

First evidence of endocrine disruption in feral carp from the Ebro River

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Abstract

Feral carps (*Cyprinus carpio*) were collected in spring 2001 from five sites along the lower course of Ebro River (Spain) with the aim of investigating the existence of endocrine-disrupting effects. Several findings (low gonadosomatic index (GSI), plasmatic vitellogenin (VTG), depressed levels of testosterone, and histological alterations in gonads) detected in male carps downstream of Zaragoza's sewage treatment plant (STP) strongly suggest that the concentration of sewage effluent in the area is a major causal factor leading to the detected estrogenic effects. Important alterations (viz. delayed maturation in females, indications of arrested spermatogenesis in males) were detected in carps from Flix, a heavily industrialized area. Low ovarian P-450 aromatase and reduced glucuronidation of testosterone and estradiol in males were observed in Zaragoza and Canal Imperial de Aragón—an agricultural area—which suggest decreased estrogen synthesis, and possibly, reduced sex hormone excretion in those organisms. These results were related to some *in vitro* assays aimed to assess the interference of model compounds (atrazin, vinclozolin, diuron, pp'-DDE, dicofol, triphenyltin, nonylphenol, and fenarimol) with the glucuronidation of testosterone and estradiol by liver microsomal fractions. The fungicide fenarimol (10–20 μM) and nonylphenol (50 μM) were found to significantly inhibit (20%) both activities at relatively low doses. Overall, this work provides the first evidence of the existence of significant alterations of the endocrine system of carps from the medium-low course of the Ebro River and demonstrates the ability of several chemicals to modulate the inactivation of endogenous steroids.

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Introduction

Over the last decade, there has been great interest in the scientific community regarding the possible alterations in the functioning of the endocrine system of humans and wildlife as a result of chemical exposure. Recent concern about a possible decline in sperm counts and increased incidence of male genital abnormalities in humans (Swan et al., 2000), alligators with abnormal male genitalia (Guillette et al., 1995), or male fish with female characteristics (Purdom et al., 1994) has emphasized the fact that many chemicals can act as endocrine disruptors (EDs) by interfering in some way with normal endocrine function. Among them are natural and synthetic hormones, plant constituents, pesticides, compounds used in the plastic industry and in consumer products, as well as industrial by-products and

effluents. The aquatic environment is the final sink for most of those chemicals, and fish, as inhabitants of aquatic systems, will inevitably be exposed. Thus, fish have been widely used as models for determining the effect of environmental pollutants on vertebrate reproduction function.

Most evidence of endocrine disruption in wildlife has come from fish living in or nearby highly contaminated effluents (paper mill effluents, sewage treatment plants (STP)) where widespread endocrine disruption and intersexuality have been reported (Crain et al., 1997; Couillard et al., 1999; Hashimoto et al., 2000; Jobling et al., 1998; Noaksson et al., 2001). These studies usually examine molecular, biochemical, and pathological responses in an attempt to associate ED exposure to the different endpoints measured, which often involve some degree of speculation. The complexity of the endocrine system, the fact that hormones and EDs may exert their effects during critical periods of development, but these effects may not be manifested until much later in the individual's lifetime, and the complex mechanisms through which EDs may exert their effects are just some of the factors that complicate the understanding of the endocrine disruption phenomena and

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the establishing of cause–effect relationships (for details, see Lister and Van Der Kraak, 2001).

Nonetheless, and despite of these limitations, an increasing number of studies have reported evidences of endocrine disruption in wild fish from UK rivers (Harries et al., 1997; Lye et al., 1997; Nolan et al., 2001; van Aerle et al., 2001), wild bream (*Abramis abramis*) from the Elbe River (Hecker et al., 2002), perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*) from a remote Swedish lake (Noaksson et al., 2001) and barb (*Barbus plebejus*) from the Po River (Viganò et al., 2001), among others. In contrast, information on the status of Spanish rivers is rather limited. The only studies available focus on two overexploited Mediterranean streams with low natural flows ($0.68\text{--}6.5\text{ m}^3\text{ s}^{-1}$), receiving extensive urban and industrial wastewater discharges with very poor dilution factor. The presence of endocrine disrupters in those waters and their effect on male carps in terms of vitellogenin (VTG) induction have been shown (Petrovic et al., 2002; Solé et al., 2000).

Thus, this study was designed to investigate potential alterations on the endocrine system of wild fish from the Ebro River, the flowest river in the Iberian Peninsula ($255\text{--}425\text{ m}^3\text{ s}^{-1}$). It receives a series of complex discharges of different origin (domestic, industrial, agricultural) that make up a relatively small proportion of its flow. Carps (*Cyprinus carpio*) were selected as sentinel species because their widespread distribution along the study area (300 km) would enable comparison between sites. Plasmatic vitellogenin was determined as a biomarker of exposure to xenoestrogens (Denslow, 1999). Total testosterone and estradiol levels were measured in fish plasma to assess potential hormonal alterations. Histologic analysis of gonadal tissue was performed to determine the reproductive development status of individual fish and potential alterations. Additionally, the steroidogenic enzyme P-450 aromatase, and testosterone and estradiol UDP-glucuronosyl transferases were measured in ovarian and liver microsomal fractions, respectively, as key enzymatic activities involved in steroid hormone synthesis and clearance. Several classes of relatively persistent pesticides such as organotin compounds, DDTs, and several imidazole-like fungicides have been shown to interfere with the activity of P450 aromatase, which catalyzes the final, rate-limiting step in the conversion of androgens to estrogens (Sanderson et al., 2002; Vinggaard et al., 2000). Inhibition of aromatase activity in mosquitofish living downstream a paper mill (Orlando et al., 2002) and carps exposed to high levels of alkylphenols (Martín et al., in press) has been reported. In contrast, no equivalent information is available for the enzymatic systems involved in hormone conjugation. Alterations of those enzymes could affect endogenous levels of steroid hormones and produce a cascade of undesirable effects for the organism (Archand-Hoy and Benson, 1998). Consequently, we determined the in vitro effects of several model compounds on the activity of testosterone and estradiol UDP-glucuronosyl transferases by incubating carp liver

microsomes with and without atrazin, vinclozolin, diuron, pp'-DDE, dicofol, triphenyltin, nonylphenol, and fenarimol. The compounds were selected because of their ability to interfere with steroid hormone function and their production or use along the Ebro River basin.

Materials and methods

Chemicals and biochemicals. Vitellogenin and rabbit anti-carp vitellogenin polyclonal antibody CT-1 were purchased from Biosense AS, Norway. Anti-rabbit IgG was purchased from Sigma. Testosterone and estradiol ^{125}I -RIA kits were from ICN Pharmaceuticals, Inc. $[4\text{-}^{14}\text{C}]$ -Testosterone and $[6,7\text{-}^3\text{H}]$ -estradiol were purchased from Amersham Pharmacia Biotech, UK. $[1\beta\text{-}^3\text{H}]$ -Androst-4-ene-3,17-dione was purchased from Perkin-Elmer Life Sciences Inc., Boston, MA.

Sample collection. Male and female carps (*C. carpio*) were collected by DC electrofishing in April to May 2001 from five locations along the Ebro River basin. The sampling stations were selected according to different environmental characteristics, namely, (C) the Canal Imperial de Aragón, an artificial irrigation channel that supplies water to the city of Zaragoza, surrounded by an agricultural area; (Z) a station located downstream of the Zaragoza sewage treatment plant; (F) a heavily industrialized area downstream the city of Flix; (L) a station in a relatively clean area, 20 km downstream the city of Lérida; (D) the irrigation channels of the Ebro Delta Natural Park, an agricultural area (Fig. 1). An effort was made to collect fish of similar size and similar reproductive stage.

Immediately after collection, 1–2 ml of blood was taken from the caudal vein using an heparinized syringe, placed into tubes containing aprotinin (2 TIU ml^{-1}), and centrifuged at $1000 \times g$ for 5 min. The plasma was then withdrawn and frozen in dry ice for transport and stored at $-80\text{ }^\circ\text{C}$ until hormones and vitellogenin (VTG) analysis. After blood extraction, animals were killed, total length and weight were recorded, and the liver immediately dissected, frozen in liquid nitrogen, and stored at $-80\text{ }^\circ\text{C}$. Gonads were weighted, frozen, and a subsample from the central part fixed with Bouin's solution for histological examination.

Vitellogenin analysis. Plasma vitellogenin was determined by Western blot as described by Solé et al. (2000). Briefly, plasma was boiled for 5 min in SDS-PAGE sample buffer and loaded into 7.5% polyacrylamide gels. Pure VTG (Biosense) was used as standard positive control. Proteins were separated in SDS-polyacrylamide gels and transferred to nitrocellulose membranes. They were probed using a 1:5000 dilution of rabbit anti-carp vitellogenin polyclonal Ab CT-1. The membranes were incubated for 1 h with

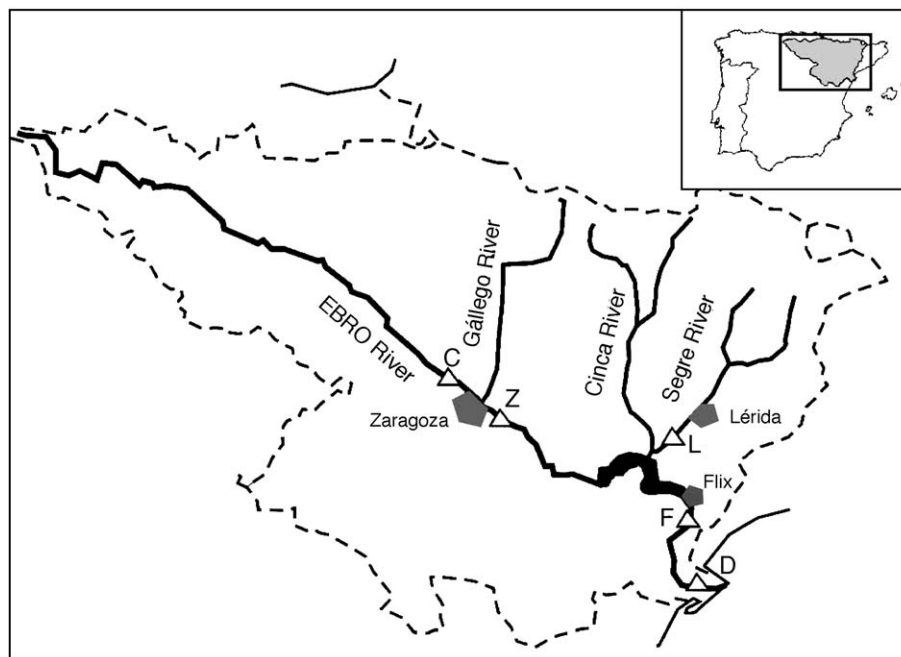


Fig. 1. Map of the sampling sites.

alkaline phosphatase-conjugated anti-rabbit IgG. Semi-quantification was carried out in a densitometer PhosphoImager (BioRad) using a Quantity One package program from BioRad and results expressed as mg ml^{-1} plasma.

Plasma steroid analysis. Total testosterone (T) and 17β -estradiol (E2) levels in plasma were assayed by radioimmunoassay using a ^{125}I RIA Kit. Individual plasma samples ($200\ \mu\text{l}$) were mixed with distilled water containing 0.05% NaN_3 at a 1:2 ratio and heated at $80\ ^\circ\text{C}$ for 1 h to liberate protein-bound steroids. Afterwards they were centrifuged at $11000 \times g$ for 30 min and the supernatant was stored at $-20\ ^\circ\text{C}$ until hormonal analysis. Diluted plasma was assayed in duplicate for T and E2 residues. The detection limits were $200\ \text{pg T ml}^{-1}$ and $10\ \text{pg E2 ml}^{-1}$. Intra-assay coefficients of variation were of 9.5% (T) and 5.5% (E2). Interassay coefficients of variation were 11.6% (T) and 7.6% (E2).

Histological analyses. Samples were fixed in Bouin's solution for up to 48 h. After dehydration and embedding in paraffin wax, gonad tissue was sectioned ($7\ \mu\text{m}$) and stained with Hematoxylin-eosin, mounted, and examined by light microscopy. Four to six tissue slides per fish were examined. The sexual maturity stages (SMS) were calculated as described by Goodbred et al. (1996). Namely, those ovaries showing a mixture of both perinucleolar and cortical alveoli oocytes were classified as stage 1, ovaries with vitellogenic oocytes of various sizes and development were classified as stage 2, and those in which most oocytes were at or near maximum size and contained numerous densely

packed vitelline granules as stage 3. For males, testes with thick germinal epithelium and diffuse pronounced proliferation and maturation of spermatozoa were classified as stage 1, and testes that mostly had a thin germinal epithelium with only scattered spermatogenic activity as stage 3.

Preparation of microsomal fractions. After weighing, gonads and livers were flushed with ice-cold 1.15% KCl and homogenized in 1:5 w/v of cold $100\ \text{mM KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer pH 7.4, containing $100\ \text{mM KCl}$, $1\ \text{mM EDTA}$, $1\ \text{mM dithiothreitol (DTT)}$, $0.1\ \text{mM phenanthroline}$, and $0.1\ \text{mg ml}^{-1}$ trypsin inhibitor. Homogenates were centrifuged at $1500 \times g$ for 15 min, the fatty layer removed, and the obtained supernatant centrifuged at $12000 \times g$ for 20 min. The 12000-g supernatant was further centrifuged at $100000 \times g$ for 60 min to obtain microsomal fractions. Ovarian microsomal pellets were washed in homogenization buffer and centrifuged again at $100000 \times g$ for 30 min. Microsomal pellets were resuspended in a small volume of $100\ \text{mM KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer pH 7.4, containing $1\ \text{mM EDTA}$, $20\% \text{ w/v glycerol}$, $1\ \text{mM DTT}$, $0.1\ \text{mM phenanthroline}$, and $0.1\ \text{mg ml}^{-1}$ trypsin inhibitor. Proteins were measured by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Ovarian aromatase activity. Aromatase activity was determined by the tritiated-water release method as described in Morcillo et al. (1999). About $0.4\ \text{mg}$ of microsomal protein was incubated at $25\ ^\circ\text{C}$ for 1 h in the presence of $100\ \text{mM Tris-HCl}$, pH 7.6, $10\ \mu\text{M}$ unlabelled androstenedione, $0.2\ \text{mM NADPH}$, and $[1\beta\text{-}^3\text{H}]\text{-androstenedione}$ (40

Table 1
Morphometric data of male and female common carp from the collection sites

	Sex	Station				
		C	Z	L	F	D
Total length (cm)	M	34.3 ± 2.5 ^{ab}	39.8 ± 0.8 ^a	37.8 ± 1.1 ^a	41.9 ± 3.2 ^a	27.2 ± 5 ^b
	F	34 ± 0.5 ^{ab}	42 ± 0.9 ^a	41.2 ± 1.5 ^a	39.7 ± 0.4 ^{ab}	31.3 ± 3.2 ^b
Body weight (g)	M	685.7 ± 93.7 ^{ab}	1195.8 ± 88.6 ^{ab}	1128.6 ± 95.8 ^{ab}	1408.3 ± 386.4 ^a	493 ± 314.4 ^b
	F	791.7 ± 30.05 ^{ab}	1579.2 ± 129.8 ^a	1462.5 ± 180.1 ^{ab}	1166.7 ± 36.3 ^{ab}	725 ± 425 ^b
CF	M	1.69 ± 0.12 ^a	1.88 ± 0.06 ^{ab}	2.07 ± 0.08 ^b	1.74 ± 0.05 ^a	1.63 ± 0.03 ^a
	F	2.01 ± 0.02	2.11 ± 0.1	2.05 ± 0.03	1.88 ± 0.12	2.13 ± 0.29
GSI	M	2.5 ± 0.7 ^a	3.9 ± 0.7 ^a	7.0 ± 0.6 ^b	3.7 ± 0.5 ^a	2.4 ± 2.1 ^a
	F	19.1 ± 3.5	15.7 ± 2.8	18.3 ± 1.7	9.7 ± 0.9	10.3 ± 8.5
SMS	M	3	3	3	3	1,3
	F	3	3	3	2	1,3

Values are mean ± SEM. CF: condition factor calculated as (weight / length³) × 100. GSI: gonadosomatic index calculated as (gonad weight / total weight) 100. Lowercase letters for statistical comparison, distinct letters indicate significant differences ($P < 0.05$).

M: males; F: females; SMS: sexual maturity stage.

pmol, 1 µCi). Assay blanks containing 100 µl of buffer instead of microsomes were used for every run. The reaction was stopped by placing the tube on ice, and organic metabolites and the excess of substrate were immediately eliminated from the aqueous phase by extraction with methylene chloride (3 × 3 ml). The possible remaining tritiated steroids were further eliminated by the addition of a suspension of 2.5% (w/v) activated charcoal and 0.25% dextran in milli-Q water (4 ml). The solution was centrifuged (60 min × 3600 rpm), and two aliquots of the supernatant (1 ml) were counted for ³H radioactivity. The lowest aromatase activity detected by the method was 60 fmol h⁻¹ mg⁻¹ protein.

UDP-glucuronosyltransferase. Testosterone and estradiol UDPGT activity was assayed in liver microsomal fractions as follows. Microsomal protein (250 µg) was incubated at 30 °C with 40 nmol [¹⁴C]testosterone or [³H]estradiol in 50 mM Tris-HCl buffer, 10 mM MgCl₂, pH 7.4. The reaction was initiated by addition of 3 mM uridine 5'-diphosphoglucuronic acid (Sigma) and terminated after 30 min of incubation by adding 2 ml ethyl-acetate. Unmetabolized steroids were extracted with 2 × 2 ml of ethyl-acetate and an aliquot (50 µl) of the remaining aqueous phase containing glucuronides was quantified by liquid scintillation counting. Blanks that consisted of all constituents except microsomes were run in every assay. Specific activity was calculated as pmol of conjugate min⁻¹ mg⁻¹ of microsomal protein.

To determine the effect of chemicals on testosterone and estradiol UDPGT activities, chemicals conveniently diluted in ethanol or methanol were added to the assay buffer and preincubated with the microsomal fraction at 30 °C for 20 min. Maximal solvent concentration in the assay was less than 2% and had no effect on UDPGT activities. Control reactions were carried out by incubation of the microsomal fraction in the presence of the carrier solvent alone. Animals from the L site were used to provide microsomes for the assay.

Statistical analyses. To keep the statistics uniform, non-parametric tests were employed due to sometimes unequal variances and variations in sample size between groups for levels of vitellogenin, testosterone, 17β-estradiol. Male and female fish were analyzed separately by the Kruskal–Wallis test and the major differences thus observed were examined further using the two-tailed nonparametric multiple comparison test of Dunn. Samples containing levels below the detection limits were considered as having 50% of the minimal values detectable. For the rest of measurements, male and female fish were analyzed separately and compared with one-way ANOVA. If an overall significance was detected, Bonferroni's tests were performed to determine differences among collection sites. Student's *t* test was used to compare two group datasets of in vitro studies.

Results

Body and organ parameters

The main characteristics of fish are given in Table 1. Individuals were adults and homogeneous (size and weight), except those from station D, where both small (150–275 g) and large (1200–1700 g) organisms were collected. The condition factor (CF) was rather similar in all organisms, except in males from station L which was higher ($P < 0.05$).

Table 2
Histological alterations in male common carp from the collection sites

Station	Macrophage and cellular aggregates	Presence of oocytes	Immature cells into the lumen	Severe destructureation
C	2/7	0/7	0/7	2/7
Z	3/6	1/6	2/6	2/6
L	0/7	0/7	0/7	0/7
F	3/6	0/6	1/6	1/6
D	0/5	0/5	0/5	0/5

Data represented as observed over studied individuals.

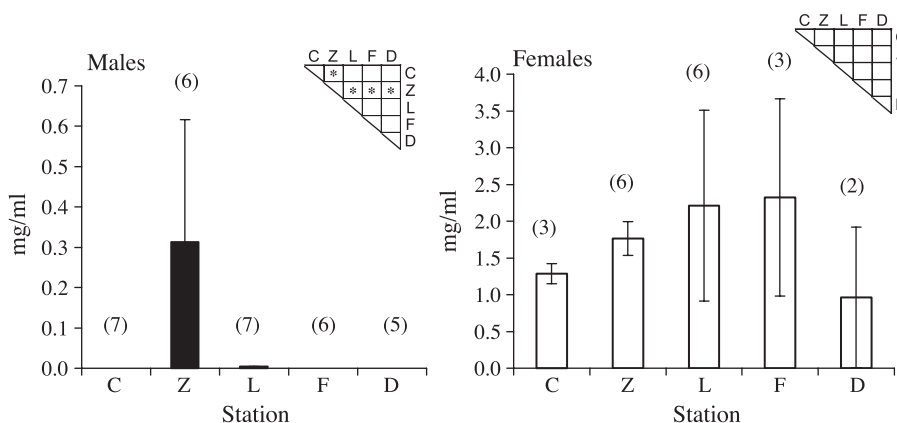


Fig. 2. Plasma vitellogenin levels in male and female carp along the Ebro River. Values are mean \pm SEM. In brackets: number of fish specimens analyzed. * denotes significant differences ($P < 0.05$) between sampling sites according to Dunn's test.

Lower CF was recorded in males (1.6–1.9) than in females (1.9–2.1).

Males from station L had the highest gonadosomatic index (GSI) ($P < 0.05$), about 2-fold higher than males of similar size from stations Z and F, and 3-fold higher than those from station C. In station D, GSI varied from below 0.5 in small specimens to 10.9 in a large one (1750 g), whose gonad was classified as SMS-3. Similarly, females from different stations exhibited different GSI according to

their different SMS. Those from station F, a heavily industrialized area, were classified as SMS-2 and had significantly low GSI. Females from station D were classified as SMS-1 and SMS-3; and their GSI varied accordingly (1.8 for SMS-1 and 18.7 for SMS-3). When only those females classified as SMS-3 were compared, the GSI was in the range of 15.7–19.1, the lowest values detected in station Z, under the influence of Zaragoza's sewage treatment plant (STP) (Table 1).

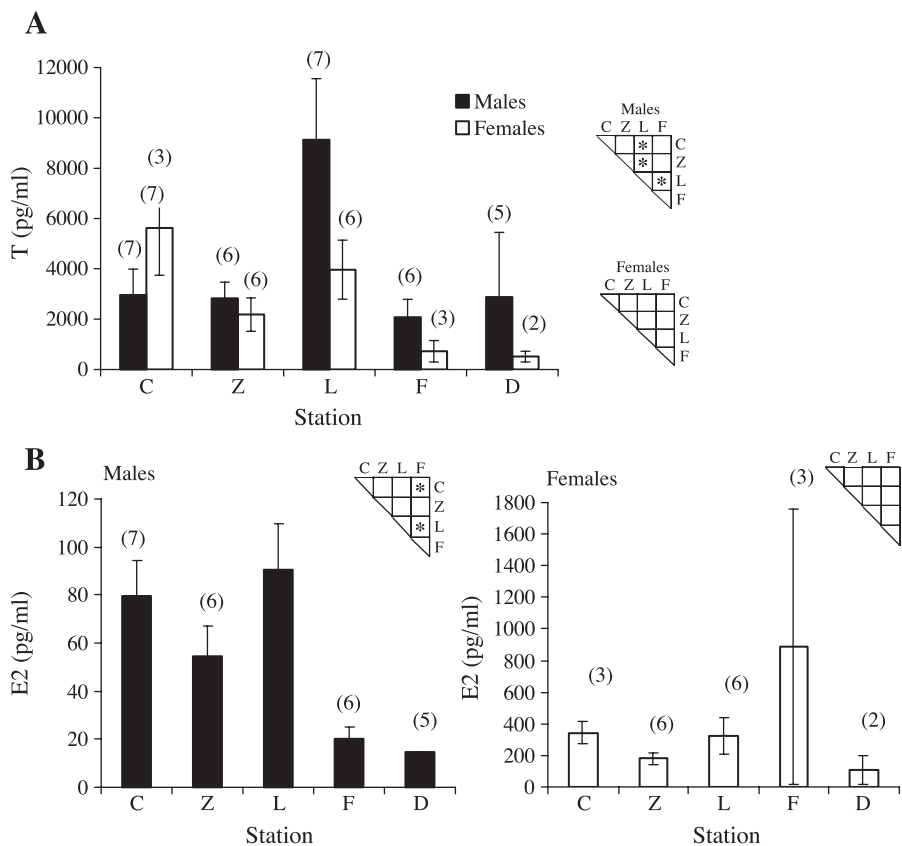


Fig. 3. Testosterone (A) and 17 β -estradiol (B) levels in plasma of male and female carp. Values are mean \pm SEM. In brackets: number of fish specimens analyzed. * denotes significant differences ($P < 0.05$) between sampling sites according to Dunn's test.

Histological observations

Some alterations were recorded in male gonads, particularly in those from stations Z (downstream of Zaragoza's sewage treatment plant) and F (highly industrialized area). Vitellogenic oocytes were observed in one out of six males from station Z. Severe destructuration of the testicular tissue was recorded in two out of six males from this location, viz. testes had the seminiferous lobules filled with high density of macrophage aggregates and granulomas. Macrophage aggregates were also observed in several individuals from station C and F (Table 2). Necrotic areas, fibrosis, and signs of vacuolation were recorded in two out of six males from station Z and two out of seven males from station C. Immature cells into the lumen occurred in two males from station Z and one male from station F. No alterations were recorded in males from stations L and D (Table 2).

All the studied ovaries had fully developed oocytes and were classified as late vitellogenic (SMS-3), with the exception of females from station F that exhibited a delay in maturation and were staged as early vitellogenic (SMS-2). Only two female carps had non-vitellogenic oocytes in their ovaries (SMS-1). One of these females was from station Z, an adult female (total length: 40.5 cm; body weight: 1225 g) with a 4.5-fold lower GSI than other females from the same location. The other pre-vitellogenic female was a small carp (total length: 22.5 cm; body weight: 275 g) from station D. No further histopathological alterations were detected in female gonads.

Plasmatic vitellogenin

Plasmatic vitellogenin was only recorded in males from stations Z and L (Fig. 2A). The highest VTG induction occurred at station Z, downstream of Zaragoza's sewage treatment plant (works serving population equivalents of 460000), where four out of six male carps had measurable levels of vitellogenin in plasma, one of them in the higher range of levels detected in females (1.8 mg ml⁻¹). This specimen had a very low GSI (0.95 against 3.9–4.1 of the rest of males) and severe alterations of the testicular tissue, which exhibited necrotic areas, fibrosis, signs of vacuolation, and destructuration. There were significant differences in circulating VTG levels between males from station Z and those from stations C, L, F, and D. VTG was also determined in females from all five stations, and no significant differences between sites were recorded (Fig. 2B).

Plasma sex steroid concentrations

Plasma-circulating hormones were determined in both males and females from all five sites. In the common carp, 11-ketotestosterone (11-KT) was reported to be the main androgen circulating in plasma (Yaron, 1995), but as levels of 11-KT have been shown to be closely related with plasma

testosterone concentrations (Goodbred et al., 1996), and methods for measuring testosterone are more readily available than those for 11-KT, this study focused on testosterone. Testosterone concentration in plasma was rather similar in males from stations C, Z, and F (2068–2960 pg ml⁻¹), about 5-fold lower than those from station L (9133 pg ml⁻¹). Because carps from station D were in a different SMS, data from this station were not included in the statistical analysis (Fig. 3). For females, the highest levels of circulating testosterone were detected in station C (5600 pg ml⁻¹), and the lowest in station F (728 pg ml⁻¹); however, no significant differences were detected among sampling sites.

Estradiol levels were comparatively higher in females (107–884 pg ml⁻¹) than in males (15–90 pg ml⁻¹). The lowest concentration of estradiol was detected in males from station F, about 4-fold lower than those from stations C and

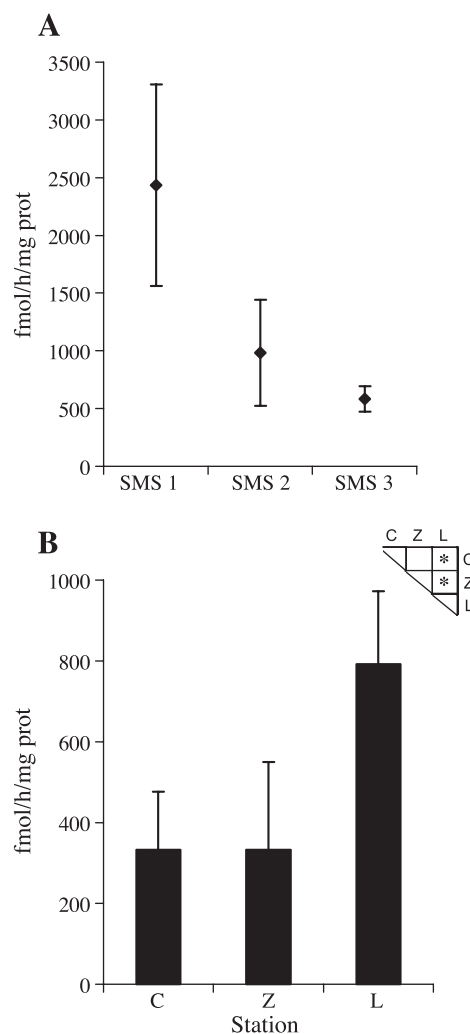


Fig. 4. (A) P450 aromatase activity in carp ovarian in function of their sexual maturity stage. (B) Spatial variation of P450 aromatase—only those females classified as SMS-3 were considered. Values are mean \pm SEM ($n = 6-7$). * denotes significant differences ($P < 0.05$) between sampling sites according to Bonferroni's test.

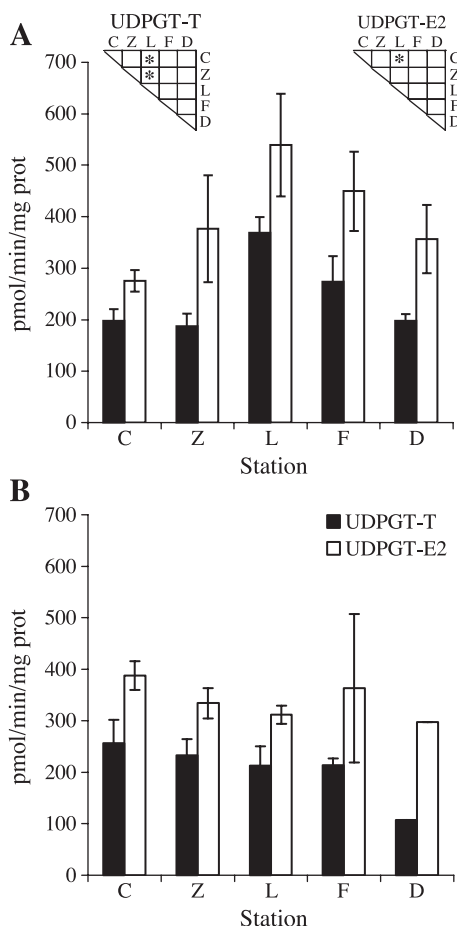


Fig. 5. UDPGT activity towards testosterone and estradiol in (A) male and (B) female common carp. Values are mean \pm SEM ($n = 6-7$). * denotes significant differences ($P < 0.05$) between sampling sites according to Bonferroni's test.

L ($P < 0.05$) (Fig. 3B). For females, no significant differences among sites were recorded due to the relatively low number of samples analyzed in some stations, and the high variability detected in station F.

Ovarian P450 aromatase

P450 aromatase converts androstenedione or testosterone to estrone or 17β -estradiol, respectively. This activity was detected in all female common carp and was tightly related to the SMS (Fig. 4), viz. those females classified as SMS-1 showed the highest P450 aromatase activity (2435 ± 872 fmol h^{-1} mg protein $^{-1}$), up to 4-fold higher than females classified as SMS-3 (583 ± 110 fmol h^{-1} mg protein $^{-1}$). P450 aromatase activities of females from stations C, Z, and L (classified as SMS-3) were further compared, and significant differences were recorded (Fig. 4), viz. females from station L showed up to 2-fold higher P450 aromatase activities (792 fmol h^{-1} mg protein $^{-1}$) than those from stations C and Z (333 fmol h^{-1} mg protein $^{-1}$).

UDP-glucuronyltransferase

Rates of glucuronidation of estradiol by liver microsomal fractions were higher than those of glucuronidation of testosterone, in both males and females (Fig. 5). Males allowed the detection of significant differences among sampling sites; the highest testosterone-UDPGT activity was observed in station L (368.4 ± 30.8 pmol min^{-1} mg protein $^{-1}$), up to 2-fold higher than males from stations C and Z ($P < 0.05$). Estradiol-UDPGT activity was also elevated in males from station L, and significant differences with station C were recorded (Fig. 5A). In contrast, no significant differences among sampling sites were recorded for females (Fig. 5B). Generally, males and females had similar glucuronidation rates of testosterone or estradiol, apart from station L, where males exhibited higher glucuronidation rates (testosterone and estradiol) than females, and station C, where females had higher estradiol-UDPGT than males. No relationship between UDPGT activities and SMS of carps was recorded.

Fig. 6 shows the in vitro effects of model chemicals on testosterone and estradiol-UDPGT activities. Product formation in the absence of xenobiotic represented 100% enzyme

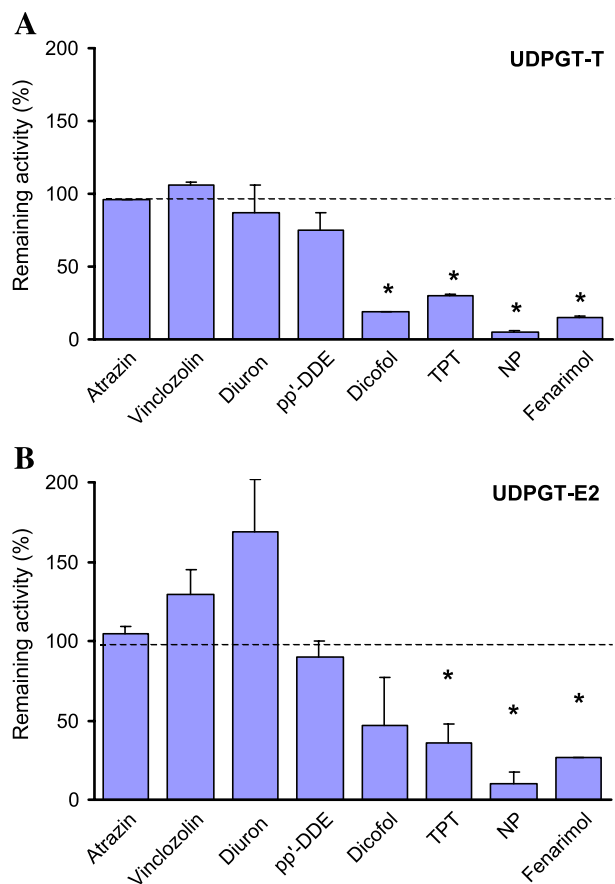


Fig. 6. Screening of selected pesticides for effects on (A) UDPGT-testosterone and (B) UDPGT-estradiol in carp liver microsomes. All compounds were tested at 1 mM. Data are presented as mean \pm SD of two determinations. * denotes significant differences ($P < 0.05$) referred to control according to Student's t test.

activity. Triphenyltin (TPT), nonylphenol (NP), and fenarimol had a strong inhibitory effect on the glucuronidation of both testosterone and estradiol. The incubation of liver microsomal fractions in the presence of those compounds at a concentration of 1 mM leads to a significant inhibition of estradiol glucuronidation, namely 64% (TPT), 74% (fenarimol), and 90% (NP). IC_{50} values were estimated at $692 \pm 134 \mu\text{M}$ for TPT, $92 \pm 62 \mu\text{M}$ for fenarimol, and $223 \pm 25 \mu\text{M}$ for NP. These compounds were even stronger inhibitors of testosterone glucuronidation, leading to 70% (TPT), 85% (fenarimol), and 95% (NP) inhibition of testosterone-UDPGT activity. IC_{50} values were estimated at 623 ± 192 , 139 ± 8 , and $71 \pm 20 \mu\text{M}$ for TPT, fenarimol, and NP, respectively. Interestingly, 1 mM dicofol significantly inhibited testosterone glucuronidation by 81% (IC_{50} value = $293 \pm 11 \mu\text{M}$) with no significant effect on estradiol-UDPGT activity. Atrazin, vinclozolin, and pp'-DDE did not have any significant effect on UDPGT activities. Diuron enhanced estradiol glucuronidation by 69%, albeit not significantly.

Discussion

The data presented show that carps living in the Ebro River have been subjected to endocrine modulation, probably because of exposure to a variety of domestic, agricultural, and industrial effluents. Differences in the endpoints measured as a consequence of water temperature—a major environmental factor steering maturation in fish—can be excluded because there were no marked regional differences in this parameter along the study area. Nonetheless, results from station D (Ebro Delta) are not directly comparable with the other stations, as carps were certainly affected by the particular hydrology of the sampling site. Carps were sampled in the irrigation channels, where they face subtle changes of water flow; viz. during the drought periods, only small fish survive and many animals (>30 cm) are caught and taken several kilometers upstream. Thus, the size of the analyzed organisms was not homogenous.

Focussing on VTG results, the detection of VTG circulating in plasma in four out of six males collected downstream Zaragoza's sewage treatment plant strongly suggests the presence of estrogenic compounds in the sewage discharge. Serum VTG was elevated without a coincident significant increase in the circulating concentration of the endogenous estrogen estradiol (Fig. 3), which further supports the fact that exposure to xenoestrogens may be responsible of the increased synthesis of VTG. Ethynylestradiol (EE₂) and alkylphenol polyethoxylates (APEs) are among the most likely estrogenic substances in domestic sewage (Purdom et al., 1994); these chemicals have been shown to bind to the estrogen receptor and induce the synthesis of VTG in fish (Jobling and Sumpter, 1993). High concentrations of nonylphenol, octylphenol, and nonylphenol mono-ethoxylates have been recorded in the bile of carps collected downstream Zaragoza STP (station Z) indi-

cating recent exposure to alkylphenols (Martín et al., in press).

Additionally, males from station Z had significantly low GSI (3.9 against 7.0 of males from station L) (Table 1) together with histopathological alterations of the gonads (fibrosis, ovotestis, immature cells in tubular lumen), which further support the hypothesis that males are exposed to complex mixtures of estrogenic compounds. Many studies have reported a significant correlation between exposure to estrogenic compounds in fish and inhibitory effects on testicular growth or altered testis structure (Jobling et al., 1996; Kinnberg et al., 2000a, 2000b; Komen et al., 1989). Also, fibrosis, atrophy of the germinal epithelium, and disappearance of spermatogenic cysts have been reported in sexually mature male carp exposed to 4-tert-pentylphenol (TPP) and estradiol (E2) (Gimeno et al., 1998).

A low GSI is not necessarily an indicator of xenoestrogen exposure because decreases have been reported in response to non-estrogenic contaminants (Sakamoto et al., 2003), but it is very often linked to pathological alteration of the gonads (Kinnberg et al., 2000b). In the present study, male fish from Flix (station F), a heavily industrialized area, exhibited a low GSI together with the presence of immature cells (spermatogonia) into the lumen, an indication of arrested spermatogenesis (Gimeno et al., 1998), and many macrophage aggregates (Table 2). This link between low GSI and pathological alterations was also evident in two males from station C that had the lowest GSI (1.8–2.5) and a high incidence of fibrosis, macrophage aggregates, vacuolation, and atrophy of the germinal epithelium.

The GSI of females was very similar in all stations (18.3–19.1) apart from stations Z (15.7) and F (9.7). The lowest GSI was detected in females from Flix that also exhibited delayed maturation (Table 1). Delayed development and spawning have been reported in female zebrafish (*Danio rerio*) exposed to ethynylestradiol (EE₂) and bisphenol A (BPA) (Segner et al., 2003); as well as in wild populations of flounder from a polluted estuary (Gill et al., 2002). Carps from Flix (station F) were exposed not only to similar levels of alkylphenols than those from Zaragoza (station Z) (Martín et al., in press), but also to high concentrations of organochlorinated compounds, viz. PCBs (95 ng g^{-1} w.w. in muscle), DDTs (29 ng g^{-1} w.w.), HCB (1.6 ng g^{-1} w.w.) (Lavado et al., unpublished results). Altogether, the mixture of industrial contaminants released to the aquatic environment in this station may lead to significant alterations of the endocrine system of fish, namely arrested gametogenesis in males and delayed maturation in females.

The above-mentioned alterations were further supported by data on sex steroids. Thus, male carps from stations C and Z had depressed levels of testosterone circulating in plasma, and those from station F had depleted levels of both testosterone and estradiol. Besides, males from all three stations (Z, F, and C) had higher E2/T ratio (0.023–0.049) than those from station L (0.011). The ratio 17 β -estradiol/

testosterone (E2/T) has often been used as an indicator of endocrine disruption because a specific estrogen to androgen ratio is necessary for sexual differentiation in developing animals and that alterations of the ratio can result in incomplete or improper gonadal development (Folmar et al., 1996). Results from this study agree with data of Folmar et al. (1996), who reported increased E2/T ratio in male carps from the more polluted areas or an increase in the estrogen/androgen ratio in male and female walleye (*Stizostedion vitreum*) collected near a STP (Folmar et al., 2001). For females, no significant differences among sampling sites were detected in terms of steroid levels circulating in plasma due to the relatively low number of samples analyzed in some stations, and the high variability observed in site F (Fig. 3B). Those females, highly exposed to industrial wastes, had a rather low and homogenous GSI, and exhibited a delay in maturation (Table 1).

However, the measurement of ovarian aromatase indicated lower activities in females from stations Z and C in comparison with those from station L. This means decreased estrogen synthesis and possibly an androgenization phenomena. Females from station Z would probably respond to high exposure to estrogen-like compounds (e.g., estradiol, ethinylestradiol, alkylphenols) by reducing estrogen synthesis in a manner that they could maintain an internal hormonal environment. One mechanism by which this could be achieved would be via alterations in aromatase activity of the gonad. Field studies are scarce, but an inhibition of aromatase activity in brook trout (*Salvelinus fontinalis*) exposed to leachate from a public refuse dump (Noaksson et al., 2002) and in carps exposed to high levels of nonylphenol (Martín et al., in press) has been reported. On the other hand, imidazole-like fungicides, among them imazalil and fenarimol, are known to strongly inhibit P450 aromatase activity in humans and rats (Hirsch et al., 1987; Sanderson et al., 2002; Vinggaard et al., 2000). Indeed, considering the wide application of these and other pesticides along the agricultural area that crosses the Canal Imperial de Aragón, and that usually less than 5% of the pesticide applied reaches the intended target, we cannot discard the hypothesis that these compounds end up in the water in a sufficient amount to alter the endocrine system of carps from station C.

Finally, xenobiotic disruption of metabolic clearance pathways of hormones was investigated by measuring the glucuronidation of testosterone and estradiol by carp liver microsomal fractions. UDPGTs are a family of isoenzymes encoded by multigenes, which play a key role in excretion of both endogenous and xenobiotic compounds (George, 1994). Thus, the hypothesis exists that induction or inhibition of UDPGTs may alter steroid metabolism, possibly with profound effects on the reproductive success of an organism (Archand-Hoy and Benson, 1998). Our in vitro studies evidenced that fenarimol, nonylphenol, and TPT inhibited the glucuronidation of estradiol at a concentration as low as 20, 50, and 100 μM , respectively. The glucuronidation of

testosterone was also significantly inhibited at 10 (fenarimol), 50 (nonylphenol and dicofol), and 100 μM (TPT). Other compounds, such as tributyltin, have been shown to selectively inhibit the glucuronidation of testosterone at a dose as low as 5 μM , but not that of estradiol (Morcillo et al., 2004). Sturm et al. (2001) also indicated that 15 μM Igepal (a mixture of nonylphenol mono- and di-ethoxylates) significantly inhibited testosterone glucuronidation in trout hepatocytes. Overall, the data available support the hypothesis that some compounds (pesticides, alkylphenols) may alter the glucuronidation of testosterone and estradiol, and this interference may account for the in vivo observed differences along the study area (Fig. 5). Hence, fish from stations highly exposed to alkylphenols and pesticides (Z and C) had relatively low testosterone and estradiol-UDPGT activities.

Although the formation of glucuronides in fish may provide a means of cessation of hormone action via their excretion and removal (Clarke et al., 1992), we did not observe a direct relationship between UDPGT activities and levels of testosterone or estradiol circulating in plasma. Nonetheless, glucuronidation is also involved in transporting steroid hormones around the body in a receptor-inactive form, and some steroid glucuronides can act as chemical messengers and are the sex pheromones of fish (Clarke et al., 1991). Hence, the sensitive inhibition of testosterone or estradiol glucuronidation by some environmental pollutants could affect those processes.

Overall, this study provides the first evidence of endocrine disruption in carps from the Ebro River and strongly suggests that the concentration of sewage effluent in the area of Zaragoza is a major causal factor leading to the detected 'estrogenic' effects in males (high levels of circulating VTG, ovotestis, depressed levels of testosterone, low GSI). Important alterations (viz. delayed maturation in females and indication of arrested spermatogenesis in males, together with depressed levels of testosterone and estradiol in males, and low GSI) were detected in carps from Flix, a heavy industrialized area. These responses are unlikely to be accounted for solely the presence of alkylphenol wastes in Flix. The chlorine industry and the high levels of mercury, PCBs, and DDTs often reported in the area can contribute to the observed effects (Friedmann et al., 1996). Further research is needed to link the observed effects in carps from the Canal Imperial de Aragón (low GSI and depressed levels of testosterone in males, low ovarian P450 aromatase) with the use of pesticides in the area. Although in vitro studies have indicated the ability of some pesticides (viz. fenarimol) to interfere with P450 aromatase, further work is needed to assess levels of exposure, uptake, and effects on aquatic fauna. Finally, this study demonstrates the ability of several chemicals to modulate the inactivation of endogenous steroids by interfering with the glucuronidation of both testosterone and estradiol. Whether in vitro and in vivo responses to those pesticides are similar, quantitatively comparable, and what are the consequences

for the organism are some of the points that remain to be investigated.

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