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17β-Estradiol Causes Abnormal Development in Embryos of the Viviparous Eelpout

Jane E. Morthorst,*[,†] Nanna Brande-Lavridsen,[†] Bodil Korsgaard,[†] and Poul Bjerregaard[†]

[†]Department of Biology, University of Southern Denmark, Campusvej 55, Odense M, DK-5230, Denmark

Supporting Information

ABSTRACT: Elevated frequencies of malformations among the offspring of Baltic eelpout (*Zoarces viviparus*) have been observed in aquatic environments receiving high anthropogenic input suggesting that manmade chemicals could be the causative agent. However, causal links between exposure to chemicals and abnormal development have never been confirmed in laboratory experiments. The purpose of this study was to investigate if exposure to 17β-estradiol (E2) causes abnormal development in larvae of the viviparous eelpout. Wild female eelpout were collected immediately after fertilization and exposed to E2 concentrations ranging from 5.7 to 133 ng L^{-1} for 6 weeks in a flow through test system. The experiment shows that E2 concentrations of 53.6 and 133 ng L^{-1} cause severe abnormal development among eelpout embryos. Reduced amount of ovarian fluid and increased weight of the ovarian sac indicate disturbance of ovarian function. Female plasma concentrations of E2 and vitellogenin increase in a monotonic concentration−response relationship with significant induction in the low concentration range. Our findings support the plausibility that the abnormal development among eelpout embryos encountered in monitoring programs may actually be caused by exposure to chemicals in the environment.

INTRODUCTION

The eelpout is one of just a few viviparous teleost fish species in Northern Europe, and it has been used as a monitoring organism for detection of harmful substances in the environment by several Baltic countries[1−3] and in international monitoring programs.[4,5] The eelpout is a suitable monitoring organism as it lives in coastal areas and has a stationary behavior, and more importantly, the viviparous life history allows detection of malformations among the offspring. Monitoring programs in several countries (i.e., Denmark, Germany, and Sweden) have revealed elevated frequencies of malformations among eelpout (*Zoarces viviparus*) embryos at certain geographic locations.[1,2,6] In wild oviparous fish species, seriously malformed larvae will most likely slip past unnoticed due to their free-living nature.

Malformations can be induced in oviparous fish species by laboratory exposure to chemicals,[7−9] and the implicit assumption in the eelpout monitoring programs is that the observed malformations might be caused by changes in environmental conditions—including exposure to chemicals. In a review of Danish data on malformations, potential sources of discharge of chemicals, oxygen depletion, etc., it was concluded that increased frequencies of malformations were associated with high anthropogenic input to the coastal areas, but no specific chemical or groups of chemicals could be pinpointed as causal agents.[10] Although exposure of eelpouts to chemicals is suspected as the causal agent, the experimental evidence linking malformations to chemical exposure is limited: Mattsson et al.[11] showed that maternal exposure to phytosterols induced malformations in newborn eelpout fry, whereas in the laboratory, exposure to contaminants abundant in the Danish areas with the highest frequencies of malformations recorded in the monitoring programmes did not result in malformations.[12] The lack of further experimental evidence is probably related to the challenges of keeping pregnant eelpout alive in the laboratory, and in addition only one single experiment can be performed annually as breeding occurs once per year.

The ovary of fish with intraluminal gestation undergoes significant changes during development of the embryos. During spring and early summer the females undergo vitellogenesis and eggs increase in size. During late August and early September the eggs are ovulated and shortly after the internal fertilization takes place; the eggs hatch approximately 3 weeks later.[13] The yolk sac provides nutrition for the embryos during the first month after hatch but in the remaining three to four months of gestation, the growth of embryos depends on nutrient transfer from the mother.[14−16] However, maternal transfer of nutrients starts already before the yolk sac is fully absorbed.[17−19] The maternal transfer may occur by means of the ovarian fluid[14,18,20,21] or, probably later in the gestation, by suckling on the postovulatory follicles[22] in which the fluid is rich in nutrients from the maternal circulation.[14]

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Unpublished observations from previous experiments in our laboratory indicate the presence of a fairly narrow sensitive window for the teratogenic effects of chemicals on eelpout larvae. To reveal teratogenic effects, it is therefore crucial that the pregnant females are caught and brought to the laboratory right after fertilization. On the other hand, one also has to be certain that the majority of females have been fertilized. The fact that ovulation and fertilization take place fairly synchronously among the females in a population[23] does, however, make it possible to bring females to the laboratory, among which the majority has been fertilized.

German investigations show that malformation frequencies among the eelpout fry[1] are highest in the areas which also have the highest frequencies[24] of feminized male eelpout (approximately $^1/_4$ of males showed intersexuality with oocytes in their testes), and the aim of the present investigation was to investigate if E2 or chemicals with estrogenic properties could be causing the malformations observed in nature, and subsequently to establish a threshold exposure concentration for teratogenic effects of E2.

EXPERIMENTAL SECTION

Experimental Animals. Eelpout were caught in seines by local fishermen in the coastal waters around the island of Birkholm (54′56″ N, 10′31″ E), Denmark during August and September 2011. Eelpout from this area have previously been used for experiments and without registration of elevated frequencies of malformations.

The reproductive status of wild female eelpout was monitored by spot checks of the ovaries twice a week from mid-August. Before August 25 no ovulated females were observed but on August 30 ovulation had occurred in most females and at the spot check on September 2 all the examined females were ovulated and fertilized. Eelpout for the experiments were collected September 1 and 3. The fish were transported to the Marine Biological Research Station in Kerteminde, Denmark. The eelpout were sexed and 11 females were transferred to each of 24 exposure tanks without acclimatization. Acclimatization was not allowed because the sensitive window for exposure is narrow and begins right after fertilization. Only healthy looking fish were used in the experiment (body length > 21 cm). The mean length and weight of all fish was 26.8 ± 0.2 cm (mean ± standard error of the mean (SEM)) and 90.1 ± 2.1 g (mean ± SEM), respectively.

Exposure. Polyethylene tanks with a volume of 170 L (water volume: 145 L) were used and the water flow was 200 L per day. Peristaltic pumps (Ole Dich) supplied the exposure tanks with seawater and chemicals. 17β-Estradiol (CAS no 50-28-2) was dissolved in isopropyl alcohol (CAS no 67-63-0), and the final solvent concentration in the exposure tanks was < 0.01%. The water was aerated during the exposure period, and submerged circulation pumps ensured maximum mixing. During the exposure period water temperature decreased from 15 to 13 °C with a mean of 14 ± 0.10 °C (±SEM), the salinity was 20.3 ± 0.14 ppt (mean ± SEM), oxygen saturation remained above 59% (average oxygen saturation for all tanks: 90.7 ± 0.65% (mean ± SEM)), and a 12:12 h light dark regime was used. Previous studies have shown that feeding activity ceases during September and October;[23] however, to avoid uneven growth due to differences in food intake, the fish were not fed during exposure. Water samples were collected regularly from the exposure tanks and frozen at −20 °C for subsequent chemical analysis (Table 1). E2 concentrations in the stock solutions were determined nine times for each stock concentration, and the average maximally deviated 7.7% from the expected value. Temperature, salinity, and oxygen saturation were measured daily in the header tanks but only weekly in each individual exposure tank because the fish are very sensitive to movement and sound disturbance.

Table 1. Actual E2 Concentrations in the Exposure Groups (Groupwise Mean ± SEM). The Values Are Based on 10 Determinations from Each Single Tank (n = 30 for Each Exposure Concentration)

nominal concentrations, ng L^{-1}	actual concentrations, ng L^{-1} (±SEM)
control	0 (±0)
solvent control	0 (±0)
12.5	5.7 (±1.17)
25	10.1 (±1.07)
50	13.3 (±1.44)
100	22.9 (±2.53)
250	53.6 (±8.96)
500	132.7 (±20.08)

Each exposure concentration had three replicates, and the following nominal concentrations were planned: 0 (control), 0 (solvent control), 12.5, 25, 50, 100, 250, and 500 ng E2 L^{-1}. Each tank was inspected every day, and dead fish were removed.

Termination of the Experiments. Owing to the very comprehensive sampling procedures, the sampling took place over five consecutive days (October 10 to 14) after 40 to 44 days of exposure. Fish from one exposure tank were sampled in a row, but tanks for sampling were randomly chosen. The pregnant eelpout were assigned a number, euthanized in tricaine methanesulfonate (MS-222; 0.1 g/L), and length (to the nearest 0.5 cm) and weight were noted. A blood sample was taken from the caudal vein with a heparinized syringe and transferred to heparinized Eppendorf tubes following centrifugation for 10 min (4 °C and 3000g). The plasma was removed and stored at −80 °C until analyses could be performed. After decapitation of the fish the liver was removed, weighed, and snap frozen in liquid nitrogen. The entire ovary was carefully dissected and placed in a plastic tray. To collect the fluid the ovary was sieved and the embryos were removed, weighed, and photographed. As the development of the vast majority of larvae within a brood is synchronous it was possible to select 10 typical individual embryos from each brood, and their weights and lengths were recorded. The following indices were calculated for the mothers:

somatic weight: body weight (g) − ovary weight (g).
gonad somatic index (GSI): (weight of embryos (g) + ovary sac weight (g) + ovarian fluid weight (g))/somatic weight (g) × 100.
condition index (CI): somatic weight (g)/length^3 (cm) × 100.
liver somatic index (LSI): liver weight (g)/somatic weight (g) × 100.
ovary sac somatic index (OSSI): weight of the ovary sac (g)/somatic weight (g) × 100.
embryo somatic index (ESI): weight of the embryos (g)/somatic weight (g) × 100.
ovary sac index (OSI): ovary sac weight (g)/ovary weight (g) × 100

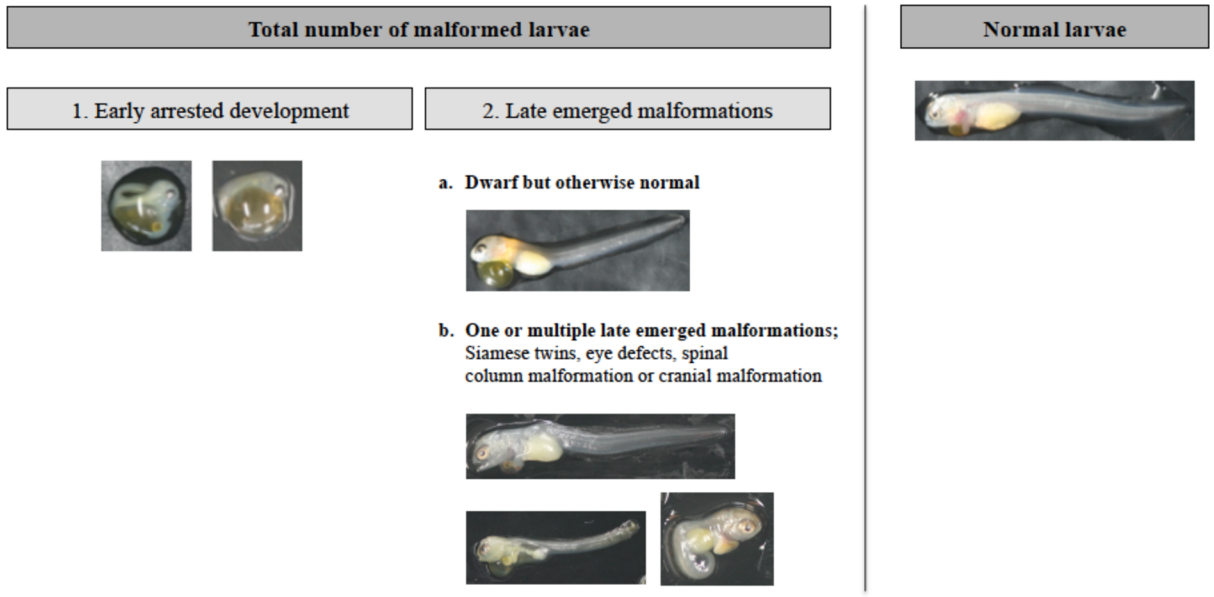

Figure 1. Malformation types. Classification of eelpout fry malformation types: type 1, early emerged malformations; type 2a, late emerged malformation—dwarf but otherwise normal; type 2b, late emerged malformation—one or multiple malformations.

ovarian fluid index (OFI): ovarian fluid weight (g)/ somatic weight (g) × 100

Condition index for the embryos (CIE) was also calculated on the basis of length and weight of 10 representative larvae from each female: Mean embryo clutch weight (g) × 100/mean embryo clutch length^3 (cm).

The anatomy of the larvae was analyzed visually based on photographs of each brood and the assessor knew only the number of the mother fish and not the exposure history. To get familiar with the developmental stages the larvae of unexposed mothers were studied regularly in the days preceding the sampling. The condition and malformation types were recorded according to the scheme in Figure 1.

Plasma Levels of Vitellogenin and E2. Vitellogenin levels were determined in plasma (5 μL of plasma diluted 1 to 8 × 10^6) by a direct noncompetitive sandwich enzyme-linked immunosorbent assay (ELISA) as described in Korsgaard and Pedersen[25] with the modifications described by Velasco-Santamaria et al.[26] The detection limit was approximately 100 ng of vitellogenin mL^{-1} (orders of magnitude below the plasma concentrations actually determined). Plasma E2 levels were determined by an enzyme immuno assay (EIA; Cayman Chemical, Ann Arbor, MI, USA) as described by Morthorst et al.[27] E2 levels in plasma from females without larvae in the ovaries were not determined.

Quantification of E2 in Water. High-performance liquid chromatography—tandem mass spectrometry (a 1200 Series HPLC and a 6410 Triple Quad LC/MS, both Agilent Technologies) was used to determine actual exposure concentrations. 17α-Ethinylestradiol (EE2) (Sigma-Aldrich) was added as internal standard. The samples were prepared using solid phase extraction on the column Strata-X 100 mg 6 mL^{-1} Polymeric RP sorbent (8b-S100-ECH, Phenomenex, Torrance, CA, USA); the samples were extracted on a Waters (Milford, Massachusetts, USA) Extraction Manifold with a maximum vacuum level of 250 mmHg. The column was conditioned with 5 mL of MeOH, 0.1% NH_4OH and equilibrated with 5 mL of H_2O, 0.1% NH_4OH. The column was washed with 5 mL of H_2O, 0.1% NH_4OH, emptied, and dried for 1 min. The column was eluted with 5 mL of H_2O, 0.1% NH_4OH and dried for 12 min in a TurboVap (Caliper Life Sciences, Hopkinton, MA, USA). Finally, the sample was redissolved in 1 mL of 70% MeOH, 0.1% NH_4OH. A 1 mL aliquot of the eluent was injected in the HPLC—MS/MS with conditions as follows: column, Zorbax SB-C18 2.1 × 30 mm; 3.5 μm Rapid Resolution HT; column temperature, 25 °C; isocratic step with 0.1% MeOH and NH_4OH in a proportion 50:50; flow, 0.3 mL min^{-1}; stop time, 9 min; injection, 40 μL; needle wash in flush port, 5 s; and negative electrospray ionization mode. Drying gas flow was 10.0 L min^{-1}, nebulizer pressure was 50 psig, drying gas temperature was 325 °C, and the capillary voltage was 4000 V. For E2 and EE2 individual setups were used: precursor ion, 271.1 (E2) and 295.1 (EE2); quantifier ion, 144.9; dwell, 200; fragmentor, 90 (E2) and 100 (EE2); and collision energy, 30. The standards were prepared using EE2 diluted in 70% MeOH, 0.1% NH_4OH. E2 extraction recoveries varied between 96.4 and 109.1%.

Actual E2 concentrations were determined in each single tank 10 times in the period September 2 to October 3.

Data Handling and Statistical Analysis. All statistical analyses were performed in Sigma Plot 12.5 or Systat 8.0. Data sets were tested for homogeneity and normality and if necessary log-transformed. One-way Analysis of Variance (ANOVA) was used to test means, but if data sets still failed the normality test after log-transformation a Kruskal—Wallis One-Way ANOVA on ranks was performed. Differences in malformation frequencies were tested by means of Chi^2-tests; where needed, Dunnet's test was used to correct for multiple comparisons. The control and solvent control groups were treated as one single control group in the statistical analyses as there was no significant difference between the groups in any of the investigated parameters. This is standard procedure in the Organisation for Economic Co-operation and Development (OECD) test guidelines; however, the groups are depicted separately in the figures. A significance level of 0.05 was used in all analyses, and the results are presented as frequencies or mean ± SEM.

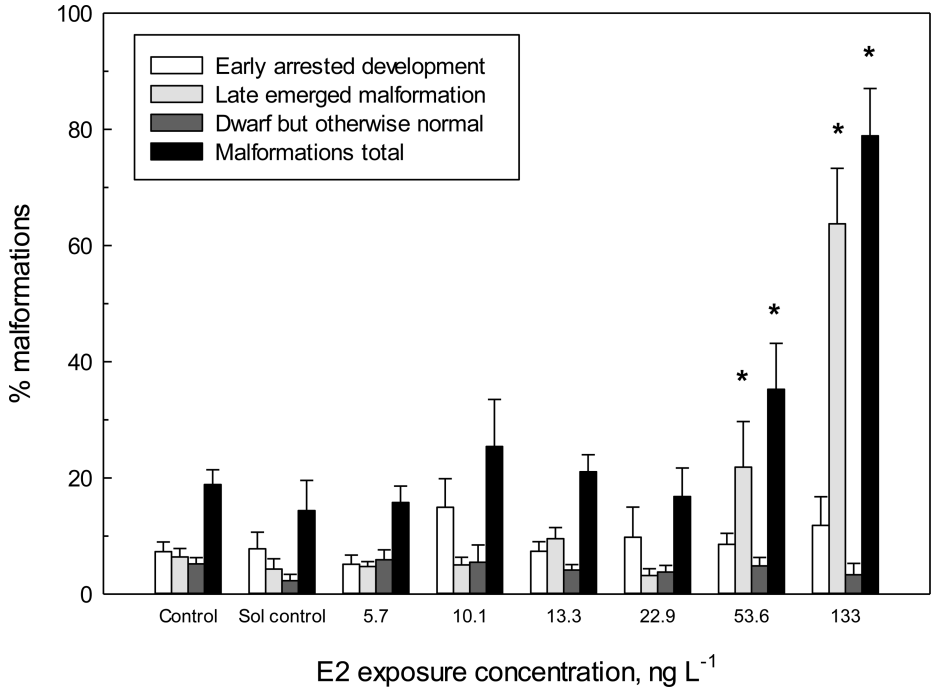

Figure 2. Frequency of fry malformations. Average malformation frequencies (±SEM) in fry of E2 exposed pregnant eelpout. Classification of malformations is explained in Figure 1. Black bars: Total number of malformed larvae (The sum of early arrested development (Figure 1 type 1) and all late emerged malformations (Figure 1 types 2a and 2b)). Dark gray bars: Dwarfs but otherwise normal (Figure 1 type 2a). Light gray bars: One or multiple late emerged malformations (Figure 1 type 2b). White bars: Early arrested development (Figure 1 type 1). The asterisk (∗) means significantly different from the control ($p < 0.05$).

RESULTS

Fry Malformations and Ovarian Fluid. The total percentage of malformed fry in broods from control mothers was 17.4% (Figure 2). The percentage of malformed larvae was significantly increased after exposure to 53.6 (35.2%) and 133 ng L^{-1} (78.9%). Further it was observed that most broods of mothers exposed to 133 ng L^{-1} were almost motionless when removed from the ovaries.

The frequencies of normal dwarfs and early arrested development are not affected by the exposure, whereas the frequency of fry with one or multiple late emerging malformations increases (Figure 2). The frequencies of fry with early arrested development (Figure 1 type 1) and dwarfs but otherwise normal (Figure 1 type 2a) are shown in Figure 2 to illustrate that the frequency of these malformation types are unaffected by the exposure. The late emerging malformations (Figure 1 type 2b) appeared very diverse and ranged from mild malformations such as smaller eye dis-pigmentations to severe malformations like coiled spinal column or multiple malformations. The brood size was not affected by the exposure (data not shown).

Vitellogenin and E2 Plasma Levels. Plasma E2 and vitellogenin concentrations increased in a monotonic concentration−response relationship with the exposure concentration; increases were statistically significant at the lowest test concentration (5.7 ng L^{-1}) (Figure 3A,B); however, when using the solvent control alone for comparison in the statistical tests, the increases in vitellogenin and E2 concentrations were significant at 13.3 and 10.1 ng L^{-1}, respectively. E2 levels were only determined in fertilized females in order to reduce costs, whereas the vitellogenin level was determined for each fish. Vitellogenin levels of fertilized and unfertilized females were only significantly different in one of the groups (exposed to 13.3 ng L^{-1}; $p = 0.016$) and therefore values for both fertilized and unfertilized females are included in Figure 3B. Average E2 concentrations in the plasma exceeded the exposure concentrations with factors between 12 and 28 (Supporting Information, Figure S1), and concentration factors tended to increase with increasing exposure concentrations.

Mortality and Fertilization Rates. In the present experiment a total of 128 out of 264 fish survived until sampling, resulting in an overall survival rate of 48.5%. The mortality within the exposure groups was 42 to 61% and not related to the exposure (Table 2). The mortality was most pronounced during the initial weeks of the experiment and very low during the weeks prior to sampling−indicating that the surviving eelpout were in a good condition. The exposure of the eelpout started just a few days after catch and damages obtained during catch, transport, or handling are not necessarily visible at the onset of the experiment. Indeed low survival rates (<50%) are common when transferring newly caught eelpout to the laboratory.[11,12]

Unexpectedly, 39% of the fish that survived until sampling were unfertilized (between 21 and 58% in the exposure groups). Animals were collected regularly during August to follow the fertilization rate but as the fishermen move around to different spots they might have caught fish from different subpopulations that are not completely synchronized. That is likely to explain why some of the females were unfertilized at sampling.

Exposure Concentrations. The actual exposure concentrations were determined and remained in the range 21 to 45% of the planned nominal concentration (Table 1).

Biometric Indices of Mothers and Fry. The amount of ovarian fluid (OFI) was significantly reduced at the highest test concentration but also an increase at 22.9 ng L^{-1} was observed

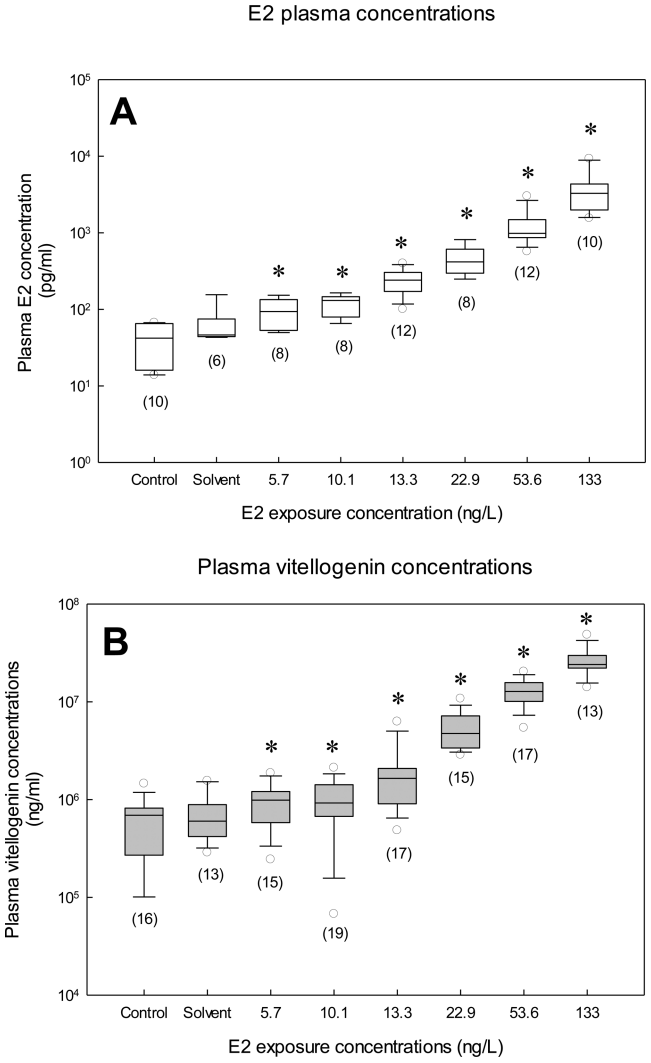

Figure 3. Female E2 and vitellogenin concentrations. E2 (A) and vitellogenin (B) plasma concentrations in E2-exposed pregnant eelpout. E2 levels were only determined in fertilized females, whereas the vitellogenin level was determined for each fish. Boxes represent the 25th and 75th percentiles, the central line within the boxes is the median and whiskers represent the 10th and 90th percentiles. Open circles are outliers. The total number of fish in each group is given in parentheses below the boxes. The asterisk (∗) means significantly different from the control ($p < 0.05$). NB! When using the solvent control alone for statistical analysis the vitellogenin increase is significantly different at 13.3 ng L^{-1} and above and the E2 increase is significantly different at 10.1 ng L^{-1} and above.

(Figure 4). The liver somatic index (LSI) and the ovarian sac somatic index (OSSI) of the mothers were increased when exposure reached 22.9 ng L^{-1} and higher (Table 2). ESI did not vary between groups and a significant difference in GSI and OSI was only observed in groups exposed to 22.9 ng L^{-1} and 133 ng L^{-1}, respectively. A significant increase in the condition index of the embryos (CIE) was only observed at the highest test concentration (Table 2) and the mean length of fry was 2.9 cm in the control group and only significantly reduced at 53.6 ng L^{-1} (2.6 cm; $p = 0.047$) and 133 ng L^{-1} (1.9 cm; $p < 0.001$).

DISCUSSION

In this study we find that maternal exposure to E2 during early pregnancy causes severe malformations in fry of the viviparous eelpout.

Fry Malformations and Teratogenic Effects of Chemicals in Fish. Maternal exposure to 53.7 and 133 ng E2 L^{-1} during early pregnancy caused increased frequency of malformations in fry (Figure 2) and the body length was also significantly reduced (data not shown). Several endocrine disrupting chemicals are able to induce severe malformations when eggs of oviparous fish are directly exposed via the water or indirectly by maternal exposure.[7−9] Estrogen has been shown to cause malformations in embryos of oviparous fish;[28] however, the exact mechanism is unknown. Maternal transferred exogenous E2 has been shown to have toxic effects in zebrafish (*Danio rerio*) embryos; increased embryo mortality was observed when female zebrafish were injected with E2.[29] Earlier eelpout experiments have shown that waterborne chemicals, including E2, accumulate in the blood of the mother fish and also in the ovarian fluid,[30] thereby exposing the embryos.

Potential Mechanisms Underlying Malformations. The biochemical or physiological mechanisms causing the fry malformations are unknown but possible explanations are listed below:

Calcium Depletion. Previous studies have shown a decrease in the content of calcium in the ovarian fluid in eelpout exposed to E2, ethinylestradiol, or octylphenol.[30−32] Depending on how the embryos obtain calcium, either through direct transfer from the mother's circulation or the ovarian fluid, their calcium status may be influenced and lead to bone abnormalities since calcium is important in embryonic bone formation.

Skeletal Estrogen Receptors. Estrogen receptors are present in bone tissue and endogenous estrogen plays an important role in the regulation of bone cell proliferation in vertebrate development. A few studies have shown severe vertebral malformations in juvenile fish exposed to exogenous estrogens during early embryonic development.[28,33] Skeletal malformations were also observed in the present experiment; however, the types of malformations observed were diverse—they ranged from dwarfs with otherwise normal morphology to severely malformed larvae with single or multiple malformations or early arrested development. Despite severe malformations most larvae were still alive at sampling except in the high exposure group where most larvae were motionless.

Reduced Amount of Ovarian Fluid. Embryonic/fetal compression due to lack of fluid causes malformations in mammals,[34] and water transporting proteins (aquaporins, occludins, and claudins) present in the ovarian wall are under steroid hormone control.[35,36] A reduction of ovarian fluid in response to estrogenic substances could explain abnormal offspring; however, ovarian fluid volume was only reduced at 133 ng L^{-1}, while increased malformation frequency was observed at 53.7 and 133 ng L^{-1}.

Osmoregulatory Imbalance and Hypoxia. Exposure of pregnant eelpout to ethinylestradiol causes a decrease in osmolarity and chloride concentrations,[37] and lack of ovarian fluid may influence gas exchange and cause hypoxia.[38] Hypoxia is a well-known inducer of embryonic malformations or early death.[2,39]

Types and Frequencies of Malformations. In this study only visible malformations were revealed and nonvisual

Table 2. Mother and Embryo Indices[a]

E2 concn (ng L^{-1})	N[b]	CI	LSI	GSI	OSI	OSSI	ESI	CIE
control	17/11	0.40 ± 0.05	1.37 ± 0.58	19.88 ± 7.88	18.55 ± 9.76	3.27 ± 1.07	11.59 ± 4.28	0.37 ± 0.05
solvent	13/6	0.41 ± 0.03	1.15 ± 0.19	16.36 ± 5.71	19.76 ± 9.12	3.01 ± 1.24	9.22 ± 4.73	0.37 ± 0.03
5.7	16/10	0.40 ± 0.04	1.27 ± 0.27	21.78 ± 7.94	17.37 ± 6.24	3.59 ± 1.28	11.70 ± 4.57	0.35 ± 0.01
10.1	19/8	0.40 ± 0.04	1.27 ± 0.13	19.82 ± 4.98	26.77 ± 16.21	5.13 ± 3.48	8.27 ± 5.18	0.35 ± 0.05
13.3	17/12	0.40 ± 0.05	1.13 ± 0.18	18.56 ± 4.66	15.48 ± 3.20	2.87 ± 0.86	10.13 ± 2.21	0.34 ± 0.03
22.9	16/8	0.40 ± 0.05	1.42 ± 0.32*	29.47 ± 7.88*	16.95 ± 4.96	4.70 ± 0.85*	14.64 ± 4.88	0.36 ± 0.04
53.6	18/12	0.41 ± 0.06	1.40 ± 0.14*	20.99 ± 9.70	27.24 ± 11.57	4.94 ± 1.53*	9.45 ± 4.29	0.37 ± 0.06
133	14/11	0.38 ± 0.03	1.52 ± 0.35*	13.77 ± 2.88	43.22 ± 10.53*	5.77 ± 1.29*	6.63 ± 2.48	0.53 ± 0.14*

[a]Biometric indices of females and embryos at the end of the experiment (mean ± SD). The asterisk (*) means significantly different from the control ($p < 0.05$). CI = condition Index, LSI = liver somatic index, GSI = gonado somatic index, OSI = ovarian sac index, OSSI = ovarian sac somatic index, ESI = embryo somatic index, and CIE = condition index embryos. [b]N is the number of surviving fish in each exposure group (all females/females with larvae) and each exposure group contained 33 females at the beginning. LSI and CI are calculated for all fish in the tanks while GSI, OSI, OSSI, ESI, and CIE are only calculated for females carrying larvae in the ovary.

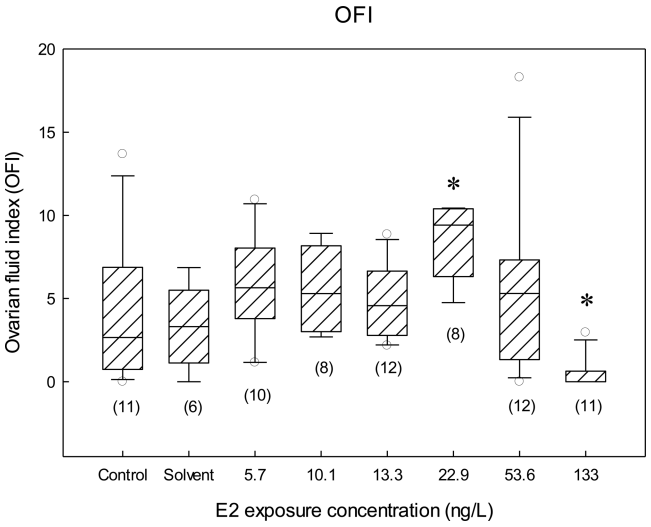

Figure 4. Ovarian fluid volume. The volume of ovarian fluid expressed as ovarian fluid index (OFI) in E2 exposed female eelpout. Boxes represent the 25th and 75th percentiles, the central line within the boxes is the median and whiskers represent the 10th and 90th percentiles. Open circles are outliers. The total number of samples is given in parentheses below the bars. The asterisk (*) means significantly different from the control ($p < 0.05$).

malformations influencing behavior, neurology, endocrinology, and histology (e.g., abnormal gonadal development) could remain undiscovered. In the monitoring programs elevated frequencies of arrested development and malformation tend to occur together.[1] The types of malformations and arrested development observed in the present experiments have also been seen in the Baltic monitoring programs.[1]

Malformation Frequency in the Control Group. In the present investigation the frequency of malformations in the control group is higher than earlier observed in fish from areas considered to be clean but different criteria for malformations have been used; the malformation frequency between broods is very different and also the sampling times varies.[1,12] Earlier experiments have shown that extreme variations in salinity, oxygen levels, and temperature might cause developmental defects in free-swimming fish larvae.[41] The potential effects of the stress imposed upon the female eelpout by the transfer from their natural habitat to the laboratory conditions are unknown.

The area from which the eelpout were collected is not considered polluted although the presence of intersex—albeit at a low frequency[42]—among the males in the population does indicate a certain contamination with estrogenic compounds. Also, while the seawater used in the experiment is not considered polluted it is drawn from the coastal zone in a small town (approximately 5000 inhabitants) and contamination cannot be excluded.

Teratogenic Window. The fry of pregnant eelpout exposed to 500 μg L^{-1} E2 (nominal) or 100 μg L^{-1} 4-*tert*-octylphenol (nominal)[30,40] at the late yolk-sac phase (mid-October to mid-November) showed no obvious, externally visible severe malformations; these exposures[30,40] were initiated more than a month later than the exposure described in the present. Therefore, for malformations to occur, the timing of the exposure to chemicals appears to be crucial.

Uptake of E2. Uptake of chemicals by fish renders actual exposure concentrations somewhat lower than nominal concentrations—even in flow through exposure systems.[43] Brown trout (*Salmo trutta*) in a slightly lower size range than the eelpout used in the present experiment took up E2 from the water phase at a rate of 20 h^{-1},[44] meaning that the amount taken up in the fish increased by a factor 20 over the concentration in the water for every hour. A simple calculation shows that the eelpout—if they have a similar uptake rate—would remove approximately two-thirds of the E2 added to each tank every day. Even if the eelpout, expectedly, have lower uptake rates than brown trout, uptake in the fish may explain why actual concentrations are lower than the nominal ones. The E2 plasma concentrations in the control group are in the same range as earlier detected during autumn.[30,32]

Vitellogenin Induction. Vitellogenin plasma concentrations increased in a monotonic concentration—response relationship (Figure 3B) and vitellogenin was increased already at the lowest test concentration (5.7 ng L^{-1}); however, if the solvent control is used instead of the pooled control in the statistical analysis, the increase is significant at 13.3 ng E2 L^{-1}. OECD test guidelines generally recommend the use of pooled controls if there is no statistically significant difference between water and solvent control; in thorough considerations on the proper use of controls in tests in aquatic systems, Green[45] recently concluded that the use of pooled controls increases the power of the statistical analysis but it may also give a slightly higher risk of identifying "false positives" than the use of the solvent control alone. The lowest observed effect concentration (LOEC) for various fish species is in the range 7.9–87 ng E2 L^{-1} and the no observed effect concentration (NOEC) is in the range 2.9–80 ng E2 L^{-1}.[46−50] The use of plasma vitellogenin

concentrations as biomarker for exposure to estrogens has mainly been aimed at investigations of male and/or juvenile fish, but it is interesting that female fish, at least eelpout, may show equal or maybe even higher sensitivity, no matter if the true LOEC is 5.7 or 13.3 ng E2 L^{-1}.

Biometric Indices of Mothers. The treatment did not influence the overall condition of the females; the CI in all groups was similar at the time of sampling; however, LSI was increased at 22.9, 53.7, and 133 ng L^{-1} (Table 2). An increase in LSI following E2 exposure has previously been observed,[51] and it is expected as the E2 induced increase in E2 plasma concentrations is followed by an increase in hepatic vitellogenin production (Figure 3A and B). The increased weight of the ovarian sac in relation to somatic weight (OSSI) at 22.9, 53.7, and 133 ng L^{-1} could be due to accumulation of fluid in the postovulatory follicles lining the ovarian wall, but the weight of the ovarian sac in relation to ovary weight (OSI) was only significantly different at 133 ng L^{-1}.

Sensitivity among Different Fish Species. The development of oviparous fish larvae is not necessarily affected by exposure to the concentrations of E2 that caused massive malformations in the eelpout of the present experiment. Exposure of eggs and larvae of brown trout to 469 ng E2 L^{-1} from fertilization until the "swim up" stage did not cause visible malformations.[52] Neither do all viviparous fish species appear to be as sensitive to chemically induced malformations among the embryos as the eelpout. Exposure of viviparous guppies (*Poecilia reticulata*), with intrafollicular development, to either 26 μg octylphenol L^{-1} or 850 ng E2 L^{-1} from mating to birth of the offspring did not result in visible malformations.[53] It is, however, also a well-established fact that Poecillidae tend to be less sensitive to estrogens[54] than most other groups of fish that have been tested.

Exposure Concentrations. The concentrations of E2 in the present experiment are fairly high but still within the range of concentrations detected in the aquatic environment. E2 concentrations up to 417 ng E2 L^{-1} have been detected in discharges from septic tanks[55] and discharges from sewage treatment plants may contain up to 147 ng E2-equivalents L^{-1}.[56] Little is known about the actual concentrations of E2 or other chemicals with estrogenic effect in the estuarine and marine environment,[57] but the fact that estrogenic effects have been detected in the coastal environment in the form of intersex and elevated levels of plasma vitellogenin in male fish,[58] for example in Danish flounders,[59] indicates that some kind of estrogenic activity finds its way to this environment. In German investigations, malformation frequencies among the eelpout fry[60] were highest in the areas which also had the highest frequencies[24] of feminized male fish (approximately $^1/_4$ of the male eelpout with oocytes in their testes). The possibility that contamination with estrogens or estrogenic chemicals induces the malformation observed among eelpout embryos in the Baltic does exist as indicated by the results of the present experiments. This investigation underlines that exposure to relatively high concentrations of E2 causes massive disturbances in the development among eelpout embryos, resulting in one or multiple late emerging malformations. Thereby our findings support the plausibility that the abnormal development among eelpout embryos encountered in monitoring programs in the Baltic may actually be caused by exposure to chemicals in the environment.

■ ASSOCIATED CONTENT

Supporting Information

Mean concentration factor for E2 in plasma calculated for each exposure group; regression line. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +45 6550 7492; fax: +45 6550 2786; e-mail: Jamor@biology.sdu.dk.

Notes

The authors declare no competing financial interest.

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