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Effects of waterborne exposure of octylphenol and oestrogen on pregnant viviparous eelpout (*Zoarces viviparus*) and her embryos *in ovario*

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Summary

Exposure to oestrogenic chemicals (xeno-oestrogens) may have severe effects on embryonic development. The present study investigates whether the oestrogenic endocrine disruptor 4-*tert*-octylphenol (4-tOP) or 17β-oestradiol (E_2) is accumulated in the viviparous fish the eelpout (*Zoarces viviparus*) and transferred to the embryos *in ovario* and subsequently affects embryonic development, including gonadal differentiation. Pregnant eelpouts were exposed to nominal concentrations of 25 μg l^{-1} or 100 μg l^{-1} 4-tOP (OP25 or OP100, respectively) or 0.5 μg l^{-1} E_2 in water. During 4-tOP exposure, the compound accumulated in both plasma and ovarian fluid in a concentration-dependent manner. In the mother fish, the oestrogenic biomarkers, vitellogenin (Vtg) in plasma, Vtg mRNA in liver and oestrogen-binding activity in liver, were all induced by 4-tOP (and by E_2) at an actual concentration of 14 μg l^{-1}. E_2 and 4-tOP were examined for their potency to disturb the maternal–foetal trophic relationship by disturbing the physiology of the ovary and by changing the distribution of essential nutrients normally transported to embryos during pregnancy. After exposure to E_2 or 4-tOP, calcium was depleted from the ovarian fluid and the level of free amino acids available in maternal plasma was decreased. A marked overall effect on ovarian components, including the ovarian sac, ovarian fluid and embryonic mass, was evident. Embryonic growth was significantly decreased, which might in part be attributed to disturbances of the maternal–foetal trophic relationship. Marked inductions of Vtg mRNA and Vtg protein, determined by RT-PCR and immunohistochemistry, respectively, were found in embryos from the OP100 group – the only group to show considerable accumulation of an oestrogenic compound in the ovarian fluid. A different pattern of gonadal development was found in embryos from the OP100 group compared with embryos from the control, OP25 or E_2 groups, in which approximately 50% had normal ovaries and 50% had normal presumptive male gonads. In the OP100 group, 46% had normal ovaries but, in contrast to controls, only 22% had normal presumptive male gonads, whereas the remaining 32% had abnormal male gonads with structures resembling the endo-ovarian cavity of a female gonad. As oestrogen receptor (ER) expression was detected by *in situ* hybridisation in early differentiating gonads, these effects could be mediated by direct interaction of the xeno-oestrogens with gonadal ER. In conclusion, this study indicates that the xeno-oestrogen 4-tOP can be transferred from the water *via* the mother fish to the ovarian fluid and can subsequently disturb the maternal–foetal trophic relationship and cause severe effects on embryonic development, including gonadal differentiation *in ovario*.

Key words: development, embryo, endocrine disrupter, oestrogen, fish, gonad, histology, maternal–foetal relationship, alkylphenol, oestrogen receptor, sex differentiation, vitellogenin mRNA, immunohistochemistry, xeno-oestrogen, viviparous, *Zoarces viviparus*, eelpout.

Introduction

Over recent years, there has been an increasing number of reports suggesting that exposure to environmental pollutants may disrupt normal endocrine functions and cause various reproductive effects in human and wildlife populations (Tyler et al., 1998). Increased levels of the yolk-precursor protein vitellogenin (Vtg), a widely accepted biomarker of exposure to oestrogens in fish, and high incidences of intersex gonads and testis abnormalities have been found in wild fish populations sampled in rivers, estuaries or coastal waters (Lye et al., 1997; Jobling et al., 1998; Allen et al., 1999). These effects have been associated with exposure in the aquatic environment to natural, synthetic and/or xenobiotic oestrogens. Laboratory studies have shown that exposure of male fish to xeno-oestrogens results in induction of circulating Vtg as well as inhibited testicular growth (Jobling et al., 1996; Christiansen et al., 1998), testis abnormalities (Lye et al., 1997; Christiansen et al.,

1998) and formation of intersex gonads (Gray and Metcalfe, 1997).

In fish, as in all other vertebrates, oestrogens play an important role in many reproductive and developmental processes, including sexual maturation and sexual differentiation (Nakamura et al., 1998). These processes are therefore likely to be susceptible to xeno-oestrogenic exposure. In particular, exposure to environmental oestrogens during the sensitive early life stages of fish, such as the period of embryonic development and gonadal differentiation, may adversely affect the later reproductive performance of adult fish.

Various groups of chemicals have oestrogen-like effects, including alkylphenol poly-ethoxylates and their degradation products, alkylphenols (APs), such as nonylphenol (NP) and octylphenol (OP) (for a review, see Servos, 1999). The alkylphenol poly-ethoxylates belong to one of the largest groups of non-ionic surfactants and are used as detergents and in many formulated products such as herbicides, pesticides and paints. The alkylphenols, mainly 4-nonylphenol (4-NP) and 4-*tert*-octylphenol (4-tOP), have been found in surface waters and sediments of both freshwater and marine habitats (for a review, see Bennie, 1999). In the UK, APs have been detected at concentrations of up to $180\,\mu g\,l^{-1}$ in river water and up to $13\,\mu g\,l^{-1}$ in an estuary (Blackburn and Waldock, 1995); however, concentrations of approximately $1–10\,\mu g\,l^{-1}$ seem to be more common.

Several of the APs, including OP, have oestrogenic activity both *in vitro* and *in vivo* (White et al., 1994; Jobling et al., 1996; Routledge and Sumpter, 1997; Servos, 1999). In fish, including the eelpout (Andreassen and Korsgaard, 2000), OP has been shown to bind to the oestrogen receptor (ER; White et al., 1994), induce Vtg synthesis in males (White et al., 1994; Jobling et al., 1996; Routledge et al., 1998), induce intersex (Gray et al., 1999a) and reduce reproductive success (Gray et al., 1999b; Gronen et al., 1999).

Sexual differentiation is the process whereby the gonadal cells begin to specialise in structure and function and, hence, the gonadal phenotype is expressed. Sexual differentiation includes the formation of the somatic components of the gonad and the formation and development of the gametes. Usually, the somatic cells of the teleost gonad differentiate before the germ cells. The mechanisms controlling and regulating the processes of sexual determination and sexual/gonadal differentiation in fish have not yet been fully elucidated and show large differences among species. However, sex differentiation is mainly thought to be under genetic control (Nakamura et al., 1998), and (sex) steroids and steroidogenic enzymes seem to play a crucial role in the regulation of the process of gonadal differentiation (Baroiller et al., 1999). Endogenous oestrogens have been suggested to act as the natural inducer of ovarian differentiation, and the enzyme aromatase, which converts androgen to oestrogen, is probably one of the key enzymes involved in this process in gonochoristic fish (i.e. sex differentiation is characterised by the early and direct establishment of one gonadal sex) (Baroiller et al., 1999), at least in some fish species (Nakamura et al., 1998; Nagahama, 2000). Recently, Baroiller and co-workers (1999) suggested that the 11-oxygenated-androgen:oestrogen ratio in fish would direct either male (excess of 11-oxygenated androgens) or female (excess of oestrogens) differentiation.

It is known that exogenous oestrogens and androgens can manipulate sex differentiation by overriding endogenous sex-determination mechanisms in the developing embryo and induce phenotypic sex reversal in some fish species (Hunter and Donaldson, 1983). Recently, it has also been demonstrated that exposure to various environmental oestrogens may alter the normal gonadal development in fish (Gimeno et al., 1997; Gray and Metcalfe, 1997).

In fish, most studies concerning effects of xeno-oestrogens on the early life stages of development, such as gonadal differentiation, have focused on oviparous species. The viviparous eelpout (*Zoarces viviparus*) is a recognised model species in aquatic toxicology (Schladot et al., 1997; Taylor et al., 1999), and there is growing interest in its application in endocrine-disruption research (Christiansen et al., 1998; Andreassen and Korsgaard, 2000; Larsson et al., 2000).

Being viviparous, the eelpout is a suitable species for studying the effect of endocrine disrupters on maternal–foetal trophic relationships as well as direct effects on embryos *in ovario*. It carries a complete brood and gives birth to well-developed young fish that have completed sexual differentiation. This makes the eelpout an outstanding model in studies of sex ratios after exposure to endocrine disruptors, especially in field studies (Larsson et al., 2000). In contrast to oviparous fish, any contaminants must be taken up by the mother fish before the eggs and embryos can be exposed. The eelpout is a non-migratory fish common in coastal (and brackish) waters in much of northern Europe. It carries its progeny inside the ovary for approximately five months. The eggs are released into an ovarian cavity, and fertilisation takes place immediately after ovulation (late August/early September). Approximately three weeks after fertilisation, the embryos hatch. The embryos lie freely in the ovary without any physical connection to the mother fish. During the yolk-sac phase (approximately one month), the embryonic growth is partly dependent on the nutritive external yolk sac. Hereafter, until parturition, the development of the embryos depends on maternal nutrients in the surrounding ovarian fluid (Korsgaard and Andersen, 1985; Korsgaard, 1986, 1992). The eelpout is a differentiated gonochorist. Gonadal differentiation appears to take place during the yolk-sac phase (T. H. Rasmussen et al., unpublished observations; Larsson et al., 2000).

In the present study, we investigated the effects of waterborne exposure to 4-tOP and the natural oestrogen 17β-oestradiol (E_2) on the mother fish and her embryos during early pregnancy. The objective of the study was to investigate whether 4-tOP and E_2 accumulate in the mother fish and are transferred to the embryos *in ovario* and subsequently disturb the maternal–foetal trophic relationship and affect embryonic development, including gonadal differentiation.

Materials and methods

Fish

The experiment was performed in the autumn of 1999. Pregnant female eelpouts, *Zoarces viviparus* L. (Teleostei, Perciformes, Zoarcidae), were caught by fyke nets at Dalby Bugt, Funen, Denmark and transferred to the Centre of Aquatic Biology, Kerteminde to large indoor tanks (300 l) with aerated running seawater pumped in directly from The Great Belt of Denmark. Fish were acclimated approximately one week before onset of the experiment. The fish were held in a 12 h:12 h light:dark photoperiod and were not fed during the experiment to avoid skewed food uptake caused by the treatment. The experiment was carried out during the period of 11 October to 15 November, beginning at the time when embryos of pregnant eelpout were in the late yolk-sac phase.

Experimental design

Fish were treated with 4-tOP (nominal concentrations of 25 μg l^{-1} or 100 μg l^{-1}), E_2 (nominal concentration of 0.5 μg l^{-1}) or isopropanol (control) in a continuous flow-through system. The system consisted of 12 aquaria (50 l), each connected to a multi-channel water pump and stock-solution pump. Fresh seawater was pumped into the aquaria at a flow rate of 200 l day^{-1}. The compounds were dissolved in 100% isopropanol and applied directly to the aquaria at a rate of 72 ml day^{-1}. To assure uniform mixing, each aquarium was fitted with a circulation pump. During the experimental period, water temperature fluctuated between 10.5°C and 13.5°C. Water samples were analysed every third day for actual 4-tOP concentrations in the control and the treatment groups. Each group (control, OP25, OP100 and E_2) consisted of 21 fish with a maximum of 7 fish per aquarium. At day 0, 10 randomly selected untreated fish were sampled. After 17 days of exposure (28 Oct), 7–8 fish from each group were selected randomly from different aquaria and sampled. At the end of the experiment, at day 35 of exposure (15 Nov), 7–8 fish from each group were chosen, as described above, and sampled.

Sampling procedure

Fish were washed in clean water and anaesthetised (0.2‰ phenoxyethanol) before the start of sampling. Fish were weighed, measured (total length) and blood was collected from the caudal vein into heparinized eppendorf tubes. Ovarian fluid was collected by gently inserting a syringe directly into the ovarian cavity of the intact ovary. Blood and ovarian fluid were centrifuged (4 min at 10 000 ***g*** at 4°C) and plasma was divided into aliquots and stored at –80°C until use. Fish were killed by decapitation, and liver and ovary were carefully removed and weighed. A small piece of the liver was quick-frozen in liquid N_2 and stored at –80°C. The embryos were carefully dissected out of the ovary, anaesthetised (0.1‰ phenoxyethanol), counted and scored for survival. Ten embryos from each mother fish were randomly chosen and weighed and measured (total length). Half of the remaining embryos were fixed in Lillies-fixative (4% neutral-buffered formalin) and the other half were snap-frozen in liquid N_2. A few representative embryos were kept alive in Ringer solution (190 mmol l^{-1} NaCl, 4 mmol l^{-1} KCl, 4 mmol l^{-1} $CaCl_2$, 10 mmol l^{-1} $NaHCO_3$, pH 7.5) and used for photographs. Finally, the ovarian sac was weighed.

Quantification of 4-tOP in water samples and body fluids

Water samples were taken from the out-flow of the aquaria and filtered. To each sample was added 50 μl internal standard (10 ng μl^{-1} *tert*-butylphenol), SDS (sodium dodecyl sulfate) to a concentration of 0.1 mol l^{-1}, and formaldehyde to a concentration of 0.4%. Samples were left at 4°C until analysis. The extraction and quantification of 4-tOP by liquid chromatography–mass spectrometry (LC-MS) followed the description in Pedersen and Lindholst (1999).

4-tOP was extracted from plasma as follows: 100 μl of plasma was added to 50 μl internal standard, diluted 10× in MilliQ-water (reverse osmosis deionised water) and applied to Sep-Pak C_{18} extraction columns previously conditioned as described by Pedersen and Lindholst (1999). 400 μl of ovarian fluid was added to 50 μl of internal standard and measured directly on the LC-MS. Tests were made to assure that this method resulted in similar results to the column extraction method described for plasma. The limit of quantification is 100 ng ml^{-1}.

Quantification of 17β-oestradiol in body fluids

E_2 was measured in plasma and ovarian fluid using a commercially available competitive enzyme-linked immunosorbent assay (ELISA) kit (DRG Instruments GmbH, Marburg, Germany). The minimal detection limit is 16 pg ml^{-1}.

Quantification of vitellogenin in plasma

Vtg in plasma was analysed using a direct ELISA as described by Korsgaard and Pedersen (1998).

Determination of specific E_2-binding in liver cytosol homogenates

Liver of mother fish was homogenised, and cytosolic liver homogenates were prepared as described by Andreassen and Korsgaard (2000). Specific binding of ^{3}H-E_2 was measured in one-point assays in triplicate as described previously (Andreassen and Korsgaard, 2000).

RT-PCR analysis on liver tissue of mother fish and embryos

RNA was extracted from mother fish liver using TRIzol® (Gibco BRL Life Technologies, MA, USA), as described by the manufacturers. 1 μg of total RNA was reverse-transcribed in 20 μl reactions using Superscript™ RNase H^- and oligo$(dT)_{12–18}$ Primer (Gibco BRL) as described in their optimized protocol. For amplification of reverse-transcribed Vtg mRNA, the following primers were used: forward primer 5′-CTG TGA AGC TGG AGA AGC AGG – 3′ and reverse primer 5′-CTT CGG CTT CAT CCC TCA GG – 3′. These primers were selected based on a partial Vtg sequence from eelpout (data not shown). As reference gene, β-actin was

amplified. Primers were selected by aligning β-actin sequences from *Sparus aurata* (X89920), *Salmo salar* (AF012125) and *Cyprinus carpio* (M24113) (GenBank data). Actin fw3: 5′-GAC GGA CAG GTC ATC ACC AT – 3′; actin revC: 5′-CAC ATC TGC TGG AAG GTG GA – 3′. Verification of the amplified products as Vtg and β-actin was obtained by sequencing the PCR product and aligning with known fish Vtgs and actins (data not shown). 1 μl of the cDNA was used for the following PCR reaction in 20 μl volumes using 1 unit of Taq DNA polymerase and 1×PCR-buffer (Sigma, St Louis, MO, USA), 25 pmol of each primer and 200 μmol l^{-1} of each dNTP. PCR was run for 4 min at 94°C followed by 15 (Vtg) or 20 (β-actin) cycles of 30 s at 94°C, 30 s at 56°C and 1 min at 72°C. A final extension step at 72°C for 7 min was added. The resulting cDNA fragments were resolved on 2% agarose gels containing 1 μg ml^{-1} ethidium bromide, and their molecular size was determined by comparison with size markers (100 bp ladder; Gibco BRL). Finally, the gel was photographed for documentation.

For determination of Vtg mRNA in embryos, the liver region of whole embryos (a pool of three per mother fish) was homogenised in TRIzol® (Gibco BRL), and total RNA was isolated as described by the manufacturers. The RT-PCR conditions and primers for Vtg and β-actin were as described above for the mother fish except that the PCR was run as follows: 4 min at 94°C followed by 28 (Vtg) cycles of 30 s at 94°C, 30 s at 56°C and 1 min at 72°C.

*Quantification of calcium in body fluid*s

The concentration of total calcium (free and bound) in plasma and ovarian fluid was measured by atomic absorption spectrophotometry (Perkin Elmer 2380, Mountain View, CA, USA; Perkin Elmer, analytical methods for atomic absorption spectrometry, 1982). The samples were diluted 200× in 0.1% La_2O_3.

Determination of free amino acid levels in body fluids

The concentration of ninhydrine-positive substances (NPS), which indicates the presence of free amino acids, was determined according to the method described by Moore and Stein (1948) and measured at 570 nm with leucine as the standard.

Immunohistochemical analysis of Vtg in embryos

The localisation of Vtg was studied in control-, E_2- and OP-treated embryos by immunohistochemical staining of Vtg using rabbit polyclonal antibody against eelpout Vtg (Korsgaard and Pedersen, 1998). The Vtg-antibody was validated for specificity by incubation on liver and ovary sections from E_2-treated female eelpouts (positive control) and liver sections from untreated male eelpouts (negative control). Optimal target/background ratio was determined by serial dilution.

Whole embryos fixed in 4% neutral-buffered formalin were processed by paraffin embedding according to standard procedures. 4 μm sections were cut and arranged on 3-aminopropyltriaethoxysilane-treated slides, dried at 37°C over night, deparaffinized in xylene, and rehydrated in alcohol. Before staining, endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide in methanol for 30 min. The slides were then rinsed (4×4 min) in 0.05% Tween in phosphate-buffered saline (PBS) at room temperature. Next, the slides were incubated with the primary Vtg antibody diluted 1:100 in 1% bovine serum albumin (BSA) in PBS for 60 min in a moist chamber at room temperature. The slides were rinsed (4×4 min) in 0.05% Tween in PBS, incubated with horseradish peroxidase conjugated swine anti-rabbit (DAKO) in 0.05% blocking serum (milk powder) in PBS for 30 min at room temperature and rinsed again for 4×4 min in PBS. Colour visualisation in sections was performed by incubation for 5 min with liquid 3,3-diaminobenzidenitetrahydrochloride (DAB) substrate (0.04% DAB in 0.05mol l^{-1} Tris/HCl, pH 7.6) (Sigma), with Mayer's haematoxylin as counterstain. The slides were evaluated qualitatively for the presence of Vtg in liver, gut and blood of the embryos using light microscopy. Negative control stainings were performed by incubating with normal rabbit sera instead of the Vtg-antibody. A minimum of four embryos from at least four different mother fish of each treatment group were analysed.

Histological examination by light microscopy

Whole embryos were fixed in Lillies-fixative and processed for histological examination by light microscopy. After 24 h, embryos were dehydrated through a series of graded ethanol (50–99%), cleared in xylene, and embedded in paraffin. Sections (5 μm) were cut so that transverse sections of the gonads were achieved. The sections were stained in Mayer's haematoxylin and eosin-Y and analysed using a light microscope.

Transverse sections of the developing gonad were investigated through the whole length of the organ. The number of sections prepared from each embryo varied between 100 and 300 depending on the size of the gonad. Gonads were staged as follows: female gonad in early differentiation, female gonad containing oocytes, presumptive male gonad and abnormal gonad (male or female) as defined in Results. Light micrographs from the anterior, central and posterior regions of the gonad were taken with a digital camera. Four to six embryos from each mother fish were examined. Only embryos from day 0 and day 35 were analysed.

In situ *hybridisation of oestrogen receptor mRNA*

Fixed, dehydrated and paraffin-embedded embryos from day 0 and after 35 days of exposure were cut on a microtome (6 μm). Transverse sections from the gonadal region were processed for *in situ* hybridisation as described elsewhere (T. K. Andreassen et al., manuscript submitted). A 515 bp [^{35}S]UTP-labelled sense and anti-sense RNA probe encoding a part of the E-domain of the cloned eelpout ERα (T. K. Andreassen et al., manuscript submitted) was prepared and used in the hybridisation experiments.

Chemicals

4-tOP and E_2 [1,3,5(10)-estratriene-3,17β-diol] were obtained from Sigma-Aldrich, Steinheim, Germany.

Statistical analysis

Values are expressed as means ± S.E.M. Data were tested for normality and homogeneity of variance and, if necessary, were $\log_{10}$-transformed prior to analysis. One-way analysis of variance (ANOVA; SYSTAT 7.0 for Windows, SPSS Inc., Chicago, IL, USA) followed by the Tukey test of multiple comparison was used to test differences between groups. Linear correlation analysis was carried out with Graph Pad Prism (ver. 1.03) software (San Diego, USA).

Results

Concentration of 4-tOP in water

The concentration of 4-tOP in the aquaria was measured throughout the experiment (Fig. 1). The concentration declined after adding the fish but thereafter the concentration in both groups remained relatively stable at a range of 8–17 $\mu g\,l^{-1}$ in the OP25 group and 57–79 $\mu g\,l^{-1}$ in the OP100 group. The mean actual water concentrations were 14 $\mu g\,l^{-1}$ and 65 $\mu g\,l^{-1}$ for the OP 25 and OP 100 groups, respectively (Table 1).

Bioconcentration of 4-tOP and E_2 in body fluids

High levels of 4-tOP were observed in plasma of pregnant eelpout after 35 days of exposure (Table 1). In the OP100 group, a mean concentration of 54.9 $mg\,l^{-1}$ 4-tOP was measured in plasma. The bioconcentration factors (BCFs) of 4-tOP in plasma were determined to be approximately 200 and approximately 550 for the OP25 and OP100 group, respectively (Table 1). Oestradiol-17β was taken up by the E_2-exposed fish, and, in plasma, a >200-fold increase was detected in comparison with control plasma after 35 days of exposure (Table 1). The BCF of E_2 in plasma was approximately 20.

In ovarian fluid, 4-tOP was also found in high concentrations. The mean concentration of 4-tOP in the ovarian fluid increased both with time (results not shown) and dose (Table 1); however, time was only a significant factor for the OP100 group. After 35 days of exposure, concentrations of

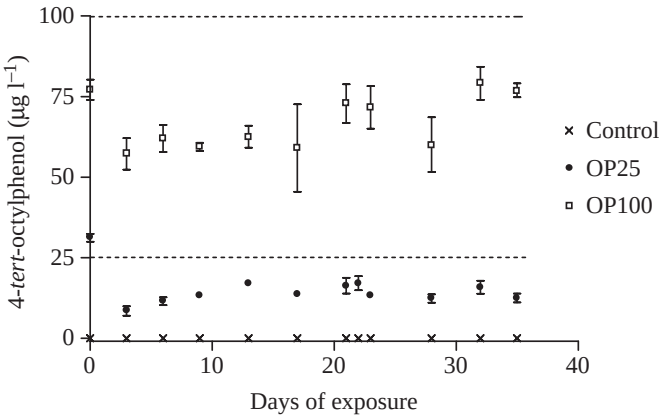

Fig. 1. Actual concentrations of 4-*tert*-octylphenol (4-tOP) in the aquaria during the experiment from 11 October to 15 November. Broken lines indicate nominal water concentrations. To the water was added isopropanol only (control) or 4-tOP dissolved in isopropanol to nominal concentrations of 25 $\mu g\,l^{-1}$ (OP25) or 100 $\mu g\,l^{-1}$ (OP100). Values are means of concentration of each group in 2–3 aquaria ± S.E.M.

Table 1. *Concentration and BCF of 4-*tert*-octylphenol and 17β-oestradiol in plasma and ovarian fluid after 35 days of water exposure of the pregnant eelpout (*Zoarces viviparus*) to the compounds*

	4-*tert*-octylphenol† ($\mu g\,l^{-1}$)			17β-oestradiol ($ng\,l^{-1}$)	
	Control (*N*=8)	OP25 (*N*=8)	OP100 (*N*=7)	Control (*N*=8)	E_2 (*N*=7)
Concentration					
Water	BD	14±2	65±3	ND	ND
Plasma	BD	5 026±874	54 904±5 357	48.3±8.1	11 074±786***
Ovarian fluid	BD	4.8±0.9	1 421±415	24.6±2.6	57.8±9.0**
BCF					
Plasma	–	201±35	549±57	–	22.2±1.6
Ovarian fluid	–	0.21±0.04	15.2±4.5	–	0.12±0.02

Values are expressed as means ± S.E.M.

Significant difference from the control is indicated by asterisks: **P<0.01 and ***P<0.001. †No statistics are applied to the data for 4-tOP, as all data in the control group were below detection limits.

Abbreviations: BCF, bioconcentration factor; 4-tOP, 4-*tert*-octylphenol; E_2, 0.5 $\mu g\,l^{-1}$ 17β-oestradiol; OP25, 25 $\mu g\,l^{-1}$ 4-tOP; OP100, 100 $\mu g\,l^{-1}$ 4-tOP; *N*, number of mother fish; BD, below detection limit; ND, not determined.

BCF was calculated as the actual concentration of the compound in the compartment compared with the nominal water concentration of the compound.

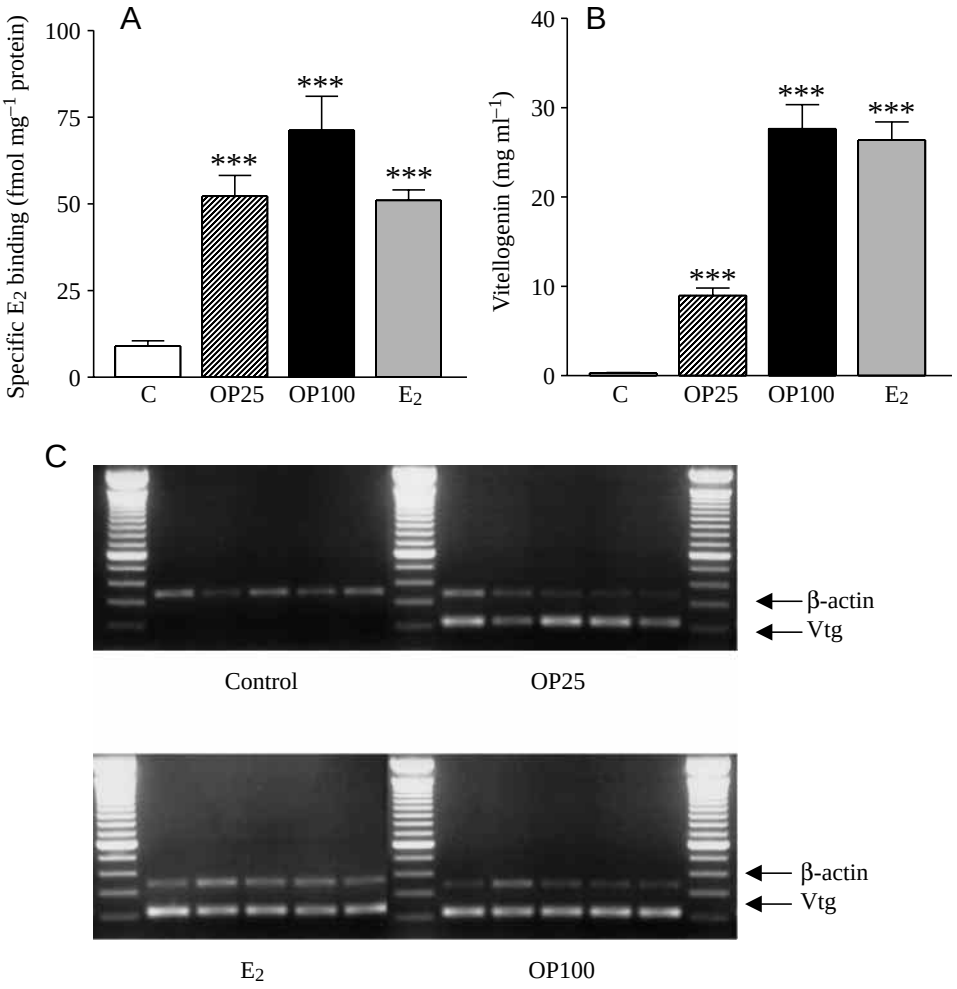

Fig. 2. Induction of oestrogenic biomarkers in pregnant eelpout: 17β-oestradiol (E_2)-binding activity, vitellogenin (Vtg) in plasma and Vtg mRNA in liver. (A) E_2-binding capacity in hepatic cytosolic extracts. E_2-binding capacity was estimated by single-point assays. (B) Vtg in plasma. (C) Vtg mRNA in liver tissue. Ethidium-bromide-stained agarose gels showing amplified RT-PCR products using Vtg-specific primers (215 bp). As an internal control for the RT-PCR reaction, eelpout β-actin mRNA was amplified (band at 340 bp) using β-actin-specific primers. Fish were exposed to isopropanol (C; control), 25 μg l^{-1} 4-*tert*-octylphenol (4-tOP) (OP25), 100 μg l^{-1} 4-tOP (OP100) or 0.5 μg l^{-1} E_2 for 17 days. Values are means ± S.E.M. Significant difference from control is indicated by asterisks (*P<0.05; **P<0.01; ***P<0.001).

up to 3.4 mg l^{-1}, with a mean concentration of 1.4 mg l^{-1}, were measured in the OP100 group in ovarian fluid, resulting in a mean BCF of 15 (Table 1). In the OP25 group, levels of 4-tOP were all lower than in the surrounding water; consequently, the BCF was <1. In the ovarian fluid, the concentration of E_2 in the E_2 group was only approximately twofold the concentration in the control group.

In both plasma and ovarian fluid, 4-tOP bioconcentrated more efficiently than E_2, but this was most apparent in the high-dose group.

Induction of oestrogenic biomarkers in pregnant eelpout: E_2-binding activity, Vtg mRNA in liver and Vtg in plasma

Waterborne exposure of 4-tOP and E_2 for 17 days (or 35 days) up-regulated the Vtg-synthesising apparatus in the pregnant eelpout, as shown by an up-regulated E_2-binding capacity in the liver (Fig. 2A), induced sequestration of the Vtg protein into the plasma (Fig. 2B) and transcription of the Vtg-encoding gene in the liver (Fig. 2C).

Oestrogen-binding activity in hepatic liver cytosol preparations was highly induced by the treatment (Fig. 2A). In the 4-tOP-treated fish, a 6–8-fold increase was observed after 17 days of continuous exposure to the compound. A comparable induction of binding activity (sixfold) was found in E_2-treated fish.

Vtg in plasma was highly induced after 17 days of exposure to the test compounds (Fig. 2B), and a clear concentration-dependent response was observed. The concentration of Vtg was threefold higher in the OP100 group than in the OP25 group but was comparable with the concentration in the E_2 group. A significant positive correlation between the actual concentration of 4-tOP in plasma and the concentration of Vtg in plasma was found (Fig. 3; r^2=0.89, P<0.0001, N=23). After 35 days of exposure, a comparable relationship was observed (results not shown).

Vtg mRNA was induced in all liver samples from E_2- as well as 4-tOP-exposed fish (Fig. 2C). A single DNA product of the expected size (215 bp) was produced in the RT-PCR reactions. β-actin (PCR product at 340 bp) was co-amplified to ascertain that RNA isolation and RT-reaction efficiency was comparable among samples. No induction of Vtg mRNA was seen in any control fish.

Changes in ovarian indices of pregnant eelpout

The gonadosomatic index (GSI) of the control fish increased throughout the experiment and more than doubled

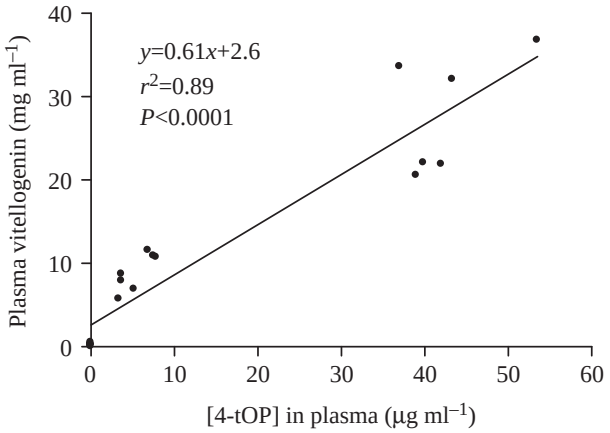

Fig. 3. The relationship between actual plasma concentration of 4-*tert*-octylphenol (4-tOP) and plasma vitellogenin concentration of pregnant eelpout exposed to isopropanol (control), 25 µg l^{-1} 4-tOP or 100 µg l^{-1} 4-tOP for 35 days.

35 days after the onset of the experiment (results not shown). In all treated groups, there was a tendency for a reduced GSI in comparison with control fish, but this was only significant for the OP25 group (Fig. 4E; $P<0.05$, $N=8$). To get a more elaborate picture of the development and condition of the ovary, four indices in relation to the ovary were calculated. These indices describe the mass of the ovarian sac, the ovarian fluid and the embryos in relation to the total body mass (excluding ovary) of the mother fish, named the ovarian sac somatic index (OSSI), the ovarian fluid index (OFI) and the embryo somatic index (ESI), respectively (Fig. 4). Finally, we calculated an index describing the proportion of the ovary made up by the ovarian sac, which we named the ovarian sac mass percentage (OSM). During the course of pregnancy (11 October to 15 November), OFI, ESI and OSM changed dramatically and significantly in the control fish (2% to 17%, 12% to 19%, and 14% to 7%, respectively; $P<0.05$, $N=8$); by contrast, OSSI was held at a remarkably constant level (2.3–2.5%). However, all treated fish showed a tendency towards lower OFI and ESI levels (Fig. 4A,B) compared with the control. Furthermore, OSSI was significantly elevated in the OP100 and E_2 groups (Fig. 4C). OSM values were significantly elevated in all treated groups (Fig. 4D) after 35 days of exposure, again showing a concentration-dependent relationship. In a similar experiment conducted in September–October, exactly the same pattern was observed (results not shown), except that OFI did not seem to change, possibly because ovarian fluid was very sparse at that time of pregnancy. The amount of ovarian fluid per embryo (OFI/embryo) for the OP100 group on day 35 was significantly lower than in controls (result not shown). The same tendency was observed in the OP25 and E_2 groups.

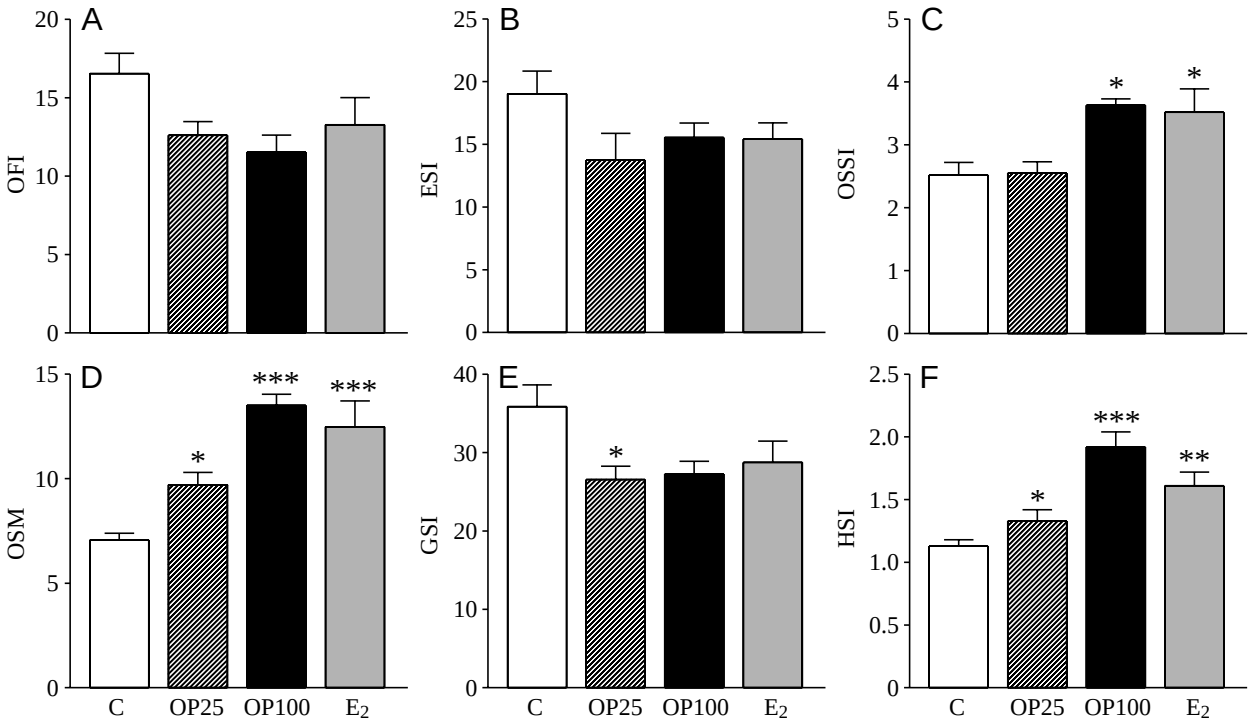

Fig. 4. Changes in five different ovarian indices and in the hepatosomatic index of pregnant eelpout after 35 days water exposure to isopropanol (control, C), 25 µg l^{-1} 4-*tert*-octylphenol (4-tOP) (OP25), 100 µg l^{-1} 4-tOP (OP100) or 0.5 µg l^{-1} 17β-oestradiol (E_2). (A) Ovarian fluid index (OFI). (B) Embryo somatic index (ESI). (C) Ovarian sac somatic index (OSSI). (D) Ovarian sac mass percentage (OSM). (E) Gonadosomatic index (GSI). (F) Hepatosomatic index (HSI). OFI, ESI, OSSI, GSI and HSI are somatic indices calculated in relation to the total mass of pregnant eelpout (excluding ovary). Values are means ± S.E.M. Significant difference from control is indicated by asterisks (*$P<0.05$; **$P<0.01$; ***$P<0.001$).

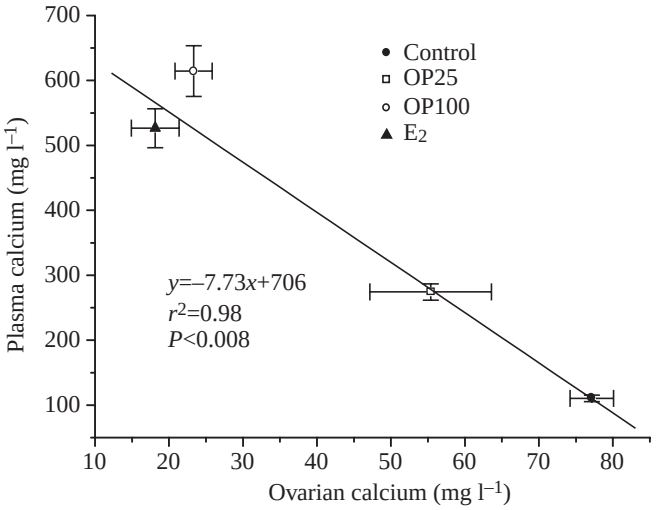

Fig. 5. The relationship between concentrations of calcium in ovarian fluid and in plasma of pregnant eelpout after 35 days water exposure to isopropanol (control, C), 25 μg l^{-1} 4-*tert*-octylphenol (4-tOP) (OP25), 100 μg l^{-1} 4-tOP (OP100) or 0.5 μg l^{-1} 17β-oestradiol (E_2). Values are means ± S.E.M.

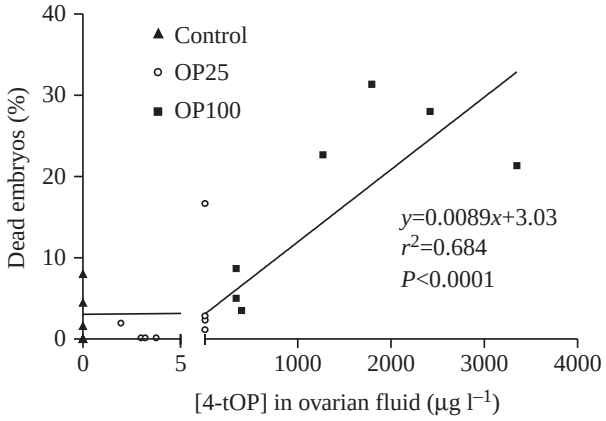

Fig. 6. The relationship between actual concentration of 4-*tert*-octylphenol (4-tOP) in ovarian fluid and the percentage of dead embryos found in the ovary of pregnant eelpout after 35 days water exposure to isopropanol (control), 25 μg l^{-1} 4-tOP (OP25) or 100 μg l^{-1} 4-tOP (OP100).

There was a significant positive linear correlation between OFI/embryo and embryonic mass (r^2=0.67, P<0.0001, N=35).

During the course of the experiment, the hepatic somatic index (HSI) of control fish declined significantly (results not shown). However, a significantly elevated HSI was observed in the OP100 (P<0.001, N=7) and E_2 (P<0.01, N=8) groups compared with the control group after 35 days of exposure (Fig. 4F).

No changes in the condition index (M_b/L^3×100, where M_b is body mass in g and L is total length in cm) among experimental groups were observed (results not shown).

Exposure to either compound did not induce any significant mortality. Only approximately 10% of the fish in the high dose 4-tOP group died. However, after approximately 14 days of exposure, we started to observe incidences of premature parturition (abortion), a factor first observed in the OP100 and E_2 groups and later in the OP25 group.

Changes in components important for the maternal–foetal trophic relationship: calcium and free amino acids

In plasma, the level of total calcium increased to high levels in the 4-tOP-treated (3–6-fold increase) and E_2-treated (fivefold increase) groups after 35 days of exposure. By contrast, in the ovarian fluid, a concentration-dependent decrease was observed during the same period in the 4-tOP-exposed fish. Fig. 5 illustrates the relationship between the calcium levels in ovarian fluid and in plasma and shows a significant negative linear correlation (P<0.008, N=31). There was, however, no significant correlation between total calcium concentration and embryonic mass (data not shown).

NPS levels (an indicator of the amount of free amino acids) in plasma of all treated groups decreased to approximately 50% of that in the control group (data not shown). In the ovarian fluid, the concentration of NPS was reduced in control fish at day 35 compared with the control fish at day 0, but comparable and very low levels, much lower than in plasma, were seen in all groups, including the control, after the 35 days (data not shown).

Effects on embryonic mortality and growth of embryos

The mortality of the embryos after 35 days of exposure was between 1.8% and 17%. Only mortality of embryos in the OP100 group was significantly higher compared with the control group. There was a positive correlation between the concentration of 4-tOP in the ovarian fluid and the mortality ratio of embryos in the ovary (Fig. 6; r^2=0.684, P<0.0001, N=23).

At day 0 of the experiment, the embryos were at the late yolk-sac stage. During the experimental period of 35 days, the control embryos had grown, as indicated by an increase in both mass and length. The 35-day control embryos had absorbed the yolk sac and appeared more pigmented compared with the control embryos at day 0. Exposure to 4-tOP or E_2 caused a decrease in embryonic growth. Compared with the control embryos at day 35, the mass of embryos from the OP25, OP100 and E_2 groups was significantly decreased (Fig. 7A; P<0.01, N=80; P<0.01, N=70; P<0.05, N=80). The length was only significantly reduced in the two 4-tOP groups (Fig. 7B; P<0.05, N=80; P<0.01, N=70). Compared with the control embryos at day 0, the embryos from the three exposed groups had not gained much in mass or length after 35 days of exposure, although they appeared similar to the 35-day control embryos in terms of their degree of pigmentation and the absorbed yolk sac.

Detection of Vtg induction and oestrogen sensitivity of the embryos

To investigate if the treatments had induced any measurable oestrogenic effect on the embryos, the induction of Vtg was

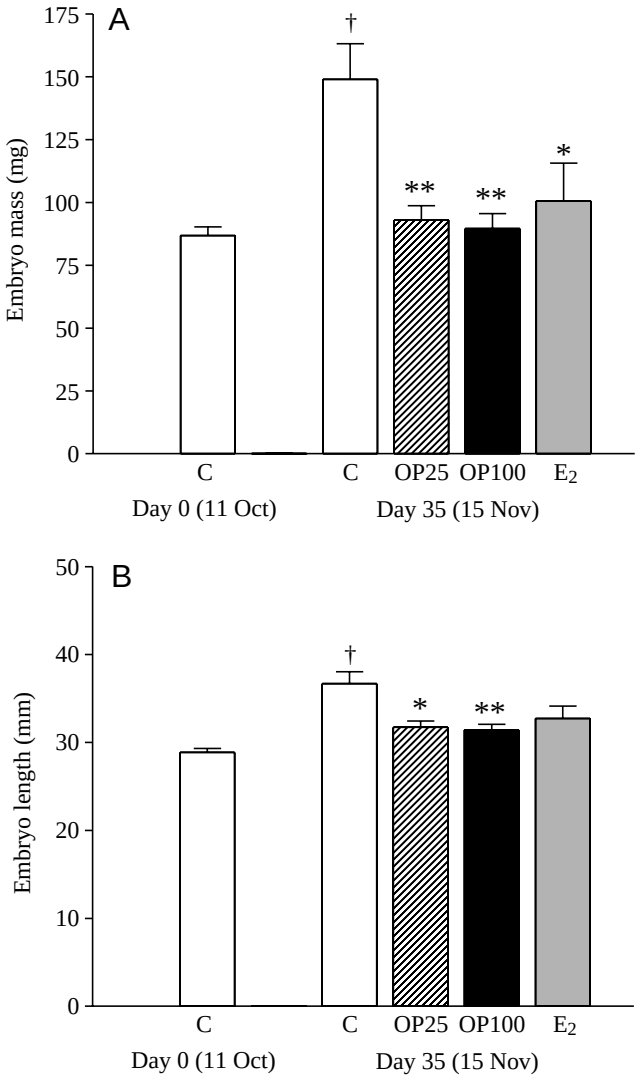

Fig. 7. Effects of treatment on embryonic growth. (A) Mass and (B) length of embryos at day 0 (11 October) and day 35 (15 November) after water exposure of pregnant mother fish to isopropanol (control, C), 25 μg l^{-1} 4-*tert*-octylphenol (4-tOP) (OP25), 100 μg l^{-1} 4-tOP (OP100) or 0.5 μg l^{-1} 17β-oestradiol (E_2). Values are means ± S.E.M. Significant difference from the respective control is indicated by asterisks (*P<0.05, **P<0.01, ***P<0.001). Significant difference of 35-day control from the 0-day control is indicated by a dagger (P<0.05).

examined by RT-PCR (Fig. 8) and immunohistochemistry (Fig. 9). Vtg cDNA was only amplified in the OP100 group, and no detectable Vtg induction was observed in the other groups (Fig. 8). In all fish, the internal standard β-actin was readily amplified, indicating that RNA isolation and RT-PCR conditions were identical for all samples.

In the liver of the OP100-treated embryos, positive immunohistochemical staining of Vtg was found in the cytoplasm of hepatocytes and the blood capillaries (Fig. 9B). By contrast, livers in the control (Fig. 9A), OP25- and E_2-treated embryos (not shown) all showed no staining.

In the hindgut, positive staining was seen in the OP100- (Fig. 9D), OP25- and E_2-treated embryos, although with a lesser intensity in the OP25 and E_2 groups. The staining was restricted to cellular debris in the lumen and to the periphery of the epithelia cells in the hindgut. There was no Vtg staining in the hindgut of the control embryos (Fig. 9C).

Fig. 10 shows the localisation of ER mRNA in early differentiating gonads (day 0), including presumptive male gonads (Fig. 10A,B), and in differentiated female gonads (control day 35; Fig. 10D,E). As a negative control, the sense probe was applied, showing no labelling in the gonads (Fig. 10C,F).

Effects on gonadal differentiation in the embryos

The gonads of newly hatched embryos appear as an undifferentiated two-lobed organ situated in a mesentery between the dorsal peritoneal wall (just beneath the kidneys) and the intestines. This undifferentiated gonad contains proliferating primordial germ cells (Fig. 11A).

At day 0 of the experiment, at the late yolk-sac phase of

Table 2. *Percentage of female, male and abnormal gonads as determined by histological examination in eelpout (*Zoarces viviparus*) embryos after 35 days of exposure to 4-*tert*-octylphenol or 17β-oestradiol*

	Day 0 (11 Oct)	Day 35 (15 Nov)			
	Control	Control	OP25	OP100	E_2
Number of mother fish	10	8	8	7	8
Number of embryos analysed*	48	44	43	40	47
Percentage of total number of gonads of the embryos					
Female gonad (early differentiating ovary)	48	0	0	0	0
Female gonad (ovary with oocytes)	0	52	58	46	49
Male gonad (presumptive)	52	48	42	22	51
Abnormal gonad – male or female?	0	0	0	32	0

*4–6 embryos from each mother fish were processed and analysed by light microscopy.
Abbreviations: 4-tOP, 4-*tert*-octylphenol; OP25, 25 μg l^{-1} 4-tOP; OP100, 100 μg l^{-1} 4-tOP; E_2, 0.5 μg l^{-1} 17β-oestradiol.

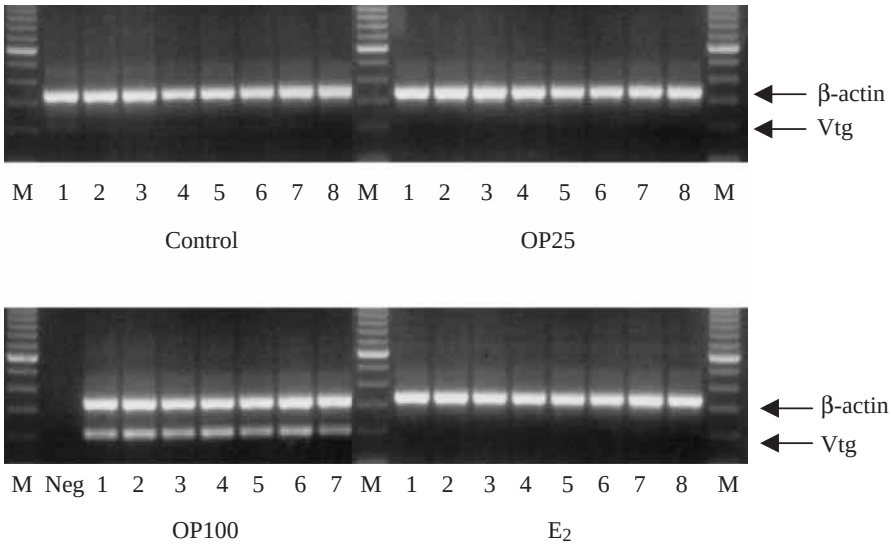

Fig. 8. Induction of vitellogenin (Vtg) mRNA in eelpout embryos exposed to 4-*tert*-octylphenol (4-tOP) *in ovario* for 35 days. Ethidium-bromide-stained agarose gel showing the amplified RT-PCR products using Vtg- (215 bp) or β-actin- (340 bp) specific primers on total RNA extracted from embryos dissected out of mother fish exposed to isopropanol (control), 25 µg l^{-1} 4-tOP (OP25), 100 µg l^{-1} 4-tOP (OP100) or 0.5 µg l^{-1} 17β-oestradiol (E_2). Three embryos were pooled per mother fish. Numbers indicate different mother fish. M, 100 bp marker; Neg, negative control (no RT-enzyme).

embryonic development, two different types of gonads were observed. Of the analysed embryos, 48% of the gonads were female gonads in an early stage of differentiation (Table 2). The undifferentiated two-lobed gonad had grown and begun to form the ovarian cavity. The ovarian cavity is formed by longitudinal growth, gradual bending towards the mesentery and final fusion of the external ends of the genital ridges in the ventral side of the gonad resulting in a single ovary with an endo-ovarian cavity (Fig. 11B–D). In the central and largest part of the gonad, the ovarian cavity was generally still separated in two parts by a central string (Fig. 11C). The anterior and the posterior ends of the gonad were smaller and hollow (Fig. 11B,D). In some of the embryos, a two-lobed structure was visible in the very posterior end but not in the

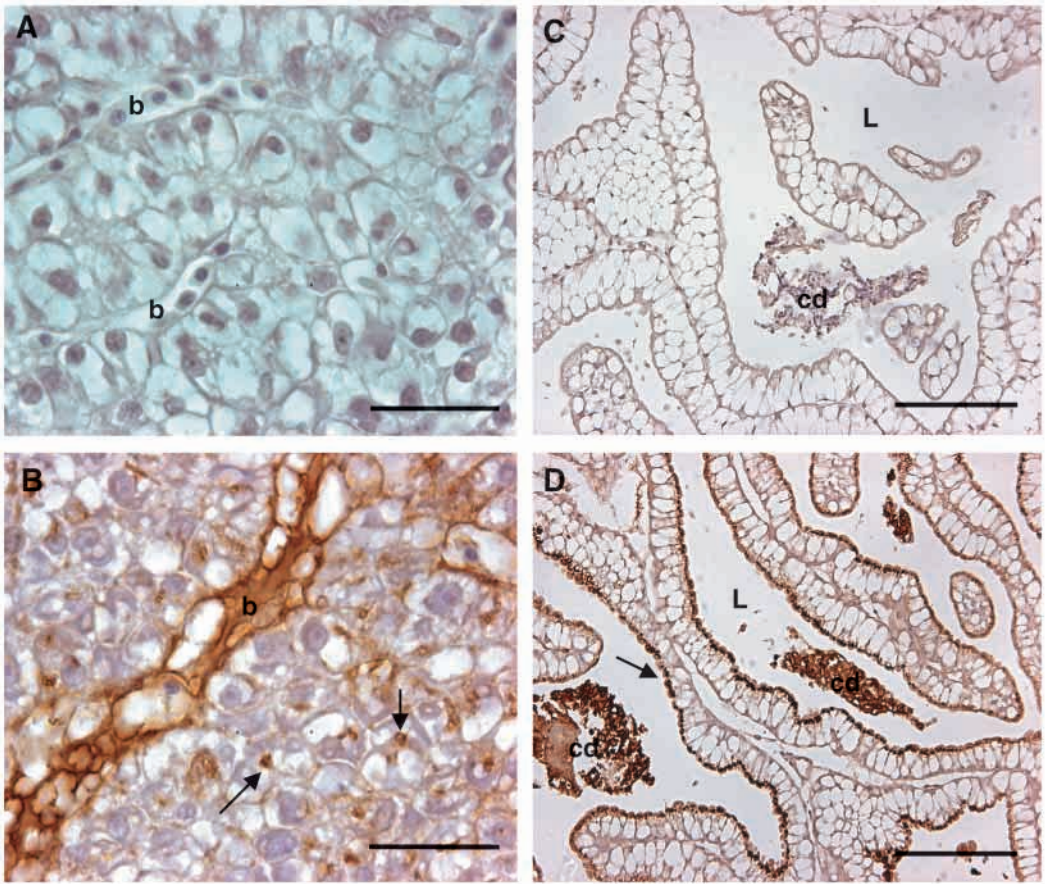

Fig. 9. Immunohistochemical localisation of vitellogenin (Vtg) in the liver and hindgut of eelpout embryos exposed to 4-*tert*-octylphenol (4-tOP) *in ovario* for 35 days. (A) Control liver: no Vtg-staining. (B) 100 µg l^{-1} 4-tOP (OP100)-treated liver: strong Vtg-specific staining in cytoplasm of hepatocytes (arrow) and blood capillaries (b). (C) Control hindgut: no Vtg-staining. (D) OP100-treated hindgut: Vtg-staining in cellular debris (cd) in the lumen (L) and the periphery of epithelial cells of the hindgut (arrow). Scale bars, A and B=20 µm; C and D=200 µm.

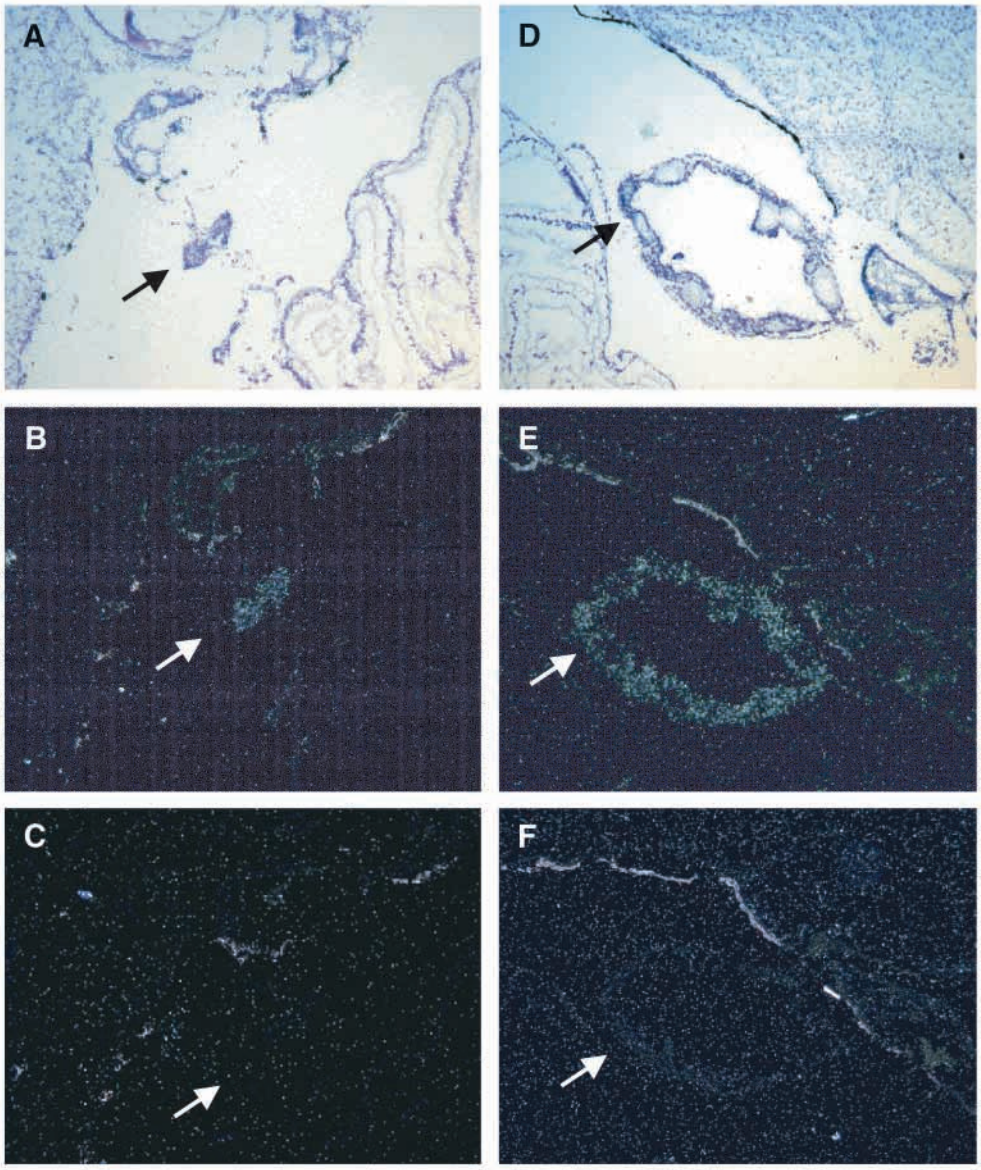

Fig. 10. *In situ* hybridisation analysis of oestrogen receptor (ER) expression in gonads of control eelpout embryos. Bright- and dark-field micrographs of representative transverse sections from the gonadal regions of embryos hybridised with anti-sense (A,B,D,E) or sense (C,F) eelpout ERα RNA probe. (A–C) Presumptive male gonad at the beginning of the experiment (11 October). (D–F) Normal female gonad (control) at the end of the experiment (15 November). Arrows indicate the position of gonads.

anterior end of the gonad. These early differentiating female gonads contained proliferating germinal cells surrounded by somatic tissue, but there was no indication of oogenesis and no oocytes were observed.

The other 52% of the embryos had presumptive male gonads (Table 2). The gonads consisted of a two-lobed organ throughout the complete gonad containing germinal cells and somatic cells (Fig. 11E–G). This two-lobed structure resembles that of the testis of the adult male eelpout. The early male gonad appears very similar to the undifferentiated gonad except for an incipient enlargement of the stromal/somatic tissue connecting the two lobes of the gonad in the posterior end. This enlargement becomes more prominent as the differentiation of the male gonad proceeds. At this early time of differentiation, it was not possible to distinguish the germinal cells in the early female gonads from those of the presumptive male gonad by light microscopy.

In the control embryos at the end of the experiment (day 35), 52% of the gonads were female gonads that had grown in size and had differentiated into single hollow ovaries containing both oogonia and primary oocytes in the perinucleolar stage throughout the ovary (Table 2; Fig. 12A–C). Only the very anterior and posterior ends of the female gonad did not contain oocytes.

The other 48% of the embryos had presumptive male gonads, which more or less appeared the same as the presumptive male gonads found at the beginning of the experiment (Table 2; Fig. 12D–F). However, in some of the gonads, the tissue connecting the two lobes at the posterior end had enlarged and the lobes had become more triangular in shape compared with the controls at the beginning of the experiment.

In the embryos from the OP25 and E_2 groups, the gonads resembled those of the control embryos, with gonads differentiated into ovaries with oocytes (58% and 49%, respectively) or with overall presumptive male two-lobed

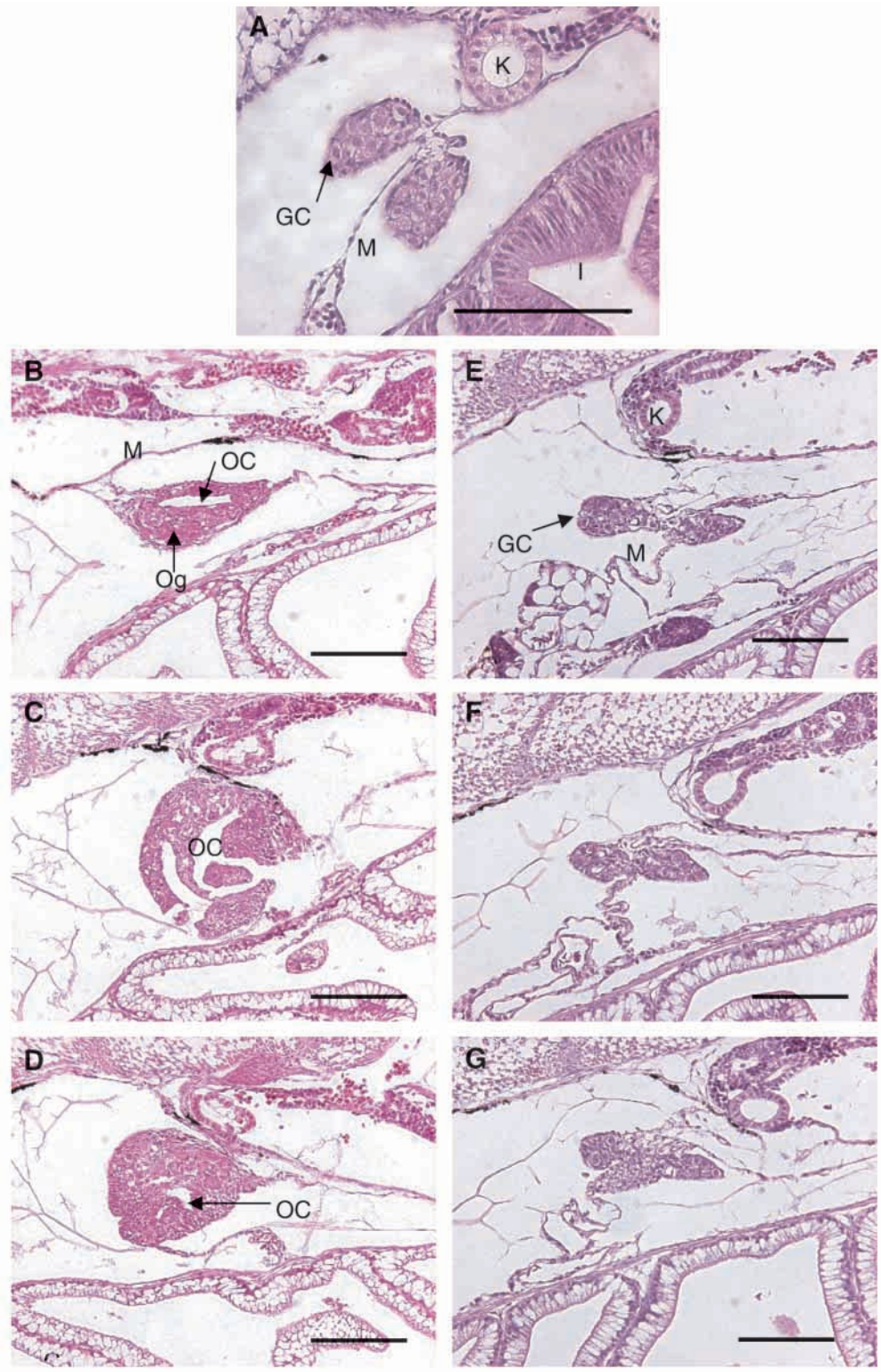

Fig. 11. Light micrographs of an undifferentiated gonad (A) and early differentiating gonads from control eelpout embryos at day 0 (11 October) (B–G), showing transverse sections from the anterior end, central part and posterior end of the gonad. Scale bars, 100 µm. Paraffin section. Haematoxylin-eosin staining. (A) Undifferentiated gonad (20 September). (B–D) Early differentiating female gonad (B = anterior end; C = central part; D = posterior end). (E–G) Early two-lobed differentiating presumptive male gonad (E = anterior end; F = central part; G = posterior end). GC, germ cell; I, intestine; K, kidney; M, mesentery; OC, ovarian cavity; Og, oogonia.

gonads (42% and 51%, respectively) (Table 2). However, in the OP100 group, different gonadal structures were found compared with the control embryos at the end of the experiment (day 35). Of the analysed embryos, 46% had normal ovaries with primary oocytes but only 22% of the embryos had normal presumptive male gonads resembling those found in the control embryos (Table 2). The remaining 32% of the embryos had abnormal gonads with atypical structures that had not been observed in the control embryos either at the beginning (day 0) or at the end (day 35) of the experiment. In most of these abnormal gonads, the anterior end of the gonad resembled that of a male gonad having two

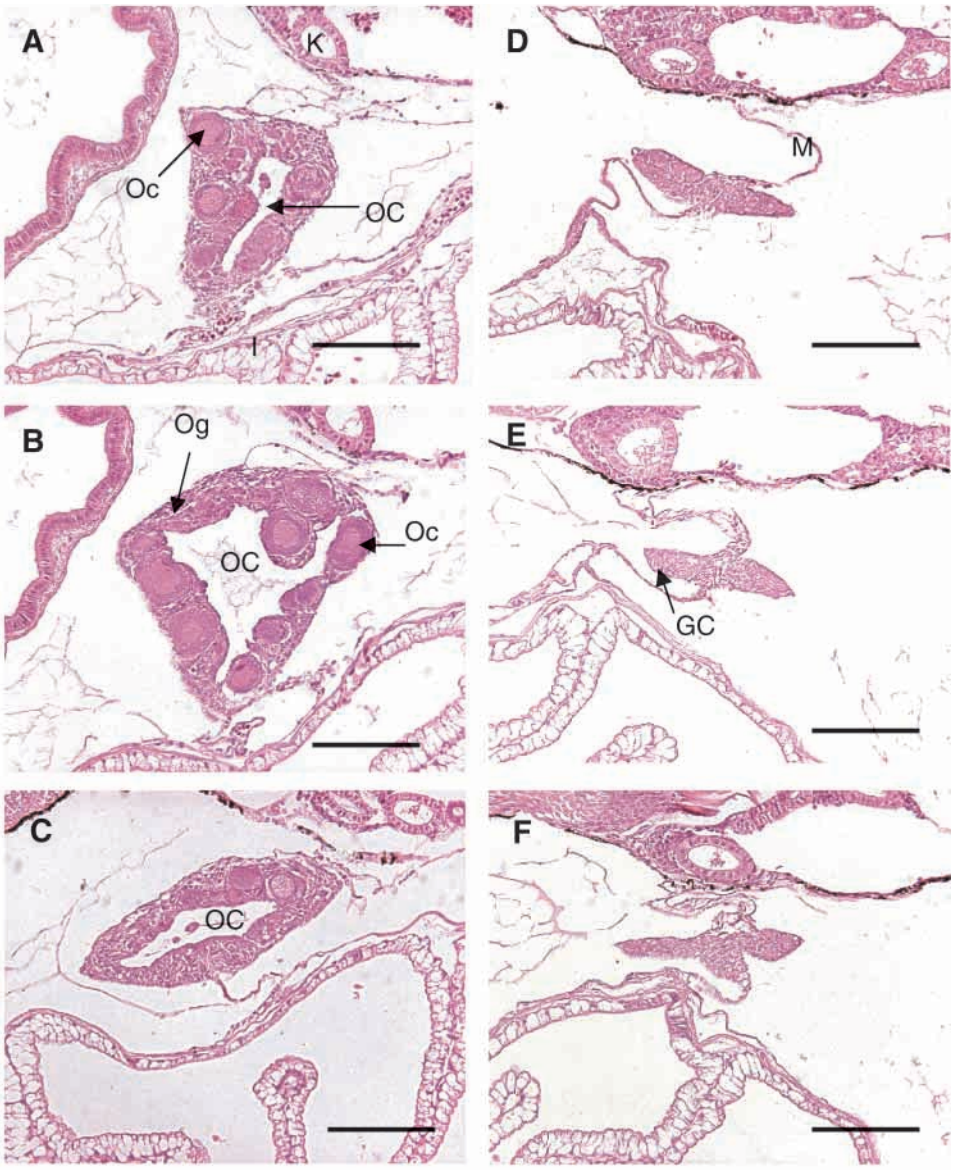

Fig. 12. Light micrographs of gonads from control eelpout embryos at day 35 (15 November) showing transverse sections from the anterior end, central part and posterior end of the gonad. Scale bars, 100 μm. Paraffin section. Haematoxylin-eosin staining. (A–C) Female gonad with a single hollow ovary containing oocytes (A = anterior end; B = central part; C = posterior end). (D–F) Presumptive male gonad, two-lobed. GC, germ cell; I, intestine; K, kidney; M, mesentery; Oc, oocyte (primary); OC, ovarian cavity; Og, oogonia.

lobes (Fig. 13A,E). At the central part, the gonad was enlarged, the two-lobed structure had disappeared and the beginning of two hollow cavities had formed in each side of the gonad (Fig. 13B,C,F,G). In some gonads, these cavities had fused into one big cavity more or less resembling the endo-ovarian cavity of the female gonad. In two of the analysed embryos, the gonads looked mostly like male gonads but had either atypical structures at the external ends of the lobes or the lobes had enlarged and started to bend down towards the central string at the posterior end (results not shown). These structurally abnormal gonads contained germinal cells and somatic cells, but no oocytes were observed in any case.

Discussion

Up-concentration of 4-tOP in body fluids

This study is the first to demonstrate that the xeno-oestrogen 4-tOP can be transferred from the water *via* the mother fish to the ovarian fluid of the ovary and can subsequently affect the embryos *in ovario* in a viviparous teleost species, the eelpout (*Zoarces viviparus*).

The continuous flow-through system developed in the present study was capable of keeping the actual water concentration of 4-tOP at a constant level throughout the experiment. The concentrations were, on average, 56–65% of the nominal concentration. These results are comparable with those obtained by other groups working with alkylphenols in similar systems (Nimrod and Benson, 1998; Harries et al., 2000). The difference from nominal concentrations can be attributed to uptake in fish, volatilisation, microbial breakdown and/or adhesion to the aquarium.

During the experiment, 4-tOP accumulated in the body fluids of the pregnant mother fish. Very high concentrations of 4-tOP were measured in the blood, and BCFs of 200–500 were measured after exposure to the actual dose of 65 $\mu g\,l^{-1}$

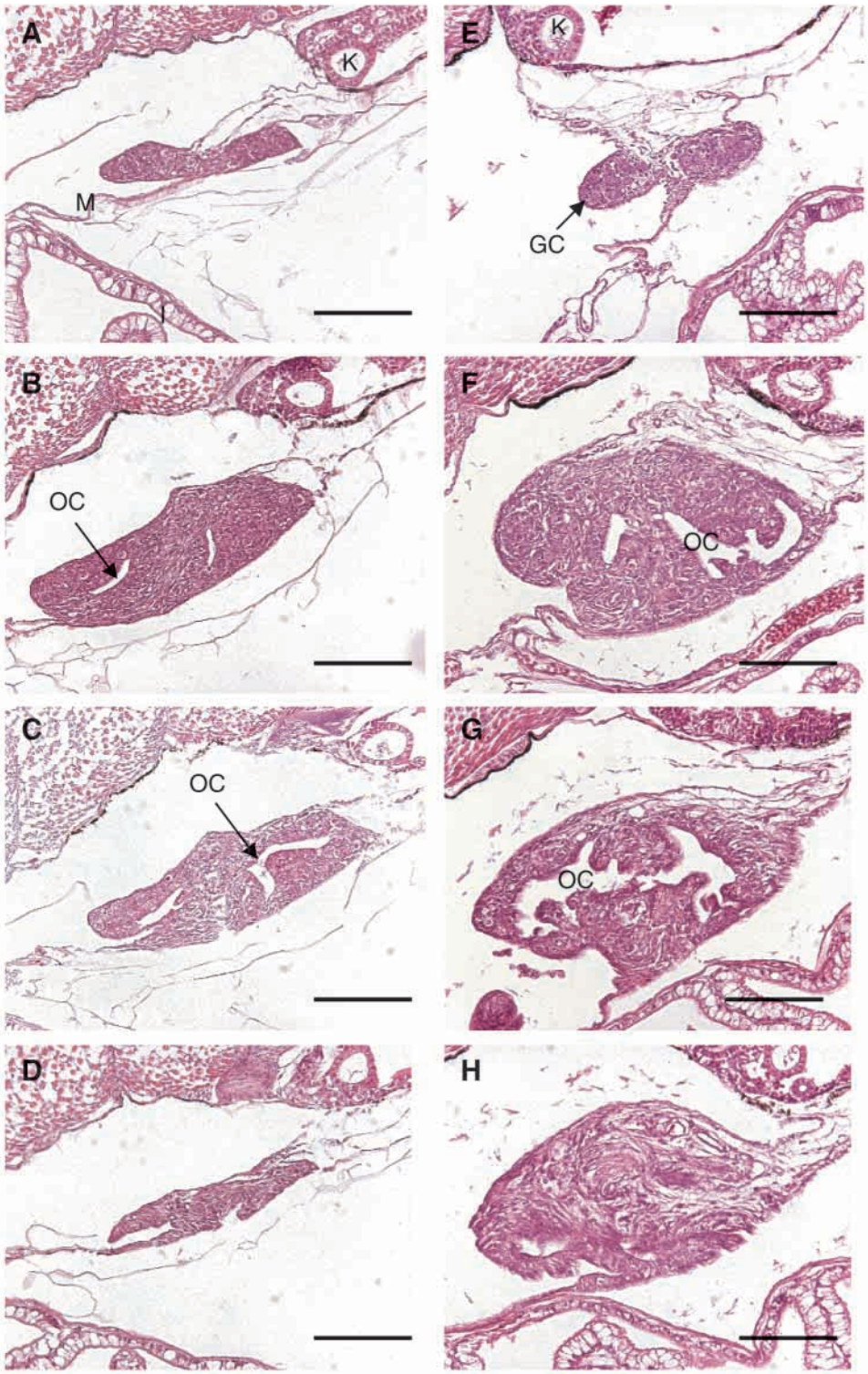

Fig. 13. Light micrographs of abnormal gonads of eelpout embryos from the OP100 (100 µg l^{-1} 4-*tert*-octylphenol) group at day 35 (15 November) showing transverse sections from the anterior end, central part and posterior end of the gonad. Scale bars, 100 µm. Paraffin section. Haematoxylin-eosin staining. (A–D) and (E–H) Abnormal gonad of embryos from two different mother fish. The abnormal gonads have structures resembling both male (two-lobed organ) and female (endo-ovarian cavity) gonads. (A,E) Anterior end; (B,C,F,G) central part; (D,H) posterior end. GC, germ cell; I, intestine; K, kidney; M, mesentery; OC, ovarian cavity.

(OP100). In trout, a BCF of 91 was measured in the blood after 10 days of exposure to 4 µg l^{-1} 4-tOP, a BCF that included OP metabolites (Ferreira-Leach and Hill, 2001). How such high BCFs are achieved in the plasma of the pregnant eelpout is unknown. Earlier studies have shown that during the experimental period (October–November) lipids are mobilised to the plasma from fat stores, making the plasma more lipid (Korsgaard and Petersen, 1979). This could increase the solubility of 4-tOP in the plasma. Furthermore, it is possible that 4-tOP that is accumulated in fat is mobilised concomitantly with the lipids, adding another source of 4-tOP. The binding of 4-tOP to different plasma proteins might contribute to an elevated solubility of the compound. But these aspects need further investigation.

We detected the presence of a conjugated metabolite in both blood and ovarian fluid but quantified only the level of the parent compound 4-tOP and not the metabolites. It is very likely that 4-tOP is conjugated in the liver after uptake in the gills. The conjugated metabolites of 4-tOP are probably not active xeno-oestrogens, as oestrogen metabolites show almost no ER agonism (Zhu and Conney, 1998).

In the ovarian fluid, a pronounced uptake of 4-tOP was observed. 4-tOP may be transferred to the ovary because alkylphenols are highly lipophilic compounds that might pass through ovarian membranes. The ovary appears to be highly vascularized during pregnancy in eelpout (Kristoffersen et al., 1973; Korsgaard, 1983), thereby offering the possibility of nutrients, oxygen and lipophilic chemicals such as 4-tOP to enter the ovarian fluid/embryos from the maternal blood. The accumulation of 4-tOP in the ovarian fluid was higher in the OP100 group than in the OP25 group, indicating that the maximal capacity of the mother fish and/or the embryos to metabolise and excrete the compound had been exceeded at the higher concentration. Fish embryos in general bioaccumulate xenobiotics to a higher degree than do adult fish, probably because of a less-efficient metabolising machinery during the earlier life stages (Monod et al., 1996). To our knowledge, no data exist on the bioaccumulation of xeno-oestrogens in other viviparous species. However, a study using guppy (*Poecilia reticulata*), a viviparous fish species with follicular gestation, showed accumulation of the xenobiotic 3,4-dichloroaniline in ovarian embryos at levels above that seen in muscle, gill, brain and skin (Hertl and Nagel, 1993).

E_2 also bioaccumulated in the body fluids, but to a lesser extent than 4-tOP. This seems reasonable as elaborate endogenous pathways for the metabolism and excretion of E_2 exist (Zhu and Conney, 1998). As 4-tOP accumulated more efficiently than E_2, its oestrogenic potency *in vivo* increases as compared with E_2.

Induction of the Vtg-synthesising apparatus in pregnant eelpout (ER, Vtg mRNA and Vtg protein)

As an indicator of the oestrogenic potency of 4-tOP, the induction of the Vtg-synthesising apparatus was examined. Vtg is widely accepted as a biomarker of oestrogen exposure in male and juvenile fish (Sumpter and Jobling, 1995), and Vtg mRNA induction is regarded as a very sensitive oestrogenic biomarker (Bowman and Denslow, 1999). The induction of Vtg mRNA in liver and also Vtg protein in plasma in all pregnant mother fish exposed to 4-tOP or E_2 was expected and has been observed in numerous experiments with fish exposed to oestrogens and xeno-oestrogens, including alkylphenols (e.g. Flouriot et al., 1995; Jobling et al., 1996; Lech et al., 1996; Christiansen et al., 1998; Andreassen and Korsgaard, 2000). The observed clear linear relationship between the actual plasma concentration of 4-tOP and Vtg indicates that the bioavailable amount of 4-tOP determines the vitellogenic response. This observation corresponds well with the assumption that 4-tOP interacts directly with the hepatic ER in eelpout (Andreassen and Korsgaard, 2000) and induces vitellogenesis. This is further supported by the fact that ER protein levels are induced in accordance with the well-known auto-regulatory effect of oestrogens at the ER level (Pakdel et al., 1991; MacKay et al., 1996). In the present study, a pronounced induction of E_2-binding capacity in the liver cytosol by both 4-tOP and E_2 indicated an up-regulation of the bio-available level of oestrogen receptors. The ability of 4-tOP to induce the Vtg-synthesising apparatus at all levels (ER, Vtg mRNA and Vtg protein) signifies the oestrogenic capacity of the compound.

Disturbance of the maternal–foetal trophic relationship: the ovary, ovarian calcium and amino acids

The ovary of the pregnant eelpout undergoes drastic changes during gestation (Korsgaard, 1986). Thus, before and during a short period after hatching, the ovary is characterised by large fluid-filled follicles and very little fluid present in the ovarian cavity. However, a sudden shift takes place in the production and/or distribution of fluid in the ovary after hatching. The amount of fluid in the ovarian cavity increases significantly, coinciding with less fluid in the follicles. The condition of the ovary and the resulting ovarian environment is likely to be an important factor for the normal functioning of the maternal–foetal trophic relationship and, therefore, the wellbeing of the embryos.

It is obvious from the present study that oestrogenic exposure has an important impact on ovarian factors, but it is difficult to interpret the consequences of these results. Usually when describing effects on fish gonads and their contents, GSI is used as a general index. However, in this context, this is not adequate as the embryo-filled ovary consists of several components, each reacting differently to oestrogenic exposure. The overall effect of E_2 and 4-tOP on the ovary is evident when looking at the OSM index, which summarises the impact on embryos (ESI), ovarian fluid (OFI) and the ovarian sac (OSSI). The changes in ESI and OFI were not statistically significant, presumably due to the large inter-individual variation of pregnant eelpout.

Oestrogen levels in plasma of eelpout peak prior to and decline throughout pregnancy (Korsgaard, 1994). Similarly, E_2 levels abruptly decline after fertilisation in the guppy *Poecilia reticulata* (Venkatesh et al., 1990), and oestrone implants have been shown to suppress ovarian and embryonic development in the viviparous fish *Neoditrema ransonneti* (Ishii, 1960). These observations indicate that oestrogen does not have an important role during pregnancy of viviparous fish or that the absence of oestrogen is important for the progression of pregnancy. Moreover, Korsgaard (1983, 1994) showed that the post-ovulatory follicles of *Z. viviparus* were able to sequester Vtg in oestrogen-treated individuals, and, in *N. ransonneti*, oestrone inhibited the normal development of the ovarian lining during gestation (Ishii, 1960). Thus, it is intriguing to speculate that exposure of pregnant eelpout to oestrogenic compounds may change the function of the ovary from an organ occupied with the nutrition and support of embryos to a Vtg-sequestering organ. The incidences of premature

parturition were only observed in the exposed fish but not in the controls. Oestradiol benzoate has been reported to induce premature birth in top minnows (*Gambusia affinis*; Ishii, 1963). Furthermore, it has been proposed that oestrogen is involved in the initiation of parturition in some viviparous fish species, when birth of the embryos and vitellogenesis for the next batch of oocytes coincide (Venkatesh et al., 1990).

The effects observed on the ovary after E_2 and 4-tOP treatment might be attributed to a direct effect mediated by the ER. ER mRNA has been shown to be highly expressed in the ovary of eelpout (T. K. Andreassen et al., manuscript submitted) and to be located in several different ovarian cell types.

The tendency towards lower levels of ovarian fluid in the ovary of the treated groups could also be of significance, as ovarian fluid is important in the effective transport of nutrients and oxygen to the embryos. Low oxygen levels are known to be able to retard the development of fish larvae (Carlson and Seifert, 1974). It is believed that nutrient (and oxygen) uptake in the ovary of *Z. viviparus* takes place *via* the highly vascularised follicles (Kristoffersen et al., 1973; Korsgaard, 1983), the so-called calyces nutriciae. This transfer of nutrients to the embryos from the mother fish might be disturbed by the oestrogenic treatment.

Previous investigations have provided evidence that free amino acids are one of the important nutrients taken up and metabolised by the embryos of *Z. viviparus* during their intraovarian development (Korsgaard and Andersen, 1985; Korsgaard, 1992). The significant decrease in the maternal plasma concentration of free amino acids (NPS) observed in both E_2- and 4-tOP-treated groups (probably due to an increased demand for amino acids for the hepatic synthesis of Vtg) may be one of the factors responsible for the observed decrease in embryonic growth, even if this was not reflected immediately in the concentration of amino acids (NPS) in the ovarian fluid. We hypothesise that the transfer of amino acids to the ovary/embryos has been negatively affected as the transfer is directly related to the embryonic amino acid availability, because, when first taken up into the ovarian fluid from the maternal circulation, amino acids are rapidly taken up by the embryos (Kristoffersen et al., 1973; Korsgaard, 1992).

A marked decline in the concentration of calcium was observed in the ovarian fluid after 4-tOP and E_2 treatment, which significantly correlated with the observed increase in the concentration of calcium in the maternal plasma. This indicates that calcium is needed by the maternal organism for the enhanced hepatic synthesis of Vtg, which is known to incorporate calcium (Nagler et al., 1987). Consequently, a different distribution of calcium is evident in the exposed fish, resulting in low ovarian calcium but high plasma concentrations. The Vtg-bound calcium in plasma is, however, not available to ovarian/embryonic uptake. Earlier studies have shown that eelpout embryos take up calcium *in ovario*, an uptake that is severely inhibited by E_2 treatment (Korsgaard, 1994). It is, however, not known if and how such a decline in calcium availability may affect embryonic development. However, as calcium is an important component necessary for the development of the skeleton and various other parts of the body, calcium may play a pivotal role during embryonic development.

Effects on embryonic growth

Exposure to 4-tOP or E_2 inhibited embryonic growth. In a similar experiment performed during very early pregnancy (September–October), growth was also significantly inhibited by 4-tOP and E_2, and deformities after E_2 treatment were observed (results not shown). Previous studies have reported negative impacts of natural oestrogens (Johnstone et al., 1978; Krisfalusi and Cloud, 1996; Gimeno et al., 1998) or xeno-oestrogens (Ashfield et al., 1998; Drèze et al., 2000) on growth in different species of fish. Such effects were suggested to be caused by disruption of the somatotropic axis (Drèze et al., 2000). Thus, the negative effects on embryonic growth observed in the present study may, in part, be a direct result of the oestrogenicity of the compounds. Being a viviparous fish in which the embryos are dependent on the ovarian environment, indirect effects involving the maternal–foetal trophic relationship of *Z. viviparous* should also be taken into account. Thus, the changed physiology of the ovary and the oestrogen-induced metabolic changes may, as explained above, have a marked effect on this relationship and hence the growth and development of the eelpout embryos. The positive correlation between the ovarian fluid index (OFI) and embryonic mass shows the importance of the ovarian fluid for the normal development of the embryos. A decrease in ovarian fluid induced by extended exposure to xeno-oestrogens may thus be critical because the ovarian fluid has a multitude of functions, such as providing oxygen and nutrients and removing waste products (Korsgaard and Weber, 1989).

Increased mortality of embryos was observed in the OP-treated groups, correlating with the concentration of 4-tOP in the ovarian fluid. This observation indicates a toxic effect of the compound on the embryos. However, with a concentration higher than $1000\,\mu g\,l^{-1}$ 4-tOP in the ovarian fluid, lethality among the embryos of this group is to be expected (Servos, 1999).

Effects on embryonic Vtg synthesis

To investigate whether the 4-tOP in the ovarian fluid had directly affected the embryos, the induction of Vtg synthesis in the embryos was analysed. As expected, no Vtg mRNA expression was found in the control embryos. Similarly, no Vtg mRNA expression was found in the OP25- and E_2-treated groups. Similar results were obtained by immunohistochemistry. However, the embryos from the OP100 group showed marked induction of Vtg mRNA and protein, thus confirming that the embryos had indeed been exposed *in ovario* to concentrations of 4-tOP high enough to elicit an oestrogenic response. The fact that no Vtg mRNA or Vtg protein induction was observed in the embryos of the E_2 and OP25 groups could be explained by the relatively low concentrations of the compounds *in ovario* and by a lower

oestrogen sensitivity of eelpout embryos compared with adult and juvenile fish.

Earlier studies have shown that the threshold concentration of E_2 for the induction of Vtg is between 27 ng l^{-1} and 272 ng l^{-1} in fathead minnow (*Pimephales promelas*; Parks et al., 1999) and between 33 ng l^{-1} and 212 ng l^{-1} in sheepshead minnow (*Cyprinodon variegatus*; Folmar et al., 2000). However, in one study, very low concentrations of E_2 (9 ng l^{-1}) were able to induce Vtg synthesis in juvenile rainbow trout (*Oncorhynchus mykiss*; Thorpe et al., 2000). In the E_2 group in the present study, an average concentration of 58 ng l^{-1} E_2 was obtained, while 25 ng l^{-1} E_2 was present in the control ovarian fluid. It is very plausible that this relatively small increase would not induce a physiological response in eelpout embryos. In the OP25 group, 5 μg l^{-1} 4-tOP was detected in the ovarian fluid, which is a fairly low concentration. A dose–response study on juvenile rainbow trout using the alkylphenol 4-NP did not induce Vtg induction at a concentration of 7 μg l^{-1} (Thorpe et al., 2000); however, Jobling et al. (1996) observed Vtg induction by exposure of male rainbow trout to 5 μg l^{-1} 4-tOP for 3 weeks.

Interestingly, strong Vtg-staining was found in the hindgut of the embryos in all the treated groups, in contrast to the liver where Vtg was induced only in the OP100 group. This indicates that the Vtg in the hindgut is of maternal origin. The Vtg probably originates from the ovary (e.g. follicular tissue containing Vtg), as embryos are known to ingest ovarian material (Kristoffersen et al., 1973). This Vtg is taken up by the epithelial cells; it is, however, unknown whether the hindgut is capable of metabolising the Vtg. Consequently, oestrogen-treated eelpout embryos might contain Vtg of both embryonic and maternal origin because of the maternal–foetal trophic relationship characteristic of the eelpout. A technical consequence is that the ELISA method widely used for the determination of Vtg in adult fish and oviparous juvenile fish is not useful for eelpout embryos (whole body homogenates).

Effects on gonadal differentiation

It has been suggested that 17β-oestradiol may act as the natural inducer of ovarian differentiation in teleost fish (Nakamura et al., 1998). The present study demonstrates that 4-tOP affects the gonadal differentiation of eelpout embryos exposed *in ovario* following exposure of the mother fish to the actual mean concentration of 65 μg l^{-1} 4-tOP (OP100 group). This result correlates well with the observation that only in this treatment group do embryos show a clear oestrogenic response (induction of Vtg mRNA and protein).

In the control group at day 0 and at day 35, the ratio of male to female embryos of the investigated embryos in the mother fish was approximately 1:1, which agrees with the reported sex ratio from field studies (Larsson et al., 2000). In the OP100 group, 46% of the analysed embryos were females; however, only 22% of the embryos had normal (presumptive) male gonads. The remaining 32% of the embryos had abnormal gonads in which the anterior end of the gonad generally appeared as a presumptive male gonad while the central parts and posterior ends appeared as an early differentiating female gonad with an endo-ovarian cavity. These observations of female-like reproductive ducts (ovarian cavities) in 'male' embryos after exposure to oestrogenic alkylphenols are supported by other studies on oviparous fish species. Gimeno et al. (1997) reported that exposure of young genetically male carps (*Cyprinus carpio*) to 4-*tert*-pentylphenol during sexual differentiation induced the formation of an oviduct in almost all fish. Gray and Metcalfe (1997) investigated Japanese medaka (*Oryzias latipes*) exposed to a range of concentrations of nonylphenol (NP) from hatching to 3 months of age. They showed that 50% of the male fish treated with 50 μg l^{-1} NP developed testis-ova. Drèze et al. (2000) reported that exposure of the viviparous mosquito fish (*Gambusia holbrooki*) to 50 μg l^{-1} 4-NP from 3 days post-parturition to 75 days post-parturition resulted in fish exhibiting female or undeveloped/atrophied gonads and no fish with normal male gonads. In accordance with our observations in the OP25 group, exposure to low concentrations of alkylphenols (0.5–1.9 μg l^{-1} 4-NP) in Japanese medaka exposed during the first month after hatching had no effect on gonadal development or sex ratio (Nimrod and Benson, 1998). However, no effects on the gonadal development and the sex ratio were found in offspring of the viviparous guppy (*Poecilia reticulata*) after exposure to octylphenol (Kinnberg et al., in press). Recently, Rodgers-Gray et al. (2001) found that exposure of roach (*Rutilus rutilus*) to graded concentrations of treated sewage effluents for 150 days during their gonadal differentiation resulted in the formation of female-like reproductive ducts (ovarian cavities) in male roach in a dose-dependent manner. The effluents contained low concentrations of alkylphenols (90–2000 ng l^{-1}), natural oestrogens (5.9–37 ng l^{-1}) and, presumably, other unidentified endocrine disrupters. Similar to the present study, Rodgers-Gray and co-workers (2001) did not find oocytes in these feminised male fish. Female-like reproductive ducts have also been reported in wild roach and gudgeon (*Gobio gobio*) in UK rivers (Jobling et al., 1998; van Aerle et al., 2001).

The oestrogenic effects on embryos observed in the present study, such as induction of Vtg mRNA and protein and the development of female-like ovarian cavities in 'males', might represent direct effects *via* the oestrogen receptors in the liver and gonadal tissues, respectively. 4-tOP is known to bind oestrogen receptors in liver tissue, causing induction of vitellogenesis in numerous fish species including the eelpout (Andreassen and Korsgaard, 2000). Recently, oestrogen receptors have also been identified in testes of adult fish, making direct effects of xeno-oestrogens such as octylphenol on testes very likely (Loomis and Thomas, 1999; Legler et al., 2000; T. K. Andreassen et al., manuscript submitted). In addition, oestrogen receptors have recently been identified during early development in both male and female transgenic zebrafish *Danio rerio* (Legler et al., 2000). In the present study, we have shown that ER mRNA expression is present in early differentiating gonads, including presumptive male gonads, indicating that functional ER may be present at this stage. This

observation makes it possible for xeno-oestrogens to act directly on the gonads during early sex differentiation in *Z. viviparus*. Thus, the critical period of gonadal differentiation may be very sensitive to the disruption of hormonal homeostasis by oestrogenic compounds. Administration of oestrogens before irreversible commitment of the gonads to sex may lead sex ratios towards the female direction in gonochoristic fish; hence, the genetic determination of gonadal sex in teleosts may be affected by exogenous sex hormones (Yamamoto, 1969; Nakamura et al., 1998). As oestrogens are known to affect the brain–pituitary–gonadal axis during development (Kah et al., 1997), it is likely that there are alternative ways in which xeno-oestrogens may affect the gonadal development in the early life stages of fish.

The timing and duration of exposure directly relates to the severity of the effects by oestrogenic compounds. Therefore, a possible explanation as to why we also observed some male embryos containing apparently normal male gonads, even in the high dose 4-tOP group, could be that the exposure was not conducted sufficiently early during the gonadal differentiation but during the late yolk-sac stage. At the late yolk-sac stage, gonadal differentiation may have progressed to a point where the xeno-oestrogens have reduced effects and hence did not induce the female-like ovarian cavity in all embryos.

Alkylphenols (including 4-tOP) have been detected at concentrations of up to $13\,\mu g\,l^{-1}$ in an estuary in the UK (Blackburn and Waldock, 1995); however, concentrations of approximately $1–10\,\mu g\,l^{-1}$ are more common (Bennie, 1999). The actual concentration of $14\,\mu g\,l^{-1}$ (OP25) is therefore an environmentally relevant concentration, while the $65\,\mu g\,l^{-1}$ concentration (OP100) is rare in the aquatic environment. In the present study, severe direct effects on embryos were only observed in the OP100 group; however, lower and environmentally relevant concentrations (e.g. OP25 group) have an effect on the viviparous mother fish, resulting in the allocation of energy from the support of embryonic growth to physiological non-essential processes (Vtg synthesis) and formation of smaller (and probably less fit) embryos or, even worse, abortions. In the wild, these kinds of effects may have consequences at the population level.

Conclusions

In conclusion, this study is the first to demonstrate that an oestrogenic endocrine disrupter, 4-tOP, can be transferred from the water *via* the mother fish to the ovarian fluid of the ovary and can subsequently affect the embryonic development *in ovario* in a viviparous teleost species. In the mother fish, the compound induced vitellogenesis, caused impairment of normal ovarian development and changed the nutritive status of maternal blood and ovarian fluid. Embryonic growth was negatively affected, which might, in part, be attributed to disturbances of the maternal–foetal trophic relationship. In the embryos, 4-tOP acted as an oestrogenic compound by inducing Vtg synthesis and causing abnormal male gonads with female-like ovarian cavities. This study contributes to the increasing evidence that xeno-oestrogens can impose severe effects on the gonadal differentiation of fish.

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