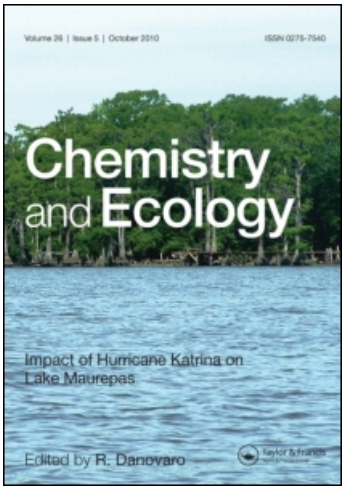


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# VALIDATION OF AN ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) FOR *CYPRINUS CARPIO* L. VITELLOGENIN, AS A BIOMARKER OF REPRODUCTIVE DISORDERS

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*(In final form 15 October 2002)*



Vitellogenin is a female sex-specific protein, and a validated and suitable method for its assay can be applied as a biomarker of reproductive disorders in male and female aquatic animals. Therefore, the present study was designed to validate an ELISA for measuring plasma vitellogenin in wild carp (*Cyprinus carpio* L.) living in the Lake of Trasimeno (Umbria, Italy); plasma samples were taken during pre-spawning, spawning, and post-spawning periods; in addition to vitellogenin, in both male and female carps, plasma changes of estradiol-17$\beta$ were monitored together with those of estrogen receptor density in the liver.

In females, VTG showed high seasonality, reaching the highest levels in March during the pre-spawning period; the VTG levels correlated with those of estradiol-17$\beta$ ($E_2$), and with the changes of gonadosomatic index (GSI), while a non parallel trend was found in the liver estrogen receptor (ER) density. In forty percent of males, VTG was found to be present in the plasma and changes of ER density in the liver were observed.

The data here reported suggest that the common carp can be a useful sentinel species for biomonitoring studies of environmental estrogens, and of their effects on its reproductive biology.

## INTRODUCTION

Recently, Colborn and Clement (1992) demonstrated that some chemical substances found in the environment without direct toxicological risk have the potential to interfere with the endocrine system of animals, including humans. In animals, the endocrine system consists of hormone producing glands that which enter the bloodstream to maintain physiological homeostasis. These molecules, called endocrine disruptors (ED), are mostly of anthropogenic origin, and the main risk is their accumulation in the waters, thus making aquatic vertebrates

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those most endangered. ED can affect reproductive biology by its (anti)estrogenic and (anti)androgenic modes of action resulting in severe effects including abnormal sexual differentiation. In this context, correlations between specific impairment of reproductive activity and elevated concentrations of xenobiotic agents have been found. Reproductive impairment is, in fact, often correlated with altered concentrations of circulating sex steroid hormones or other critical reproductive factors (Mosconi *et al.*, 2001). Suitable models for the study of ED are fish, and many studies have been conducted on a variety of contaminated aquatic ecosystems by assessing peripheral sex steroid changes (Kime, 1998).

The endocrine system is responsible for the regulation of estrogen and androgen levels in both sexes, via negative feed-back mechanisms throughout the hypothalamus-pituitary-gonadal (HPG) axis. Several inputs lead to the secretion of gonadotropin releasing hormone (GnRH), which is produced by cells of the hypothalamus. In its turn, GnRH stimulates the pituitary gonadotrophs to synthesize and secrete luteinizing hormone (LH) and follicle stimulating hormone (FSH) into the blood circulation. Gonadotrophins increase the synthesis and release of estradiol ($E_2$) by the follicular cells of the ovary. $E_2$ is produced by granulosa cells through aromatase activity that triggers the conversion into $E_2$ of the androgen testosterone (T), produced by the thecal cells.

Decreased blood levels of $E_2$ act via negative feed-back on the hypothalamus and pituitary axis by inhibiting the secretion of GnRH and gonadotrophins. Besides sex steroid hormones, vitellogenin (VTG), an estrogen-inducible phosphoglycolipoprotein, can also be used as a biomarker for contaminant exposure in fish (Kime *et al.*, 1999) and in other oviparous species (Kloas *et al.*, 1999). Vitellogenin is normally synthesized (Wallace, 1985) by the liver of female oviparous vertebrates during oogenesis, upon multihormonal control (Carnevali and Mosconi, 1992; Carnevali *et al.*, 1995) and is a precursor of egg yolk. Estrogen reaches target organs including liver, where cytosolic estrogen receptors are present; free $E_2$ penetrates the cell membrane via passive diffusion, entering the cytosol where it binds to the estrogen receptor in order to induce estrogen specific gene expression. The $E_2$-estrogen receptor complex is translocated into the nucleus, where a dimer of $E_2$-estrogen receptor complexes binds to estrogen responsive elements in DNA, and initiates the transcription of $E_2$ specific genes (Beato *et al.*, 1995). $E_2$ specifically elevates the transcription of mRNA VTG (Wahli *et al.*, 1979; Perlman *et al.*, 1984), and induces the transcription of further genes such as the estrogen receptor (Tata, 1987), and the retinol binding protein (McKearin *et al.*, 1987; McKearin and Shapiro, 1988). ED, by behaving as a sex steroid, may impair reproductive process in exposed organisms.

Vitellogenin, as described above, is a female sex-specific protein, and a validated and suitable method for its assay can be applied as a biomarker of reproductive disorders in male and female aquatic animals. Therefore, the present study was designed to validate an ELISA for measuring plasma vitellogenin in wild carp living in the Lake of Trasimeno (Umbria, Italy); sampling was performed during pre-spawning, spawning, and post-spawning periods; moreover, plasma changes of estradiol-17$\beta$ in female carps were monitored, and estrogen receptor density in the liver was measured in both male and female carps.

## MATERIALS AND METHODS

### Animals

The Lake of Trasimeno is of tectonic origin, and is located near Perugia (Umbria, Central Italy). It is the largest lake of the central Italian peninsula in extension (126 $km^2$) and the catchment basin covers an area of 360 $km^2$. This laminar lake is characterized by an average

depth of 4.72 m and a theoretic water return time of 24 years. It is very productive from the trophic point of view and the water temperature is almost the same as that of the air.

In the Lake of Trasimeno, the average length and weight of *Cyprinus carpio* is about 40–50 cm and 2–3 kg, respectively. The carp is an omnivorous bottom-dwelling fish preferring water temperatures between 15 °C and 25 °C. Reproduction occurs between May and July, when the water temperature reaches 20 °C. In general, sexual maturation is reached in 2–3 years for males and one year later for females.

The carps were sampled during the post-spawning (October 1999), pre-spawning (March 2000), and spawning (April 2000) periods. Ten animals of each sex were captured and rapidly anesthetized in a tank containing water plus 100 mg/L of 3-amino benzoic acid ethyl ester (Sigma, St. Louis, MO); blood was immediately collected from the caudal vein with a syringe and put into heparinized tubes containing PMSF (1 mM); tubes were then centrifuged (1500 g for 15 min at 4 °C) and plasma was stored at −20 °C. The animals were weighed, gonads and livers were removed and weighed also; portions of liver were processed for ER evaluation; the gonadosomatic indexes (GSI) were calculated as a ratio of gonadal weight to body weight.

## VTG Purification

Carp vitellogenin was previously purified and characterized, and a specific antibody against VTG was raised in New Zealand rabbits (Carnevali and Belvedere, 1991).



## ELISA

The test was validated using the method of Mosconi *et al.* (1998), and VTG titers were assayed in the carp plasma.

*Antigen Coating* The coating was performed in 96-well microtiter plates (Greiner, medium affinity, SIAL Rome, Italy) in 200 µl of carbonate buffer pH 9.6 containing VTG. Blank values were obtained by coating 8 wells with lyophilized male plasma proteins or bovine serum albumin (BSA, Sigma) at the same concentration as that of the VTG. The plates were then covered and incubated for 16 h at 4 °C. The content of the wells was discarded by inverting the plates, and three successive washes of 30 sec each were applied using 0.01 M phosphate buffer (pH 7.4), 0.15 M NaCl, and 0.05% Tween 20 (PBS-T). The saturation of nonspecific binding sites was achieved by incubating the plates with 2% normal pig serum in PBS-T (PBS-T-NPS). The plates were placed in an oven for 30 min at 37 °C followed by a three-wash cycle with PBS-T.

*Specific Antibody Incubation* In separate tubes, the specific antibodies diluted in PBS-T-NPS were preincubated with serial dilutions (factor 2) of samples (diluted at least 1:100) or of VTG reference preparation for 16 h at 4 °C. The content of these tubes was then distributed in duplicate (200 µl/well) and the plates were incubated for 3 h at 37 °C, followed by a three-wash cycle with PBS-T.

*Second Antibody Incubation* Each well received 200 µl of goat IgG anti-rabbit IgG (Sigma; diluted 1:2000 in PBS-T-NPS). The plates were incubated for 1 h at 37 °C and rinsed as before.

*Peroxidase Anti-peroxidase (PAP) complex incubation* As in the previous step, the PAP complex (Sigma) obtained in rabbits (diluted 1:3000 in PBS-T-NPS) was distributed in the wells, and the plates were incubated for 45 min at 37 °C and then washed.

*Visualization of the Reaction* Each well received 200 µl of the following solution prepared immediately before use: 20 ml of 0.1 M citrate-phosphate buffer (pH 5), containing 10 mg of o-phenylene diamine (Sigma) and 10 µl of 30% hydrogen peroxide.

Color development reached its maximum after 15 min in the dark at 20 °C, and the reaction was stopped by adding 50 µl/well of 4 M sulphuric acid. The absorbance of each well was measured at 492 nm using a Titertek EIA reader (Titertek Multiskan plus).

## Expression of the Results

Linearization of the standard curve was performed by logit transformation to obtain a linear equation as $Y = a + bX$, where $Y = -\ln (B_o - B/B - N)$, and where $B$ represents the binding of each point, $B_o$ the maximum binding, and $N$ the non-specific binding. The parallelism between the regression curves was tested by analysis of the covariance (ANCOVA).

## Estradiol-17$\beta$ Assay

The assay of plasma estradiol was performed by radio immunological analyses (RIA) as previously described by Polzonetti-Magni *et al.* (1984). The sensibility of the assay was 7 pg (intra- and interassay coefficients of variation 4.5% and 7.5%, respectively).



## Western Blot Analysis for Estradiol Receptor Evaluation

Total proteins from liver samples were extracted and resuspended in 1% SDS, quantified using a Hitachi U2000 spectrophotometer (Tokyo, Japan) and the bicinchoninic acid (BCA) method for protein quantification (Pierce, Rockford, IL). 10 mg of total proteins were loaded per lane of gel. The protein samples were resolved on a 10% resolving gel and a 3.75% stacking gel. Samples were electrophoresed at 150 V constant until the bromophenol blue dye front reached the bottom of the gel. Gels used for western transfer were run with Amersham molecular weight markers (rainbow markers) and used for transfer immediately without staining. Western Blotting-Proteins were transferred to PVDF plus membrane (MIcron SEparations Inc.) in a Bio Rad MIni-Protean 2 TM cell apparatus at 100 V for 1 h. ECL western blotting system (Amersham LIfe SCiences) was used for detection purposes. The primary antibody used was polyclonal antibody raised against human ER alpha in rabbit (Sigma) at a dilution of 1:1000 for a minimum time of 1 h. Horse radish peroxidase labelled secondary antibody (anti-rabbit conjugated) was used at a dilution of 1:3000 for 1 h. Exposure was performed on KODAK autoradiography sensitive film for as little as 40 sec, and a clear signal was detected. The dilution of the second antibody was optimized to give a clear background.

## Morphological Studies

Small pieces of male and female gonads were dissected from anterior, central, and posterior parts of the gonads and immersed in Bouin's fixative, dehydrated with ethanol and subsequently embedded in paraffin in a vacuum stove. Six-micrometer sections were stained with Mallory's tricrome modified by Galgano (Mazzi, 1977) for 24 h at 4 °C.

## Statistical Analysis

The comparison of regression curves was based on covariance analysis (ANCOVA) as described by Sokal and Rohlf (1981) using the statistical software, ANCOVA Mac, provided by Dr. M. Angeletti (University of Camerino, Italy). VTG and $E_2$ results were analyzed by

two-way ANOVA with the statistical software package, Stat View 512 + TM (Brain Power Inc., USA). Results were expressed as mean ± SE of data. A probability level of 0.05 was taken to indicate a statistical difference between means.

## RESULTS

### ELISA Validation

The optimal assay concentrations for VTG and antibody and the working conditions were determined to obtain a maximum absorbance value ($B_o$) near 1.5, since the EIA plate reader gave linear responses in the range of 0–2.0 O.D. units. A VTG coating concentration of 260 ng/ml and an antibody dilution of 1:14,000 were chosen. The range of the standard curve corresponds to a VTG concentration of between 1500 ng/ml and 2.92 ng/ml.

Figure 1a shows different displacement curves obtained with serial dilutions of plasma from a vitellogenic female, from a male containing VTG, from a normal male, and serial



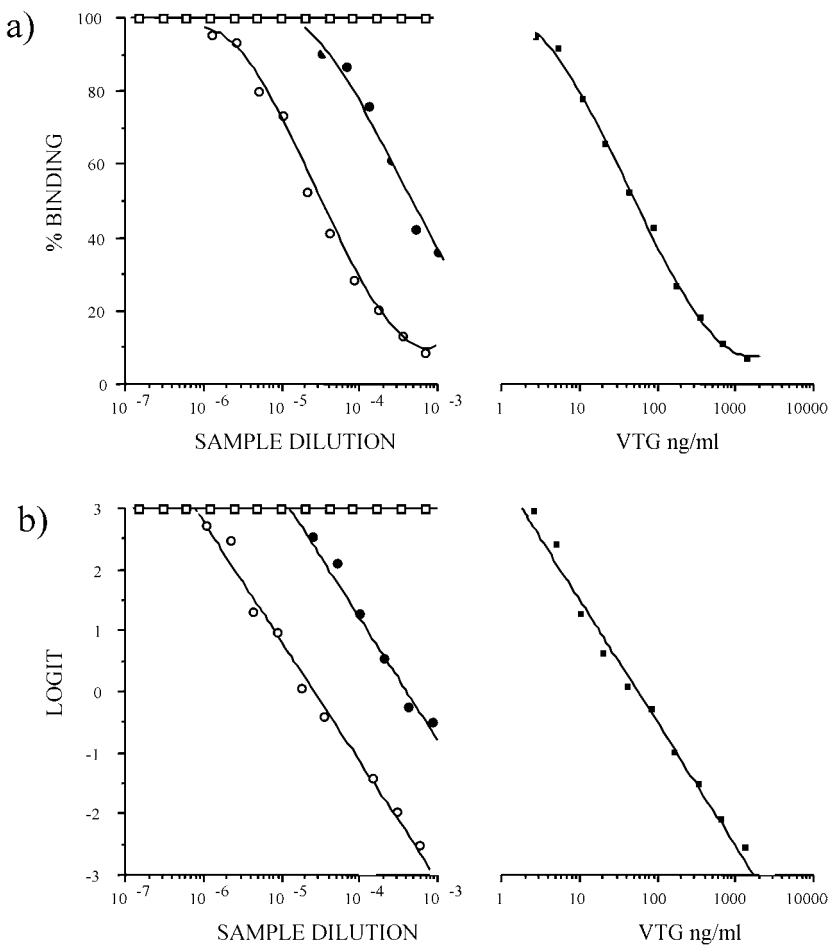


FIGURE 1 (a) Binding curves obtained with the following antigens: carp VTG reference preparation (■), serial dilution of vitellogenic female (○), male with VTG (●), and normal male (□) plasma carp. (b) Logit transformation of the binding curves.

concentrations of the purified VTG as standard. No significant displacements were observed with the control male plasma. Figure 1b shows the linearization of the displacement curves using the logit/log transformation. The parallelism between these curves was tested by analysis of the covariance (ANCOVA) and shows that the antibody recognized in the same manner ($F_s = 1.711 < F0.001 = 9.339$ with 2, 24 df) VTG present in the standard solution and in the plasma of both the female and male containing VTG. The sensitivity (the amount of VTG which gave 90% of binding) was about 8 ng/ml with an intra-assay variation of 4.8% ($n = 16$) and an inter-assay variation of 7.2% ($n = 12$) around 50% of binding. Parallelism of 12 different standard curves was assessed by ANCOVA test. No statistical differences were observed between the different regression lines ($F_s = 0.370 < F0.001 = 3.191$ with 11, 96 df).

## Vitellogenin and Estradiol-17$\beta$ in the Plasma, and Changes in Liver Estrogen Receptor

Plasma VTG and $E_2$ changes were assessed in female carp during the years 1999–2000 (Fig. 2a,b). VTG titers, assessed by validated ELISA, were found highest ($14.9 \pm 2.235$ mg/ml) in pre-spawning period (March), when also the highest ($800 \pm 78$ pg/ml) $E_2$ plasma levels were present. In spawning period (April), both plasma VTG and $E_2$ significantly ($P < 0.05$) decreased to $3.0 \pm 0.55$ mg/ml and $147 \pm 13$ pg/ml, respectively. The evaluation of GSI (Fig. 2c) was consistent with the highest ovary weight found both in March and April corresponding to the pre-spawning and spawning periods, while it showed

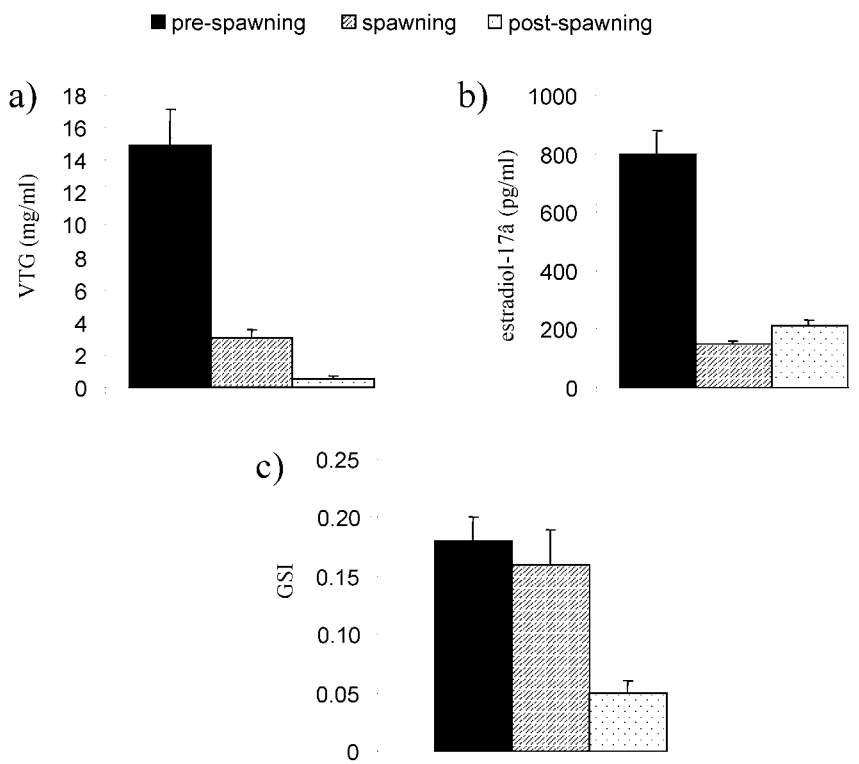


FIGURE 2 Plasma levels of vitellogenin (a), and estradiol-17$\beta$ (b), and gonadosomatic index (c) in *Cyprinus carpio* females. Results are expressed as mean ($n = 10$) of data $\pm$ SD.

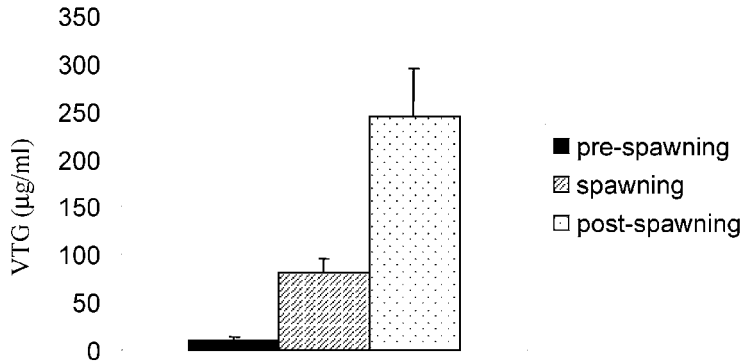


FIGURE 3 Plasma levels of vitellogenin in *Cyprinus carpio* males. Results are expressed as mean ($n = 4$) of data ± SD.

a statistically ($P < 0.05$) significant decrease in non-breeding season (October), in parallel with the lowest plasma levels of VTG ($0.546 \pm 0.16$ mg/ml) and $E_2$ ($211 \pm 18.9$ pg/ml).

When VTG was assessed in male carp plasma samples, in forty percent of them VTG was found to be present at $10 \pm 2.80$, $80 \pm 16$, and $245 \pm 49$ µg/ml in March, April, and October, respectively (Fig. 3); in the remaining male plasma samples, VTG was not detectable in their plasma (data not shown). The morphological observations of testis including those from males producing VTG, did not show any changes in their histology (data not shown).

The estradiol receptors were also tested in male and female livers. In the females, ER was found at low levels in pre-spawning period (March), the maximum expression was observed during spawning (April), while it dramatically decreased in autumn post-spawning; in males showing VTG in the plasma, ER density significantly ($P < 0.05$) increased in March (Fig. 4).



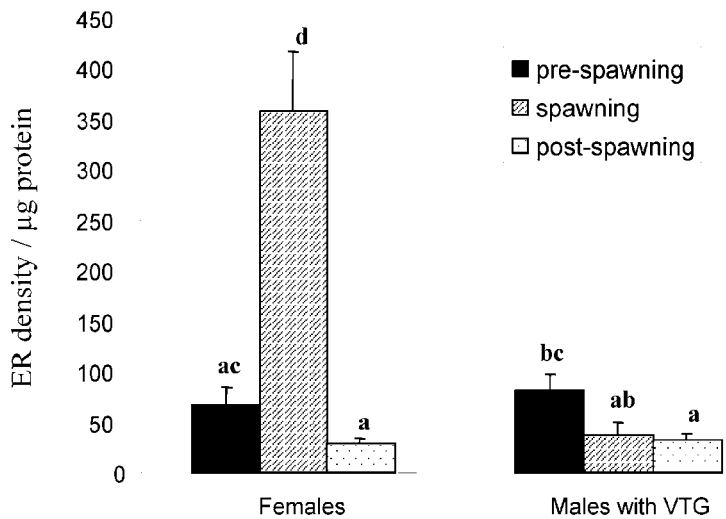


FIGURE 4 $E_2$ receptor density in liver of *Cyprinus carpio* females and males with VTG. Results are expressed as mean ($n = 4$) of data ± SE. The different letters as suffixes indicate significant differences.

## DISCUSSION

In this study, *Cyprinus carpio* L. vitellogenin plasma changes were assessed by validated ELISA together with those of plasma estradiol-17$\beta$ and hepatic estradiol receptor density, providing evidence on some biological responses very useful for environmental hormones biomonitoring studies. Fish represent the largest and most diverse group of vertebrates, and they provide an excellent model for assessing the impacts of contaminants on biological functions such as reproduction. In addition, their intimate association with the aquatic environment makes them an excellent early warning system for environmental health problems that could potentially lead to human health concerns.

Recent data indicate that compounds such as alkylphenol-ethoxylates, frequently present in river and lake water, have estrogenic activity. In particular, within the phenol chemical group, the alkylated phenols have been shown to bind to the estrogen receptor, displaying estrogenic effects, such as vitellogenin induction in male fish (Kime *et al.*, 1999).

In the present study, attention was focused on some key molecules for reproduction: vitellogenin, estradiol-17$\beta$, and its receptor. The sex specific protein, VTG, is the egg yolk precursor protein synthesised in the liver, under the control of estradiol-17$\beta$ secreted into the vascular system and selectively taken up by the growing oocytes. Once in the oocytes, VTG is proteolytically cleaved in lipovitellin and phosvitin, (Wallace, 1985; Carnevali *et al.*, 1999a,b), the main sources of food supply for future embryo development. The other molecule investigated was the ER, which is part of the steroid receptor super family and is under the control of estradiol and of growth factors (Carnevali *et al.*, 2001). The levels of these three key molecules involved in reproduction show seasonal variations related with the reproductive cycle.



For measurement of plasma VTG, a specific ELISA allowing rigorous and reliable quantitative analysis was validated. In females, the changes in vitellogenin and estradiol-17$\beta$ plasma levels were consistent with those found in other teleost species living both in fresh- and seawater. It is noteworthy, in comparison with plasma VTG levels found in other piscine species, that, in carp, VTG in the blood was very high; notwithstanding this, the data here reported, fit well with those previously found for this species (Kime *et al.*, 1999).

In the pre-spawning period, plasma VTG and $E_2$, widely accepted as being the parameters related with oocyte vitellogenic growth and maturation, were compared with GSI and found well correlated. During spawning, plasma VTG, sequestered from the circulation, rapidly decreased, and therefore GSI values were found still high.

Moreover, the presence of VTG in some male plasma was found, and, since no displacements were observed in the ELISA when control male plasma was analyzed, the VTG amount can be considered specific, and, its changes to be dependent on the concentration of estrogenic contaminants present in the Lake of Trasimeno waters and on the time of fish exposure. In fact, plasma VTG in male carp has been found to be xenoestrogen dependent (Solé *et al.*, 2002). These authors described increasing level of plasma VTG (up to 20 μg/ml) related with the content of EDCs, and in particular of NP (nonylphenol) measured in the water and in the sediment of two Spanish rivers; the reported level of NP at 6 μg/l in water and 380 μg/kg in the sediment were found high enough to exhort estrogenicity, a phenomenon due to interaction of estradiol-17$\beta$, or to compounds which mimic this natural hormone (xenoestrogens), with its cellular receptor. Therefore, the mechanism responsible for the changes of estrogen receptor density in male liver is unknown and the interference with other xenobiotics, perhaps those known to act as antiestrogenic compounds, can only be speculative.

The changes of estradiol receptor density in the liver of female carps were found not parallel with those of plasma VTG and estradiol-17$\beta$, even it is worthy of note that estradiol up-regulates its own receptor. Notwithstanding, multihormonal control of hepatic vitellogenin synthesis has recently been suggested by the work on the marine teleost,

*Sparus aurata* (Mosconi *et al.*, 2002), in which it was found that not only does estradiol-17$\beta$ induce liver vitellogenin synthesis, but also growth hormone is able to do that, and the magnitude of the effects of these hormones depends on the seasons.

In conclusion, it seems that the common carp can be considered a useful sentinel species for biomonitoring studies of environmental estrogens, and of their effects on its reproductive disorders.

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