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Severe malformations of eelpout (*Zoarces viviparus*) fry are induced by maternal estrogenic exposure during early embryogenesis

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Abstract

Pregnant eelpout were exposed via the water to known endocrine disrupting compounds (EDCs) to clarify if EDCs could be causing the increased eelpout fry malformation frequencies observed in coastal areas receiving high anthropogenic input.

Both 17α-ethinylestradiol (EE2) (17.8 ng/L) and pyrene (0.5 μg/L) significantly increased fry malformation frequency whereas 4-*t*-octylphenol (4-*t*-OP) up to 14.3 μg/L did not. Vitellogenin was significantly induced by EE2 (5.7 and 17.8 ng/L) but not by 4-*t*-OP and pyrene. A critical period for estrogen-induced fry malformations was identified and closed between 14 and 22 days post fertilisation (dpf). Exposure to 17β-estradiol (E2) between 0 and 14 dpf caused severe malformations and severity increased the closer exposure start was to fertilization whereas malformations were absent by exposure later than 14 dpf. Data on ovarian fluid volume and larval length supported the suggested teratogenic window. Larval mortality also increased when exposure started right after fertilization.

1. Introduction

The eelpout (*Zoarces viviparus*) is a benthic live-breeder and a common inhabitant of Northern European marine coastal areas. Fertilization takes place in September and approximately five months later each female releases up to 400 larvae. The frequency of malformed eelpout fry has been observed to increase in marine areas receiving high anthropogenic inputs (Gercken et al., 2006; Strand et al., 2004). Because of the viviparous and prevailingly stationary behaviour of eelpouts (Kinitz et al., 2013) changes in their physiological conditions, reproduction etc. may be linked to local environmental conditions. Developing embryos are more sensitive to exogenous hormones than the adult (Bern, 1992) and it has been suggested that endocrine disrupting compounds (EDCs) could be a contributing factor to the increased in fry malformations as intersex has also been observed in male eelpout caught in areas with increased fry malformations frequencies (Gercken and Sordyl, 2002).

17β-estradiol (E2) is the primary female sex hormone in vertebrates and play an important role in the reproductive system e.g. sex development and in fish the synthesis of yolk protein, vitellogenin, is regulated by E2. However, non-reproductive tissues and processes are also influenced by E2 e.g. heart development (Allgood et al., 2013), bone formation (Gao et al., 2013), and development of cartilage (chondrogenesis) (Fushimi et al., 2009).

Polyaromatic hydrocarbons (PAH) like pyrene are formed by incomplete combustion of organic compounds, fossil fuels, oil and wood and enter the aquatic environment by runoff and atmospheric deposition. Benzo(a)pyrene or fluoranthene (Le Bihanic et al., 2014) (additional references in (Corrales et al., 2014)) induced larval abnormalities in rainbow trout (*Oncorhynchus mykiss*) and Brande-Lavridsen (2013) found indications that pyrene injections of pregnant eelpout could induce fry malformations. Alkylphenol polyethoxylates are used as detergents, cosmetics, pesticides and constituents of paints and they are degraded into

54 alkylphenols like nonylphenol (NP) and octylphenol (OP) (reviewed by (Sharma et al.,
55 2009)). Alkylphenols are stable and accumulate in sediments and filter-feeding organisms
56 like bivalves. In marine molluscs NP and OP have been detected in concentrations up to
57 thousands of ng/g tissue but normally concentrations are in the low ng/g range (reviewed by
58 David et al. (2009)). Eelpout are likely to be exposed to alkylphenols via water and food as
59 they are bottom-dwelling and feed on benthic crustaceans, worms and bivalves. Laboratory
60 experiments showed that alkylphenols feminize male fish (Gimeno et al., 1998; Gray and
61 Metcalfe, 1997) and impair embryo development in oviparous fish (Chaube et al., 2013).
62 By conducting a small-scale pilot study it became clear that maternal exposure to E2 and 4-
63 *tert*-octylphenol (4-*t*-OP) during certain periods of pregnancy impaired larval growth and
64 increased the malformation frequency and later we showed that E2 (≥53.6 ng/L) caused
65 severe malformations in eelpout fry (Morthorst et al., 2014). If environmental xenoestrogens
66 have similar properties and if severity of the effects depends on timing of the exposure
67 remains unknown. When pregnant eelpout were exposed to EDCs several weeks after
68 fertilization the malformation frequency was not increased (Rasmussen et al., 2002) but
69 sensitive windows for xenoestrogens and E2 exposure have been suggested for *Xenopus*
70 *laevis* embryos (Nishimura et al., 1997). Knowledge about sensitivity and responsiveness are
71 important as eelpout are used as biomonitoring organisms in several environmental programs
72 and both the Regional Conventions Oslo-Paris (OSPAR, 2007) and the Helsinki Commission
73 (HELCOM, 2008) have suggested using eelpout as sentinel species for monitoring effects of
74 toxic compounds in marine environments.
75 In the present experiments we investigate if maternal exposure to common environmental
76 EDCs induce malformations in eelpout fry and if malformation is delimited by exposure
77 during a temporal sensitive window.

78 The goals were (1) to determine the duration of the terotogenic window for E2-induced fry
79 malformations and (2) to clarify if waterborne exposure of known EDCs could lead to
80 abnormal embryo development.
81

2. Materials and methods

2.1 Animals

Feral eelpout (*Zoarces viviparus*) were caught in seines in the coastal areas around the island of Birkholm (54’56’’N, 10’31’’E), Denmark during August and September 2012 and 2013 and transported to the Marine Biological Research Centre in Kerteminde, Denmark. Eelpout from this area have previously been used for experiments and elevated frequencies of fry malformations have not been observed. Beginning in mid-August the reproductive status of females was monitored weekly by spot checks of the ovaries. After arrival at the Research Centre the fish were sexed immediately and 10 or 11 females were randomly distributed in each experimental tank (Table 1). Acclimatization was not allowed because the assumed sensitive window for exposure begins right after fertilization. Ovulation and fertilization takes place in late summer and early autumn but may shift a few weeks from year to year. An overview of the reproductive periods, experimental timing and setup is presented in Table 1. In 2012 the fish were put in tanks over a 4-day period due to low catchment success on some days. Four fish were put in all tanks on the first and second day and three fish on the fourth day.

2.2 Exposure

Both experiments were set up according to Morthorst *et al.* (2014) and with triplicates of each exposure concentration. In 2012 all of the fish were put in the exposure tanks on the same day. In 2013 the exposures were started at different days to study the sensitive window for exposure. Experimental details and exposure concentrations are given in Table 1. Briefly, twenty-four polyethylene tanks were set up in a flow-through system with a water exchange of 200 L per day and each tank was provided with two air stones and two circulation pumps. To provide hiding and shade during light hours pieces of drainpipe were put in the tanks and the tanks were partly covered by black plastic. Stock solutions of 17β-estradiol (CAS 50-28-

2), 17α-ethynylestradiol (CAS 57-63-6), pyrene (CAS 129-00-0) and 4-*tert*-octylphenol (CAS 140-66-9) were prepared in 10% isopropanol (CAS no 67-63-0) and the final solvent concentration in the exposure tanks was <0.01 %.

Water samples were collected regularly from the exposure tanks and frozen at -20°C for subsequent chemical analysis (Table 2). The concentration of stock solutions was monitored regularly during the experimental period to ensure accurate exposure concentrations.

Temperature, salinity and oxygen saturation were measured regularly in the header tanks but only weekly in each individual exposure tank because the fish are very sensitive to movement and sound disturbance.

2.3 Pilot studies – early observations and range finding

In an earlier pilot study we observed severe malformations in fry of mothers (n=4-6 females per group) exposed to E2 (nominal 500 ng/L) and 4-*t*-OP (nominal 100 µg/L and actual 31 µg/L) from fertilization and 29 days onwards. The maximum amount of isopropanol in the tanks was 0.036%. Both larvae weight and length were significantly reduced and the larvae had various types of malformations. The malformation frequency in the E2 group was 99% and 91% in the 4-*t*-OP group compared to 23 % in the solvent control.

To avoid the risk of toxic effects of the solvent it was decided to reduce the solvent concentration according to OECD test guideline recommendations in subsequent experiments.

As the toxicity of pyrene in fish and especially viviparous fish has not been investigated a pilot study was set up. In August 12 fish of mixed sex were exposed to a nominal pyrene concentration of 5 µg/L. After one week of exposure the mortality was 42% and after 15 days it was 75% and the nominal exposure concentration was reduced to 500 ng/L in the subsequent experiment. The exposure setup and conditions were as described previously.

131 *2.4 Sampling procedure*

132 The sampling procedures and the calculations of biometric indices are described in detail in
133 Morthorst *et al.* (2014). After removing the ovary, the larvae were removed and categorised
134 as live or dead (presence/absence of heart beat), placed on the side on plastic boards and
135 photographed. The total number of live, dead and deform larvae was also recorded.
136 The various types of abnormal development (early death, malformations of eyes and spinal
137 cord etc.) were evaluated under a stereo microscope and double checked on photographs.
138 Plasma vitellogenin levels were determined by an enzyme-linked immunosorbent assay
139 (ELISA) as described in Morthorst *et al.* (2014).

140 *2.5 Quantification of chemical concentrations in the water*

141 The actul exposure concentratons (Table 2) were determined by high-performance liquid
142 chromatography-tandem mass spectrometry (a 1200 Series HPLC and a 6410 Triple Quad
143 LC/MC, both Agilent Technologies). Determination of E2 and EE2 concentrations was
144 performed as described in Morthorst *et al.* (2014) and Madsen *et al.* (2013) respectively and
145 procedures for determination of 4-*t*-OP and pyrene are described below.
146 17α-ethinylestradiol (EE2) (Sigma-Aldrich) was added as internal standard. The samples
147 were prepared using solid phase extraction; Strata PAH 750 mg 6 mL^{-1} columns were used
148 for pyrene (8b-S130-WCH, Phenomenex, Torrance, CA, USA) and Oasis HLB 200 mg
149 columns were used for 4-*t*-OP (WAT106202, Waters). The samples were extracted on a
150 Waters Extraction Manifold (Milford, Massachusetts, USA).
151 The Oasis columns were conditioned with 5 mL ethyl acetate and 5 mL MeOH, equilibrated
152 with three times 5 mL H_2O, washed with 10 mL H_2O:MeOH (9:1), emptied and dried for 10
153 min. Further, the columns were eluted with 10 mL ethyl acetate and dried for 12 min in a
154 TurboVap (Caliper Life Sciences, Hopkinton, MA, USA). Finally, the sample was re-
155 dissolved in 0.5 mL 80% MeOH. The sample was injected in the HPLC-MS/MS with

156 conditions as follows: column Zorbax SB-C18 2.1x30mm, 3.5µm Rapid Resolution, column
157 temperature 25º C, eluation with a gradient H_2O, 0.1% NH_4OH : MeOH, 0.1% NH_4OH (0
158 min, 30%; 3 min, 70 %; 6 min, 100%; 10 min, 100%; 10.1 min, 30% and 11 min, 30%), flow
159 0.3 mL min^{-1}, stop time 11 min, injection 40 µL, needle wash in flush port 5 sec, and negative
160 Electro Spray Ionisation mode. Drying gas flow 10.0 L min^{-1}. Nebulizer pressure 50 psig,
161 drying gas temperature 325º C and capillary voltage of 4000 V. Setup steps for 4-*t*-OP:
162 Precursor ion 205.3, quantifier ion 133.1, dwell 200, fragmentor 120 and collision energy 15.
163 The Strata PAH columns were conditioned with 5 mL dichloromethane and 5 mL MeOH,
164 equilibrated with 5 mL H_2O, washed with 5 mL H_2O, emptied and dried for 1 min. Further,
165 the columns were eluted with 5 mL dichlorormethane and dried for 12 min in a TurboVap
166 (Caliper Life Sciences, Hopkinton, MA, USA). Finally, the sample was re-dissolved in 1 mL
167 90% MeOH. The sample was injected in the HPLC-MS/MS with conditions as follows:
168 column Zorbax SB-C18 2.1x30mm, 3.5µm Rapid Resolution, column temperature 25º C,
169 isocratic elution with 90% MeOH, flow 1.0 mL min^{-1}, stop time 2 min, injection 20 µL,
170 needle wash in flush port 5 sec, and positive Electro Spray Ionisation mode. Drying gas flow
171 10.0 L min^{-1}. Nebulizer pressure 35 psig, drying gas temperature 300º C and capillary voltage
172 of 4000 V. Setup steps for pyrene: Precursor ion 204, quantifier ion 176, dwell 200,
173 fragmentor 150 and collision energy 60.
174 For E2 and EE2 individual setups were used: precursor ion 271.1 (E2) and 295.1 (EE2),
175 quantifier ion 144.9, dwell 200, fragmentor 90 (E2) and 100 (EE2) and collision energy 30.
176 The standards were prepared using EE2 diluted in 70% MeOH, 0.1% NH_4OH.
177 *2.6 Data handling and statistical analyses*
178 Mortality and malformation frequencies were analyzed for significant differences using a
179 Chi^2 test. Data on biometric indeces and vitellogenin concentrations were analyzed for
180 significant difference using t-test (single comparison) or a one-way ANOVA followed by

Dunnets post hoc test (multiple comparisons). When significance was achieved, a Bonferroni-Holm adjustment was used to identify significance in the remaining exposure groups in the dose-response experiment. When data did not meet the criteria of equal variance and normality, data were logtransformed and if the criteria were still not fulfilled a Mann-Whitney on ranks with Dunn's posthoc test was run.

When the control and solvent groups did not differ significantly the groups were poooled however, the control and solvent control groups are shown separately in figures and tables. If the groups were different the solvent control was used as control group in the statistical tests. Significance was considered at $P<0.05$.

3. Results

We present data on two experiments conducted on pregnant eelpout in 2012 and 2013, respectively.

Mortality and reproductive status

The overall mortality in the experiment was respectively 52% (2012) and 53% (2013) with the majority of fish dying within 2 weeks from exposure start. A high mortality is normal when newly caught eelpout are transferred to the laboratory (Brande-Lavridsen et al., 2013; Mattsson et al., 2001; Morthorst et al., 2014) and the mortality in the experimental groups did not vary significantly from the control group. Some females had an empty ovary or an ovary only containing eggs or up till 4/6 (live/dead) larvae. The number of such females was 11 out of 126 (2012) and 11 out of 102 (2013). These females appeared in control as well as exposure groups and were removed from the dataset. The smallest number of fry in a brood included in the calculations was 35 (2012) and 28 (2013).

The experimental setup and exposure concentrations

During the exposure periods the water temperature decreased from 15.1 to 12.3°C (2012) and from 15.2 to 12.0°C (2013) with a mean (± SEM) of 13.5±0.17°C (2012) and 13.5±0.23°C (2013). The salinity was 19.6±0.14 ppt (2012) and 12.6±0.15 ppt (2013) (mean ± SEM) and the average oxygen saturation for all tanks was 74.7±1.4 % (2012) and 86.7±0.99 % (2013) (mean ± SEM).

Table 1: Nominal exposure concentrations and experimental timing and setup (2012, 2013)

The exposure groups with nominal 4-*t*-OP concentrations of 25 and 50 µg/L had similar actual concentrations of 14.2 and 14.3 µg/L, respectively. The average E2 concentration in the exposure groups was in the range 324-363 ng/L (Table 2).

215

216 **Table 2:** Actual exposure concentrations (2012, 2013)

217 The condition index (CI) and liver somatic index (LSI) of mothers and various ovarian
218 indices (GSI, OSI, OSSI, ESI and OFI) were not significantly different from the control in
219 any of the exposure groups in the 2012 experiment (data not shown). The condition index of
220 fry (CIE) was significantly different for the groups exposed to 4-*t*-OP concentrations at 5.1
221 µg/L and above but fry length and weight were not significantly different (data not shown).
222 In 2013 the LSI and OSI was significantly increased in all exposure groups and OSSI was
223 increased in all E2 groups (Table 3). The increase in OSI and OSI became more pronounced
224 when exposure was started close to fertilization. GSI and ESI were significantly reduced in
225 the EE2 group (17.8 ng/L) and in the E2 groups exposed from 0 and 9 dpf.
226 Also length and weight of fry exposed to EE2 and E2 from 0 and 9 dpf were significantly
227 reduced and fry exposed from 14 dpf also had reduced length (Table 4). When the exposure
228 started at 22 dpf fry length and weight were unaffected. CIE was only significantly increased
229 in the E2-groups exposed from 0 and 22 dpf.

230

231 **Table 3:** Biometric indices 2013

232 **Table 4:** Fry length and weight 2013

233

234 **Figure 1A and B: Fry malformation frequencies and mortality**

235 The brood size was not significantly different between the pooled control and exposure
236 groups (data not shown).
237 The malformation frequency was significantly higher in the solvent control compared to the
238 control because two females had many malformed larvae (Figure 1A). The number of

malformed larvae was only significantly higher in the pyrene group both when tested against the pooled control ($P<0.001$) and when tested against the solvent control alone ($P=0.004$). The malformation frequencies of the control and solvent control groups in 2013 were not significantly different and therefore the groups were pooled (Figure 1B). The EE2 and E2 groups exposed at 14 dpf and earlier were significantly different ($P<0.001$) from the pooled control whereas the E2 group exposed at 22 dpf was not ($P=0.06$).

The mortality of the control and solvent control groups was not significantly different in 2012 and therefore the groups were pooled (Figure 1A). The mortality was significantly lower in the EE2 group ($P<0.001$) and one of the 4-*t*-OP groups (14.3 µg/L) ($P=0.02$), but significantly higher in the group with 14.2 µg/L ($P=0.003$).

The number of dead larvae was significantly different between the control and solvent control in 2013 ($P< 0.001$, higher in the control, however, with no biological reasonable explanation) and the mortality was higher in all exposure groups compared to the solvent control (Figure 1B).

Figure 2A and B: Vitellogenin

Plasma vitellogenin levels are only significantly different in the group exposed to EE2 (5.7 ng/L) in 2012 (Figure 2A) but in 2013 all the exposed groups were significantly different from the control (Figure 2B).

Figure 3: Ovarian fluid 2013

The amount of ovarian fluid was similar in the control and exposure groups in 2012 (data not shown) but in 2013 the ovarian fluid index was significantly decreased in the EE2 group (17.8 ng/L) and the E2 groups when exposure was initiated on 0, 9 and 14 dpf (Figure 3).

4. Discussion and conclusion

The present data demonstrate the presence of a teratogenic window for E2-induced malformations in eelpout fry and that the window closes between 14 and 22 dpf (days post fertilization). Firstly, the fry malformation frequency was significantly increased when exposure of pregnant eelpout females was started 14 dpf or earlier, and the frequency increases the earlier exposure was started. The ovarian fluid volume and fry length were also reduced when exposure started at 14 dpf or earlier and again the severity increased when exposure was started close to the time of fertilization. By postponing the exposure start to 22 dpf the above-mentioned endpoints were not significantly different from the control. We also show, that by exposing pregnant females from 0 dpf both EE2 (17.8 ng/L) and pyrene (0.5 µg/L) induce malformations in fry, whereas 4-*t*-OP concentrations up to 14.3 µg/L and EE2 (5.7 ng/L) did not.

Actual exposure concentrations of 4-*t*-OP

The actual concentration of 4-*t*-OP in tanks with nominal concentrations of 25 and 50 µg/L were surprisingly low despite thorough preparations for the experiment. The LC-MS and LC-MSMS method for detection of 4-*t*-OP has been running routinely in our laboratory for 15 years and actual 4-*t*-OP concentrations of 8-17 and 57-79 µg/L were detected when nominal concentrations were 25 and 100 µg/L, respectively (Rasmussen et al., 2002). However, the solvent concentration in older studies was 0.036% (Jespersen et al., 2010; Rasmussen et al., 2002) and in the present experiments we used a solvent concentration of 0.01% in order to meet the OECD recommendations (OECD, 2012). Actual 4-*t*-OP concentrations above 14.3 µg/L could not be obtained regardless of the nominal concentration (25 or 50 µg/L) and is most likely explained by the reduced solvent concentration. The water solubility of 4-*t*-OP at

287 room temperature is in the low mg/L range and even poorer at the present experimental
288 conditions (12-15°C).

289 **Fry mortality**

290 Larval mortality was significantly increased when pregnant females were exposed to E2 and
291 the mortality increased when exposure start was close to fertilization (Figure 1B). EE2 (17.8
292 ng/L) also had a weak but significant negative influence on mortality whereas at 5 ng/L the
293 influence was positive (Figure 1A). Mortality was also significantly increased at a 4-*t*-OP
294 concentration of 14.2 µg/L but decreased at 14.3 µg/L (Figure 1A) but this can most likely be
295 ascribed to randomness. The increase in larval mortality after exposure to high E2
296 concentrations is in concordance with studies on *Xenopus laevis* embryos (Nishimura et al.
297 1997). E2-injections of female zebrafish caused increased embryo mortality (Westerlund et
298 al., 2000) and E2 exposure of hatched trout larvae caused increased mortality (Krisfalusi et
299 al., 1998). Also, E2 have been shown to affect hatching of fish embryos (Schubert et al.,
300 2014). The toxic mechanism behind E2-induced fry mortality is unknown but could involve
301 premature hatch if E2 affects hatching enzymes or embryo metabolism and mobility leading
302 to early penetration of the chorion.

303 **Fry malformations**

304 Some chemicals are transferred maternally to the eggs after oral exposure (Halden et al.,
305 2010) and developing embryos in viviparous fish may be exposed to chemicals via two
306 routes. Chemicals are incorporated into the eggs as vitellogenin accumulates in the eggs and
307 hence the embryos are exposed to the chemicals as they feed on proteins and lipids in their
308 yolk sac. Also, chemicals absorbed in the blood of the mother enter the ovarian fluid, which
309 the larvae feed on when the yolk sac is depleted. The close relationship between fry and
310 mother also makes the fry sensitive to effects of the chemical in the mother fish. If the mother

311 fish is comprised by exposure to the chemical it might have an indirect effect on the fry if the
312 nutrient transfer to the ovary or ovarian fluid is affected.
313 *Teratogenic window*
314 Prior to hatch the embryos move around within the egg until hatching occurs approximately
315 21 dpf (A figure showing eelpout embryonic development is provided in Rasmussen *et al.*
316 (2006)). Before hatch cartilage and bone development have started and the larvae have well-
317 pigmented eyes, beating heart, vascular blood circulation, a large yolk sac and pectoral fins.
318 After hatch the liver and intestine develop and the yolk sac decrease gradually until fully
319 absorbed two weeks after hatch. Changes in fry malformation frequency, ovarian fluid
320 volume and larval growth revealed that the sensitive window for induction of malformations
321 closes around the time of hatch – between 14 and 22 dpf. Rasmussen (2002) showed that
322 pregnant eelpout accumulate up to 55 mg OP/L in their plasma after 35 days of exposure to
323 65 ug/L. However, when females were exposed to 100 µg 4-*t*-OP/L and 500 ng E2/L
324 (nominal concentrations) from September 20 (approximately 20 dpf) and for 14 days neither
325 fry mortality nor fry malformation frequency increased (Jespersen et al., 2010). This is in
326 concordance with the results from the present experiment and clearly illustrate that in the
327 eelpout the early embryonic developmental stages are particularly sensitive to E2 exposure.
328 Exposure to high concentrations of alkylphenols and E2 (10-20 µM) during early
329 development caused abnormal development and increased mortality in *X. laevis* embryos but
330 the effects seemed to depend on timing of the exposure and hence, an early critical window
331 for E2 induced toxicity in *X. laevis* embryos was suggested (Nishimura et al., 1997; Sone et
332 al., 2004). The observed reduction of fry length when E2 exposure was initiated at 14 dpf and
333 earlier (Table 4) is in concordance with a short-term E2 exposure of brown trout embryos
334 (Schubert et al., 2014).

335 The mechanism behind non-reproductive embryotoxic effects of estrogens is not very well
336 investigated. During early embryonic development the teleost embryo seems unable to
337 produce hormones but rely on maternally delivered sex steroids. These hormones are
338 metabolised during early embryo development and E2 levels have been found to decrease
339 substantially during the first two weeks of trout embryonic development (Yeoh et al., 1996)
340 and also estrogen receptor (ER) mRNA is reduced prior to organogenesis in zebrafish
341 (Westerlund et al., 2000).
342 Regulation of circulating E2 by ERs is an important regulator of ovarian development in fish
343 and is interlinked with growth and development (Devlin and Nagahama, 2002). ERs increase
344 in number during development, and in particular are likely to increase rapidly prior to and
345 during sexual differentiation and reproduction (Duffy et al., 2014; Liao et al., 2009).
346 The specific role of sex steroids on general embryonic development is also not well described
347 but additional exogenous E2 during early embryonic development is likely to alter steroid
348 metabolism and the ER expression pattern and thereby lead to embryonic developmental
349 disturbances because E2 affects neural crest development and bone and cartilage
350 development by influencing the differentiation and proliferation of chondrocytes and
351 osteocytes. The teratogenic effect of E2 on non-reproductive tissues has been investigated in
352 a few studies. Direct exposure to moderate and high E2 concentrations caused craniofacial
353 abnormalities in zebrafish embryos (Cohen et al., 2014) and in concordance with the present
354 study the sensitivity of embryo skeletal development to E2 depended on developmental stage
355 - the effect of E2 decreased with the delay of initiation of treatment (Fushimi et al., 2009).
356 The neural crest is a transient, pluripotent and migratory cell population unique to embryonic
357 development in vertebrates. This cell population is able to generate a variety of cells and
358 gives rise to neurons of the peripheral nervous system, melanocytes, (melanin-producing
359 cells), the dorsal fin and elements of the craniofacial skeleton. By exposing *X. laevis* embryos

360 to estrogenic compounds Bevan *et al.* (2003) showed, that abnormalities in developing
361 tissues derived from the neural crest were induced by estrogenic compounds. The
362 interference of estrogenic compounds with neural crest development could explain the
363 observed sensitive window for estrogen-induced malformations in eelpout (Figure 1B).
364
365 The amount of ovarian fluid at the time of sampling was significantly reduced but only if the
366 exposure was started before hatch (Figure 3). Before hatch the ovary sac contains fluid-filled
367 follicles and almost no ovarian fluid in the ovarian cavity whereas after hatch the follicles
368 decrease in size and the amount of ovarian fluid increases, probably because the individual
369 yolk sac of the larvae in this period gradually is depleted and the ovarian fluid becomes the
370 major nutritional source for the developing fry (Korsgaard, 1986). After depletion of the yolk
371 sac nutrients are transferred from mother to embryos via the ovarian fluid (Korsgaard and
372 Andersen, 1985) and/or the postovulatory follicles (Skov et al., 2010). The morphology of the
373 ovarian sac is also affected by E2 exposure as illustrated by the increase in ovarian sac index
374 (OSI) and ovarian sac weight/somatic weight index (OSSI) (Table 3). The effect is higher the
375 earlier exposure is started and even when exposure is started at 22 dpf the increase is
376 significant. It was clear upon sampling that the ovarian sac of exposed females had fluid-
377 filled follicles and ovarian fluid was lacking. This could indicate that the transfer of fluid
378 from the follicles to the inner ovarian cavity is impaired by E2 and thereby also transport of
379 essential nutrients and gasses.
380 *Exposure and malformations*
381 Pyrene significantly increased the malformation frequency of fry whereas 4-*t*-OP in
382 concentrations up to 14.3 µg/L did not (Figure 1A). In an earlier experiment Brande-
383 Lavridsen (2013) also found that pyrene had a non-significant tendency towards an effect.

Pyrene might affect aromatase enzyme function (Booc et al., 2014; Dong et al., 2008) and thereby steroid concentrations but the mechanism behind reproductive toxicity of PAHs in fish is unknown (Hoffmann and Oris, 2006). Body length of medaka fry is affected by embryonic pyrene exposure (Barjhoux et al 2014) and pyrene induced bone malformations, cardiovascular impairments and yolk sac resorption defects. Dysfunction of the cardiovascular system has been linked to decreased absorption of the yolk sac – reduced larvae growth due to dysfunctioning of the cardiovascular system and thereby malabsorption of nutrients from the yolk sac.

The synthetic estrogen EE2 (17.8 ng/L) also reduces length and weight of the larvae and both mortality and malformation frequency is significantly increased. From the experiment in 2012 it was shown that 5.4 ng/L of EE2 did not cause increased malformations (Figure 1A). EE2 decreased body size and induced yolk sac and pericardial edema in fathead minnow larvae (Johns et al., 2009).

Vitellogenin and LSI

Some of the investigated parameters are affected in all exposure groups. Both plasma vitellogenin levels (Figure 2B) and LSI (Table 3) are increased in all exposure groups and the increase in LSI is probably a consequence of increased vitellogenin synthesis in the liver. Vitellogenin has calcium binding capacities and the increased vitellogenin plasma levels could also explain the observed skeletal malformations as calcium increases in the plasma and decrease in ovarian fluid when mothers are exposed to E2 and EE2 (Korsgaard et al., 2002). The calcium concentration in the ovarian fluid has been reported to decrease in response to chemical exposure. Deformities in the skeleton could be a consequence of insufficient calcium levels in the ovarian fluid. However, pyrene induces fry malformations but vitellogenin is not induced (Figure 2A) which contradicts this hypothesis.

409 A teratogenic window for E2-induced malformations in eelpout fry is established and lasts
410 from fertilization until it closes between 14 and 22 dpf. Both pyrene and EE2 are able to
411 induce malformations whereas 4-*t*-OP up to 14.3 µg/L is not. These data illustrate that
412 eelpout embryonic development is particularly sensitive to estrogenic exogenous exposure
413 during the embryonic period before hatch but fry mortality is still increased when exposure
414 start is postponed until 22 dpf.
415

416 # 5. Acknowledgements

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References

Allgood, O.E., Hamad, A., Fox, J., DeFrank, A., Gilley, R., Dawson, F., Sykes, B., Underwood, T.J., Naylor, R.C., Briggs, A.A., Lassiter, C.S., Bell, W.E., Turner, J.E., 2013. Estrogen prevents cardiac and vascular failure in the 'listless' zebrafish (Danio rerio) developmental model. Gen Comp Endocr 189, 33-42.

Bern, H.A., 1992. The Fragile Fetus. In: Colborn, T. and Clement, C. eds. Chemically-Induced Alterations in Sexual and Functional Development : The Wildlife / Human Connection. Princeton Scientific Publishing Co., NJ, U.S. 21, 9-15.

Bevan, C.L., Porter, D.M., Prasad, A., Howard, M.J., Henderson, L.P., 2003. Environmental Estrogens alter early development in Xenopus laevis. Environ Health Persp 111, 488-496.

Booc, F., Thornton, C., Lister, A., MacLatchy, D., Willett, K.L., 2014. Benzo[a]pyrene effects on reproductive endpoints in Fundulus heteroclitus. Toxicol Sci 140, 73-82.

Brande-Lavridsen, N., Korsgaard, B., Dahllof, I., Strand, J., Tairova, Z., Bjerregaard, P., 2013. Abnormalities in eelpout Zoarces viviparus upon chemical exposure. Mar Environ Res 92, 87-94.

Chaube, R., Gautam, G.J., Joy, K.P., 2013. Teratogenic Effects of 4-Nonylphenol on Early Embryonic and Larval Development of the Catfish Heteropneustes fossilis. Arch Environ Con Tox 64, 554-561.

Cohen, S.P., LaChappelle, A.R., Walker, B.S., Lassiter, C.S., 2014. Modulation of estrogen causes disruption of craniofacial chondrogenesis in Danio rerio. Aquat Toxicol 152, 113-120.

Corrales, J., Thornton, C., White, M., Willett, K.L., 2014. Multigenerational effects of benzo[a]pyrene exposure on survival and developmental deformities in zebrafish larvae. Aquat Toxicol 148, 16-26.

David, A., Fenet, H., Gomez, E., 2009. Alkylphenols in marine environments: Distribution monitoring strategies and detection considerations. Mar Pollut Bull 58, 953-960.

Devlin, R.H., Nagahama, Y., 2002. Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. Aquaculture 208, 191-364.

Dong, W., Wang, L., Thornton, C., Scheffler, B.E., Willett, K.L., 2008. Benzo(a)pyrene decreases brain and ovarian aromatase mRNA expression in Fundulus heteroclitus. Aquat Toxicol 88, 289-300.

Duffy, T.A., Iwanowicz, L.R., McCormick, S.D., 2014. Comparative responses to endocrine disrupting compounds in early life stages of Atlantic salmon, Salmo salar. Aquat Toxicol 152, 1-10.

468 Fushimi, S., Wada, N., Nohno, T., Tomita, M., Saijoh, K., Sunami, S., Katsuyama, H., 2009.
469 17 beta-Estradiol inhibits chondrogenesis in the skull development of zebrafish
470 embryos. Aquat Toxicol 95, 292-298.
471
472 Gao, Y.H., Huang, E.Y., Zhang, H.M., Wang, J.H., Wu, N.N., Chen, X., Wang, N., Wen, S., Nan,
473 G.X., Deng, F., Liao, Z., Wu, D., Zhang, B.S., Zhang, J.H., Haydon, R.C., Luu, H.H., Shi, L.L., He,
474 T.C., 2013. Crosstalk between Wnt/beta-Catenin and Estrogen Receptor Signaling
475 Synergistically Promotes Osteogenic Differentiation of Mesenchymal Progenitor Cells.
476 Plos One 8.
477
478 Gercken, J., Forlin, L., Andersson, J., 2006. Developmental disorders in larvae of eelpout
479 (Zoarces viviparus) from German and Swedish Baltic coastal waters. Mar Pollut Bull 53,
480 497-507.
481
482 Gercken, J., Sordyl, H., 2002. Intersex in feral marine and freshwater fish from
483 northeastern Germany. Mar Environ Res 54, 651-655.
484
485 Gimeno, S., Komen, H., Gerritsen, A.G.M., Bowmer, T., 1998. Feminisation of young males
486 of the common carp, Cyprinus carpio, exposed to 4-tert-pentylphenol during sexual
487 differentiation. Aquat Toxicol 43, 77-92.
488
489 Gray, M.A., Metcalfe, C.D., 1997. Induction of testis-ova in Japanese medaka (Oryzias
490 latipes) exposed to p-nonylphenol. Environ Toxicol Chem 16, 1082-1086.
491
492 Halden, A.N., Nyholm, J.R., Andersson, P.L., Holbech, H., Norrgren, L., 2010. Oral
493 exposure of adult zebrafish (Danio rerio) to 2,4,6-tribromophenol affects reproduction.
494 Aquat Toxicol 100, 30-37.
495
496 HELCOM, 2008. Manual for Marine Monitoring in the COMBINE Program. HELCOM
497 Commission.
498
499 Hoffmann, J.L., Oris, J.T., 2006. Altered gene expression: a mechanism for reproductive
500 toxicity in zebrafish exposed to benzo[a]pyrene. Aquat Toxicol 78, 332-340.
501
502 Jespersen, A., Rasmussen, T.H., Hirche, M., Sorensen, K.J.K., Korsgaard, B., 2010. Effects
503 of Exposure to the Xenoestrogen Octylphenol and Subsequent Transfer to Clean Water
504 on Liver and Gonad Ultrastructure During Early Development of Zoarces viviparus
505 Embryos. Journal of Experimental Zoology Part a-Ecological Genetics and Physiology
506 313A, 399-409.
507
508 Johns, S.M., Kane, M.D., Denslow, N.D., Watanabe, K.H., Orlando, E.F., Villeneuve, D.L.,
509 Ankley, G.T., Sepulveda, M.S., 2009. Characterization of Ontogenetic Changes in Gene
510 Expression in the Fathead Minnow (Pimephales Promelas). Environ Toxicol Chem 28,
511 873-880.
512
513 Kinitz, T., Quack, M., Paulus, M., Veith, M., Bergek, S., Strand, J., Tuvikene, A., Soirinsuo, A.,
514 Hochkirch, A., 2013. Strong isolation-by-distance in the absence of genetic population
515 structure in the eelpout (Zoarces viviparus, Linnaeus 1758). Ecol Indic 27, 116-122.

516 Korsgaard, B., 1986. Trophic Adaptations during Early Intraovarian Development of
517 Embryos of Zoarces-Viviparus (L). J Exp Mar Biol Ecol 98, 141-152.
518
519 Korsgaard, B., Andersen, F.O., 1985. Embryonic Nutrition, Growth and Energetics in
520 Zoarces-Viviparus L as Indication of a Maternal-Fetal Trophic Relationship. J Comp
521 Physiol B 155, 437-444.
522
523 Korsgaard, B., Andreassen, T.K., Rasmussen, T.H., 2002. Effects of an environmental
524 estrogen, 17 alpha-ethinyl-estradiol, on the maternal-fetal trophic relationship in the
525 eelpout Zoarces viviparus (L). Mar Environ Res 54, 735-739.
526
527 Krisfalusi, M., Eroschenko, V.P., Cloud, J.G., 1998. Methoxychlor and estradiol-17 beta
528 affect alevin rainbow trout (Oncorhynchus mykiss) mortality, growth, and
529 pigmentation. B Environ Contam Tox 61, 519-526.
530
531 Le Bihanic, F., Morin, B., Cousin, X., Le Menach, K., Budzinski, H., Cachot, J., 2014.
532 Developmental toxicity of PAH mixtures in fish early life stages. Part I: adverse effects in
533 rainbow trout. Environmental science and pollution research international 21, 13720-
534 13731.
535
536 Liao, C.L., Lee, M.Y., Tyan, Y.S., Kok, L.F., Wu, T.S., Koo, C.L., Wang, P.H., Chao, K.C., Han,
537 C.P., 2009. Progesterone receptor does not improve the performance and test
538 effectiveness of the conventional 3-marker panel, consisting of estrogen receptor,
539 vimentin and carcinoembryonic antigen in distinguishing between primary
540 endocervical and endometrial adenocarcinomas in a tissue microarray extension study.
541 J Transl Med 7.
542
543 Madsen, L.L., Korsgaard, B., Pedersen, K.L., Bjerregaard, L.B., Aagaard, T., Bjerregaard, P.,
544 2013. Vitellogenin as biomarker for estrogenicity in flounder Platichthys flesus in the
545 field and exposed to 17 alpha-ethinylestradiol via food and water in the laboratory. Mar
546 Environ Res 92, 79-86.
547
548 Mattsson, K., Tana, J., Engstrom, C., Hemming, J., Lehtinen, K.J., 2001. Effects of wood-
549 related sterols on the offspring of the viviparous blenny, Zoarces viviparus L. Ecotox
550 Environ Safe 49, 122-130.
551
552 Morthorst, J.E., Brande-Lavridsen, N., Korsgaard, B., Bjerregaard, P., 2014. 17-beta-
553 estradiol causes abnormal development in embryos of the viviparous eelpout. Environ
554 Sci Technol 48, 14668-14676.
555
556 Nishimura, N., Fukazawa, Y., Uchiyama, H., Iguchi, T., 1997. Effects of estrogenic
557 hormones on early development of Xenopus laevis. J Exp Zool 278, 221-233.
558
559 OECD, 2012. Fish toxicity testing framework. Series on Testing and Assessment. No.
560 171. .
561
562 OSPAR, 2007. JAMP guidelines for general biological effects monitoring. In: OSPAR (Ed.),
563 OSPAR Agreement 1997-7.

564 Rasmussen, T.H., Andreassen, T.K., Pedersen, S.N., Van der Ven, L.T.M., Bjerregaard, P.,
565 Korsgaard, B., 2002. Effects of waterborne exposure of octylphenol and oestrogen on
566 pregnant viviparous eelpout (Zoarces viviparus) and her embryos in ovario. J Exp Biol
567 205, 3857-3876.
568
569 Rasmussen, T.H., Jespersen, A., Korsgaard, B., 2006. Gonadal morphogenesis and sex
570 differentiation in intraovarian embryos of the viviparous fish Zoarces viviparus
571 (Teleostei, Perciformes, Zoarcidae): A histological and ultrastructural study. J Morphol
572 267, 1032-1047.
573
574 Schubert, S., Peter, A., Schonenberger, R., Suter, M.J.F., Segner, H., Burkhardt-Holm, P.,
575 2014. Transient exposure to environmental estrogen affects embryonic development of
576 brown trout (Salmo trutta fario). Aquat Toxicol 157, 141-149.
577
578 Sharma, V.K., Anquandah, G.A., Yngard, R.A., Kim, H., Fekete, J., Bouzek, K., Ray, A.K.,
579 Golovko, D., 2009. Nonylphenol, octylphenol, and bisphenol-A in the aquatic
580 environment: a review on occurrence, fate, and treatment. Journal of environmental
581 science and health. Part A, Toxic/hazardous substances & environmental engineering
582 44, 423-442.
583
584 Skov, P.V., Steffensen, J.F., Sorensen, T.F., Qvortrup, K., 2010. Embryonic suckling and
585 maternal specializations in the live-bearing teleost Zoarces viviparus. J Exp Mar Biol
586 Ecol 395, 120-127.
587
588 Sone, K., Hinago, M., Kitayama, A., Morokuma, J., Ueno, N., Watanabe, H., Iguchi, T., 2004.
589 Effects of 17 beta-estradiol, nonylphenol, and bisphenol-A on developing Xenopus laevis
590 embryos. Gen Comp Endocr 138, 228-236.
591
592 Strand, J., Andersen, L., Dahllof, I., Korsgaard, B., 2004. Impaired larval development in
593 broods of eelpout (Zoarces viviparus) in Danish coastal waters. Fish Physiol Biochem
594 30, 37-46.
595
596 Westerlund, L., Billsson, K., Andersson, P.L., Tysklind, M., Olsson, P.E., 2000. Early life-
597 stage mortality in zebrafish (Danio rerio) following maternal exposure to
598 polychlorinated biphenyls and estrogen. Environ Toxicol Chem 19, 1582-1588.
599
600 Yeoh, C.G., Schreck, C.B., Feist, G.W., Fitzpatrick, M.S., 1996. Endogenous steroid
601 metabolism is indicated by fluctuations of endogenous steroid and steroid glucuronide
602 levels in early development of the steelhead trout (Oncorhynchus mykiss). Gen Comp
603 Endocr 103, 107-114.
604

Table 1 Overview of the experimental setup in 2012 and 2013. Days post fertilization (dpf).

	2012	**2013**
Ovulation observations	Sept 6 4/6 females ovulated	Sept 4 No ovulation in 6/6 females
		Sept 12 Small larvae in 5/5 females
Date of catchment	Sept 11, 12 and 13	Sept 13
Fish per tank	11	10
Exposure start	Sept 11 (0 dpf), 12 and 14 All tanks	Sept 14: Control, EE2 and E2-group 1 (Started 0 dpf)
		Sept 23: E2-group 2 (Started 9 dpf)
		Sept 28: E2-group 3 (Started 14 dpf)
		Oct 6: E2-group 4 (Started 22 dpf)
Experiment ended	Oct 22-26 (41-45 dpf)	Oct 26-28 (42-44 dpf)
Nominal exposure concentrations		
4-*t*-OP (µg/L)	6.25, 12.5, 25 and 50	-
EE2 (ng/L)	5	20
E2 (ng/L)	-	500
Pyrene (ng/L)	500	-
Solvent control	Solvent <0.01%	Solvent <0.01%
Seawater control	Yes	Yes

Table 2 Actual 4-t OP, pyrene and EE2 concentrations in the exposure groups (group wise mean ± SEM). N is the total number of water samples from three replicate tanks.

Exposure	N	Nominal concentration	Actual concentration	± SEM
2012				
4-*t*-OP	15	6.25 µg/L	3.4 µg/L	0.20
	15	12.5 µg/L	5.1 µg/L	0.33
	15	25 µg/L	14.2 µg/L	1.11
	15	50 µg/L	14.3 µg/L	1.12
Pyrene	17	0.5 µg/L	0.5 µg/L	0.07
EE2	18	5 ng/L	5.7 ng/L	0.001
2013				
E2-0 dpf	37	500 ng/L	346 ng/L	13.7
E2-9 dpf	34	500 ng/L	332 ng/L	16.0
E2-14 dpf	21	500 ng/L	363 ng/L	14.2
E2-22 dpf	13	500 ng/L	324 ng/L	22.5
EE2 (ng/L)	34	20 ng/L	17.8 ng/L	0.84

Poul har beregnet de aktuelle eksponeringskoncentrationer 23/12/14 for 2012-forsøget. Data fra 2013 er beregnet i kopi af Bentes regneark (skrivebord/ålekvabbe manuskript/MS-målinger)

Table 3: Mother and embryo indices 2013. 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 and ESI = Embryo Somatic Index.

Exposure	N	CI	LSI	GSI	OSI	OSSI	ESI
Control	18	0.36 ± 0.04	1.29 ± 0.22	34.02 ± 5.27	11.0 ± 2.30	3.72 ± 0.94	18.60 ± 2.42
Solvent	12	0.38 ± 0.05	1.31 ± 0.31	32.48 ± 8.76	11.75 ± 2.13	3.83 ± 0.87	18.20 ± 5.56
E2-0 dpf	13	0.36 ± 0.04	2.10 ± 0.39*	17.06 ± 5.25*	47.53 ± 16.2*	8.24 ± 4.48*	7.98 ± 2.22*
E2-9 dpf	10	0.35 ± 0.03	2.18 ± 0.42*	26.31 ± 11.1*	32.42 ± 15.5*	7.55 ± 2.66*	13.70 ± 5.47*
E2-14 dpf	12	0.33 ± 0.02*	2.22 ± 0.49*	32.25 ± 9.34	20.91 ± 4.96*	6.57 ± 2.17*	17.14 ± 4.73
E2-22 dpf	11	0.36 ± 0.03	2.28 ± 0.28*	39.1 ± 13.77	15.72 ± 3.08*	5.97 ± 1.83*	20.44 ± 7.81
EE2 17.8 ng/L	15	0.37 ± 0.03	1.52 ± 0.20*	27.2 ± 8.30*	15.45 ± 4.16*	4.16 ± 0.90	14.99 ± 3.43*

Table 4 Fry length and weight. Mean length and weight (Mean ± SEM) of fry (2013). The asterisk (*) means significantly different from the control ($p<0.05$).

Exposure	N	Fry length (cm)		Fry weight (g)	
Control	18	3.047 ± 0.034		0.0949 ± 0.003	
Solvent	12	3.124 ± 0.085		0.102 ± 0.008	
E2-0 dpf	13	2.258 ± 0.118*	P<0.001	0.050 ± 0.007*	P<0.001
E2-9 dpf	10	2.602 ± 0.136*	P=0.007	0.0663 ± 0.008*	P=0.003
E2-14 dpf	12	2.941 ± 0.049*	P=0.0499	0.0866 ± 0.004	
E2-22 dpf	11	2.938 ± 0.057		0.0907 ± 0.005	
EE2 17.8 ng/L	15	2.733 ± 0.078*	P<0.001	0.0721 ± 0.004*	P<0.001

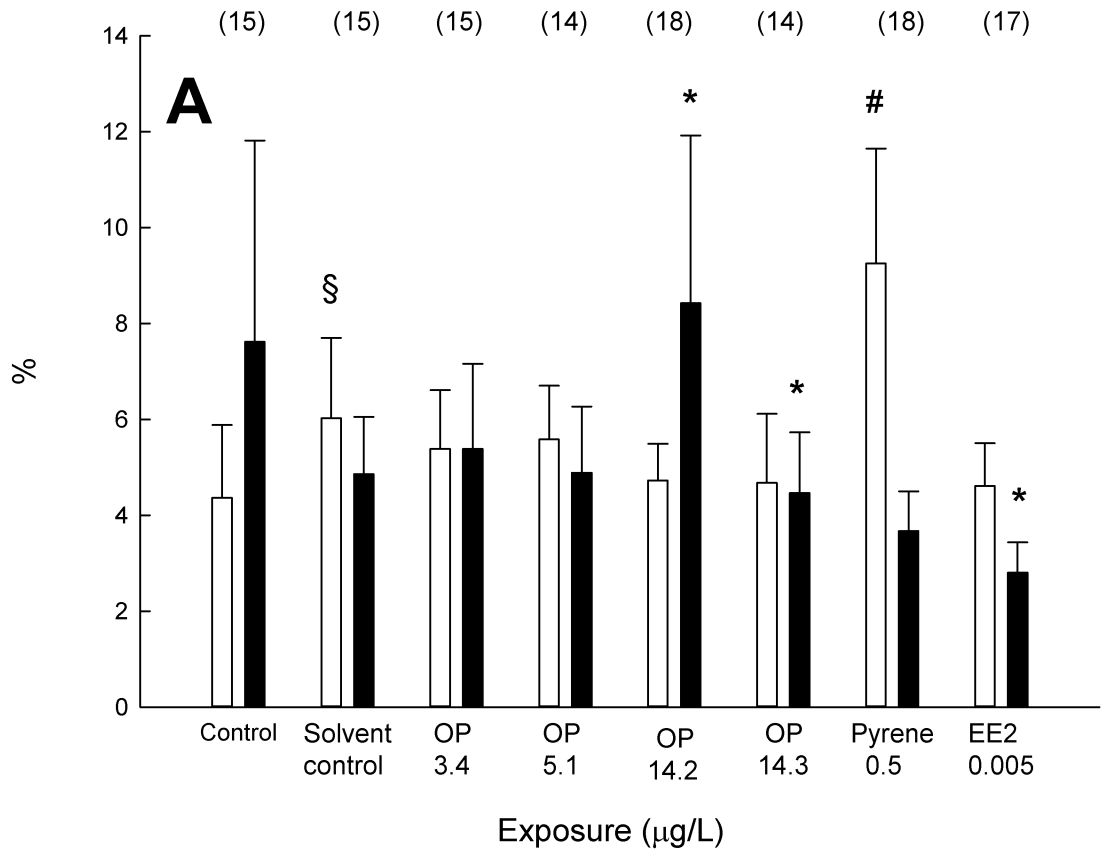

Figure 1A: Fry mortality and malformations (2012)

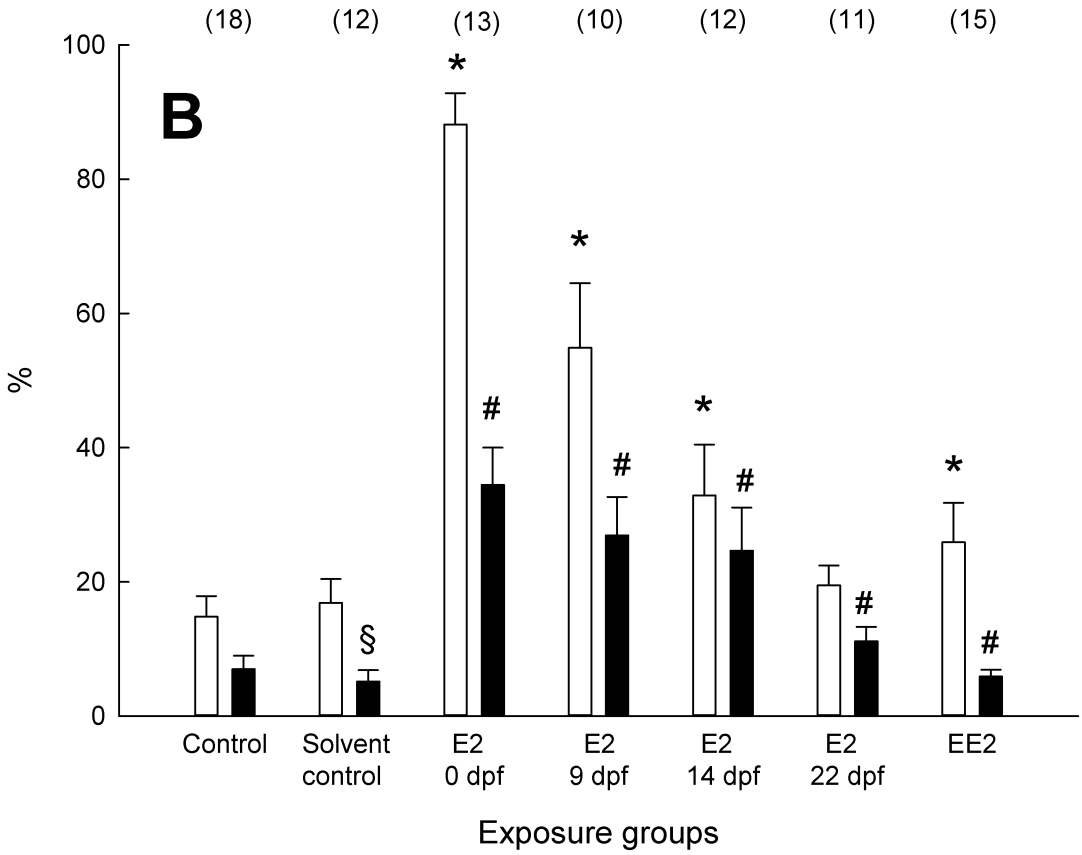

Figure 1B: Fry mortality and malformations (2013)

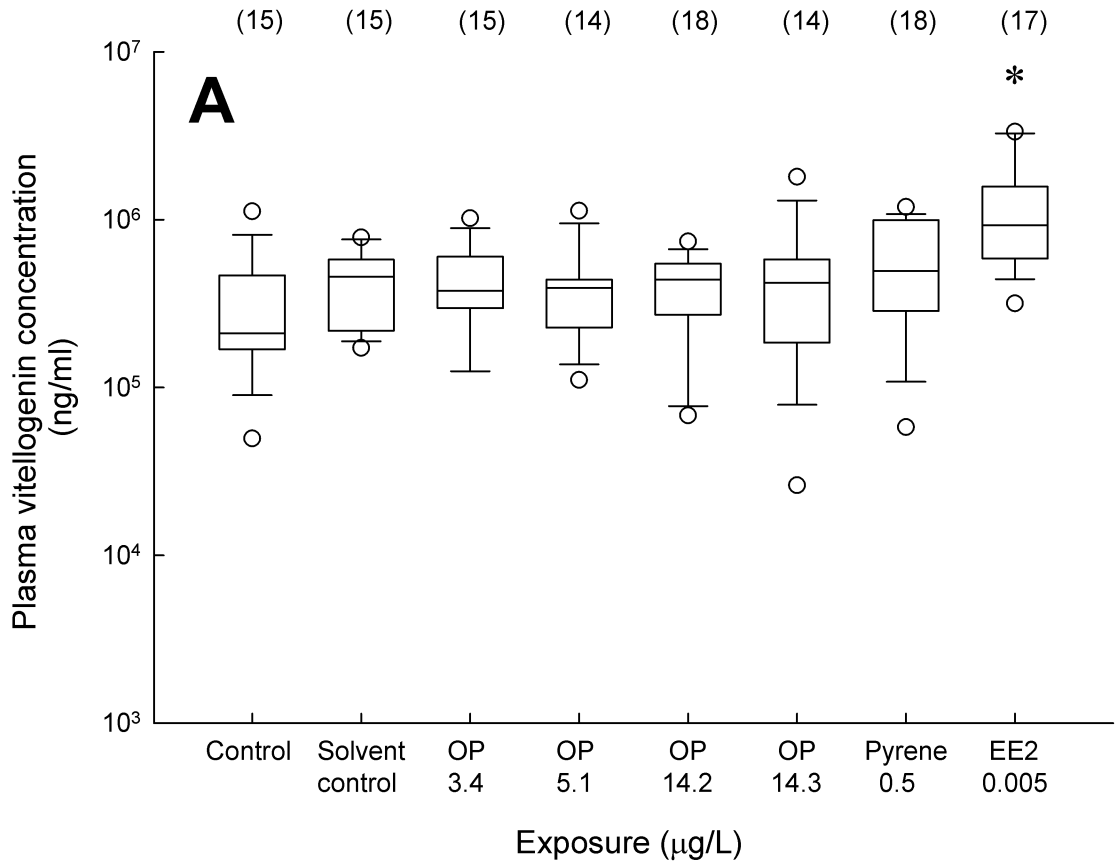

Figure 2A: Plasma vitellogenin levels of females (2012)

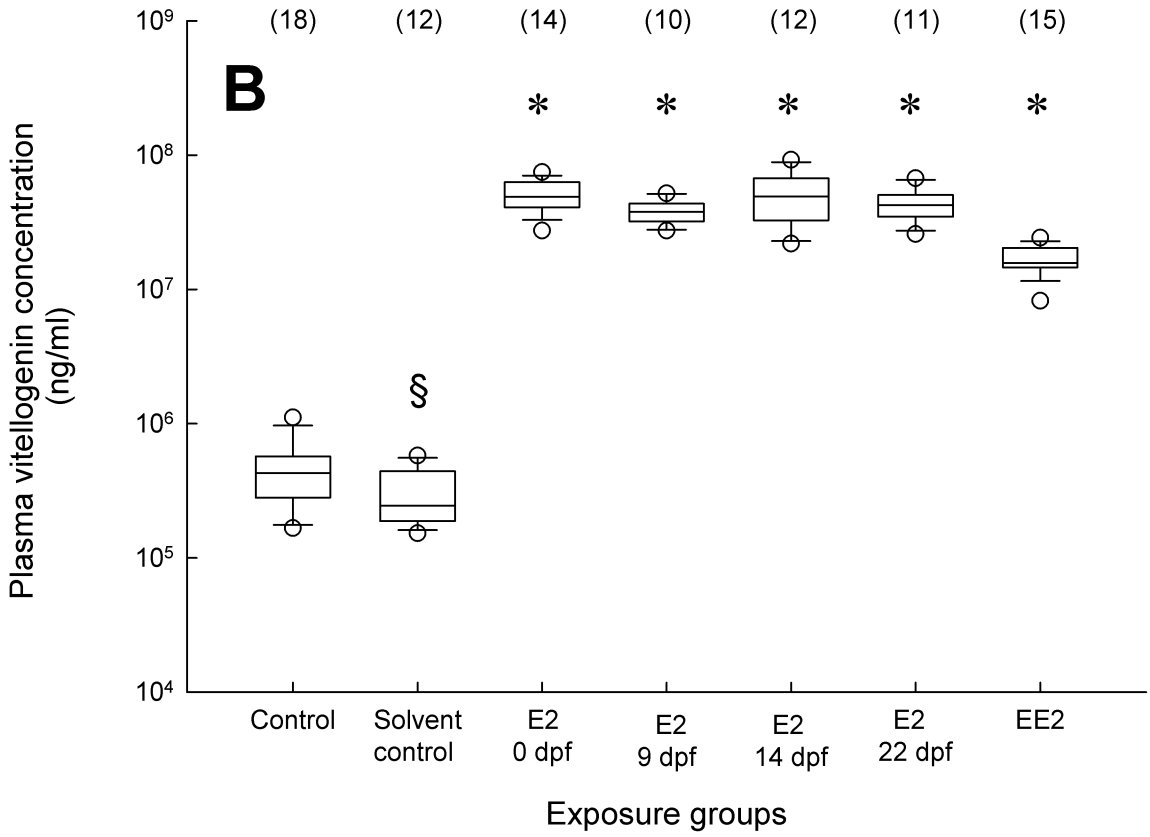

Figure 2B: Plasma vitellogenin levels in females (2013)

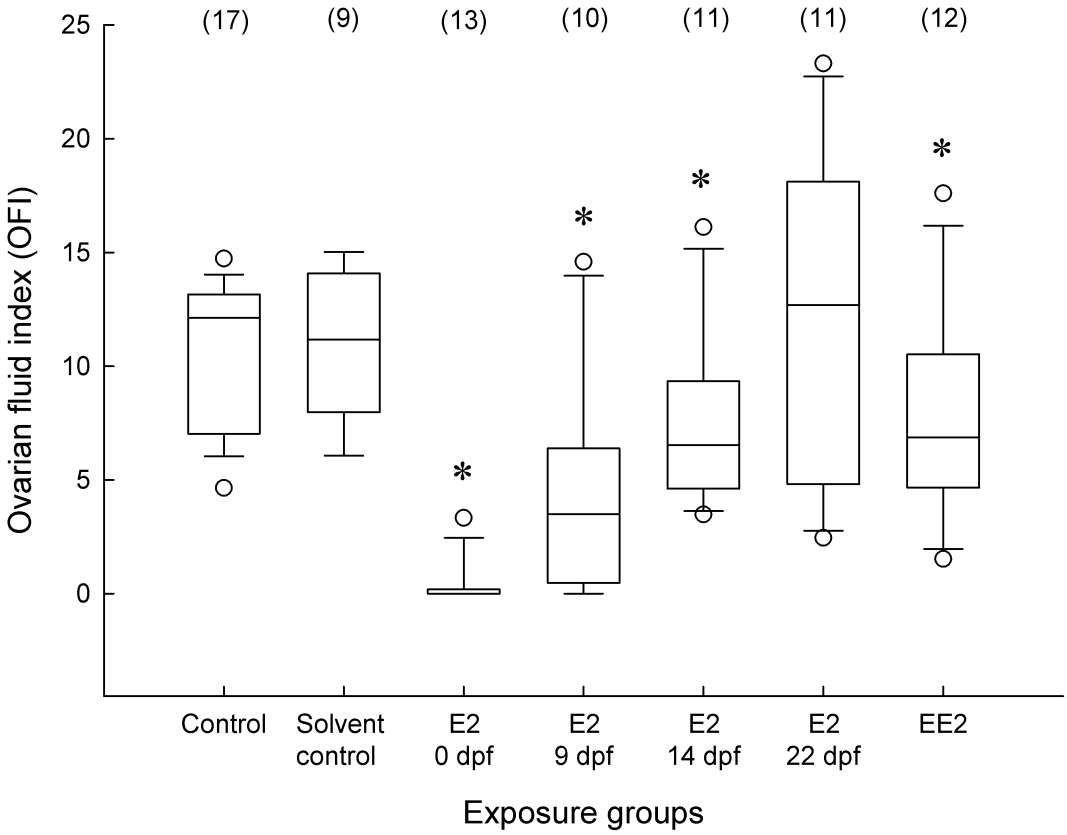

Figure 3: Ovarian fluid index of females (2013)

Figure legends

Figure 1 Mortality and malformations in 2012 and 2013
A) The percentage of dead larvae and deform live larvae in 2012 and **B)** the percentage of dead larvae and deform live larvae in 2013. Days post fertilization (dpf).
The total number of fish in each group is given in parentheses above the boxes.
The asterisk (*) means significantly different from the pooled control/solvent control group ($p<0.05$), the hash tag (#) means significantly different from the solvent control ($p<0.05$) and the section sign (§) means significantly different from the control.

Figure 2 Plasma vitellogenin in 2012 and 2013
Female plasma vitellogenin concentrations in **A)** females exposed to 4-t-OP, pyrene and EE2 (2012) **B)** females exposed to EE2 or E2 during different periods of pregnancy (2013). Days post fertilization (dpf).
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 above the boxes.
The asterisk (*) means significantly different from the pooled control/solvent control groups ($p<0.05$) and the section sign (§) means significantly different from the control ($p<0.05$).

Figure 3 Ovarian fluid in 2013
Ovarian fluid index in females exposed to EE2 or E2 during different periods of pregnancy (2013). Days post fertilization (dpf).
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 above the boxes.
The asterisk (*) means significantly different from the pooled control/solvent control groups ($p<0.05$).