showed an intense immunoreactivity restricted to the apical region of enterocytes. Labelling was never observed at nucleus and goblet cell level. The axial connective tissue weakly reacted. The only strong staining observed in the distal intestine was localized at connective tissue level. Enterocytes exhibited an apical modest binding while goblet cells did not react (Fig. 5).

Umbrina cirrosa

The gastric glands of shi drum stomach exhibited the

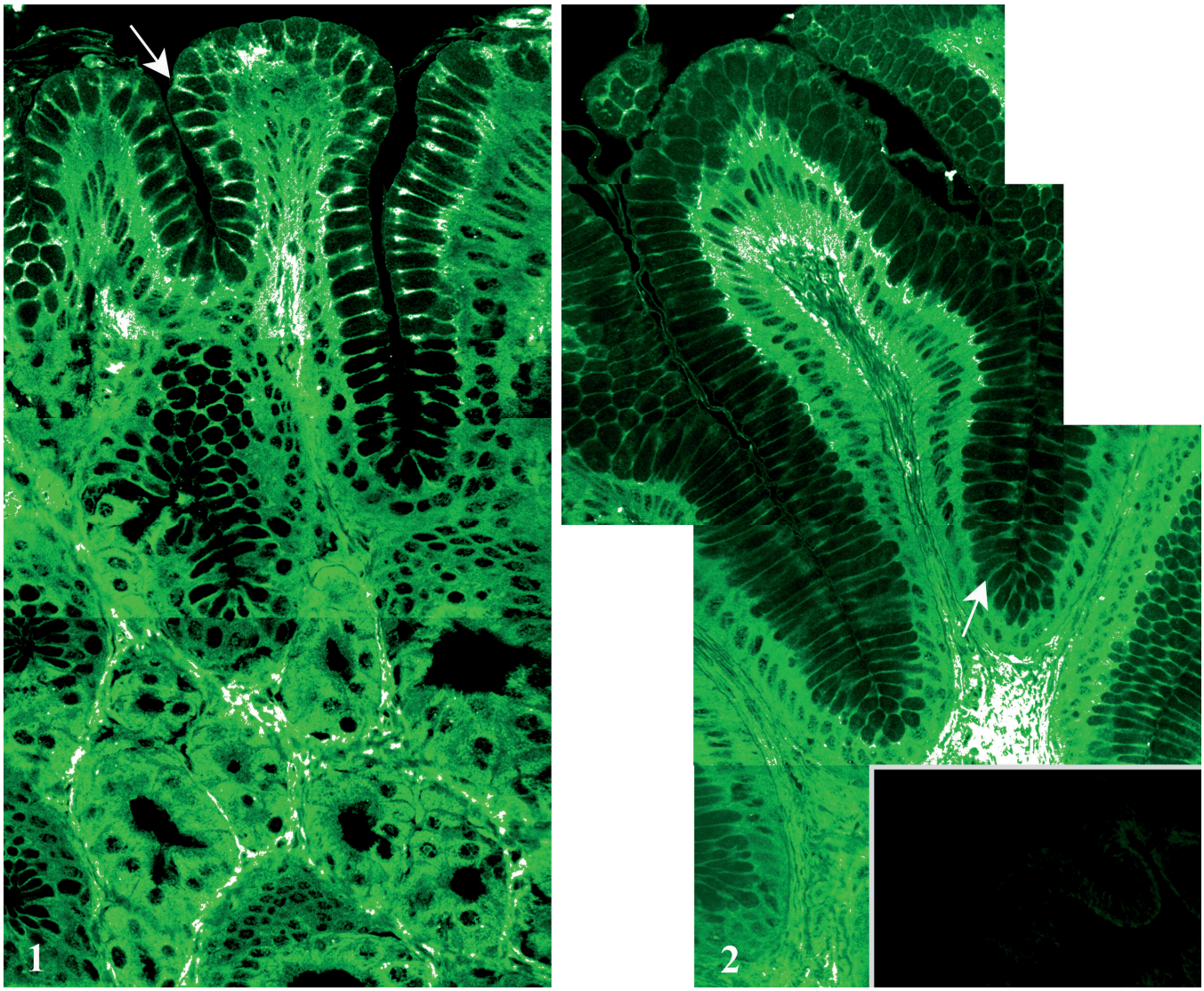

Fig. 1. Rainbow trout. Stomach - Cardiac region. Montage of optical sections derived from confocal laser scanning microscopy (CLSM) showing the o-phthalate ester distribution. Gastric glands as well as connective tissue exhibited intense immunostaining fluorescing in the green scale, with white being the most intense. Reactive sites for o-phthalate esters were also found in cytoplasm and intercellular boundaries (arrows) of epithelial lining. x 730

Fig. 2. Rainbow trout. Stomach - Pyloric region. Montage of optical sections from CLSM showing the o-phthalate ester distribution. The connective tissue showed the most intense binding patterns fluorescing in the green scale, with white being the most intense. In contrast, nuclei appeared always unreactive. Similarly to figure 1, cytoplasm and intercellular boundaries (arrows) of epithelial lining showed reactive sites. Control section did not present appreciable fluorescence (inset). x 730

most intense reactivity. The axial connective tissue of plicae showed binding (Fig. 6).

The intestinal tract lacked macroscopic zonal differences even if recent studies showed the identification of three fundamental segments: proximal, medium and distal intestine (Pedini et al., 2001; Parillo et al., 2002). The strongest reactivity was observed in the apical region of the enterocytes in all intestinal tracts examined while the connective tissue showed moderate binding. Goblet cells lacked staining (Figs. 7-9). In the control section, the exchange of the primary antibody with non-immune rabbit IgG produced no specific staining (inset Fig. 9).

Discussion

For several years researchers have been studying the presence and fate of phthalate esters in fish (Metcalf et al., 1973; Stalling et al., 1973; Williams, 1973). Many authors investigated the aquatic toxicity of several plasticizers (Sanborn et al., 1975; Group, 1986; Wams, 1987; Kim et al., 2002; Tollefsen, 2002).

The alimentary canal represents a very efficient surface for absorption but, so far, very little is known about EDs distribution and its potential toxic effects on digestive tracts in fish.

The metabolism, biodistribution and excretion of

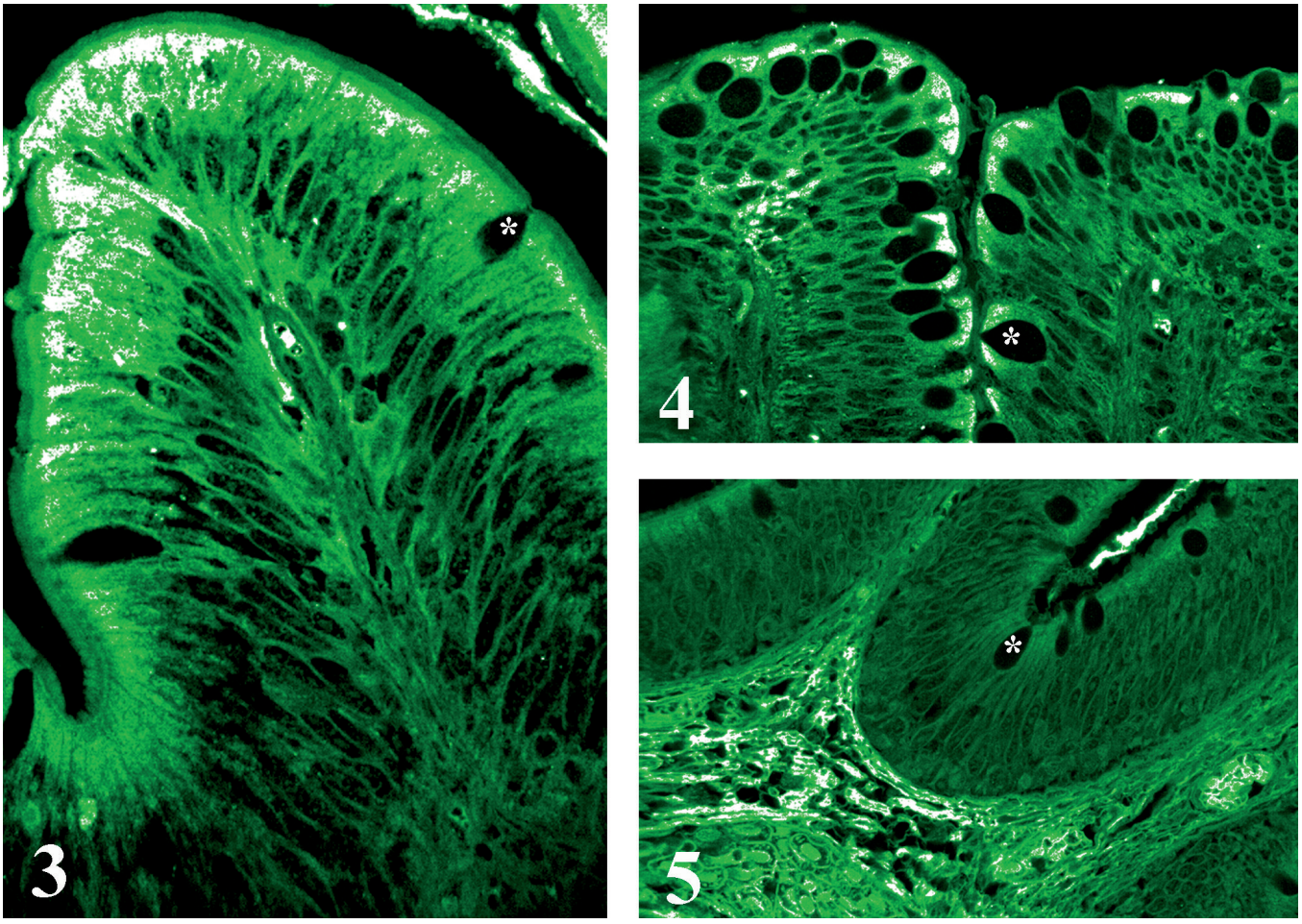

Fig. 3. Rainbow trout. Intestinal cecae - A scanning confocal immunofluorescence microscopic image showing the distribution of o-phthalate esters in an optical section from a 5 μm paraffin section. Reactive sites, fluorescing in the green scale with white being the most intense, were only located at the supranuclear region of enterocytes. Goblet cells (asterisk) as well as nuclei did not react. x 850

Fig. 4. Rainbow trout. Intestine - Proximal tract. Optical section from CLSM. Phthalate containing sites were restricted to the apical region of enterocytes. Mucous goblet cells (asterisk) and nuclei were unstained. The qualitative evaluation of immunofluorescence was always done in the green scale with white being the most intense and black the less intense. x 850

Fig. 5. Rainbow trout. Intestine - Distal tract. Optical section from CLSM. Intense staining for o-phthalate esters (white colour) occurred at connective tissue level. The supranuclear region of enterocytes showed only a modest binding (green colour). Goblet cells were not reactive (asterisk). x 850

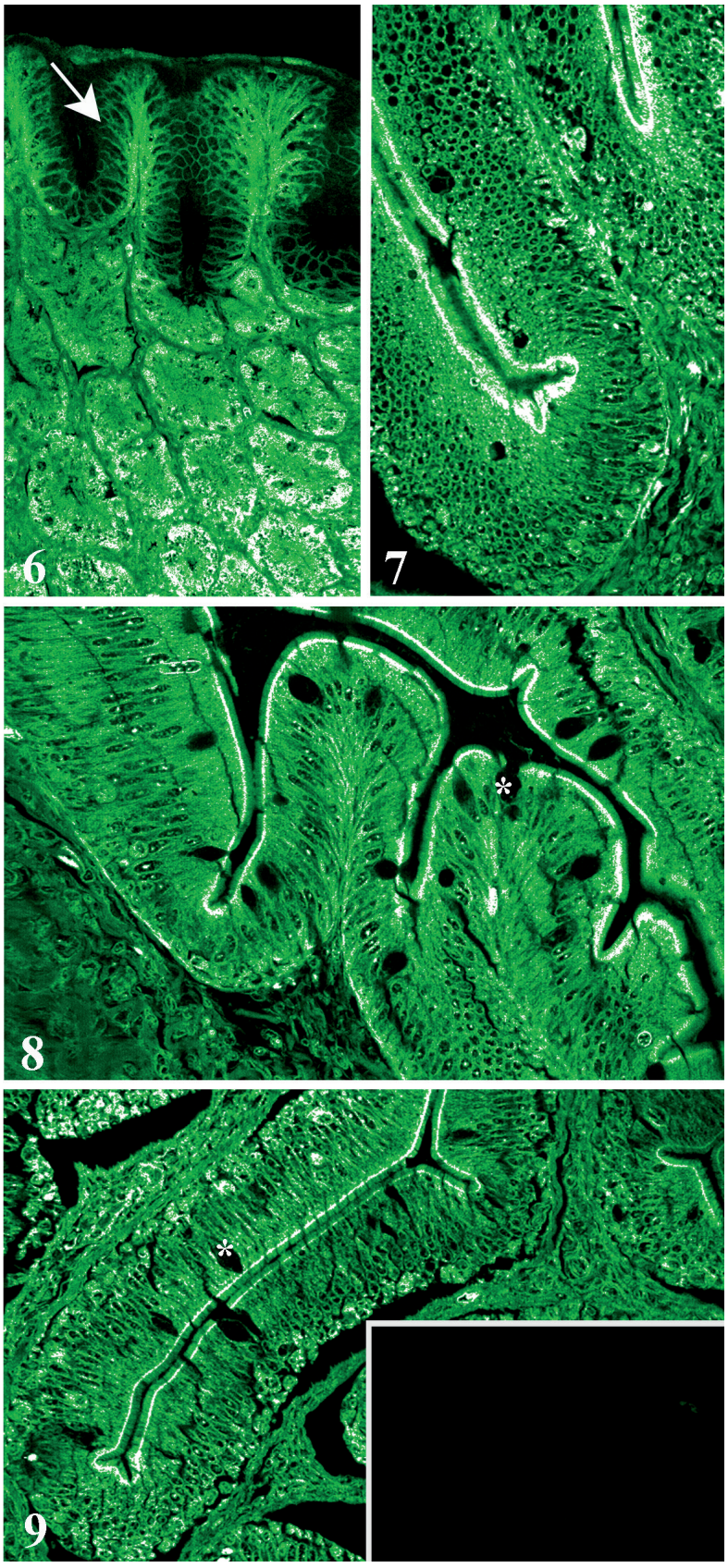

Fig. 6. Shi drum. Stomach. Montage of optical sections from CLSM showing the o-phthalate ester distribution. The highest amount of labelling (white colour) was observed at gastric gland level. Specific immunofluorescence was also observed at connective tissue level. Cytoplasm and intercellular boundaries (arrow) of epithelial lining showed reactive sites (green colour). x 650

Figs. 7, 8, 9. Shi drum. Intestine - Proximal, medium and distal tracts. A scanning confocal immunofluorescence microscopic image showing the distribution of o-phthalate esters in an optical section from a 5 μm paraffin section. Strong staining occurred in the supranuclear region of the absorptive cells (white colour). Only a modest binding was observed at connective tissue level along the entire intestine (green colour). Goblet cells failed to stain (asterisk). Control section showed no appreciable staining (inset Fig. 9). x 850

xenobiotics varies to some extent among the organs, tissues and different phthalates. The toxicological profile of several phthalates are known to be affected by the route of administration and the level and length of exposure (White et al., 1994; Sugita et al., 2003). By actual immunoistochemical approach we were unable to establish the main route of exposure, i. e. feed or water.

In previous works the occurrence of phthalate esters was reported in *Tilapia* ssp and in *Rana esculenta* and it was ascertained that these oestrogen-mimicking substances are not only present in the epithelial lining, i.e. the most external layer, but also in internal structures such as gastric glands (Menghi et al., 2002, 2003). The endocrine disrupting chemicals enter via the alimentary canal and then not only reach the target cells but also persist differently in specialized cells. The peculiar micro-environment responsible for the maintenance of adequate pH probably allows the phthalate concentration with effects on pepsinogen activation and consequential reduction of peptic activity and loss of digestive efficiency. In an *in vitro* model it was also demonstrated that phthalates could evoke metabolic effects without entering the osteoblast cell nucleus (Sabbieti et al., 2000; Menghi et al., 2001) and actin cytoskeleton modifications (Marchetti et al., 2002); it was also shown that phthalates act in a dose- and time-dependent way with transient effects analogously to peroxisome proliferation. These findings and the previous ones imply that the teleost intestine could be a favoured site for phthalate absorption from water/feed.

The present study demonstrated the occurrence and biodistribution of phthalate esters in freshwater and marine aquacultural species such as rainbow trout *Oncorhynchus mykiss* and shi drum *Umbrina cirrosa*, respectively. Qualitative evaluation of results revealed that, as far as the stomach is concerned, the *Umbrina cirrosa* exhibited a very intense immunoreactivity at gland level in contrast to a modest staining in *Oncorhynchus mykiss*. These differences reflect a diverse occurrence of glycoconjugates involved in transport processes associated with gastric secretion and with dilution of ingested seawater (Pedini et al., 2001). Conversely, the trout revealed the strongest reactivity at connective tissue level in both the anterior and the posterior region of the stomach. In the intestinal tract of both species, goblet cells failed to stain, whereas enterocytes showed the highest binding of phthalates only restricted to the apical cytoplasm. However, strong cytoplasmic immunohistochemical staining was only observed in enterocytes of trout intestinal cecae and proximal intestine. Control experiments, carried out on sections from gastrointestinal tract of both species, supported the specificity of the antibody used.

Phthalates and other compounds act as peroxisome proliferators in different organisms, in particular in mussels and in aquatic organisms living in coastal and estuarine areas which are exposed to a variety of pollutants of industrial, agricultural and urban origin (Cajaraville et al., 2003).

The gastrointestinal occurrence of phthalates partially reflects the cellular localization and expression patterns of specific cytochrome P450 (Cok et al., 1998; Lee et al., 2001). The concomitant presence of phthalates and CYP450 may suggest a possible role of cytochrome P450 system in phthalate fate. In teleosts, as in mammals, the cytochrome P450 system plays important roles not only in the metabolism and excretion of endogenous compounds but also in the biotransformation of certain xenobiotics (Buhler and Williams, 1988; Goksøyr and Förlin, 1992; Buhler and Wang-Buhler, 1998).

Gastrointestinal metabolism results in the formation of derived products that in turn can be absorbed and transported to the liver and other tissues where they can produce physiological, pharmacological or toxicological effects or undergo conjugation prior to excretion. Intestinal biotransformation reactions can also convert some chemicals into reactive metabolites, leading to a greater toxicity than the parent chemicals (Lee et al., 2001).

The two teleost fish investigated in this study showed several differences linked to species, habitat, alimentation and metabolism, but in spite of such differences, we surprisingly found that the target organ-tissues were the same. This may be due to the exclusive microenvironment of gastric glands; also, the positivity of apical enterocytes is compatible with their specific role in the unrefined nutrient absorption. Phthalates were confirmed to be ubiquitary water contaminants and this immunohistochemical approach may contribute to a "biomonitoring system" for sea and freshwater endocrine disruptor assessment.

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