Sexual re-programming and estrogenic sensitization in wild fish exposed to ethinylestradiol

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Brief: Environmental concentrations of EE_2 feminize roach and exposure during early life sensitizes fish to subsequent estrogen exposure.

ABSTRACT

Globally, feminization responses in wild male freshwater fish are caused by exposure to estrogenic chemicals, including natural and synthetic estrogens, contained in effluents from wastewater treatment works (WwTW). In UK rivers, feminization responses, including intersex, are widespread in wild roach (*Rutilus rutilus*) populations and severely affected fish have a reduced reproductive success. We exposed roach to environmentally relevant concentrations of the contraceptive estrogen, 17α-ethinylestradiol (EE_2) for up to two years, including intermittent and repeated exposures, to determine effects on sexual development and subsequent responsiveness to estrogen. Exposure of roach to EE_2 (at 4 ng/L) for 2 years resulted in sex reversal in males leading to an all-female population with two cohorts in terms of their stages of ovarian development, one paralleling the control females and one at a significantly less advanced stage, which we propose were sex-reversed males. Differing developmental and maturing rates of the putative sex-reversed males compared with control females, would question their functional capability as females in the wild. Early life exposure to environmentally relevant concentrations of EE_2 sensitized females to estrogen, as determined by the measurement of the responses of estrogen-sensitive genes in a further EE_2 challenge 398 days after the original exposure. In the wild, exposure to environmentally relevant concentrations of EE_2 during early life has significantly wider implications for the sexual physiology in fish than has thus far been determined.

INTRODUCTION

Endocrine disrupting chemicals (EDCs) including natural and synthetic steroid estrogens, alkylphenol ethoxylates, various pesticides, phthalates and bisphenols, are widespread in the environment and many have been shown to disrupt endocrine function in wildlife and humans *(reviewed in 1)*. Wildlife living in and/or closely associated with freshwater ecosystems is especially at

risk of EDC exposure because many freshwaters receive discharges, principally via WwTWs and in the UK, it is not uncommon for half of the flow of a river to be comprised of treated WwTW effluent *(2)*. Globally, exposure to WwTW effluents has been associated with a range of deleterious effects on reproduction in fish and fish populations (e.g. UK *(3,4)*, France *(5)*, Germany *(6)* or USA *(7)*). There are other studies, however, that have found no adverse effects of WwTW effluents on fish *(8,9)*.

Extensive studies carried out on wild roach (*Rutilus rutilus*) living downstream of WwTWs have shown a range of feminization responses, including elevated concentrations of blood vitellogenin (VTG, an estrogen-dependent yolk precursor) in males and immature females, the presence of a female-like ovarian cavity in the testis of males, and a high incidence of intersex *(3)*. We would emphasize that the prevalence of intersex reported in wild roach is considerably lower in rivers in other European countries compared with the UK *(10,11)*. Although some species of fish are hermaphrodites, containing both male and female sex cells in their gonads or undergo changes in sex as part of their normal sexual development, roach are normally gonochorists (single sexed). Disruption in sexual development in roach has been shown to impact negatively on the reproductive success of affected fish *(12,13)* with the potential for population-level ramifications. All of the feminized phenotypes seen in wild fish can be induced experimentally by controlled exposure to WwTW effluents *(2,14,15)*.

In fish, sex determination and sexual differentiation are controlled by a delicate balance of genetic and environmental factors, and any alteration created by other exogenous influences, including EDCs, can ultimately impact on sex assignment, even in gonochoristic species *(e.g.16)*. Estrogens play key roles in sexual differentiation and gametogenesis, and exposure to estrogen-mimicking chemicals during critical periods of differentiation can affect sexual development and have consequences for subsequent reproductive capabilities. It has been hypothesized that steroidal estrogens, both natural and synthetic, present in effluents play a major role in the disruption of sexual function in wild roach in UK rivers. Of these estrogens, the pharmaceutical, 17α–ethinylestradiol (EE_2), used in the contraceptive pill is the most potent *(17)*, with high persistence and a tendency to bioconcentrate in organisms *(18)*.

EE_2 thus has the potential to disrupt reproductive processes in fish at relatively low (ng/L) concentrations that occur in the aquatic environment. In Europe, EE_2 has been measured at concentrations from below the detection limit up to 15 ng/L in effluents and up to 5 ng/L in surface waters *(14,18-26)*. Laboratory exposures of fish to EE_2 have been shown to disrupt normal sexual development and differentiation *(e.g.27)*, alter reproductive behaviors *(e.g.28)*, and reduce reproductive success via effects on fecundity *(e.g.29)* or fertilization success/embryo viability *(e.g.30,31)*, but often at exposure concentrations exceeding those measured in the aquatic environment. In mammals, neonatal exposure to estrogens and their mimics can alter regulation of gene transcription, producing long-term changes in a number of signaling pathways including those regulating cell proliferation, differentiation, and survival and some of these changes in gene regulation, or imprinting, have been implicated in the susceptibility to environmentally-related diseases, including cancer *(32,33)*. Whether environmental estrogens produce permanent changes in gene function or "imprint" on the endocrine system in non-mammalian species is not known, but this could have serious implications for wildlife populations.

In this study we conducted exposures of roach to environmentally relevant concentrations of EE_2 for up to two years, and show complete sex reversal and delayed gonadal maturation in roach. Furthermore, and a particularly novel finding was that roach exposed during early life to EE_2 become more sensitive and responsive when re-challenged with EE2, more than a year later after the original exposure.

MATERIALS AND METHODS

Fish source and husbandry

Pre-spawning, sexually mature male and female roach were obtained from the Environment Agency's National Coarse Fish Farm (Calverton, Nottinghamshire, UK) and brought into the aquarium facility where they were induced artificially to spawn using established procedures with carp pituitary extract *(12)*. The fertilized eggs were deployed into glass aquaria under flow-through conditions.

Embryos hatched 7-10 days post fertilization and the resulting fish were provisioned with dietary requirements according to their age *(34)*. In brief, fish were fed with Cyprico Crumble EX dry food (Coppens International bv, Helmond, The Netherlands) and at all life stages, the diet was supplemented with live food.

Fish were maintained at ambient water temperature (18±1°C) with a 16hour light:8hour dark photoperiod in their first year. Between December and February of their second year, the temperature of the incoming water gradually decreased to 12±2°C before progressively increasing again up to 18±2°C in April. With decreasing temperature, the photoperiod regime was also reduced in a stepwise manner to 12h:12h light:dark and then in a stepwise manner back to 16h:8h light:dark by May.

Exposure systems and experimental design

Roach were exposed continuously to one of three concentrations of EE_2 (Sigma-Aldrich, Poole, UK; nominal concentrations of 0.1, 1.0 and 10 ng/L) from fertilization up to 720 dph in flow-through conditions, in duplicate tanks (Figure 1). EE_2 was made up in ethanol and the solvent dosing to all the tanks was less than 0.0001% v/v. Control roach were maintained in dilution water tanks. Dilution water and the EE_2 dosing stock solution were both delivered to the tanks using peristaltic pumps. Water flows and EE_2 dosing rates were monitored regularly and the EE_2 dosing stock solution was renewed every 4 to 7 days. Fish were sampled at regular intervals for assessments on sexual development.

In a further experiment roach from this EE_2 exposure were transferred into clean water at 120 dph, subsequently depurated for 398 days (when the status of their sexual development was assessed). Fish were then re-exposed to one concentration of EE_2 (nominal 5 ng/L EE_2) for 10 days (Figure 1) and effects on the rate and level of plasma VTG induction and transcription of a suite of estrogen-responsive genes determined.

Water samples were collected periodically from each tank throughout the experiment to determine the actual exposure concentrations of EE_2. The extraction method and the applied radioimmunoassay were as described by Katsu et al. *(35)*.

Fish sampling and biological analyses

All fish were sacrificed humanely by terminal anesthesia with benzocaine followed by cervical dislocation as approved by the UK Home Office (Animals (Scientific Procedures) Act 1986). In the long-term exposure to EE_2, fish (n=12-30) were sampled at random from the exposure populations and analyzed for gonadal development at 250, 518 and 720 dph and VTG induction (250 and 518 dph only). Fish exposed to EE_2 during early life and then maintained in clean water were analyzed for gonadal development and for VTG induction at 518 dph, and also subsequently for plasma VTG induction and the transcription of gonadal aromatase (*cyp19a1a*), estrogen receptor alpha (*esr1*) and estrogen receptor beta 2 (*esr2b*) using qRT-PCR, at 522 and 528 dph, after 4 and 10 days of re-exposure to a single concentration of EE_2. These genes were chosen for study because of their fundamental role within estrogen-responsive pathways.

At all sampling points, total wet body weight and standard length were determined for each fish. The condition factor was calculated by expressing the cube of the fish length as a percentage of the body weight. At the final sampling point for EE_2 exposure (720 dph), the gonadosomatic index (GSI) was calculated as a measure of gonadal growth by expressing the dissected gonad weight as percentage of the total body weight.

At all time points, gonads excised were divided in half and one half used for histological analysis and, in the re-exposure study, one half for molecular analysis. For histopathology, gonads were first preserved in Bouin's fixative for 4–24 hours, depending on the size of the gonad, processed as described previously *(35)* and analyzed for the presence of sex cells and their developmental stages. Excised gonadal tissue for molecular analyses was immediately snap frozen in liquid nitrogen and

stored at -80°C until analysis. Total RNA was extracted from the gonadal tissue and reverse transcribed as described previously *(35)*. Subsequently, the mRNA expression of the target genes was established by qRT-PCR and normalized to the endogenous reference gene ribosomal protein L8 (*rpl8*) as described previously *(27,35)*.

For VTG analysis, blood samples were collected from the caudal sinus into heparinized haematocrit tubes or heparinized syringes, transferred into microfuge tubes containing aprotinin (Sigma-Aldrich) and centrifuged. The resulting plasma was stored at –20°C until analysis. VTG was determined using an ELISA originally established for common carp VTG which has been validated for measuring VTG in roach *(36)*.

Statistical Analysis

Unless stated otherwise, data are presented as mean±SEM and a probability level of $p<0.05$ was considered to be statistically significant. Data were examined for conformity with the assumptions of normality. If these were not met, data were transformed, as appropriate.

For the continuous EE_2 exposure, statistical analyses were carried out using SigmaStat® 3.1 (Systat Software, Inc) and effects on each endpoint were analyzed by one-way ANOVA if data met the assumptions of normality and homogeneity of variance, followed by Tukey's all pair-wise multiple comparison procedures. Data not meeting the assumptions of normality after transformation were analyzed by Kruskal–Wallis ANOVA on Ranks followed by Dunnett's or Dunn's all pair-wise multiple comparison procedures.

Data of the EE_2 re-challenge experiment were analyzed using two-way ANOVAs (in the software R 2.5.1) to assess the effect of exposure concentration during early life and duration of estrogen re-challenge on the chosen endpoints and the interaction between both factors. Between-treatment comparisons within each time point were carried out using Tukey's Honest Significant Differences test (Tukey's HSD).

RESULTS

Effects of long-term exposure to EE_2

EE_2 was not detected in water in the control tank and EE_2 in the nominal 0.1 ng/L treatment was below the detection limit of the radioimmunoassay (the detection limit for the assay in this study was 40 pg/L). The mean measured exposure concentrations of EE_2 in the nominal 1.0 and 10 ng/L test tanks were 0.3±0.1 and 4.0±0.3 ng/L, respectively *(as reported previously in 27,35)*. No significant mortalities occurred during the course of the two year exposure experiment and no concentration-dependent mortality rates were observed. There were no concentration-related effects of EE_2 on length, weight or condition factor in male or female roach exposed to EE_2 (Figure S1). After 720 days of exposure, no effect was observed on the GSI in male or female roach exposed to nominal 0.1 and measured 0.3 ng/L EE_2. The GSI of fish exposed to 4 ng/L EE_2 was highly variable (Figure S2) and all fish in this treatment group had an ovarian morphology (see below) and it was not possible to assign fish as females or (putative sex-reversed) males and consequently these fish were excluded from the statistical analyses for effects of EE_2 on growth.

In control females, ovaries at 250 dph contained all sex cells up to primary oocytes at the Balbiani body stage and in males, testes had well defined lobules with all stages of sex cells up to, and including spermatogonia A and spermatogonia B (Figure S3). At 518 dph, the ovaries in females included early vitellogenic oocytes and in males, testes contained spermatocytes. At 720 dph, all females contained vitellogenic oocytes, and all males contained spermatocytes, with one male containing spermatozoa. There was a male bias in the population throughout the study: at 250 dph, there were 60% males versus 40% females (n=53); at 518 dph, 59% males versus 41% females (n=17), at 720 dph, 69% males versus 31% females (n=26).

There were no histological differences in the gonadal status of fish exposed to nominal 0.1 ng/L EE_2 compared with control fish throughout the 720-day exposure. In fish exposed to measured 0.3 ng/L EE_2

at 250 dph the sex ratio was equally balanced (49% males and 51% females; n=35), but at 720 dph there was a female sex bias (42% males and 58% females; $\chi^2(1, n=24)=8.05$, $p<0.01$) compared to the control population at 720 dph. At each time point, gonads of fish exposed to 0.3 ng/L EE_2 contained sex cells at the same developmental stage as gonads of control fish throughout, with the exception of a single male fish in this treatment group at 720 dph that was intersex (Figure S4).

Exposure to 4 ng/L EE_2 feminized the population completely (Figure S3) for all life stages sampled. At 250 dph (n=22), most ovaries contained sex cells up to and including primary oocytes at the Balbiani body stage, comparable to the control fish at this time, but few fish in this treatment group were less developed. At 518 dph (n=12), there was a far greater degree of variation in gonadal development between individuals in this treatment group compared with control females and this persisted in these fish at 720 dph (n=24) where ovaries from 58% of the fish analyzed were comparable to the control females, containing all stages of sex cells, including, vitellogenic oocytes, and 42% had predominantly primary oocytes with just a few sex cells at a more advanced stage of development.

Concentrations of VTG in controls at 250 dph were similar in males (142.8±36.6 ng/mL) and females (147±46.6 ng/mL), but at 518 dph they were widely divergent, and in accordance with sex (20.3±6.0 ng/mL in males compared with 5416±4875 ng/mL in females). Significantly higher concentrations of VTG occurred in fish exposed to 4 ng/L EE_2 compared with controls. Exposure to 4 ng/L EE_2 resulted in VTG inductions of 472- and 489-fold after 250 dph (data not shown) and 103581-fold and 388-fold after 518 dph compared with control female and male roach, respectively (Figure S5).

Gonadal impacts of early life exposure to EE_2

There were no significant differences in length or weight of fish exposed to EE_2 during early life until 120 dph and maintained in clean water thereafter to 518 dph compared with fish at 518 dph that had been exposed continuously to EE_2 or maintained in clean water throughout. (Figure S6). There were also no effects on gonad development in roach exposed during early life (encompassing the

period of gonadal sex differentiation) to a nominal concentration of 0.1 and a measured concentration of 0.3 ng/L EE_2. Some fish exposed to 4 ng/L EE_2 during early life did not differ from control fish (unexposed throughout) at 518 dph in terms of the stages of germ cells present (Figure 2A–B). In this treatment group, however, there were some intersex fish (4 out of 11, 36%) at 518 dph (Figure 2C). The gonads of intersex roach were characterized by the presence of a few primary oocytes scattered throughout gonad sections of the testicular tissue in a multifocal arrangement. The primary oocytes occurred singly or in clusters. The gonads of these fish were dissected out prior to histological analysis and thus it was not possible to observe any malformations of the reproductive ducts.

At 518 dph, there was a male bias in the depurated fish population, as occurred for the unexposed controls, and the percentages of males ranged between 56 (0.3 ng/L EE_2 during early life) and 69% (4 ng/L EE_2) (Figure 2D).

Exposure of roach to 4 ng/L EE_2 during early life to 120 dph significantly induced plasma VTG in exposed fish (2116±726 ng/g) compared to 40±6 ng/g in controls, but after 398 days in clean water, VTG concentrations did not differ from control fish (unexposed throughout; Figure S5).

Estrogenic sensitization

During the course of the re-challenge experiment, the EE_2 exposure concentration was 2.3±0.2 ng/L EE_2. Overall, and as expected, re-exposure to EE_2 induced VTG synthesis and the duration of the EE_2 re-challenge had a significant effect on the level of the VTG induction in both females and males (females: $F_{1,47}$ =46.45; $p<0.0001$; males: $F_{1,43}$ =78.55; $p<0.0001$). In females, the EE_2 exposure concentration during early life appeared to have an effect on the level of induction of VTG in the estrogen re-challenge (plasma VTG levels in females exposed to 4 ng/L EE_2 during sexual differentiation were almost twice those in fish exposed to 0.3 ng/L EE_2 – 5.0- and 9.9-fold difference on day four and 1.7- and 3.3-fold on day ten compared to re-exposed control fish), however, there was no statistically significant difference ($F_{2,48}$ =1.39; $p=0.74$) due to the high variability in the responses

between individuals. In males, there was no effect of EE_2 treatment during early life on the VTG response on re-challenge to estrogen ($F_{2,44}$ =0.76; p=0.47), and males appeared to be less responsive generally to estrogen at this time compared with females (Figure 3A+B).

The expression of *esr1*, *esr2b* and *cyp19a1a*, genes key in estrogen signaling, appeared to show an enhanced responsiveness to estrogen in fish that had been exposed to estrogen during early life when compared with control fish previously unexposed, i.e. there was a sensitization in the responses of these genes as a consequence of prior exposure to EE_2 during early life. This apparent trend was more pronounced in females compared with males (Figure 3). Overall, the effect of exposure concentration during early life was significant for the expression of *esr1*, *esr2b* and *cyp19a1a* in ovaries and the expression of *esr2b* in testes (Table S1). In females, the gonadal responses of *esr2b* and *cyp19a1a* in the EE_2 re-challenge experiment was dependent on the EE_2 exposure concentration during early life, e.g. the expression of *cyp19a1a* in the ovary differed significantly from controls (fish not previously exposed to estrogen) with 5.1-fold and 10.5-fold elevations in females exposed previously (during early life) to 0.3 and 4.0 ng/L EE_2, respectively, compared to fish that received no estrogen exposure during early life (and were exposed during the estrogen re-challenge experiment).

DISCUSSION

This study shows that life-long exposure of roach to concentrations of EE_2 found in some of the more polluted aquatic environments resulted in complete feminization of the population, and exposure during early life encompassing the phase of sex determination and sex differentiation induced the development of testis-ova. For the first time, we also show that exposure to estrogen during early life sensitizes fish to subsequent (and environmentally relevant) estrogen challenge, as shown by the responses in estrogen-responsive genes.

In most reported exposures of fish to environmental estrogens that have feminized gonadal development and/or caused complete sex reversal, pharmacological concentrations of estrogens have

been used that bear no environmental relevance *(37)*. Here we show that long-term exposure to a concentration of only 4 ng/L EE_2, (a concentration found in some of the more polluted WwTW effluents) feminizes roach completely, resulting in an all-female population, an effect that would result in population failure in the wild. Populations of wild roach living in UK rivers heavily polluted with estrogenic WwTW effluents have been found with a sex ratio that is skewed towards females *(38)*, and in some cases are all-female (S.Jobling, personal communication). The lack of a genetic sex marker for roach, however, has excluded a definitive answer at to whether this bias in females is due to the presence of sex-reversed males. If sex-reversed roach occur in UK rivers and even if they were able of produce functional gametes, a retarded maturation rate, as occurred in this study, would likely mean the gametes were released at an inappropriate time in the wild; timing of reproduction and gamete release is critical in this seasonally spawning fish.

Generally, concentrations of EE_2 in UK WwTW effluents are measured at concentrations between 0.15 and 2.85 ng/L *(39)*, lower than that required to cause complete sex reversal in roach (here 4 ng/L), however, these effluents also contain biologically active concentrations of natural steroidal estrogen and other estrogenic EDCs that are additive in their feminizing effects *(40)*, and, therefore, roach populations may be at risk, given the overall estrogenic loadings entering UK rivers.

In mammals, (xeno)estrogens can induce effects via both genomic and non-genomic mechanisms *(41)*. Genomic mechanisms include direct effects on the expression of genes involved with estrogen signaling that define the pathways of development for the two sexes and/or effects on co-regulators of ER function. Imprinting of genes by estrogens during critical periods of development has been suggested as an important mechanism for functional defects later in life *(42,43)*. Epigenetic (non-genomic) mechanisms include effects on DNA methylation controlling selectively transcription or silencing tissue-specific genes. The mechanism by which sexual reprogramming occurs in roach as a consequence of exposure to steroidal estrogens is not known. In fish, however, exposure to E_2 and EE_2 have both been shown to lead to global genomic hypermethylation in the testis and to induce tissue-

and sex-specific changes in the methylation pattern of ER and aromatase genes *(44,45)*. It is therefore possible, that exposure of roach to EE_2 during early life led to persistent changes in the DNA methylation pattern of these and/or other crucial sex determining genes resulting in alterations in the regulation of their gene expression. This might explain the induction of intersex (and even possibly the differences in the subsequent responsiveness of estrogen-controlled genes to stimulation in later life), but the fact that exposure of roach to 4 ng/L EE_2 during the window of sexual differentiation did not induce complete sex reversal, which occurred only for the continuous exposure to EE_2, suggests another operational mechanism(s) for this effect. Our findings in this study showing that sex reversal occurs only for prolonged exposures to estrogen, support other studies showing longevity of exposure affects the magnitude (and severity) of the effects seen for sexual disruption, including for the vitellogenic response *(15)* and the intersex condition *(46)*, and emphasizes the need to consider the exposure period when assessing the possible hazards posed by steroidal estrogens. Our study also shows that detrimental effects of long-term exposure to EE_2 (at 4 ng/L) were not limited to the males, and they also occurred for females, where the ovaries (at 720 dph) were relatively smaller compared with 'normal' females.

Early life stage windows are especially sensitive to estrogenic (and other EDC) effects, and this is especially so for many fish species *(e.g.47)*. In some cases, effects induced by exposure during early life do not become manifest until puberty or sexual maturation *(47,48)*. In our studies on the roach too, we showed the induction of intersex (ovotestis) occurs as a consequence of exposure to 4 ng/L EE_2 during early life. Our most novel finding, however, was that exposure to EE_2 during early life altered the subsequent responsiveness of roach to estrogen in later life. Females in particular showed enhanced responses on re-challenge to EE_2 and this occurred in a concentration dependent manner for the exposures during early life. These findings strongly indicate that multiple exposures to estrogen, even with considerable time intervals between the successive exposures, can markedly affect the dynamics of the response to an environmental estrogen. If this response also occurs for endogenous estrogen (as

is likely), then the reproductive dynamics could be altered, given the importance of steroidal estrogens such as E_2 in both fecundity and timing of maturation *(49,50)*. These findings have further implications for assessing the hazards and health risks associated with exposure to this synthetic steroidal estrogen as, for instance, thresholds for biomarker responses may be altered. Again, the mechanism(s) in operation that enhance estrogen responsiveness in later life in roach is (are) not known, but potentially they might include a molecular priming effect, where the sensitivity of e.g. ERs and aromatases is enhanced. These molecular markers studied are known for their functional role as part of estrogen-responsive pathways: ER expression has implications for receptor availability and regulation, whereas aromatase expression has implications for the regulation of the ratios between estrogens and androgens *(27,35 and references within)*.

This study demonstrates that life-long exposure of roach to environmental concentrations of EE_2 (4 ng/L) induced sex reversal of males resulting in an all-female population and further, that the induction of ovotestis is a consequence of (re-)programming during early life. These data strongly support the hypothesis that EE_2 plays a major role in the induction of feminization responses in wild male fish living in rivers contaminated with estrogenic WwTW effluents. A further significant finding is that exposure to EE_2 during early life at concentrations as low as 0.3 ng/L induced more subtle disruptions on normal sexual programming altering the subsequent responsiveness to estrogens in later life and with wider potential health implications.

FIGURE CAPTIONS

Figure 1. Experimental design of the long-term exposures of roach to EE_2. Roach were exposed from fertilization to 720 dph to environmental concentrations of EE_2 (nominal 0.1, 1.0 and 10 ng/L). Some fish from each treatment were transferred to clean water after gonadal sex differentiation at 120 dph, and allowed to depurate for 398 days, when a sub-population was sampled. The remaining depurated fish were then re-exposed to one concentration of EE_2 (nominal 5 ng/L) and sampled at day four and ten of the re-challenging experiment.

Figure 2. Gonad histopathology and sex ratio of roach at 518 days post hatch (dph). Sections of ovary (A), testis (B) and intersex testis (C) of roach exposed to measured 4 ng/L EE_2 until 120 dph and then kept in clean water. po, primary oocyte; so, secondary oocyte; vo, vitellogenic oocyte; sgA, spermatogonia A; sgB, spermatogonia B; sy, spermatocytes; Bars: 50 μm. (D) Sex ratios of roach exposed to EE_2 until 120 dph and then kept in clean water for 398 days.

Figure 3. Effects of an estrogen re-challenge on plasma vitellogenin and expression of genes key in estrogenic signaling. (A+B) Concentrations of plasma vitellogenin and relative mRNA expression of *esr1* (C+D), *esr2b* (E+F) and *cyp19a1a* (G+H) in female (left) and male (right) roach exposed to measured 2.3 ng/L EE_2 for 10 days following early life exposure to different concentrations of EE_2 until 120 dph and subsequent depuration period of 398 days. Each column represents mean ±SEM and numbers in columns indicate the number of samples analyzed. Asterisks indicate a significant difference from the control at that specific time point. +: Note in graphs for gene expression in males that at day 10 only one male fish was analyzed from the 4 ng/L EE_2 early life treatment group and was therefore excluded from any analysis.

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SUPPORTING INFORMATION

Statistical results of two-way ANOVAs, histopathology of continuously EE_2-exposed roach as well as growth data and vitellogenin results for continuous exposure and depuration experiment. This information is available free of charge via the Internet at http://pubs.acs.org.

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