

EFFECTS OF NONYLPHENOL AND 17 β -ESTRADIOL ON VITELLOGENIN SYNTHESIS AND TESTIS MORPHOLOGY IN MALE PLATYFISH *XIPHOPHORUS MACULATUS*

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Summary

Nonylphenol has been found to exert estrogenic effects in fish and may influence the fertility of male fish. In the present study, the effects of nonylphenol and 17 β -estradiol on vitellogenin synthesis and testis morphology in platyfish *Xiphophorus maculatus* were investigated. Vitellogenin was observed in the plasma of all fish exposed to nonylphenol or 17 β -estradiol. Exposure to 17 β -estradiol resulted in a significant reduction in the gonadosomatic index. A tendency for a dose-dependent reduction in the gonadosomatic index in the nonylphenol exposed groups was observed. Histological examination revealed dose-dependent effects of nonylphenol on the testis structure. The testes of control fish contained numerous cysts with spermatogenic cells. The testes of fish exposed to nonylphenol or 17 β -estradiol showed a decrease in the number of cysts concomitant with an increase in the

amount of hypertrophied Sertoli cells present. Formation of spermatozeugmata is compulsory for this species, but free spermatozoa were observed in the efferent ducts of the treated fish. The study indicates that nonylphenol has estrogenic potency, and that both nonylphenol and 17 β -estradiol have marked effects on the testis morphology of *X. maculatus*. The ambient concentration of nonylphenol was measured by high pressure liquid chromatography during the experiment. The measurements revealed that the actual concentrations of nonylphenol in the water were about 30–40 % of the nominal concentrations.

Key words: estrogen, high pressure liquid chromatography (HPLC), nonylphenol, platyfish, Sertoli cell, spermatogenesis, testis histology, vitellogenin, *Xiphophorus maculatus*.

Introduction

There has been increasing public concern that man-made chemicals in the environment affect reproductive health by disrupting normal endocrine function in wildlife populations and humans. Notable among these chemicals are those with estrogenic activity.

Alkylphenols (including nonylphenol) are the final biodegradation products of alkylphenol polyethoxylates, which are non-ionic surfactants widely used in detergents, paints, pesticides, cosmetics and other formulated products (Jobling et al., 1996; White et al., 1994). A number of studies have demonstrated the estrogenic effects of nonylphenol in teleosts (Jobling and Sumpter, 1993; Routledge and Sumpter, 1996, 1997; Toppari et al., 1996; White et al., 1994). *In vivo* studies have shown an increase in the plasma vitellogenin level of fish exposed to nonylphenol (Christiansen et al., 1998; Jobling et al., 1996). Vitellogenin is the precursor of the egg yolk proteins. It is produced by the liver of female fish under the influence of 17 β -estradiol secreted by the ovary (Mommensen and Walsh, 1988). Vitellogenin is normally found in the blood of female fish, whereas the levels in male fish are very low.

However, vitellogenin synthesis can be induced in male fish if they are exposed to exogenous estrogens (Mommensen and Walsh, 1988). The presence of vitellogenin in the plasma of a male fish is therefore considered a sensitive biomarker of exposure to an estrogenic chemical (Sumpter and Jobling, 1995). In addition to the effect on vitellogenin synthesis, exposure to natural estrogens (Chang et al., 1995; Colombo and Grandi, 1995) and nonylphenol (Christiansen et al., 1998; Jobling et al., 1996) also affect the testes of teleosts.

In the present study, the platyfish *Xiphophorus maculatus*, a cyprinodont of the family Poeciliidae native to the fresh waters of Central America, was used as the experimental animal. It was chosen because it reproduces continually, is viviparous and has a short generation time, which makes it feasible to conduct future experiments over two generations. The effects of nonylphenol on the testis morphology of mature males were examined. The testis of Poeciliidae is of the restricted spermatogonial testis type (Grier, 1981). Numerous tubules radiate from the central cavity towards the periphery of the testis. Spermatogonia are located in the blind end of the tubule,

where they are associated with Sertoli cells (Billard, 1990; Grier, 1981), which reorganise to form cysts when the spermatogonia transform into primary spermatocytes (Grier, 1981). As spermatogenesis proceeds, the cysts migrate along the tubule to the efferent ducts in the centre of the testis (Billard, 1990; Grier, 1981). The secondary spermatocytes in the cysts transform into spermatids, which differentiate into spermatozoa (Grier, 1981). With the flagella pointing inwards, sperm nuclei become associated with the surrounding Sertoli cells to form spermatozeugmata (Grier et al., 1978). At the time of spermiation, the cysts open and the spermatozeugmata are voided into the efferent duct system, which ends in a central cavity (Billard, 1990; Grier et al., 1978). The Sertoli cells that formed the cyst hypertrophy and transform into efferent duct cells (Grier, 1981).

The aim of the present study was to investigate the effects of nonylphenol on vitellogenin synthesis and testis morphology and cytology of adult male *X. maculatus*. An experiment with 17 β -estradiol was included to test the hypothesis of an estrogenic effect of nonylphenol.

Materials and methods

Chemicals

Technical 4-nonylphenol was obtained from Fluka (Buchs, Switzerland). It contains approximately 85% *p*-isomers (a mixture of isomers with differently branched nonyl side chains). Main impurities are 2-nonylphenol (*o*-isomer), decylphenol and dinonylphenol (together approximately 10%) (Ahel and Giger, 1993). 17 β -estradiol was obtained from Sigma (St Louis, USA). Stock solutions of nonylphenol and 17 β -estradiol in acetone were stored in the dark at 5°C.

Animals and experimental protocol

Adult, male platyfish *Xiphophorus maculatus* (Günther), of the yellow comet variety, purchased from Fyns Fugle and Akvarie Centrum, Odense, were used as experimental animals. During the experiments fish were kept in glass aquaria with stagnant aerated tap water (21 per fish) at 28°C, approximately pH 8.1, under a 12 h:12 h light:dark cycle. Faeces accumulating at the bottom of the aquaria were siphoned off and 30% of the water was changed twice a week. Fresh amounts of the stock solutions corresponding to the volume of water changed were added to the water while stirring with a glass rod. Fish were fed once daily with 20 mg TetraRubin dry flake food (TetraWerke, Germany) per fish.

Experiments were conducted in November and December 1996. Male *X. maculatus*, body mass 0.62–1.15 g, were used. Five treatment groups were included: (1) control, (2) 4-nonylphenol, 80 $\mu\text{g l}^{-1}$, (3) 4-nonylphenol, 640 $\mu\text{g l}^{-1}$, (4) 4-nonylphenol, 960 $\mu\text{g l}^{-1}$ and (5) 4-nonylphenol, 1280 $\mu\text{g l}^{-1}$. The concentration of acetone in the water in all five groups was 25.5 $\mu\text{l l}^{-1}$. Initially there were 10–15 fish in each group. Daily observations were made of mortality. After 28 days of exposure all fish were individually anaesthetised in 0.4‰ 2-

phenoxyethanol, blot-dried with a laboratory tissue and weighed. Blood was sampled from the caudal vein by capillary aspiration using NH₄-heparinized microhaematocrit tubes. Plasma was separated by centrifugation and stored at –80°C until electrophoresis. The fish were decapitated and testes quickly removed, weighed and their size expressed as a percentage of the total body mass (gonadosomatic index: GSI). Testes were either fixed for subsequent histological examination or frozen in liquid nitrogen and stored at –80°C for later protein and enzymatic analysis.

To examine whether the effects of nonylphenol corresponded to the effects of the natural estrogen 17 β -estradiol, an additional experiment was conducted in October and November, 1997, using male *X. maculatus*, body mass 0.41–1.56 g. Four treatment groups were included: (1) control, (2) 17 β -estradiol, 96 $\mu\text{g l}^{-1}$, (3) 4-nonylphenol, 320 $\mu\text{g l}^{-1}$ and (4) 4-nonylphenol, 960 $\mu\text{g l}^{-1}$. The concentration of acetone in the water in all four groups was 25.5 $\mu\text{l l}^{-1}$. Initially there were 9–16 fish in each group. On day 0 of the experimental period all fish were weighed. After 28 days of exposure, all fish were sampled following the same procedure as for the previous experiment.

Gel electrophoresis

Vitellogenin was identified using gel electrophoresis, which is a semiquantitative method. Because of the small size of *X. maculatus* it was not possible to use quantitative methods such as ELISA or RIA.

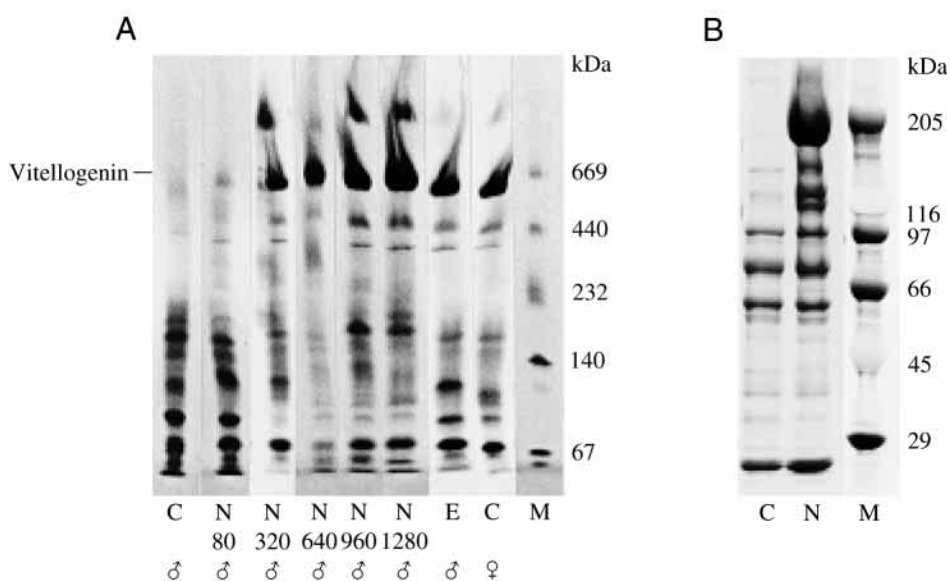
Native PAGE

Intact nondenatured vitellogenin was identified by native polyacrylamide gel electrophoresis (native PAGE) using a PhastSystem (Pharmacia LKB Biotechnology) with PhastGel Gradient 4-15 and PhastGel Native Buffer Strips. The plasma samples were diluted 1:3 in 0.9% NaCl and 0.3 μl of diluted plasma was loaded onto each lane of the gels. PhastSystem™ Separation Technique File No. 120 (Pharmacia LKB Biotechnology) was used for the separation process. Upon completion of electrophoresis, the proteins were stained with the Coomassie dye PhastGel Blue R according to the PhastSystem™ Development Technique File No. 200 (Pharmacia LKB Biotechnology). The molecular masses of the proteins were determined using a Pharmacia HMW Electrophoresis Calibration Kit.

SDS-PAGE

Estimation of the molecular mass of the subunits of the vitellogenin was made by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in a 4–20% gradient gel. Electrophoresis was carried out at 25 mA (constant current). Before electrophoresis, plasma was diluted 1:1 with reducing sample buffer (125 mmol l⁻¹ Tris/HCl, pH 6.8, 0.2 mmol l⁻¹ dithiothreitol, 4% SDS, 40% glycerol, 0.04% Bromophenol Blue) and boiled for 3 min. 5 μl of diluted plasma in sample buffer was loaded into each well on the gel. The gel was stained with Coomassie Brilliant Blue R-250. Molecular masses were

Fig. 1. (A) Native PAGE gel showing the migration pattern of the proteins in the plasma of representative control, nonylphenol exposed and 17 β -estradiol exposed *Xiphophorus maculatus*. C, control; N, nonylphenol (at the indicated nominal concentrations in $\mu\text{g l}^{-1}$); E, 96 $\mu\text{g l}^{-1}$ 17 β -estradiol; M, marker proteins: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), albumin (67 kDa). (B) SDS-PAGE gel showing the migration pattern of the proteins in pooled plasma of control and nonylphenol exposed male *Xiphophorus maculatus* from the dose-response experiment. C, control; N, nonylphenol; M, marker proteins: myosin (205 kDa), β -galactosidase (116 kDa), phosphorylase b (97.4 kDa), bovine albumin (66 kDa), egg albumin (45 kDa), carbonic anhydrase (29 kDa).



determined by comparing the mobility of the polypeptides in the plasma with those of standard proteins (Sigma HMW Standard Mixture SDS 6H).

Histological examination

Light microscopy

Testes were fixed in Lillie's Neutral Buffered Formalin (McManus and Mowry, 1964). Following dehydration in increasing ethanol concentrations (50–99.9%), the testes were cleared in xylene and embedded in paraffin wax. Sections of 5 μm were cut, mounted on glass slides and stained in Mayer's Haematoxylin and Eosin-Y.

Electron microscopy

Testes were fixed in 2% glutaraldehyde in 0.1 mol l^{-1} cacodylate buffer for 2 h, rinsed in buffer and postfixed for 1 h in 1% OsO_4 in 0.1 mol l^{-1} cacodylate buffer. Before postfixation, the tissue was cut into smaller pieces. Following fixation, the tissue was dehydrated in a graded ethanol-propyleneoxide series and embedded in Araldite. Sections (1 and 2 μm thick) were stained with Toluidine Blue and used for orientation. Ultrathin sections were stained with uranyl acetate and lead citrate and studied in a Jeol JEM-100 SX transmission electron microscope.

Enzyme and protein measurements

The frozen testes from the second experiment were homogenised (1:49, w/v) on ice in ice-cold phosphate buffer (10 mol l^{-1} , pH 7.0) with a motor-driven Potter S homogeniser. The homogenates were centrifuged at 4 $^{\circ}\text{C}$ for 10 min at 16,000g. The supernatant was used for assaying γ -glutamyl transpeptidase activity (γ -GTP) and for measuring total protein content. The activity of γ -GTP was determined using a commercial kit ' γ -GT MPR 2' from Boehringer-Mannheim (Szasz et al., 1974). The enzyme activity was

assayed at 28 $^{\circ}\text{C}$. The reaction mixture contained Tris buffer (100 mol l^{-1} , pH 8.25), L- γ -glutamyl-3-carboxy-4-nitroanilide (2.9 mol l^{-1}) and glycylglycine (100 mol l^{-1}). The amount of 5-amino-2-nitrobenzoate produced by the enzymatic reaction was measured at 405 nm over a period of 300 s. The enzymatic activity was expressed as units per gram of testis (U g^{-1} testis) and as milliunits per milligram of total testis protein (mU mg^{-1} protein). One milliunit is defined as the amount of enzyme that forms 1 nmol of product per minute under assay conditions. The concentration of total protein in the supernatant was determined utilizing the principle of protein-dye binding (Bradford, 1976).

Solid-phase extraction of nonylphenol from water and reverse-phase HPLC analysis

In the second experiment the temporal variation in the actual concentration of nonylphenol in the aquarium water during the third week of the experiment was monitored using reverse-phase high pressure liquid chromatography (HPLC). Prior to HPLC analysis the nonylphenol in the water samples was extracted using solid-phase extraction techniques. To a 5 ml water sample drawn from the middle of the aquarium were added 10 μl of internal standard (25 $\text{ng } \mu\text{l}^{-1}$ tert-octylphenol), 5% NaCl, 0.1 mol l^{-1} SDS and 50 μl formaldehyde (37%, v/v). Nonylphenol was extracted using a Sep-Pak C18 cartridge. Analysis for nonylphenol was performed by reverse-phase HPLC using an HP 1100 Series LC. The mobile-phase solvents were 20% methanol (A) and 100% methanol (B). The initial conditions with 45% A and 55% B were run for 5 min, then a linear program to 100% B in 15 min was used. A hold time of 5 min with 100% B was followed by a re-equilibration time of 5 min with the initial conditions before the next injection. The flow rate was 0.4 ml min^{-1} . Sample injection volume was 30 μl . Peak areas and retention times were electronically recorded.

Statistical analysis

Values are expressed as means \pm S.E.M. Data were checked for assumptions of normality using the Kolmogorov–Smirnov test, and homogeneity of variances using Bartlett's test. If the assumptions were met the data were analysed by a parametric one-way analysis of variance (ANOVA) followed by Tukey's test. In cases where normality and homogeneity of variances could not be achieved with log, square root or arcsine transformations, the data were analysed by the nonparametric Kruskal–Wallis test (one-way analysis of variance by ranks) followed by the Dunn Multiple Comparison Procedure. $P < 0.05$ was chosen as the level of significance.

Results

Survival and growth

In the first experiment, which lasted 28 days, 80% of the control fish, 86% of the fish exposed to $80 \mu\text{g l}^{-1}$ nonylphenol, 13% of the fish exposed to $640 \mu\text{g l}^{-1}$ nonylphenol, 60% of the fish exposed to $960 \mu\text{g l}^{-1}$ nonylphenol and 20% of the fish exposed to $1280 \mu\text{g l}^{-1}$ nonylphenol survived.

In the second experiment, also 28 days, all of the control fish and the fish exposed to $320 \mu\text{g l}^{-1}$ nonylphenol survived while 73% of the fish exposed to 17β -estradiol and 56% of the fish exposed to $960 \mu\text{g l}^{-1}$ nonylphenol survived to the end of the experiment.

In the second experiment, where the body masses were recorded at the beginning and the end of the experiment, statistical analysis indicated that there were no significant differences in the growth of the fish in the various treatment groups (data not shown).

Gel electrophoresis

Native PAGE

Fig. 1A shows the mobility of the plasma proteins separated by native polyacrylamide gel electrophoresis. A very distinct protein band migrating in a position corresponding to an estimated molecular mass of approximately 600 kDa was found in the plasma of all nonylphenol treated and 17β -estradiol treated fish. A corresponding protein band was observed in the plasma from female *Xiphophorus maculatus* exhibiting natural vitellogenesis. In contrast, this protein band was absent from the plasma of control males. Gel electrophoresis is not a quantitative method for identification of proteins; however, the density of the band did appear to correlate with the exposure concentration of nonylphenol.

A less distinct protein band was observed migrating in a position well above the marker protein at 669 kDa. This band was found in the plasma of some of the treated fish. None of the fish had this band without also having the 600 kDa band, whereas the reverse was occasionally observed. For the control males no corresponding protein band was ever seen.

SDS-PAGE

Fig. 1B shows the mobility of the plasma proteins in SDS-polyacrylamide gel electrophoresis. One major protein with a

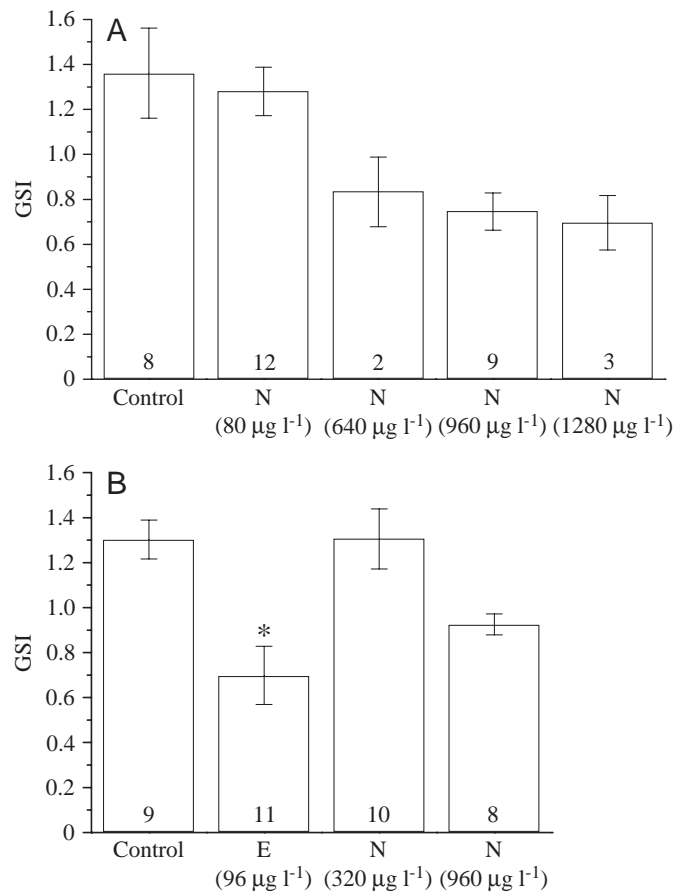


Fig. 2. Effect of nonylphenol (N) and 17β -estradiol (E) on gonadosomatic index (GSI) in the dose–response experiment (A) and in the experiment including 17β -estradiol (B). Values are means \pm S.E.M. *Significant difference ($P < 0.05$) from the control value from the respective experiment. The number of fish sampled is indicated in each column.

molecular mass of approximately 200 kDa appeared in the plasma of nonylphenol exposed fish. In contrast, this protein band was absent from the plasma of control males.

Gonadosomatic index

Fig. 2 shows a dose-related tendency for reduction of the gonadosomatic index (GSI) in the nonylphenol exposed fish. In the first experiment the fish exposed to $960 \mu\text{g l}^{-1}$ nonylphenol had significantly smaller ($P < 0.05$) GSI values compared with the fish exposed to $80 \mu\text{g l}^{-1}$ nonylphenol. Although the control fish had a higher mean GSI than the fish exposed to $80 \mu\text{g l}^{-1}$ nonylphenol there was no significant difference between the control fish and the fish exposed to $960 \mu\text{g l}^{-1}$ nonylphenol at the $P < 0.05$ level. However, the difference was significant at the $P < 0.10$ level.

Exposure to $96 \mu\text{g l}^{-1}$ 17β -estradiol resulted in a significant reduction ($P < 0.05$) in the GSI as compared with controls.

Histological examination of the testes

Light microscopy

Histological examination of the testes revealed moderate to

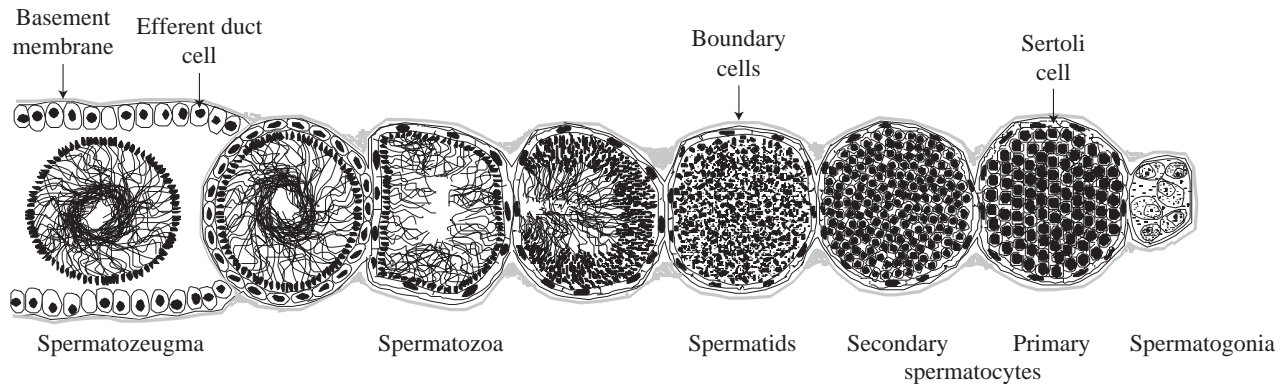


Fig. 3. Structure of testicular tubule in platyfish *Xiphophorus maculatus*. The tubule contains cysts with spermatogenic stages from spermatogonia (right) to spermatozeugmata surrounded by Sertoli cells (left). At the time of spermiation the boundary cells and associated basement membranes between the encysted spermatozeugma and the efferent duct lumen separate and reorganise to form a spermiation channel (Grier et al., 1980; Grier, 1981). This involves the fusion of tubule and efferent duct basement membranes. Cyst Sertoli cells and efferent duct cells separate and the spermatozeugma enters the efferent duct. The Sertoli cells hypertrophy and become continuous with the efferent duct epithelium.

severe effects on the testicular structure by exposure to nonylphenol or 17β -estradiol.

Testes from the control fish (Figs 3, 4A) contained regularly organized cysts with all spermatogenic stages: spermatogonia peripherally, and cysts with primary spermatocytes, secondary spermatocytes, spermatids and developing spermatozeugmata closer to the centrally situated efferent ducts containing mature spermatozeugmata. Transformed hypertrophied Sertoli cells were incorporated in the efferent duct epithelium (Fig. 5A).

In testes from fish exposed to nonylphenol the decreased number of cysts were irregularly arranged (Fig. 4B–E). An increased number of hypertrophied Sertoli cells, which were not included within the efferent duct epithelium, was observed (Fig. 5B). The testes showed different degrees of release of free spermatozoa (Fig. 5C,D). The testicular ducts were enlarged and cavities in the centre of the testes were observed (Fig. 5C). All these changes were more pronounced with higher doses of nonylphenol.

Exposure to $96\mu\text{g l}^{-1}$ 17β -estradiol resulted in effects on the testis structure similar to those observed in fish exposed to high concentrations of nonylphenol (Fig. 4F). In some of the testes the effects were even more pronounced. These testes were almost denuded of germ cells and contained large central cavities.

Electron microscopy

Transmission electron microscopy was used to examine the cytology of the Sertoli cells lining the cysts in testes from the second experiment. In the control fish the Sertoli cells were flattened with ovoid nuclei and few organelles. Their basic morphology and size remained essentially unaltered from the formation of the cysts until mature spermatozoa were formed. The Sertoli cells enlarged slightly when the sperm nuclei were embedded in the Sertoli cell cytoplasm during spermatozeugmata formation (Fig. 6A).

In the testes of fish exposed to $960\mu\text{g l}^{-1}$ nonylphenol the cytology of the Sertoli cells lining the cysts with developing spermatozeugmata was distinctly different (Fig. 6B). These cells were greatly hypertrophied and cuboidal to columnar. They contained larger and more spherical nuclei, vacuoles of different shapes and sizes, and abundant dilated rough endoplasmic reticulum (RER) cisternae (Fig. 6B). Underlying the plasma membrane, a heavily folded basement membrane was present (Fig. 6E). Apart from the abnormal cysts with spermatozeugmata, cysts with free spermatozoa (Fig. 6C) and empty cysts (Fig. 6D), both with hypertrophied Sertoli cells, were found in the testes of fish exposed to $960\mu\text{g l}^{-1}$ nonylphenol.

Protein and γ -GTP activity in testes

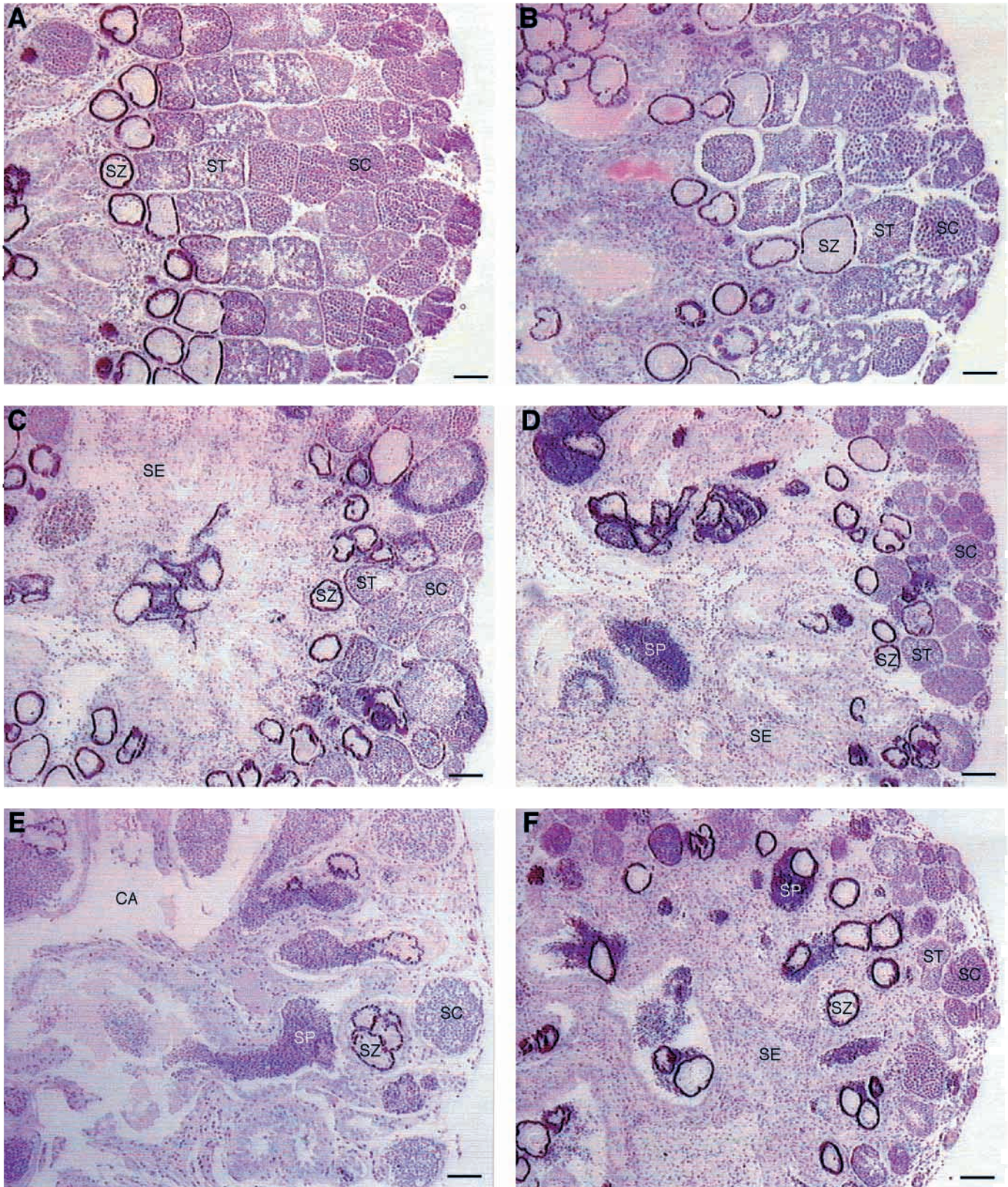
In mammals, γ -glutamyl transpeptidase has been suggested as a putative Sertoli cell marker. In the present study, the activity of γ -GTP and the protein concentration were measured in the testes of the fish. Treatment with nonylphenol or 17β -estradiol had no significant effect on protein concentration ($46.6\pm 1.8\text{ mg g}^{-1}$, $N=19$) or γ -GTP activity ($4.2\pm 0.4\text{ mU mg}^{-1}$ protein, $N=15$) in the testes, although there was a tendency for increased γ -GTP activity in the treated groups.

Actual concentration of nonylphenol in the water

The results of the analysis of the actual concentration of nonylphenol in the aquaria in the second experiment are shown in Table 1.

Discussion

In the present study, exposure of *Xiphophorus maculatus* to nonylphenol or 17β -estradiol resulted in the induction of two different high molecular mass proteins in the plasma. The protein with an estimated molecular mass of 600kDa is identified as vitellogenin, on the basis of three characteristics:



(1) the protein is sex specific, being present in the plasma of females exhibiting vitellogenesis but absent from the plasma of control males, (2) the protein is estrogen-inducible and (3) the estimated molecular masses of the native protein (approximately

600 kDa) and its subunits (approximately 200 kDa) are in close agreement with the molecular masses of native vitellogenin and its subunits from other teleosts (Mommensen and Walsh, 1988; Specker and Sullivan, 1994). SDS-PAGE revealed only one

Fig. 4. Light micrographs of testes from *Xiphophorus maculatus*. (A) Control fish. The radiating tubules contain numerous cysts of spermatogenic cells, and the efferent ducts contain spermatozeugmata. (B) Fish exposed to $80\mu\text{g l}^{-1}$ nonylphenol. The number of cysts are reduced, but the testicular ducts contain spermatozeugmata. (C) Fish exposed to $320\mu\text{g l}^{-1}$ nonylphenol. Only a few cysts are present, but the testicular ducts contain spermatozeugmata. The amount of hypertrophied Sertoli cells/efferent duct cells is pronounced. (D) Fish exposed to $960\mu\text{g l}^{-1}$ nonylphenol. Only a few cysts are present and the testicular ducts contain free spermatozoa and disintegrating

spermatozeugmata. The amount of hypertrophied Sertoli cells/efferent duct cells is pronounced. (E) Fish exposed to $1280\mu\text{g l}^{-1}$ nonylphenol. The testis is almost denuded of cysts and the testicular ducts contain free spermatozoa and a few spermatozeugmata. (F) Fish exposed to $96\mu\text{g l}^{-1}$ 17β -estradiol. The testis contain only a few cysts, and free spermatozoa and spermatozeugmata are present in the testicular ducts. The amount of hypertrophied Sertoli cells/efferent duct cells is pronounced. CA, cavity; SC, spermatocytes; SE, hypertrophied Sertoli cells/efferent duct cells; SP, free spermatozoa; ST, spermatids; SZ, spermatozeugma. Scale bars, $50\mu\text{m}$.

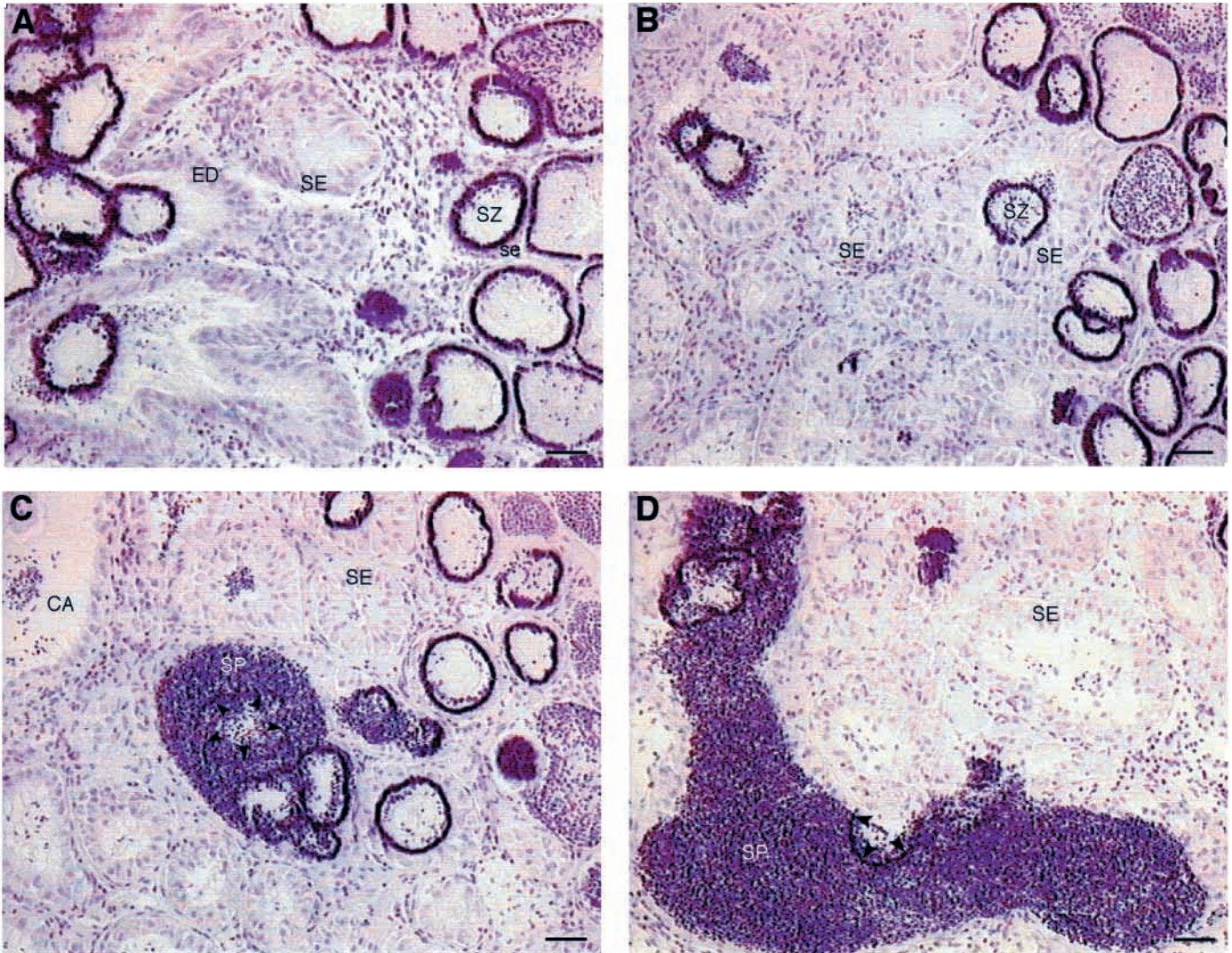


Fig. 5. Higher magnification light micrographs of testes from *Xiphophorus maculatus*. (A) Control fish. Flattened Sertoli cells are surrounding the developing spermatozeugmata. Hypertrophied Sertoli cells are being incorporated in the efferent duct epithelium. (B-D) Fish exposed to $960\mu\text{g l}^{-1}$ nonylphenol. (B) Hypertrophied Sertoli cells are not incorporated in the efferent duct epithelium but still form cysts, some containing spermatozeugmata. (C) Free spermatozoa with a disintegrating spermatozeugma faintly seen in the middle (arrowheads). (D) A large amount of free spermatozoa, and a disintegrating spermatozeugma (arrowheads). CA, cavity; ED, efferent duct cells; se, Sertoli cells; SE, hypertrophied Sertoli cells/efferent duct cells; SP, free spermatozoa; SZ, spermatozeugma. Scale bars, $25\mu\text{m}$.

induced protein band, which indicates that the native vitellogenin consists of homologous monomers. Native vitellogenin in *X. maculatus* is therefore assumed to be a homodimer of subunits with molecular masses of approximately

200kDa , bound with some lipids and carbohydrates, giving a molecule with a molecular mass of approximately 600kDa . In addition to the protein identified as vitellogenin, another plasma protein migrating in a position well above the marker protein at

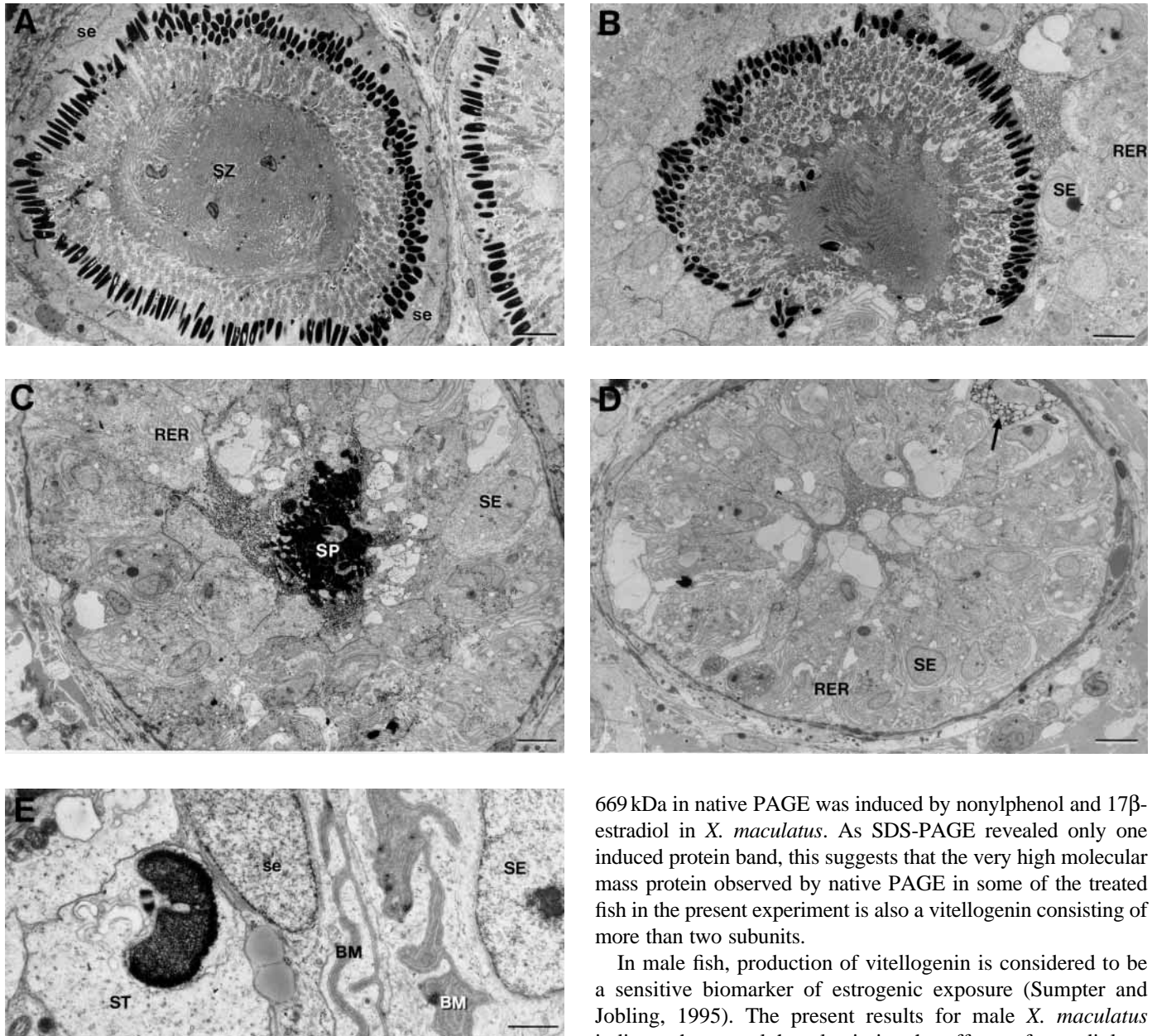


Fig. 6. Electron micrographs showing the cyst Sertoli cells in testes from *Xiphophorus maculatus*. (A) Control fish. Spermatogonium surrounded by a single layer of flat Sertoli cells with ovoid nuclei. (B–F) Fish treated with $960 \mu\text{g l}^{-1}$ nonylphenol. (B) Spermatogonium surrounded by hypertrophied cuboidal to columnar Sertoli cells with spherical nuclei, vacuoles, and abundant dilated RER-cisternae. (C) Free spermatozoa surrounded by hypertrophied Sertoli cells with spherical nuclei, vacuoles and abundant dilated RER-cisternae. (D) Empty cyst consisting of hypertrophied Sertoli cells with roughly spherical nuclei, vacuoles, and abundant dilated RER-cisternae. The cyst contains a degenerating Sertoli cell (arrow). (E) A Sertoli cell lining a cyst with spermatids (left), and a hypertrophied Sertoli cell with a strongly folded thick basement membrane (right). BM, basement membrane; se, Sertoli cell; SE, hypertrophied Sertoli cell; SP, free spermatozoa; ST, spermatid; SZ, spermatogonium. Scale bars, $5 \mu\text{m}$ (A–D); $1 \mu\text{m}$ (E).

669 kDa in native PAGE was induced by nonylphenol and 17β -estradiol in *X. maculatus*. As SDS-PAGE revealed only one induced protein band, this suggests that the very high molecular mass protein observed by native PAGE in some of the treated fish in the present experiment is also a vitellogenin consisting of more than two subunits.

In male fish, production of vitellogenin is considered to be a sensitive biomarker of estrogenic exposure (Sumpter and Jobling, 1995). The present results for male *X. maculatus* indicate that nonylphenol mimics the effects of estradiol on vitellogenin synthesis and acts as an estrogen. Nonylphenol appeared to induce the synthesis of vitellogenin in a dose-dependent manner.

A dose-related tendency for reduction of the testis mass (GSI) was observed after nonylphenol treatment. In the dose-response experiment the GSI of the fish exposed to $960 \mu\text{g l}^{-1}$ nonylphenol was significantly lower ($P < 0.05$) than the GSI of the fish exposed to $80 \mu\text{g l}^{-1}$ nonylphenol. However, although the control fish had a higher mean GSI than the fish exposed to $80 \mu\text{g l}^{-1}$ nonylphenol, there was no significant difference between the control fish and the fish treated with $960 \mu\text{g l}^{-1}$ nonylphenol. This is due to the larger standard error on the GSI in the control group compared to the group exposed to $80 \mu\text{g l}^{-1}$ nonylphenol. Treatment with $96 \mu\text{g l}^{-1}$ 17β -estradiol resulted in a significant ($P < 0.05$) reduction in the testis mass (GSI).

Table 1. Actual concentrations of nonylphenol in the aquarium water during the third week of the second experiment

Time (h)	Nominal nonylphenol concentration ($\mu\text{g l}^{-1}$)			
	320		960	
	Actual	%	Actual	%
0	305 \pm 34 (2)	95 \pm 11 (2)		
1			804 (1)	84 (1)
24	187 \pm 128 (2)	59 \pm 40 (2)	289 \pm 21 (2)	30 \pm 2 (2)
48	57 \pm 9 (2)	18 \pm 3 (2)	244 \pm 90 (2)	25 \pm 9 (2)
72	47 \pm 22 (2)	15 \pm 7 (2)	220 \pm 27 (2)	23 \pm 3 (2)
96	43 \pm 12 (2)	14 \pm 4 (2)	75 (1)	8 (1)

Measurements were made 0, 1, 24, 48, 72 and 96 hours after addition of nonylphenol. Values are means \pm S.E.M. (N).

% are values for the actual nonylphenol concentration expressed as a percentage of the nominal concentration.

The histological examinations of the testes of *X. maculatus* revealed that treatment with nonylphenol or 17 β -estradiol had pronounced effects on testicular structure. Exposure to nonylphenol or 17 β -estradiol resulted in a reduction of the number of cysts containing all the different spermatogenetic stages. In fish exposed to high concentrations of nonylphenol or to 17 β -estradiol the testes were more or less denuded of cysts, hence in these fish spermatogenesis must be expected to be almost totally impaired. An increased number of hypertrophied Sertoli cells, which were not incorporated in the efferent duct epithelium, was also observed in the treated fish. The sperm ducts of fish exposed to high concentrations of nonylphenol or to 17 β -estradiol contained free spermatozoa, which may be due to incomplete formation of the spermatozeugmata before extrusion into the efferent ducts. This could cause rupture of the spermatozeugmata, indicated by the observation of incomplete spermatozeugmata in the area of free spermatozoa in the efferent ducts. It is assumed that if the transfer of sperm from the gonopodium of the male to the genital opening of the female is not carried out by way of the spermatozeugmata, the sperm will be lost in the water and no fertilization will occur (Tavolga, 1949). Therefore, the changes observed after exposure to high concentrations of nonylphenol or to 17 β -estradiol strongly indicate that these compounds are capable of decreasing male fertility in *X. maculatus*.

A possible explanation for the observed changes in testis structure is an effect on the Sertoli cells. Several roles have been attributed to the Sertoli cells, including formation of cysts in which spermatogenesis takes place and spermatozeugmata morphogenesis (Grier, 1981). In the present study, the formation of cysts was impaired in testes from fish exposed to high concentrations of nonylphenol or to 17 β -estradiol, resulting in gonads more-or-less denuded of cysts. Electron microscopy revealed an effect of nonylphenol on the cytology of the Sertoli cells lining the cysts with developing spermatozeugmata. In the testes of control fish the Sertoli cells

were flattened with ovoid nuclei and only a few organelles. In the testes of fish exposed to 960 $\mu\text{g l}^{-1}$ nonylphenol, the Sertoli cells were greatly hypertrophied with larger and more spherical nuclei, vacuoles, dilated RER cisternae, and with a strongly folded basement membrane present. These features are all characteristic of efferent duct cells (Hurk et al., 1974). It thus appears that the cyst Sertoli cells hypertrophy and transform into efferent duct cells before spermiation in the treated fish. As the formation of spermatozeugmata always involves the evolution of a Sertoli cell-spermatid association (Grier, 1981), this precocious transformation could result in impaired formation of spermatozeugmata and release of free spermatozoa into the efferent duct system. The observed effects are, therefore, likely to result from an altered function of the Sertoli cells.

The mechanisms whereby nonylphenol causes the effects on the testes – including the Sertoli cells – are not clear. Nonylphenol may act indirectly via the hypothalamus-pituitary axis to alter gonadotropin synthesis and secretion, or it may act directly on the testes. Altered gonadotropin secretion, resulting in disruption of sex steroid production, can have secondary effects on the testicular cells (e.g. Sertoli cells), which are dependent upon the correct hormone levels for normal functioning. Direct effects on the testes can be either cytotoxic, when the disruption is caused by damage to the testis cells in general, or endocrine, in which the function of specific cells (e.g. Sertoli cells) are disrupted due to an endocrine malfunction (Kime, 1999). Recently an estrogen receptor – with affinity for nonylphenol – has been identified in the testis of the Atlantic croaker *Micropogonias undulatus* (Loomis and Thomas, 1999). The nonylphenol-induced effects on the testicular structure observed in the present study could be mediated via such testicular estrogen receptors. However, the mechanisms underlying the observed effects need further investigation.

In the testicular tissue of mammals, γ -glutamyl transpeptidase (γ -GTP) is primarily found in the Sertoli cells and has been used as a Sertoli cell marker (Carreau et al., 1996; Gupta et al., 1997; Lu and Steinberger, 1977). It is not known if this also applies to Sertoli cells in fish. A marked decrease in the activity of γ -GTP concomitant with an altered Sertoli cell structure in the testes of the eelpout *Z. viviparus* after treatment with nonylphenol or 17 β -estradiol has been observed (Christiansen et al., 1998). However, in the present study no significant difference in the activity of γ -GTP in the testes of *X. maculatus* was seen after exposure to nonylphenol or 17 β -estradiol, although there was a tendency for increased activity in the treated groups. The relationship between testis structure and γ -GTP activity will be more closely examined in future experiments.

The actual concentrations of nonylphenol in the water were somewhat lower than the nominal concentrations. The results indicate that the fish on the average were exposed to about 30–40% of the nominal concentration during the experiment. The mechanisms for loss of nonylphenol from the aquarium water could be (1) uptake in fish, (2) adsorption to the glass,

(3) microbial degradation (Ekelund et al., 1993) and (4) photodegradation (Ahel et al., 1994).

The concentrations of nonylphenol used in the present study are relatively high compared to the concentrations found in nature. In general, concentrations of nonylphenol in rivers and lakes rarely exceed $10\ \mu\text{g l}^{-1}$ (Ahel et al., 1987; Bennie et al., 1997; Blackburn and Waldock, 1995), although in rivers receiving significant amounts of industrial effluents, concentrations may reach $1000\ \mu\text{g l}^{-1}$ (Warhurst, 1995). In nature, however, many other factors must be taken into consideration when assessing the estrogenic exposure of fish. These include duration of exposure, bioaccumulation and the presence of other interfering chemicals. In nature fish may spend long periods in contaminated water. The severe effects on the testes reported here occurred at high concentrations of nonylphenol but the fish were exposed for 4 weeks only. It is possible that similar effects may also be observed after long-term exposure to lower concentrations. Nonylphenol is strongly lipophilic (Ahel and Giger, 1993) and tends to accumulate in aquatic organisms. Bioconcentration factors between 13 and 1300 have been reported for fish (Ahel et al., 1993; Ekelund et al., 1990). Other estrogenic chemicals in the environment may have the potential to produce effects similar to those of nonylphenol. Indeed a combination of several estrogenic chemicals may have an additive effect (Sumpter and Jobling, 1995). Thus, it is quite possible that nonylphenol, either by itself or through its contribution to the pool of environmental estrogens, may have adverse effects on the reproductive system of male fish.

In conclusion, the present study indicates that nonylphenol exerts estrogenic action in male *X. maculatus*. This is based on the marked increase in vitellogenin synthesis, which corresponds to the effect seen with 17β -estradiol. Furthermore, the effects on the testes strongly indicate that nonylphenol is capable of decreasing male fertility in *X. maculatus*. Nonylphenol and 17β -estradiol exerted similar effects on the testes. It is therefore likely that the observed effects of nonylphenol on the testes are estrogenic.

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