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In situ visualization of o-phthalate esters in gastrointestinal tract of the frog *Rana esculenta*

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Summary. The regional distribution and relative occurrence of phthalates were studied immunohistochemically by confocal laser scanning microscopy in the alimentary tract of the green frog, *Rana esculenta*, using an antibody against o-phthalate esters. Many positive sites indicating the basal presence of phthalate esters were identified. The immunoreactive cells were located in the gastric glands of the stomach and in the intestinal epithelium regions with variable frequencies. The regional distribution of phathalate-accumulating cells resembled that of fish and demonstrated that these endocrine disruptors not only enter via the alimentary canal, but also bioaccumulate inside cells specialized in secretion as well as absorption functions.

Key words: Phthalates, Immunohistochemistry, Confocal microscopy, Digestive tract, *Rana esculenta*

Introduction

Phthalates, a class of chemicals used in a whole range of plastic goods to impart flexibility, can be also found as ingredients in many formulations such as paints, inks and adhesives; phthalates are now ubiquitous global contaminants and specific phthalates, considered to be hormone disruptors, include di-ethylhexyl phthalate, butyl benzyl phthalate, di-n-butyl phthalate, and diethyl phthalate (Jobling et al., 1995; Harris et al., 1997).

There is large information about endocrine disruptor effects in fish (Group, 1986; Sumpter and Jobling, 1995; Arukwe, 2001); conversely, data about their efficacy and occurrence in amphibians are lacking although there is an universal decline in amphibian populations (Blaunstein and Wake, 1990; Carey and Bryant, 1995; Kloas et al., 1999; Carey, 2000; Houlahan et al., 2000; Kloas 2002).

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In this study we used a selective antibody, developed for a time-resolved fluoroimmunoassay for o-phthalate esters in water (Ius et al., 1993), in order to immunodetect and localize their basal bioaccumulation within organ tissues; at the moment, we restricted our attention to the *Rana esculenta* gastrointestinal tract that is involved in introduction, elaboration and absorption of chemicals contained in food and water. The potential importance of investigating phthalates in different organs and species (Sabbieti et al., 2001; Menghi et al., 2002) represents a powerful tool in view of individualizing pathologies or abnormalities correlated with massive presence of environmental phthalates.

We previously developed a method *in vitro* and monitored the phthalate internalization and induced modifications in Py1a cells by differential scanning calorimetry and confocal microscopy (Sabbieti et al., 2000a); also the effects of butyl benzyl phthalate and di-butyl phthalate on FGF-2 in the rat Py1a osteoblasts were studied and it was demonstrated that both chemicals strongly and reversibly affect the nuclear translocation of the growth factor in a dose-dependent and time-related manner (Menghi et al., 2001).

Materials and methods

Tissue collection

Ten adult green frogs, *Rana esculenta*, were collected in the mountain pond of Colfiorito, Italy. The animals were sacrificed without any additional treatment under ether anaesthesia and their digestive tract was quickly removed. Some samples were fixed in Carnoy's fluid for 24 h and post-fixed in a mixture of 2% calcium acetate and 4% paraformaldehyde (1:1) for 3 h in order to obtain the best preservation of antigenic properties and carbohydrate components as previously detailed (Gabrielli and Menghi, 1994). Tissues were then dehydrated in a series of graded ethanol and embedded in paraffin wax. Stomach and intestine tracts were serially cut (5 μm thickness) and sections were collected on SuperFrost/Plus slides (Bio-Optica, Milano, Italy). The choice of the fixative mixtures was based on a

previous investigation (Sabbieti et al., 2001).

Immunohistochemistry

De-waxed sections were re-hydrated and immersed in 0.1 M phosphate-buffered saline (PBS), pH 7.4, with added 0.5% bovine serum albumin (BSA) and 0.3% Triton X-100, for 20 min at room temperature before incubation with the primary antibody (diluted 1:50), that selectively recognizes o-phthalate esters (Ius et al., 1993), for 2 h at room temperature. The antibody was kindly provided by Prof. Roda, University of Bologna, Italy. After rinsing (three washes, 5 min each) in 0.1 M 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 for 2 h at room temperature. Control experiments included omission of the primary antibody from the staining protocol or the incubation with a nonimmune rabbit immunoglobulin G. After three washes, coverslips were mounted on slides with PBS-glycerol (1:1). The optimization of the procedure was previously carried out on fish tissues (Menghi et al., 2002).

Additional de-waxed and routinely re-hydrated sections were treated with BSA-Triton X-100 for 2 h at room temperature to reveal the background staining.

Confocal analysis and image acquisition

Transverse sections of stomach and intestine from each animal were analyzed. Fluorescing patterns were scanned using an Ar/Kr confocal laser scanning microscope (Bio-Rad MRC-600, Hertfordshire, UK), which was connected to a Nikon Diaphot-TMD-EF inverted microscope equipped with Plan Apo x10 and x60 oil-immersion objectives. The standard BHS block (excitor filter 488 DF) was used. Images were obtained using a Bio-Rad Comos software and then image acquisition was performed by a PIC format file then printed with Epson Stylus photo 890 on Epson glossy photo paper (Sabbieti et al., 2000b).

Results

Stomach

The stomach of *Rana esculenta* was found to be lined with a thick mucosa with a few longitudinal folds. A single layer of mucus-secreting cells lined the luminal surface of the stomach and the gastric pits. The epithelial lining was of the simple columnar type and the gastric pits were surrounded by foveolar cells. Glands were of the simple or ramified tubular type and consisted mainly of mucous neck cells and oxyntic-peptic cells (Palatroni et al., 1988).

Superficial cells and foveolar cells of the epithelial lining as well as mucous neck cells of gastric glands did not react with the antibody to o-phthalate esters. The connective tissue was faintly stained; the whole cell cytoplasm of oxyntic-peptic cells of glands was found to be the most strongly stained structure of the stomach (Fig. 1). Controls demonstrated the specificity of the immunoreaction and evidenced a minimal basal staining due to an autofluorescence at blood vessel (bottom inset Fig. 1) and serosa level.

Intestine - Proximal tract section

In this region the intestine of the frog *Rana esculenta* was lined by a simple columnar epithelium that mainly consisted of absorptive cells with intercalated goblet cells. The intestinal mucosa was organized in longitudinal folds (Gabriel, 1990). Goblet cells failed to stain; absorptive cells reacted in all the cytoplasm and in particular in the supranuclear region where immunostaining showed the highest degree of intensity. Nuclei never reacted (Fig. 2). In control sections, omission of the primary antibody produced no specific staining; only blood vessels showed a faint autofluorescence (top inset Fig. 2).

Intestine - Distal tract section

No significant differences were observed between the proximal and distal regions except for height and number of longitudinal folds, which increased in the distal tract. As far as phthalate immunodetection is concerned, appreciable, but not intense, staining was restricted to the supranuclear region of the columnar cells within the epithelial lining (Fig. 3). Controls supported the above immunostaining as specific and confirmed the modest autofluorescence of blood capillaries (left inset Fig. 3).

Discussion

The aim of the present study was to describe the distribution of o-phthalate esters in the gastrointestinal tract of the most common frog, *Rana esculenta* L. It has been previously demonstrated that changes caused by pesticides pertain chiefly to the alimentary canal, in addition to other organ tissues, and are connected with the developmental stage of the animals (Jordan et al., 1977). Thus, information about the occurrence and location of phthalate esters in amphibians will help understand the potential effects of these chemicals on modification of amphibian reproductive biology and metamorphosis. To this end, we tested an antibody that selectively recognizes o-phthalate esters and has been standardized to a direct time-resolved fluoro-immunoassay for these esters in water (Ius et al., 1993); we previously optimized the conditions to use this antibody for in situ immunohistochemical studies (Menghi et al., 2002). This antibody is useful to visualize the most widely diffused phthalic esters; in fact, cross-reactivity tests demonstrated similar affinity for dimethylphthalate, diethylphthalate, dibutylphthalate,

butylbenzylphthalate, and dioctylphthalate, whereas little or no interference was found for phthalic acid or isomeric esters (Ius et al., 1993).

Although the gut is homologous among different vertebrates, structural differences exist between species. The most obvious variation in the guts of extant vertebrates appears in the stomach; in particular, gastric glands of fishes, amphibians, reptiles, and birds are mainly composed of oxyntic-peptic cells capable of producing pepsinogen and hydrochloric acid, whereas mammalian gastric glands have individual peptic and oxyntic cells. The global patterning of the gut is remarkably similar among the different vertebrate lineages (Smith et al., 2000). Accordingly, we found, in stomach and intestine, a location of o-phthalate esters as occurs in the corresponding to organ tissues (Menghi et al., 2002), but a more marked occurrence of these substances in this amphibian species was observed. The

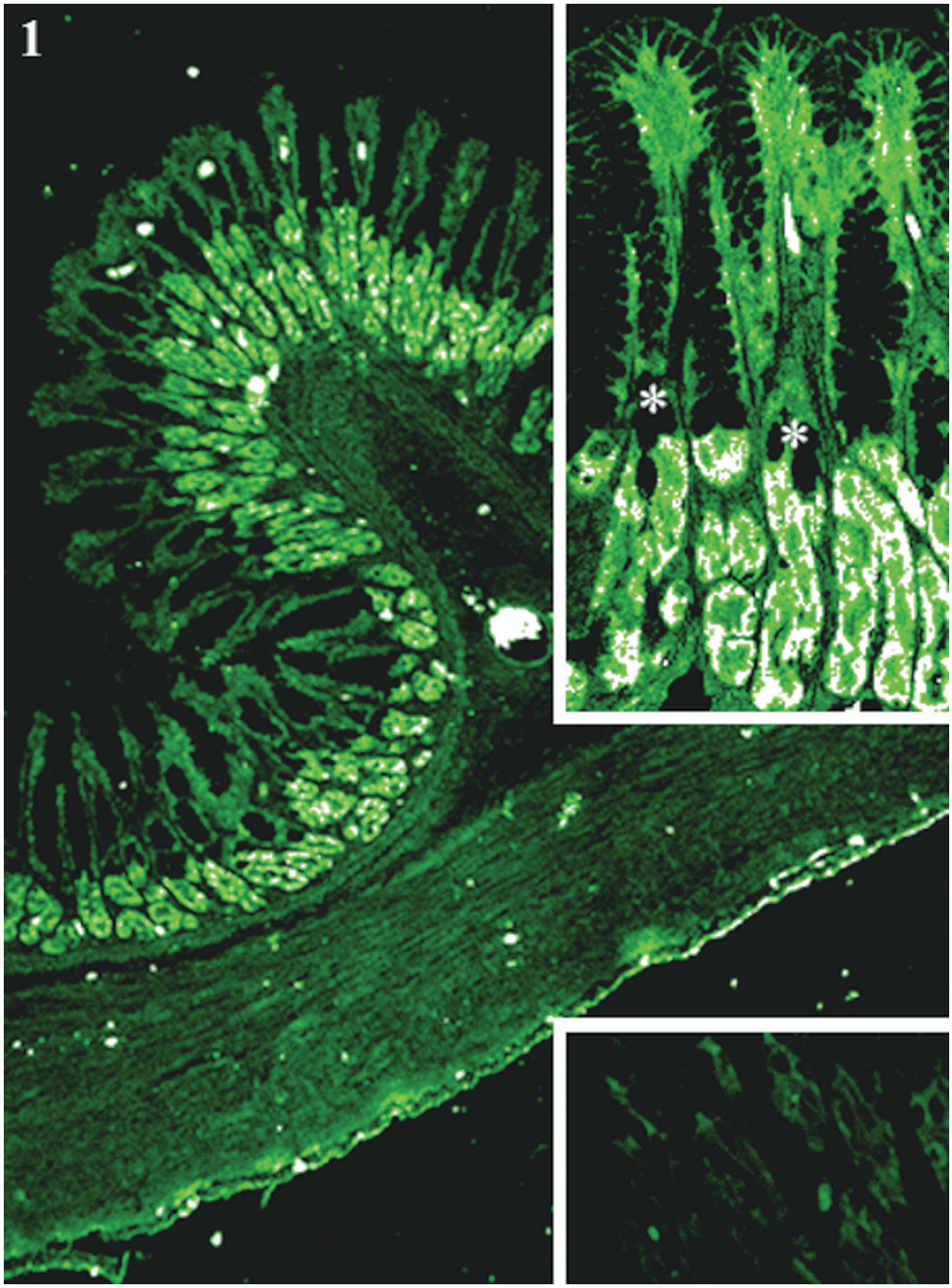

Fig. 1. Stomach. Montage of cross section of a longitudinal fold showing the o-phthalate ester distribution. Reactive sites are mainly located in the oxyntic-peptic cells of gastric glands; also the connective axis of mucosa and serosa shows immunostaining. Both superficial and foveolar epithelial cells as well as neck cells (asterisk) of gastric glands do not react except for intercellular boundaries (montage of top inset). Control sample only presents a weak autofluorescence at blood capillaries (bottom inset). x 10; top inset, x 60; bottom inset, x 60

massive bioaccumulation of phthalate esters in distinct compartments of untreated subjects that we found in all green frogs examined may be correlated with the exposition to both aquatic and terrestrial environment.

Also in this study we noted the lack of accumulation of phthalates inside the nucleus of reactive cells; however, it is to be considered that phthalates which were demonstrated do not enter into the nucleus of the rat osteoblasts Py1a greatly influence the FGF-2 nuclear translocation with the correlated effects on cell proliferation and collagen type I synthesis and glycosylation (Menghi et al., 2001). Thus, phthalates may influence nuclear activities without entering inside but by modulating growth factors and pathways of signal transduction.

The regional bioconcentration of phthalate esters in cells differentially involved in the organ functions could be of particular concern since these environmental

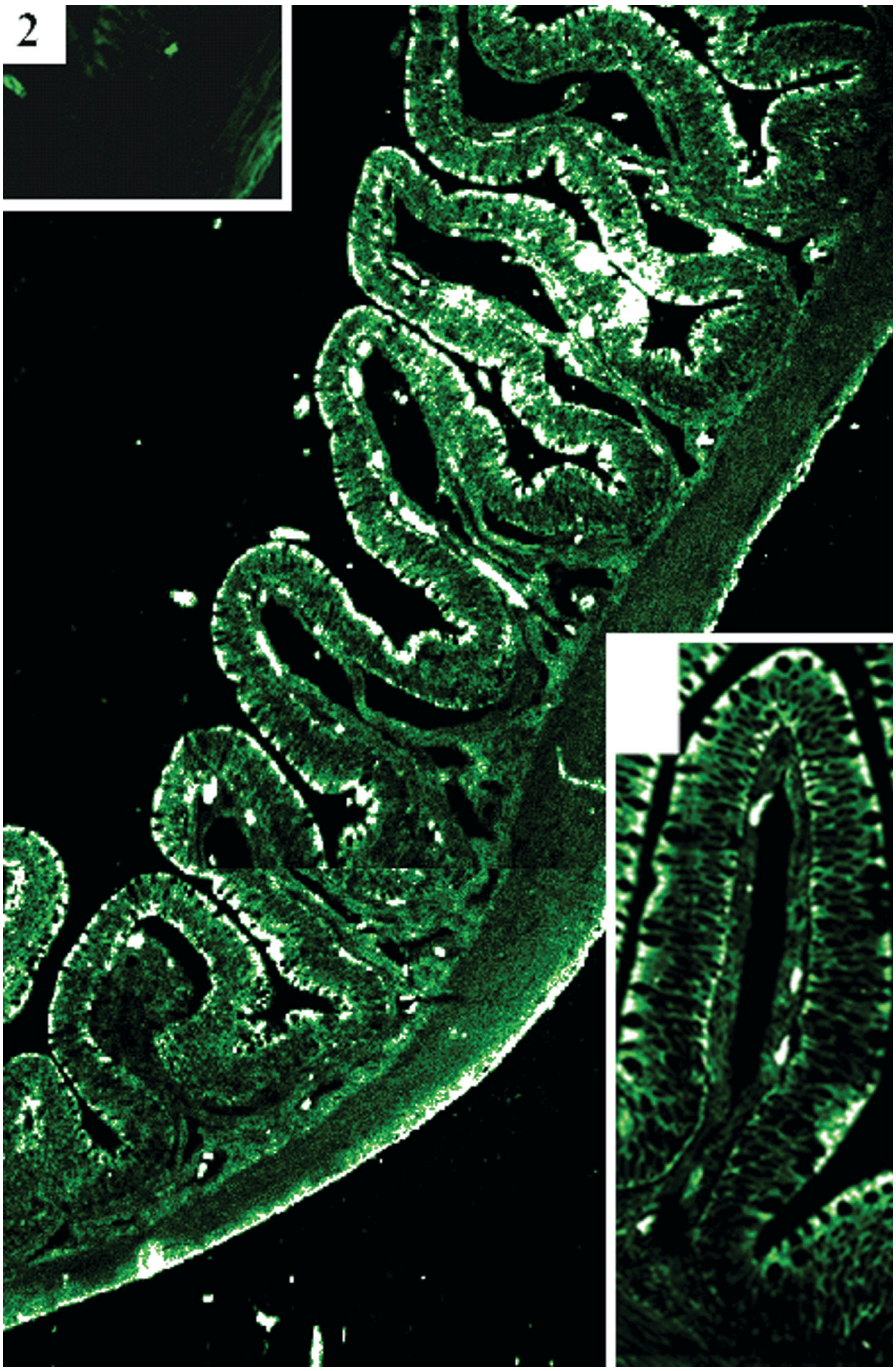

Fig. 2. Intestine - Proximal tract. Montage of cross section. Phthalate-containing sites, displayed in the gradient green color scale with white being the most intense, are restricted to the columnar absorptive cells exhibiting the strongest staining in the supranuclear region as clearly emerged in the magnification (montage of bottom inset). Mucous goblet cells are unstained. Control section confirms the specificity of the antibody used (top inset). x 10; bottom inset, x 60; top inset, x 60

contaminants, in addition to other widespread compounds such as atrazine (Hayes et al., 2002), may be relevant factors in global amphibian declines. The bioconcentration of o-phthalate esters, regarded as global contaminants *in vivo* and *in vitro*, which after digestion, absorption and accumulation in the enterocytes could perturb the endocrine system is of interest; in particular, phthalate esters can affect reproduction by estrogenic modes of action that produce severe effects including abnormal sexual differentiation (Jobling et al., 1995; Harris et al., 1997). Recently, the potential of autonomous hormonal steroidogenesis in liver and small intestine of frog, *Rana esculenta*, has been investigated and it has been found that both liver and intestine can be independent sources of hormonally active steroids (Belvedere et al., 2001). In addition, knowledge of amphibian biology and endocrinology indicated that aquatic vertebrates such as amphibians are very suitable models for the study of endocrine disruptors mainly of anthropogenic origin and distributed in surface water (Kloas, 2002); probably, among other endocrine disruptors, phthalates could contribute to changes of amphibian populations via adverse effects on reproduction by abnormal sexual differentiation and on the thyroid system by acceleration or retardation of metamorphosis. Indeed, the effects of dibutyl phthalate on gonadal sex differentiation of genetically male tadpoles of *Rana rugosa* have been examined and it has been found that the histological examination of the gonads showed the typical structure of testes in the control tadpoles; conversely, treatments with dilute solutions of dibutyl phthalate and 17 beta-estradiol caused the undifferentiated gonads to develop into gonads of complete or partial ovarian structure. Dibutyl phthalate, at concentrations of 0.1, 1 or 10 μm, was estimated to be 1,000-fold less potent than 17 beta-estradiol; nevertheless, this phthalate ester is an environmentally dangerous hormone that disrupts the

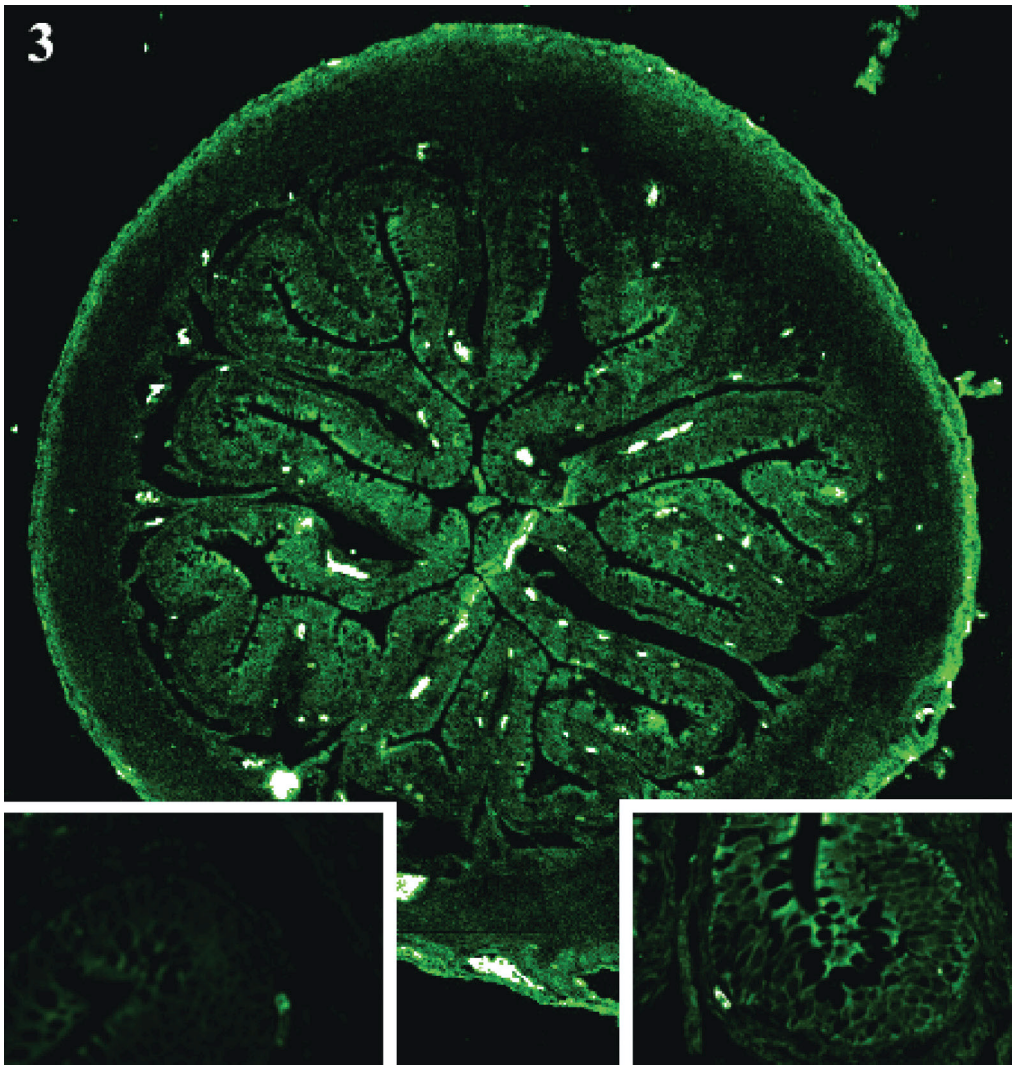

Fig. 3. Intestine - Distal tract section. Moderate staining occurs in the supranuclear cytoplasm of absorptive cells; serosa also reacts. Goblet cells lack staining (right inset). Control sample showing no appreciable staining (left inset). x 10; right inset, x 60; left inset, x 10

pathways of testicular differentiation in genetically male animals (Ohtani et al., 2000). Similar concentrations were found to be noxious and markedly but transiently affect the actin cytoskeleton in Py1a rat osteoblasts (Marchetti et al., 2002). Recently, the effects of an estrogenic compound, 4-nonyl-phenol, on the amphibians *Rana esculenta* and *Triturus carnifex* have been described together with those on sexual differentiation in *Xenopus laevis* and it has been found that 4-nonyl-phenol increases plasma vitellogenin in male frogs and newts in a dose-related manner, produces inhibitory effects on gonadotropin and prolactin secretion by pituitary together with an elevation of plasma androgens; moreover, it has been observed that both 4-nonyl-phenol and bisphenol A at concentrations ranging from 10^{-7} M to 10^{-8} M cause feminization and the in vivo effects were more pronounced than those of estradiol-17beta (Mosconi et al., 2002). It has also been demonstrated that some of the phthalates, which are estrogenic in vitro but not in vivo, cause malformations in male rats that appear to result from antagonism of androgens in utero (Gray, 1998). In addition, although the precise mechanism of sclerosing peritonitis, caused by organic compounds (i.e., plasticizers) from plastic tubing and dialysis bags, is unknown these accessories have been suggested to be a cause of the syndrome. Accordingly, the effects of some phthalate acid esters on water and sodium transport in vitro have been studied on toad bladder and it has been found that these compounds significantly inhibit vasopressin-stimulated water flow, while basal water flow was not affected; also sodium transport was decreased to an equivalent degree by all compounds (Sabatini et al., 1989).

Ongoing investigations of the occurrence and distribution of phthalate esters on various organ tissues of this and other species of amphibians, fishes, birds, and mammals will assess the real impact of these widespread compounds in animal tissues.

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