EFFECTS OF NONYLPHENOL AND 17β-OESTRADIOL ON VITELLOGENIN SYNTHESIS, TESTICULAR STRUCTURE AND CYTOLOGY IN MALE EELPOUT *ZOARCES VIVIPARUS*

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Summary

Nonylphenol has been found to be oestrogenic in fish and may influence the reproductive system of male fish. In the present study, the effects of low ($10\,\mu\mathrm{g\,g^{-1}\,week^{-1}}$) and high ($100\,\mu\mathrm{g\,g^{-1}\,week^{-1}}$) doses of nonylphenol and of 17β-oestradiol on the synthesis of vitellogenin and on testicular structure and cytology were investigated in male eelpout *Zoarces viviparus* during active spermatogenesis (May) and late spermatogenesis (June). Twenty-five days after injection, a significant dose-dependent increase in the plasma vitellogenin concentration, measured by enzyme-linked immunosorbent assay, was observed in the treated groups. A highly significant reduction in the gonadosomatic index was observed concomitant with the increase in the plasma vitellogenin concentration. Macroscopically, milt was observed to be present in the control fish, but was sparse or absent in the treated fish. Histological examination using light microscopy revealed severe effects of nonylphenol as well as of oestradiol treatment on testicular structure. Control fish had seminiferous lobules containing spermatogenic cysts and only a few spermatozoa (May) or had the walls of their seminiferous lobules lined with cuboidal Sertoli cells (June). In the treated fish, the seminiferous lobules were degenerated (May) or were filled with numerous spermatozoa and the Sertoli cells appeared very squamous (June). Electron microscopy revealed greater numbers of phagocytozed spermatozoa in these Sertoli cells. In rats, γ-glutamyl transpeptidase (γ-GTP) has been used as a specific marker of Sertoli cell function. In the present study, both nonylphenol and 17β-oestradiol treatment resulted in a reduction in the activity of this enzyme. The study provides evidence that nonylphenol is oestrogenic, as indicated by the large increase in vitellogenin synthesis, and that both nonylphenol and oestradiol have marked effects on the testicular structure and cytology of germ cells and Sertoli cells of male *Z. viviparus*.

Key words: nonylphenol, oestrogens, testis, vitellogenin, Sertoli cell, spermatogenesis, γ-glutamyl transpeptidase, histology, eelpout, *Zoarces viviparus*.

Introduction

During the last couple of decades, increases in several pathological disorders in the human male reproductive system, including cryptorchidism (maldescent of the testis), falling sperm counts, decreased semen quality and testicular cancer, have been observed (Sharpe and Skakkebœk, 1993; for a review, see Toppari *et al.* 1995). Abnormalities in the male reproductive system of fish, reptiles and mammals have also been observed (Purdom *et al.* 1994; Guillette *et al.* 1994; Facemire *et al.* 1995; for a review, see Toppari *et al.* 1995). Recently, it has been suggested that the increasing incidence of male reproductive abnormalities may be a result of environmental pollution by man-made chemicals that have oestrogenic effects (Colborn *et al.* 1993; Sharpe and Skakkebœk, 1993; Toppari *et al.* 1995). Such reproductive abnormalities not only impair the individual, but may also threaten populations as a whole (Colborn *et al.* 1993; Toppari *et al.* 1995).

No clear connection has been found between disorders in the human reproductive system and exposure to environmental oestrogens, but a number of effects reported in wild animals have been connected to environmental oestrogens (for a review, see Toppari *et al.* 1995). Aquatic animals are especially affected. In male alligators from lakes in Florida, which have been contaminated with DDT (an oestrogenic pesticide), smaller penis size, poorly organised testes and lower plasma testosterone levels have been reported (Guillette *et al.* 1994). In England and Wales in water near effluents from sewage-treatment works, an increased prevalence of hermaphroditism in roach (*Rutilus rutilus*) was reported (Sumpter and Jobling, 1995). This discovery initiated a nationwide field study in and near effluents from sewage-treatment works. The study revealed a pronounced increase in the plasma vitellogenin level – a protein normally found only

in females – of male rainbow trout *Oncorhynchus mykiss* held in such water (Sumpter and Jobling, 1995). In some of the fish, retarded testicular growth was also observed (Harries *et al.* 1997). The identity of the oestrogenic compound(s) in the sewage effluents that may have contributed to these effects is still largely unknown, but alkylphenolic chemicals may be responsible for part of the effect (Harries *et al.* 1997). Elevated vitellogenin levels and testis abnormalities are also found in flounders (*Platichthys flesus*) living in estuaries receiving effluent from sewage-treatment works and other industrial discharges (Lye *et al.* 1997).

In female fish (and other oviparous vertebrates), oestrogens play an important role in vitellogenesis – the process whereby yolky eggs are produced. Vitellogenin is a yolk precursor protein of high molecular mass produced in the liver of oviparous females (for reviews, see Mommsen and Walsh, 1988; Specker and Sullivan, 1994). Male fish also possess the vitellogenin gene, but in males the gene is normally silent. Vitellogenin synthesis in males can, however, be induced *de novo* by treatment with exogenous oestrogens, resulting in an increase in the vitellogenin level in the blood (Korsgaard-Emmersen and Petersen, 1976; Campbell and Idler, 1980; Mommsen and Walsh, 1988; Korsgaard, 1990). Therefore, the production of vitellogenin in male fish is considered to be a sensitive biomarker for oestrogenic exposure (Sumpter and Jobling, 1995; Toppari *et al.* 1995).

Recently, alkylphenols were found to be oestrogenic (Sumpter and Jobling, 1995; Toppari *et al.* 1995). Alkylphenols are the final biodegradation products of the widely used non-ionic surfactant alkylphenol polyethoxylates used in detergents, paints and cosmetics. After sewage treatment, alkylphenol polyethoxylates and alkylphenols (e.g. nonylphenol) end up in the aquatic environment (Ahel *et al.* 1994; Blackburn and Waldock, 1995). Like many other environmental oestrogenic contaminants, alkylphenols are lipophilic, relatively persistent and have a strong tendency to bioaccumulate, particularly in aquatic organisms (Ekelund *et al.* 1990; Ahel *et al.* 1993).

Nonylphenol (and other alkylphenols) exerts oestrogenic effects both *in vitro* and *in vivo* (Soto *et al.* 1991; Jobling and Sumpter, 1993; White *et al.* 1994; Jobling *et al.* 1996). It has been demonstrated using *in vitro* oestrogen assays in mammals, birds and fish that the action of nonylphenol (and of other oestrogenic chemicals) is mediated by the oestrogen receptor in a similar way to that of 17β-oestradiol (Jobling and Sumpter, 1993; White *et al.* 1994). Nonylphenol has been shown to induce the growth of human breast cancer cells in culture (Soto *et al.* 1991; White *et al.* 1994). *In vitro* studies using cultured hepatocytes from male rainbow trout *Oncorhynchus mykiss* show that nonylphenol increases vitellogenin synthesis in a dose-dependent manner (Jobling and Sumpter, 1993). *In vivo* alkylphenols have been demonstrated to exert oestrogenic effects in female rats (Soto *et al.* 1991). In male rainbow trout, four different alkylphenolic chemicals, including nonylphenol, are found to be oestrogenic *in vivo* on the basis of their induction of vitellogenin synthesis

(Jobling *et al.* 1996). In our laboratory, it has previously been shown that intraperitoneally injected nonylphenol causes an increase in the plasma vitellogenin level in male flounder *Platichthys flesus* (Christensen *et al.* 1995) and Atlantic salmon *Salmo salar* (Madsen *et al.* 1997).

The aim of the present study was to investigate whether low as well as high doses of nonylphenol can induce *de novo* vitellogenin synthesis and affect testicular growth and spermatogenesis in adult male eelpout *Z. viviparus*. The eelpout is an excellent indicator of pollution of the marine environment, being a fairly stationary teleost in the near-shore coastal area. The testicular structure and the Sertoli cells of male *Z. viviparus* were analysed microscopically (using light and electron microscopy), and the testicular activity of γ-glutamyl transpeptidase (γ-GTP) – a potential Sertoli cell marker enzyme – was analysed to assess whether Sertoli cell function might be affected.

Materials and methods

Animals and experimental protocol

Sexually mature male *Zoarces viviparus* (L.) (Teleostei, Percifomes, Zoarcidae) (N=18, mean body mass 59.9±5.1 g; mean ± S.E.M.) were caught in the sea south of Funen, Denmark. The fish were transported to the laboratory and separated into two groups. They were kept in two large indoor tanks containing 300 l of filtered, ultraviolet-treated, recirculated and aerated sea water (salinity 20 ‰, temperature 14–15 °C) and exposed to a 12 h:12 h L:D photoperiod. The fish were acclimated to these conditions for 1 week before the experiment. They were not fed during the acclimation period or during the experimental period (June 1995). On day zero of the experimental period, the fish were marked with liquid nitrogen, weighed and given the first treatment under anaesthesia (0.2 ‰ phenoxyethanol). The fish were injected intraperitoneally as follows: (1) control (C), peanut oil (0.1 ml per fish); or (2) nonylphenol (N), 100 µg g^{-1} week^{-1} dissolved in peanut oil (0.1–0.2 ml per fish). The injections were given in half doses twice a week. The experimental period was 25 days. The fish were sampled on day 25. The fish were weighed, and blood was taken from the caudal artery and heparinized. Plasma was separated by centrifugation (4 min at 20 000 g at 4 °C) and stored at −80 °C until use. The fish were killed by decapitation. The livers were quickly excised, weighed, frozen in liquid nitrogen and stored at −80 °C. Their size was expressed as a percentage of total body mass (hepatosomatic index, HSI). The testes were quickly excised, weighed (to give the total mass of a pair of testes), and their size was expressed as a percentage of the total body mass (gonadosomatic index, GSI). Macroscopic observations of testis structure, colour and amount of milt were noted. The right testis was fixed and stored in Lillie's fixative (10 % buffered formalin) for light microscopical examination. Small parts from the middle portion of the left testis were fixed in glutaraldehyde for electron microscopy. The caudal and cranial parts of the left testis were frozen in liquid nitrogen and stored at −80 °C for later protein and enzymatic analysis.

To investigate the effect of nonylphenol and of oestradiol during an earlier stage of spermatogenesis, an additional experiment was performed in May of the following year. Male *Z. viviparus* ($N=60$, mean body mass 70.7 ± 2.1 g; mean $\pm$ S.E.M.) were separated into four groups and injected intraperitoneally as follows: (1) control group (C) receiving the vehicle only, peanut oil (0.1 ml per fish); (2) low-dose nonylphenol (n), $10\,\mu g\,g^{-1}\,week^{-1}$; (3) high-dose nonylphenol (N), $100\,\mu g\,g^{-1}\,week^{-1}$; or (4) oestradiol (E$_2$), $0.5\,\mu g\,g^{-1}\,week^{-1}$. Photographs of the testes were taken for three representative fish from the control group, the $100\,\mu g\,g^{-1}$ nonylphenol group and the oestradiol group.

To ensure that the vitellogenic response to nonylphenol was also induced by a more realistic exposure to nonylphenol in the ambient sea water, an additional experiment was performed. Male fish ($N=4$) were kept in an aquarium containing sea water (salinity 20‰, temperature 12 °C) supplemented with $1\,mg\,l^{-1}$ nonylphenol dissolved in acetone ($33\,\mu l\,l^{-1}$). During the experimental period of 3 weeks (August 1996), the water was changed three times and supplemented with $1\,mg\,l^{-1}$ nonylphenol. On day 21, the fish were sampled following the same procedure as for the previous experiments. Fish ($N=7$) caught shortly before the end of this experiment were sampled and used as control group. Only the vitellogenin response and GSI are included in the results from this experiment.

Determination of vitellogenin concentration
Gel electrophoresis

Vitellogenin was identified by native polyacrylamide gel electrophoresis (PAGE) using a PhastSystem (Pharmacia LKB Biotechnology). Plasma samples from each group of fish were compared with a sample of purified vitellogenin. PhastSystem separation Technique File no. 130 (Pharmacia LKB Biotechnology) was used for the separation process, and the protein bands were stained with a Coomassie dye according to PhastSystem Development Technique File no. 200 (Pharmacia LKB Biotechnology). Before loading, the plasma samples were diluted 10-fold in 0.9 % NaCl.

ELISA method

Vitellogenin was isolated by gel filtration and ion-exchange chromatography from the plasma of oestradiol-treated males or females in natural vitellogenesis. Electrophoretic patterns of vitellogenin were investigated by native PAGE at each purification step. The purified vitellogenin was used to raise anti-vitellogenin antibodies in rabbits. The purity of the antigen (vitellogenin) and the specificity of the affinity-purified antibody raised against the vitellogenin were assessed by western blot analysis. No cross-reactivity was observed with plasma from non-oestradiol-treated control males. A non-competitive enzyme-linked immunosorbent assay (ELISA) was developed using the anti-vitellogenin antibody. The detection limit for the purified standard vitellogenin by the ELISA method was $10\,ng\,ml^{-1}$. Plasma samples were diluted a minimum of 100-fold, and the detection limit was therefore $1\,\mu g\,ml^{-1}$.

Histological examination
Light microscopy

After fixation in Lillie's fixative for at least 1 week, the testes were dehydrated through a series of graded ethanol solutions (50–99 %) and toluene. The testes were embedded in paraffin, and $5\,\mu m$ sections were cut starting from the side opposite the sperm duct and through the testis, resulting in transverse sections of the seminiferous lobules. The seminiferous lobules from some of the testes were also cut longitudinally. The sections were stained in Mayer's haematoxylin and eosin-Y. The sections were analysed using a Leitz microscope and were categorised into one of four groups as shown in Table 1.

Electron microscopy

Small (2–3 mm thick) slices of testis tissue were fixed in 3 % glutaraldehyde in $0.1\,mol\,l^{-1}$ cacodylate or phosphate buffer for 2 h, rinsed in the same 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–propylenoxid series and embedded in Araldite. Sections (1 and $2\,\mu m$ thick) were stained with Toluidine Blue and used for orientation. Ultrathin sections were contrasted with uranyl acetate and lead citrate and studied in a Jeol JEM-100 SX transmission electron microscope.

Enzyme and protein measurements

A sample (25–35 mg) from the cranial end of the frozen testis was weighed and homogenized on ice in 0.5 ml of ice-cold phosphate buffer ($10\,mmol\,l^{-1}$, pH 7.0). The homogenates were centrifuged for 10 min at $20\,000\,g$ at 4 °C. 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 with a modification of the method of Szasz *et al.* (1974) using a commercial kit 'γ-GT new-MPR 2' from Boehringer Mannheim. The enzyme activity was assayed at 37 °C, which is a high temperature for fish. However, the enzyme assay performed in our laboratory had been validated in preliminary experiments by measuring enzyme activity in the supernatant of a testicular homogenate from rats. When the assay was subsequently performed on fish supernatant at 37 °C, the reaction was still stable and linear. Testing at 25 °C did not result in higher or lower enzyme activity. The reaction mixture contained Tris buffer ($100\,mmol\,l^{-1}$, pH 8.25), L-γ-glutamyl-3,4-nitroanilide ($2.9\,mmol\,l^{-1}$) and glycylglycine ($100\,mmol\,l^{-1}$). The amount of 3-carboxy-4-nitroaniline 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 (units g^{-1} testis) and as milliunits per milligram of total testis protein (munits mg^{-1} protein). One milliunit is defined as the amount of enzyme that forms 1 nmole of product per minute under assay conditions. The concentration of total protein in the supernatant was determined according to the method described by Bradford (1976).

Chemicals

Nonylphenol: technical nonylphenol (4-t-nonylphenol hydroxyl, no. 253) was obtained from Fluka Chemika. Estradiol: 17β-oestradiol [1,3,5(10)-estratriene-3,17β-diol] was obtained from Sigma Chemical Co.

Statistical analyses

Values are expressed as means ± S.E.M. Data were tested for normality and variance homogeneity and, if necessary, were log_{10}-transformed prior to analysis. HSI and GSI were square-root-arcsine-transformed to obtain normality. One-way analysis of variance (ANOVA) followed by a Tukey test was used to determine the effect of different treatments on vitellogenin concentration, GSI, HSI and γ-GTP activity. Correlation analysis (Pearson) was carried out to examine the significance of the relationships between vitellogenin concentrations and GSI and between total testis protein concentration and γ-GTP activity. In order to make statistical analysis possible, control values of plasma vitellogenin concentration (measured using ELISA) lying below the detection limit were set to $1 \mu g \, ml^{-1}$.

Results

Vitellogenin

A qualitative identification of vitellogenin was made by gel electrophoresis (Fig. 1). For all the nonylphenol- and oestradiol-treated groups, electrophoresis revealed a distinct protein band between the protein markers at 440 kDa and 669 kDa and at the same molecular mass as for purified vitellogenin from a female *Z. viviparus* exhibiting natural vitellogenesis (positive control).

For the control males, no corresponding protein band was seen. Gel electrophoresis is not a quantitative method for identification of vitellogenin. However, the vitellogenin band from the fish treated with $10 \mu g \, g^{-1}$ nonylphenol (Fig. 1, n) appeared less dense than the bands from the other treated fish, indicating a smaller response to $10 \mu g \, g^{-1}$ nonylphenol treatment than to $100 \mu g \, g^{-1}$ nonylphenol treatment.

For quantitative measurements of vitellogenin, the ELISA method was used. This analysis demonstrated a significant ($P<0.001$) increase in vitellogenin concentration for all the treated groups (Fig. 2). In control fish, the vitellogenin concentration was $1–5 \mu g \, ml^{-1}$, whereas in the groups treated with $100 \mu g \, g^{-1}$ nonylphenol or oestradiol, the vitellogenin concentration was $8–11 \, mg \, ml^{-1}$. This corresponds to an approximately 10 000-fold increase. Treatment with $10 \mu g \, g^{-1}$ nonylphenol resulted in a significant ($P<0.001$) increase in vitellogenin concentration to approximately $2 \, mg \, ml^{-1}$, corresponding to an approximately 1000-fold increase. The fish exposed to $1 \, mg \, l^{-1}$ nonylphenol in sea water in August 1996 had high vitellogenin concentrations of approximately $8 \, mg \, ml^{-1}$. In the experiment of June 1995, two nonylphenol-treated fish had much lower vitellogenin concentrations than the other treated fish and showed no response in the other analyses presented in this paper. Because of this, they were not included in the data analysis.

Testis mass and macroscopic appearance

No significant difference in the hepatosomatic index (HSI) was observed for any of the nonylphenol- or oestradiol-treated groups compared with controls in any of the experiments.
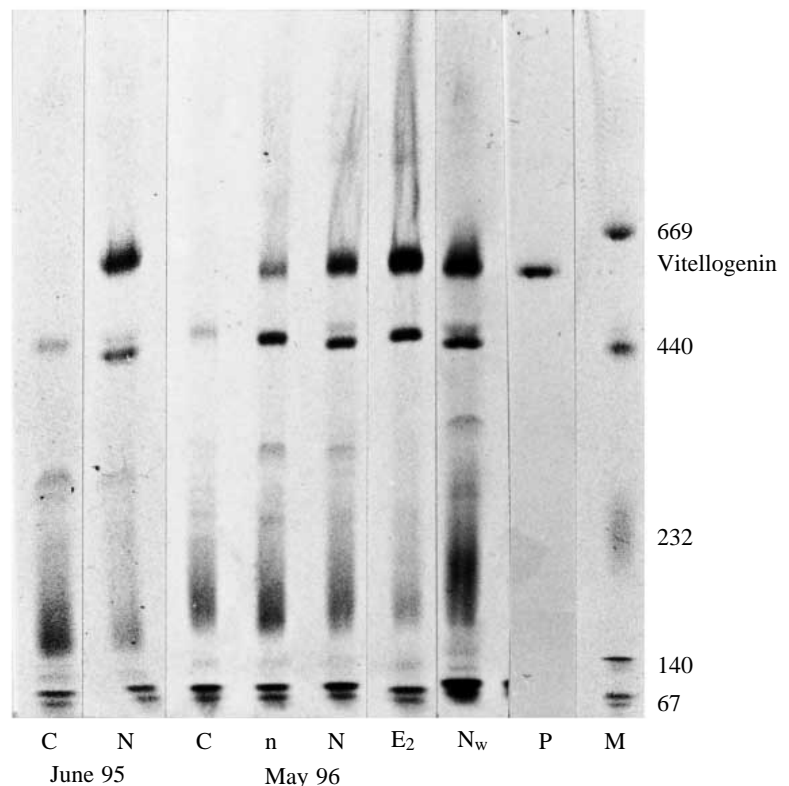

Fig. 1. Electrophoresis gel showing the electrophoretic protein fractions (including vitellogenin) in the plasma from representative male *Zoarces viviparus* of each experimental group in June 1995, May 1996 and August 1996. C, control; n, $10 \mu g \, g^{-1}$ nonylphenol; N, $100 \mu g \, g^{-1}$ nonylphenol; E_2, $0.5 \mu g \, g^{-1}$ oestradiol; N_w, $1 \, mg \, l^{-1}$ nonylphenol from August 1996; P, purified vitellogenin from female *Z. viviparus*. M, marker containing the following proteins: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa) and albumin (67 kDa).

Fig. 2. Effects of nonylphenol and oestradiol on plasma vitellogenin concentration in male *Zoarces viviparus* for the experiments in June 1995 and May 1996 after 25 days of treatment and in August 1996 after 21 days of treatment. Values are means ± S.E.M. *** denotes a significant difference from the control value (*P*<0.001) for the respective experiment. Groups are identified as in Fig. 1. The number at the base of the column denotes the number of fish.
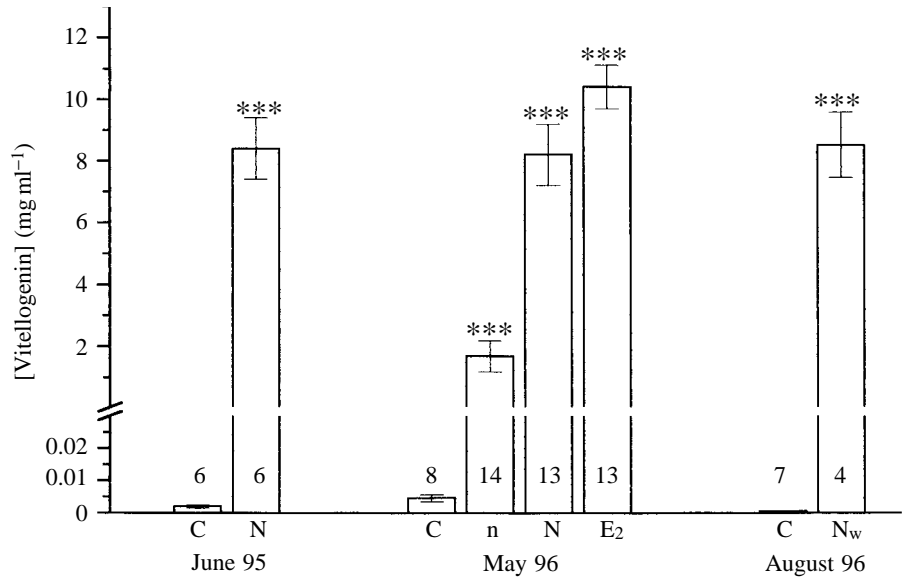
Treatment with nonylphenol ($100 \mu g\,g^{-1}$ and $1\,mg\,l^{-1}$) or oestradiol resulted in significant (*P*<0.001) reductions in the gonadosomatic index (GSI) compared with the respective control value (Fig. 3). Fish treated with the low dose ($10 \mu g\,g^{-1}$) of nonylphenol also showed a significant (*P*<0.01), but less marked, reduction in GSI. In this group, seven of the 15 fish had normal GSIs, while six had a GSI that was approximately one-third of the control value.

Fig. 4 shows the gross external morphology of the testis of control and treated fish. Macroscopically, the control fish in June 1995 and May 1996 had large, white and soft testes (Fig. 4, C) and milt was present. The fish treated with $100 \mu g\,g^{-1}$ nonylphenol, the oestradiol-treated fish and six of the 15 fish treated with $10 \mu g\,g^{-1}$ nonylphenol had small, grey and firm testes (Fig. 4, N and E$_2$). The amount of milt

was reduced compared with that in control fish, or milt was absent.

A plot of the gonadosomatic index as a function of the plasma vitellogenin concentration (Fig. 5) showed that these parameters were significant negatively related (*r*=−0.825, *P*<0.001, *N*=60).

Histological examination

Light microscopy

Histological examination of the testes revealed severe effects on testicular structure following injection with nonylphenol or oestradiol. Features of the structure of the seminiferous lobules of the testes in control and treated fish are listed in Table 1, and representative light micrographs of testes from control and treated fish are shown in Fig. 6.

Fig. 3. Effects of nonylphenol and oestradiol on the gonadosomatic index (GSI) in male *Zoarces viviparus* for the experiments in June 1995 and May 1996 after 25 days of treatment and in August 1996 after 21 days of treatment. Values are means ± S.E.M. Asterisks denote a significant difference from the control value for the respective experiment: **P*<0.01; ***P*<0.001. Groups are identified as in Fig. 1. The number at the base of the column denotes the number of fish.
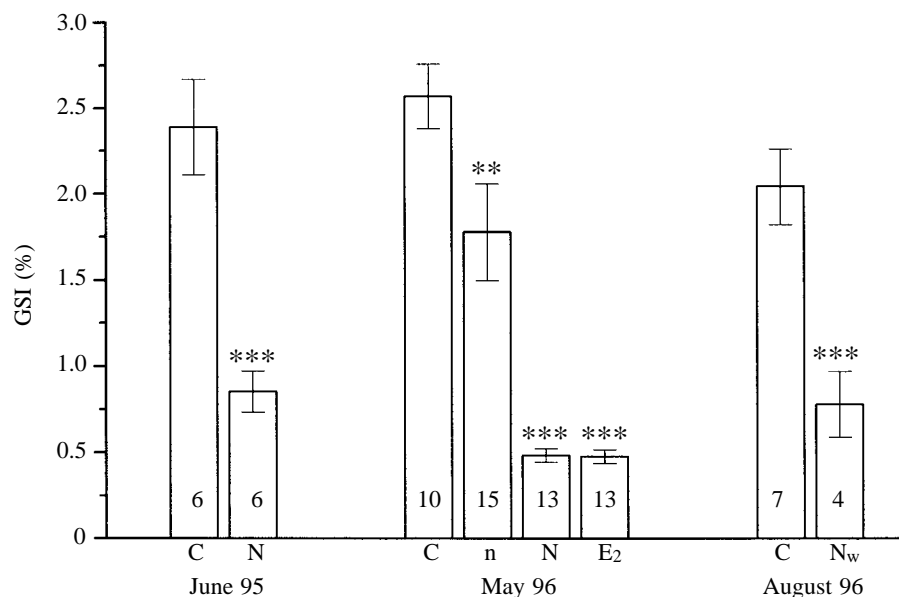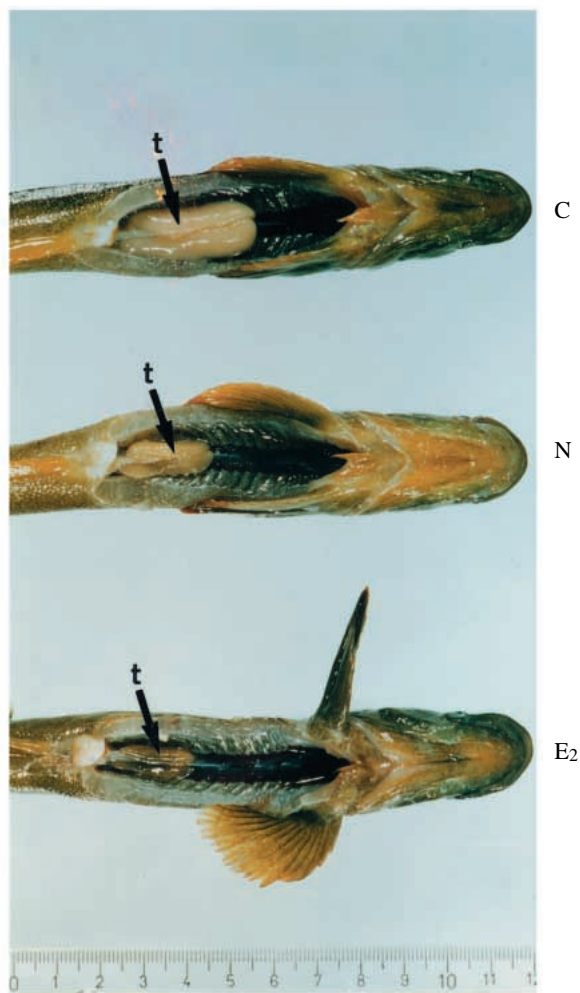
Fig. 4. Photographs of male *Zoarces viviparus* showing the external morphology of the testis (t) of representative fish from three experimental groups in May 1996: control (C), $100\,\mu g\,g^{-1}$ nonylphenol (N) and oestradiol (E$_2$). The scale is in centimetres.
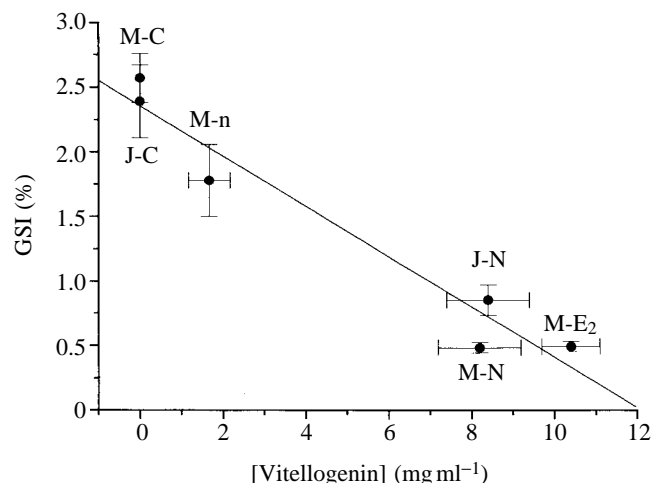

Fig. 5. The relationship between plasma vitellogenin concentration and the gonadosomatic index (GSI) ($r=-0.825$, $P<0.001$, $N=60$). Values are means ± S.E.M. Data from both experiments in June 1995 (J) and May 1996 (M) are included. Groups are identified as in Fig. 1.

In the testes of *Z. viviparus*, the germinal compartments (seminiferous lobules) terminated in the periphery of the testis (Fig. 6A). Single spermatogonia were occasionally seen throughout the seminiferous lobules, but spermatogonia were mainly located at the terminal end of the lobules (Fig. 6A). This indicated that the testis of *Z. viviparus* is of the unrestricted spermatogonial type as opposed to the restricted spermatogonial type typical of viviparous fish (Grier *et al.* 1980; Grier, 1993).

Light micrographs of the seminiferous lobules of control fish sampled in June indicated that, at this time of the year, spermatogenesis is either nearly complete or complete, because no (or very few) spermatocysts containing maturing germ cells were seen (Fig. 6A,B). The lumen of the seminiferous lobules contained few spermatozoa or was empty. This could be an artefact, because part of the sample might have been lost as a result of the liquidity of the milt when the testis was cut for fixation. Fixation of testicular tissue in coagulative fixatives

followed by processing through paraffin may also result in tissue-shrinkage artefacts (H. J. Grier, personal communication). The epithelium of the seminiferous lobules was lined with cuboidal or even columnar epithelial cells (Fig. 6B), which we identified as Sertoli cells (see electron microscopic results below). From some Sertoli cells (especially the columnar ones), it seemed that material was secreted into the lumen of the lobules. In the epithelial layer of Sertoli cells, single spermatogonia were occasionally embedded (Fig. 6B). The nonylphenol-treated fish had a different testicular structure from the control fish (Table 1). In these treated fish, which also showed a reduction in testis mass, the seminiferous lobules were filled with spermatozoa (and/or late spermatids) (Fig. 6D). Very few or no spermatocysts were seen. Spermatogonia were located in the periphery of the lobules. A few single spermatogonia were occasionally observed along the lobules. The Sertoli cells lining the seminiferous lobule walls were very squamous (Fig. 6E). These Sertoli cells seemed to have phagocytozed many residual spermatozoa.

The majority of the control fish sampled in May were in active spermatogenesis. Of the control fish, 70 % had testes containing numerous spermatocysts with maturing germ cells at various stages (in each cyst, all germ cells were in the same developmental stage) (Fig. 6C). The cysts contained predominantly spermatocytes and/or spermatids. The lumen of the lobules contained some spermatozoa and/or late spermatids. Squamous Sertoli cells surrounded the germ cells forming the cysts. Cuboidal Sertoli cells were also observed lining the walls of the seminiferous lobules (Fig. 6C). The remaining 30 % of the fish had a testicular structure similar to that of the June control fish (Fig. 6B). Fish from the beginning of the experiment (results not shown) had testes in which the lobules contained many spermatocysts with germ cells at an early stage (spermatogonia or spermatocytes). Nonylphenol as well as oestradiol treatment resulted in an altered testicular

structure (Table 1). After treatment with $100\,\mu\mathrm{g\,g}^{-1}$ nonylphenol or oestradiol, some fish (38 % and 31 %, respectively) had the same testicular structure as the nonylphenol-treated fish from the June sampling (Fig. 6E). The majority showed degeneration of the testes, and normal lobular arrangements were difficult to locate or absent (Fig. 6G). Most of these testes contained very few or no spermatozoa. In some of the testes, the cells occupying the lumen could be identified

as degenerated Sertoli cells and/or germ cells (Fig. 6F). The overall histological appearance of testes from the control and the high-dose nonylphenol- and oestradiol-treated fish in the May experiment indicated that approximately 30 % of these fish were at late spermatogenesis and had therefore responded like the fish from the June experiment (see Table 1).

After low-dose ($10\,\mu\mathrm{g\,g}^{-1}$) nonylphenol treatment, approximately half of the fish showed no change in GSI or in
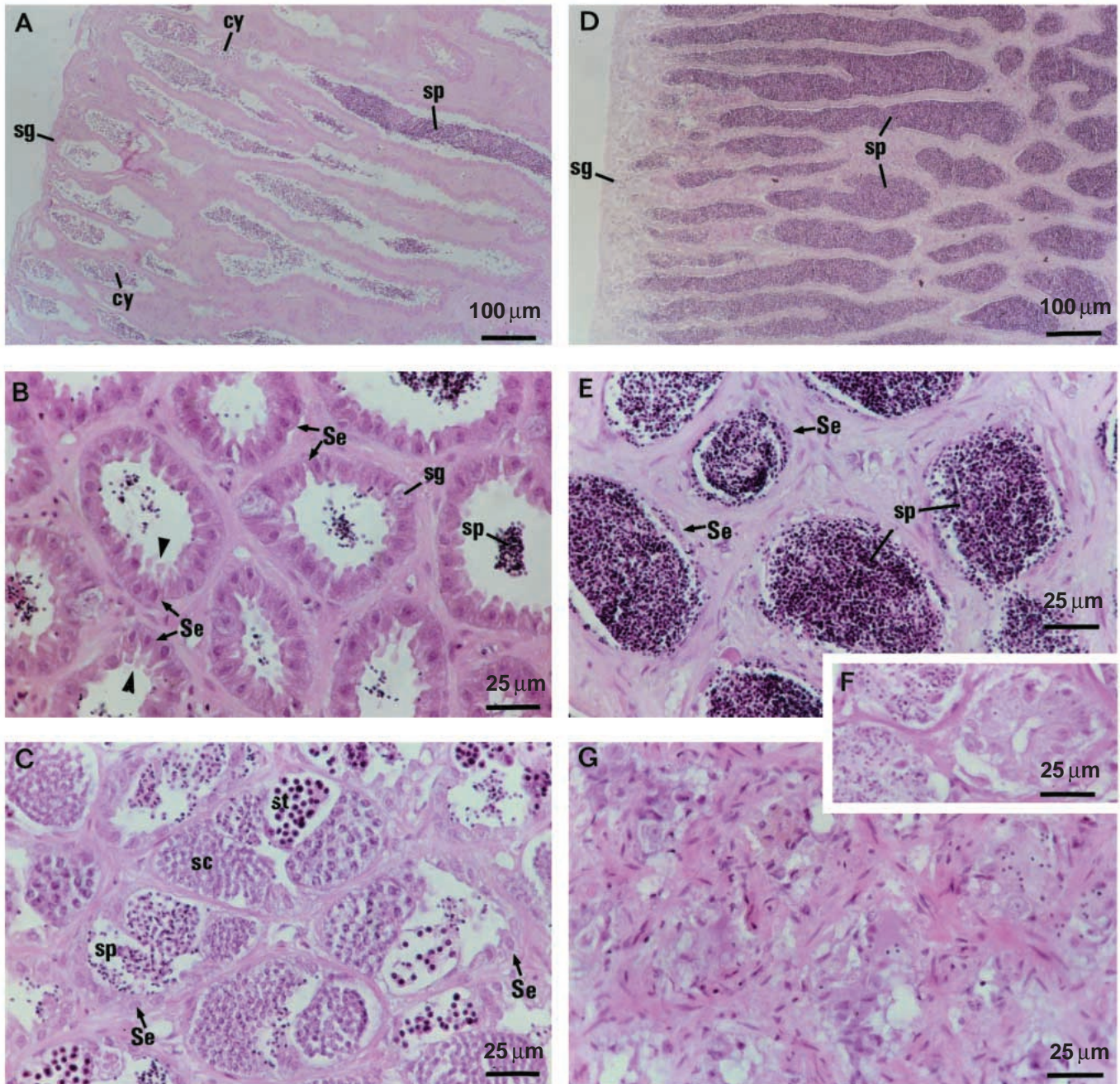

Fig. 6. Light micrographs of the testis of male *Zoarces viviparus* from the experiments in June 1995 and May 1996 showing the seminiferous lobules in control fish (A–C) and in nonylphenol- or oestradiol-treated fish (D–G). (A) Longitudinal section (control, June). (B) Transverse section (control, June). (C) Transverse section (control, May). (D) Longitudinal section (nonylphenol $100\,\mu\mathrm{g\,g}^{-1}$, June). (E) Transverse section (nonylphenol $100\,\mu\mathrm{g\,g}^{-1}$, May). (F) Transverse section (nonylphenol $100\,\mu\mathrm{g\,g}^{-1}$, May). (G) Transverse section (oestradiol, May). cy, spermatocysts; sp, spermatozoa; sg, spermatogonium; Se, Sertoli cell; sc, spermatocytes; st, spermatids. Arrowheads in B indicate possible secretory material from Sertoli cells.

Table 1. *Structure of the seminiferous lobules of the testis of* Zoarces viviparus *for the experiments carried out in June 1995 and May 1996*

| | Percentage of total number of testes | | | | | |
| | June 1995 | | May 1996 | | | |
Observed structures of the seminiferous lobules	C (N=6)	N (N=6)	C (N=10)	n (N=13)	N (N=13)	E$_2$ (N=13)
(1) SLs with cuboidal and/or columnar Sertoli cells; few or no spermatocysts; low to medium numbers of spermatozoa (see Fig. 6B)	100	0	30	8	0	0
(2) SLs with medium to high number of spermatocysts with germ cells at various stages (sg, sc and st); low to medium numbers of spermatozoa; many of the SLs also contain cuboidal Sertoli cells (see Fig. 6C)	0	0	70	46	0	0
(3) SLs filled with spermatozoa; few or no spermatocysts; very squamous or no Sertoli cells (in some testes, some areas of SLs are also degenerated) (see Fig. 6E)	0	100	0	23	38	31
(4) Degenerated SL structure; no spermatocysts; very few or no spermatozoa (see Fig. 6G)	0	0	0	23	62	69

After light microscopical examination, all the testes were placed into one of four different categories (1–4). Categories 1 and 2 are testes with a normal appearance and categories 3 and 4 are testes with an abnormal appearance.

The results are expressed as a percentage of the total number of testes examined in each group.

C, control; n, $10 \mu g\,g^{-1}$ nonylphenol; N, $100 \mu g\,g^{-1}$ nonylphenol; E$_2$, $0.5 \mu g\,g^{-1}$ oestradiol.

sg, spermatogonia; sc, spermatocytes; st, spermatids; SL, seminiferous lobule.

macroscopic appearance, and there appeared to be no effect on the testicular structure (8 % looked similar to June controls and 46 % looked similar to the majority of the May controls) (see Table 1). In the other half, which all had a reduced testis mass, the testes were either degenerated (Fig. 6G) or had lobules filled with spermatozoa and/or late spermatids (Fig. 6E).

Electron microscopy

Electron microscopy was used to investigate the cytology of the cells of the wall of the testis lobule. It was possible to distinguish between germ cells and Sertoli cells in the seminiferous epithelium in both the control and the treated fish.

In the control fish from June, the only cell type found in the single-layered testis lobule epithelium was the Sertoli cell (Fig. 7A–C). The wall consisted of cuboidal cells resting on the basement membrane (the cells were sometimes detached from the membrane). Spermatozoa were seen in the lumen of the lobule. Adjacent Sertoli cells were attached to each other by a well-developed junctional complex consisting of prominent tight junctions and desmosomes. The nucleus of the cell was round, mostly regular in shape and contained a distinct nucleolus. In the cytoplasm, small spherical or oblong mitochondria and well-developed rough endoplasmic reticulum were found. In a few specimens, occasional phagocytozed spermatozoa were observed.

The testes of the control fish from May contained germ cells (Fig. 7D) at various spermatogenic stages, organized into cysts. In these testes, the Sertoli cells were easily recognized (Fig. 7D,E). They were found in the periphery of the cyst, surrounding the germ cells with long cytoplasmic processes. Their cytoplasm was dense and they appeared darker than the germ cells. They were resting on the basement membrane and pushed the germ cells towards the centre of the lumen. The nucleus of the Sertoli cell at this stage was irregular in shape and deeply indented. Well-developed rough as well as smooth endoplasmic reticulum were present, and spherical mitochondria were scattered in the cytoplasm. Lysosomes of different sizes and shape were seen. The Sertoli cells were attached to each other by tight junctions and desmosomes.

The spermatogonia from the control male (Fig. 8A) appeared pale compared with the Sertoli cells and were regular in shape with rounded nuclei lacking indentations. The nucleoli were distinct. The spermatogonia were surrounded by Sertoli cells and thus not in contact with the basement membrane.

In the fish treated with either nonylphenol or oestradiol, the Sertoli cells contained numerous phagocytozed spermatozoa (Fig. 8B,C). In some of the specimens, the entire content of the lumen seemed to be Sertoli cells with 'ingested' sperm cells. Vacuoles of different shape and content were present in the cytoplasm. A well-developed rough endoplasmic reticulum and numerous tight junctions between the cells were observed after treatment.

γ-GTP activity

In rats, γ-glutamyl transpeptidase has been used as a specific marker of Sertoli cells. In the present study, the activity of γ-GTP was measured in the testes of the experimental fish and expressed both as units g^{-1} testis and as munits mg^{-1} protein (Fig. 9). Treatment with either $100 \mu g\,g^{-1}$ nonylphenol or

oestradiol resulted in a significantly ($P<0.001$) lower activity of γ-GTP expressed either as units g^{-1} testis or as munits mg^{-1} protein. Treatment with the low dose of nonylphenol (10 μg g^{-1}) had no significant effect on γ-GTP activity expressed as units g^{-1} testis. However, when expressed as munits mg^{-1} protein, a significant ($P<0.05$), but smaller,

reduction was observed compared with fish treated with the high dose of nonylphenol (100 μg g^{-1}).

The total testis protein concentration was significantly increased after treatment with 100 μg g^{-1} nonylphenol or oestradiol compared with the respective controls (results not shown). Testes showing a degenerated structure and testes
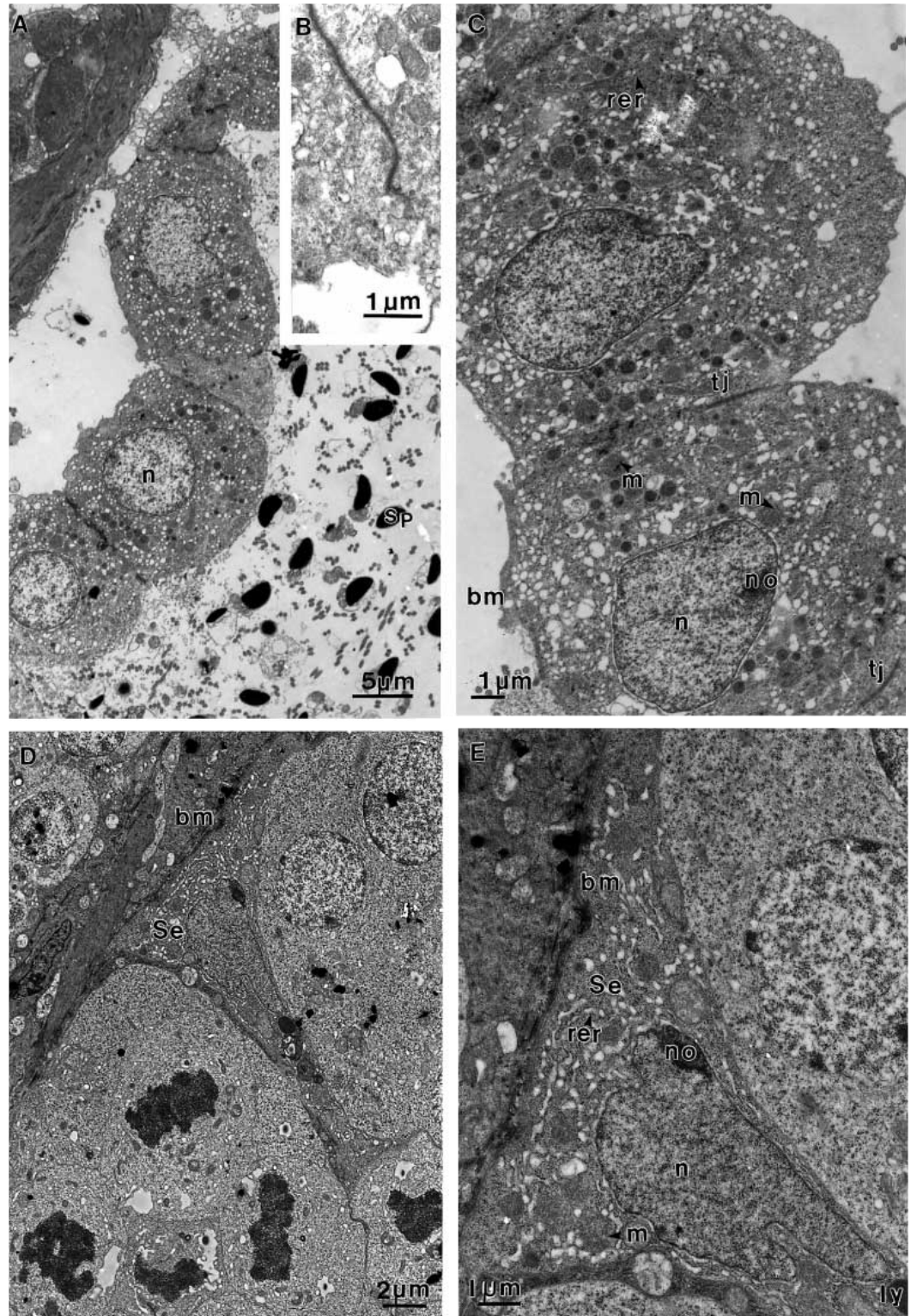

Fig. 7. Electron micrographs showing the Sertoli cells in testes from control male *Zoarces viviparus* from the experiments in June 1995 (A–C) and May 1996 (D–E). (A) A single layer of cuboidal Sertoli cells. (B) High magnification of a prominent tight junction between two adjacent cuboidal Sertoli cells. (C) Two adjacent cuboidal Sertoli cells. (D) A Sertoli cell. (E) Higher magnification of the Sertoli cell from D. n, nucleus; m, mitochondrion; rer, rough endoplasmic reticulum; tj, tight junction; bm, basement membrane; no, nucleolus; Se, Sertoli cell; ly, lysosome; sp, spermatozoon.
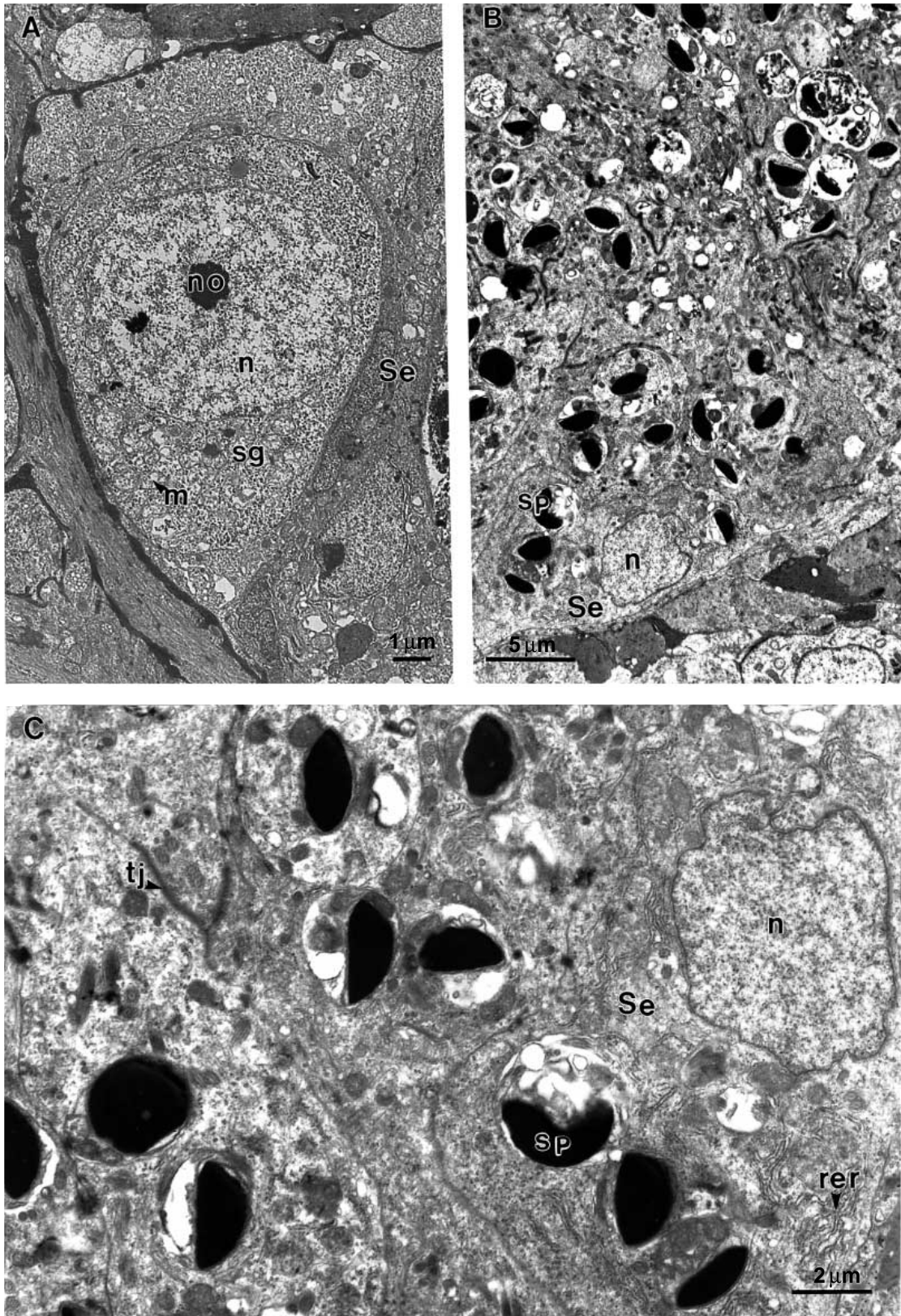
Fig. 8. Electron micrographs showing (A) a spermatogonium (control, June), (B) Sertoli cells (nonylphenol $100\,\mu g\,g^{-1}$, June) and (C) a higher magnification of the Sertoli cells from B with a phagocytosed spermatozoan. sg, spermatogonium; n, nucleus; m, mitochondrion; rer, rough endoplasmic reticulum; tj, tight junction; no, nucleolus; Se, Sertoli cell; sp, spermatozoon.
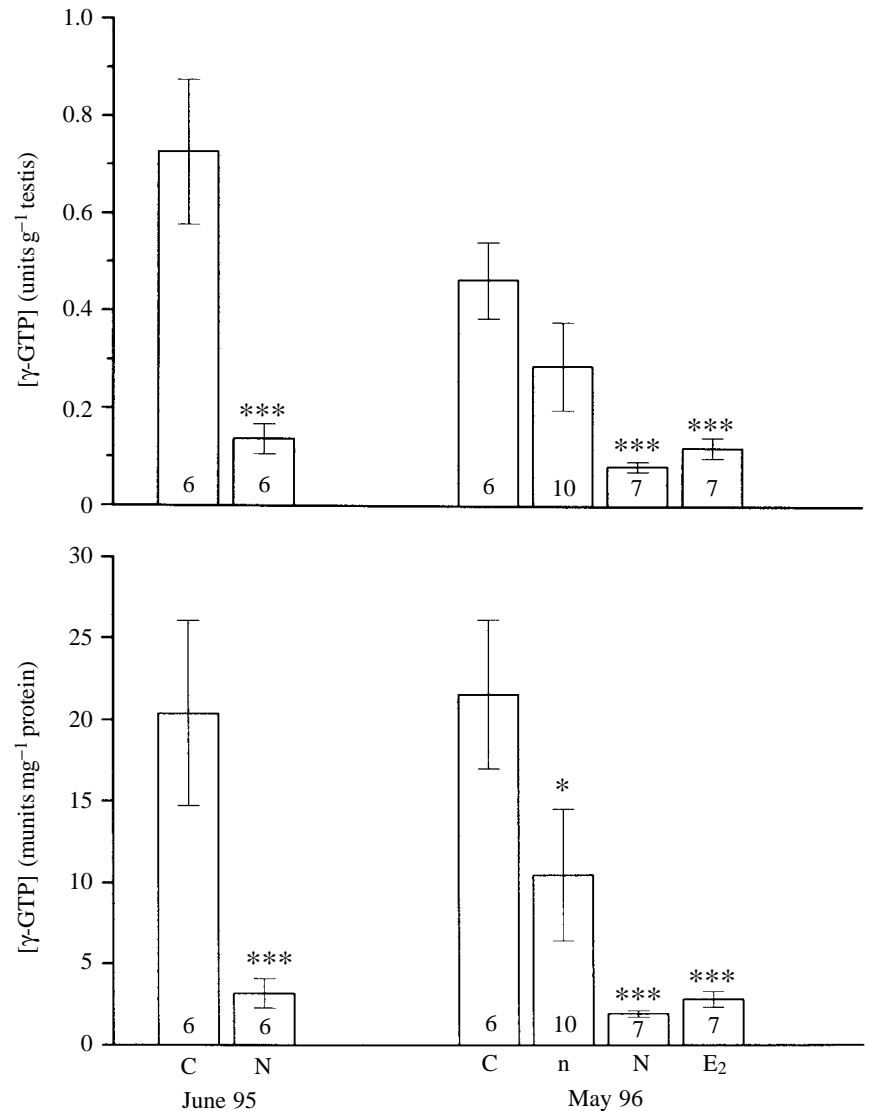
Fig. 9. Effects of nonylphenol and oestradiol treatment on γ-glutamyl transpeptidase (γ-GTP) activity expressed as units g^{-1} testis and as munits mg^{-1} protein for the experiments in June 1995 and May 1996 after 25 days of treatment. Values are means ± s.e.m. Asterisks denote a significant difference from the control value for the respective experiment: *$P<0.05$; ***$P<0.001$. Groups are identified as in Fig. 1. The number at the base of the column denotes the number of fish.

filled with spermatozoa in the treated groups showed the same pattern of total testis protein concentration. The concentration was generally higher (for both control and treated fish) in the June experiment than in the experiment performed in May. There was a significant negative correlation between total testis protein concentration and γ-GTP activity in both the June ($r=-0.855$, $P<0.001$, $N=12$) and May ($r=-0.600$, $P<0.001$, $N=30$) experiments.

Discussion

In the present study, treatment by injections with low as well as high doses of nonylphenol and exposure to nonylphenol in the water resulted in a marked increase in the plasma vitellogenin concentration in male *Z. viviparus*. Our results for male *Z. viviparus* provide confirmation that nonylphenol mimics the effects of oestradiol on vitellogenin synthesis and acts as an oestrogen. This observation supports previous work concerning the effect of nonylphenol in other teleosts: rainbow trout *Oncorhynchus mykiss* (Jobling *et al.* 1996), Atlantic salmon *Salmo salar* (Madsen *et al.* 1997) and flounder *Platichthys flesus* (Christensen *et al.* 1995). The increase in plasma vitellogenin concentration in response to the low dose of nonylphenol (10 μg g^{-1}) was less marked. This indicated that nonylphenol may stimulate vitellogenin synthesis in a dose-dependent manner as reported for rainbow trout (Jobling and Sumpter, 1993; Jobling *et al.* 1996).

A marked reduction in the testis mass (GSI) in the fish during active spermatogenesis and at late spermatogenesis was observed after nonylphenol as well as oestradiol treatment. The effect was more pronounced in the fish during active spermatogenesis (in May). Other studies have shown that oestrogen treatment causes reduced testis mass in male fish (Billard *et al.* 1981; Chang *et al.* 1995). The study of Jobling *et al.* (1996) reported that nonylphenol exposure (30 μg l^{-1}) for 3 weeks results in a decrease in testis mass in rainbow trout during active spermatogenesis. Only approximately half of the *Z. viviparus* treated with the low dose of nonylphenol

($10 \mu g\, g^{-1}$) showed a change in testis mass (GSI). However, there was a significant correlation between the oestrogenic potency (based on the vitellogenin level) and the reduction in testis mass. Nonylphenol ($100 \mu g\, g^{-1}$) had the same effect as oestradiol. These findings indicate that nonylphenol may have an oestrogenic effect on testis mass.

Few studies have been carried out on the histology of the testis of male *Zoarces*. Kristofferson and Pekkarinen (1975) reported that in *Z. viviparus* in June/August the lobular walls of the testis are coated with a 'lobule boundary cell epithelium'. A recent study of the very similar species *Zoarces elongatus* presented light micrographs showing cuboidal epithelial cells lining the lobular walls (Koya *et al.* 1993). Our microscopical examination of control male *Z. viviparus* from June revealed similar cuboidal or even columnar epithelial cells lining the lobular walls. On the basis of the known ultrastructural characteristics of Sertoli cells (Grier, 1993; Pudney, 1993), we identified these cells as Sertoli cells. They contained phagocytozed sperm cells, they had prominent tight junctions between adjacent cells, their cytoplasm contained lysosomes and they formed the spermatocysts. The presence of such cuboidal (or even columnar) Sertoli cells is unusual for perciform fish (H. J. Grier, personal communication). They have been observed in the viviparous perciform fish *Cymatogaster aggregata* (Wiebe, 1969) and in other viviparous teleosts, for example the poeciliids (Grier *et al.* 1980; Pudney, 1993).

The histological examination of the testes of *Z. viviparus* revealed severe effects on the testicular structure of the seminiferous lobules in response to the high dose nonylphenol as well as to oestradiol treatment. It should be emphasized that the histological alterations in the high-dose group were also observed in approximately 50 % of the fish injected with the low dose of nonylphenol. The period of spermatogenesis, when the germ cells are developing, seemed to be more sensitive to oestrogenic treatment. This is supported by other studies of the effects of alkylphenol (Jobling *et al.* 1996) and of natural oestrogens (Billard *et al.* 1981), both of which reported an absence of effects on spermatogenesis in fully mature and regressing male trout, while oestrogen treatment (*via* diet) during active spermatogenesis resulted in marked testicular regression (Billard *et al.* 1981). Species, age, dose, duration of treatment, type of oestrogen and method of application may be expected to cause variations in the effects. The period of sex differentiation in fish has also been demonstrated to be sensitive to oestrogenic compounds such as 4-nonylphenol (Gray and Metcalfe, 1997) and 4-*t*-pentylphenol (Gimeno *et al.* 1996).

Both nonylphenol and oestradiol treatment affected the Sertoli cells. The failure of Sertoli cell function is believed to be especially critical during spermatogenesis, when the germ cells are under development, as regulation of spermatogenesis is one of many important roles of the Sertoli cells. It is possible that the degeneration of the germ cells observed in *Z. viviparus* during active spermatogenesis (May) might be a result of the altered function of the degenerated Sertoli cells. The Sertoli cells secrete the seminiferous tubule fluid in mammals and are also believed to secrete part of the seminal fluid in some fish (Billard *et al.* 1982). The observation that the amount of milt in the treated groups was reduced during late spermatogenesis indicated that a seminal fluid synthezising/secretory function of the Sertoli cells might also be inhibited by nonylphenol and oestradiol treatment.

The mechanism(s) whereby oestrogens and oestrogenic chemicals such as nonylphenol cause inhibitory and/or degeneratory effects on testicular development and structure is still unknown. A possible explanation is a direct effect on the Sertoli cells that may result in the changes described above. Such a direct effect on Sertoli cells is supported by the fact that oestrogen receptors are found in Sertoli cells in mammals (Nakhla *et al.* 1984), and preliminary evidence suggests that they are also found in the dogfish *Squalus acanthias* (Dubois and Callard, 1989). In the eel *Anguilla anguilla*, ethynyloestradiol inhibits development or causes degeneration of the Sertoli cells, and oestrogen appears to affect first the somatic components of the gonad and then the germ cells (Colombo and Grandi, 1995). Oestrogenic chemicals may also exert their effects directly on the testis *via* inhibition of androgen synthesis (Trudeau *et al.* 1993). Oestrogenic chemicals may act indirectly *via* the hypothalamo-pituitary-gonad axis, inhibiting (or stimulating) the synthesis and secretion of gonadotropin releasing hormone and/or gonadotropin(s). Regardless of the mechanism(s) of action, it is obvious that nonylphenol had severe effects on the testicular structure and affected the Sertoli cells of male *Z. viviparus*, resulting in impairment of spermatogenesis. Oestradiol had the same effect on the testis, indicating that the effects of nonylphenol were oestrogenic. However, the possibility of toxic effects cannot be ruled out.

The activity of γ-glutamyl transpeptidase (γ-GTP) in the testis of *Z. viviparus* showed a marked reduction after treatment with the high dose of nonylphenol and with oestradiol. A significant effect was also observed in response to the low dose of nonylphenol when enzyme activity was expressed as munits mg^{-1} protein. In mammalian testis, prolonged oestrogen therapy can cause a decrease in γ-GTP activity (Steinberger and Steinberger, 1977). In fish, the presence of γ-GTP has been reported in various tissues (Teh and Hinton, 1993), but to our knowledge no study has reported the presence of γ-GTP in the testis of fish. γ-Glutamyl transpeptidase in the testes is primarily found in the Sertoli cells of mammals and has been used as a Sertoli cell marker in rats (Hodgen and Sherins, 1973; Lu and Steinberger, 1977; Fukuoka *et al.* 1990). The observed decrease in γ-GTP activity in the testis of *Z. viviparus* indicated that Sertoli cell function might be affected. The consequences of this altered enzyme activity are not known. The finding of a decrease in γ-GTP activity was in accordance with the histological results of altered Sertoli cell structure after nonylphenol as well as oestradiol treatment. Cytochemical studies are needed, however, to localize the enzyme specifically to the Sertoli cells in fish.

In conclusion, on the basis of the significant increase in vitellogenin synthesis, the present study shows that nonylphenol is oestrogenic in fish. Furthermore, our results clearly show that low as well as high doses of nonylphenol have severe effects on testis growth, on testicular structure as a whole and on the cytology of the Sertoli cells in male *Zoarces viviparus*. Nonylphenol and 17β-oestradiol exerted similar effects on the testes. It is therefore likely that the observed effects of nonylphenol on the testes are oestrogenic.

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