Effects of maternal exposure to environmentally relevant concentrations of 17α-ethinyloestradiol in a live bearing freshwater fish, *Xenotoca eiseni* (Cyprinodontiformes, Goodeidae).

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Abstract

The viviparous teleost redtail splitfin (*Xenotoca eiseni*) is a live bearing fish that presents a novel freshwater model for investigating the effects of maternally derived micropollutants on vulnerable early developmental life stages. Here, adult female *X. eiseni* were exposed to 17α-ethinyloestradiol (EE2), a potent a contraceptive oestrogen, at environmentally relevant concentrations, to investigate for effects on sex partitioning and development. Pregnant and non-pregnant females were exposed for four-weeks to EE2 at measured concentrations of 0.9 and 3.4 ng/L EE2 and offspring from gravid females kept in clean water for a further four weeks. Only pregnant females were seen to respond to 3.4 ng/L EE2 with an increase in the transcription of hepatic vitellogenins (*vtgA*, *vtgB* and *vtgC*). Offspring of exposed mothers showed no obvious effects on somatic growth, gonadal development, sex partitioning or development. However, there was a higher rate of deformities and developmental abnormalities in offspring of EE2-exposed females. The work presented provides the foundation for the development of *X. eiseni* as a new freshwater model for studies on maternal transfer of chemical pollutants in live bearing animals.

Keywords: Maternal transfer, 17α-ethinylestradiol (EE2), sexual development, viviparity, Goodeidae

1 Introduction

Small oviparous fish species including zebrafish (*Danio rerio*), fathead minnow (*Pimephales promelas*), Japanese medaka (*Oryzias latipes*) and three-spined stickleback (*Gasterosteus aculeatus*) are widely used in aquatic ecotoxicity testing (Ankley and Johnson, 2004; Helfman et al., 2009; Lange et al., 2012), but so far little attention has been directed towards studies on viviparous fish species. Maternal transfer is potentially a major route for chemical contaminant uptake into developing embryos and early life stages are likely to be amongst the most susceptible to adverse effects of toxicants (Brande-Lavridsen et al., 2013; Brion et al., 2004; Cazan and Klerks, 2014; Hedman et al., 2011; Hutchinson et al., 1998; Rasmussen et al., 2002; Wourms and Lombardi, 1992). Live bearing fish also offer possible supplementary models to mammals for toxicological studies on maternal transfer (Ankley and Johnson, 2004). Further supporting live bearing fish as study models, gravid viviparous fishdisplaying intraluminal gestation may contain tens of developing embryos that develop at the same rate and share the same environment for studies into chemical uptake and effect analysis. Transfer of nutrients from the mother to embryo in matrotrophic species means that the mother can also transfer xenobiotics present in her environment to her developing embryos (Guerrero-Estévez and López-López, 2016). Live bearing fish species that have been applied in ecotoxicology include the marine eelpout (*Zoarces viviparus*) (Brande-Lavridsen et al., 2013; Hedman et al., 2011; Rasmussen et al., 2002), the least killifish (*Heterandria formosa*) and mosquitofish (*Gambusia affinis*), both viviparous freshwater poeciliids (Cazan and Klerks, 2014), and, albeit in a very limited manner, the goodeids, darkedged splitfin (*Girardinichthys multiradiatus*), blackfin goodea (*Goodea atripinnis*) and barred splitfin (*Chapalichthys encaustus*) (De La Vega Salazar et al., 1997; Guerrero-Estévez and López-López, 2016). In the poeciliid *Gambusia affinis*, both essential and non-essential metals have been shown to transfer from exposed gravid females to developing young and maternal exposure to various contaminants has been shown to adversely affect the embryos' life history (Cazan and Klerks, 2015). Furthermore, in *G. multiradiatus*, it has been shown that for some pollutants that cross the placental barrier they can reach

concentrations in the developing embryos several orders of magnitude higher than in maternal tissue (De La Vega Salazar et al., 1997). Many endocrine disrupting chemicals (EDCs), which affect embryonic development and in particular sex differentiation and gonadal development in oviparous fish species (e.g. Baumann et al., 2013; Lange et al., 2009), are known to bioaccumulate in fish but there is little understanding on the maternal-embryo transfer of these xenobiotics in viviparous teleosts (Guerrero-Estévez and López-López, 2016).

The eelpout, least killifish and mosquitofish all have relatively long gestation periods of between four to more than six months (Brande-Lavridsen et al., 2013; Cazan and Klerks, 2015, 2014; Hedman et al., 2011; Rasmussen et al., 2002; Skov et al., 2010) and highly complex reproductive strategies including sperm storage and superfetation, which complicates their use as models in ecotoxicological studies. In contrast, the redtail splitfin (*Xenotoca eiseni*), a goodeid fish, has a gestation time of a few weeks only (Parenti, 2005; Tinguely et al., 2020) and is potentially practically more suitable as a species for testing effects of maternally transferred toxicants. A shorter gestation however does mean a shorter time window for assessing the bioaccummulative effects of contaminants.

X. eiseni possess a trophotaenial placenta, which includes a modified ovarian lumen epithelium as maternal component and trophotaeniae as an embryonal structure (Schindler, 2015; Uribe et al., 2005; Wourms, 2005; Wourms and Lombardi, 1992). A specialised ovarian epithelium is used to transport nutrients from the maternal vascular system to the ovarian fluid (Uribe et al., 2005) from where they are taken up by the hatched embryos within the ovary (Schindler, 2015). Embryonic waste is assumed to be transported in the opposite direction and subsequently removed via the maternal vascular system (Uribe et al., 2005). In the Goodeinae, as for most live bearing fish there is little information on the effect of maternally derived exposure to pollutants or their effects on the subsequent offspring (as reviewed by Guerrero-Estévez and López-López, 2016).

The synthetic oestrogen 17α-ethinyloestradiol (EE2) is a highly potent synthetic oestrogen with high environmental persistence and a tendency to bioconcentrate in organisms (Larsson et

al., 1999) and can disrupt reproductive processes in fish at low (ng/L) concentrations that occur in the aquatic environment. Globally, EE2 has been measured in sewage effluent and surface waters at concentrations from below the detection limit up to 15 ng/L (Cargouët et al., 2004; Olivares-Rubio et al., 2015; Ternes et al., 1999; Williams et al., 2003). Exposure to environmental concentrations of EE2 has been shown to induce a wide range of reproductive effects in fish spanning induction of vitellogenin (VTG), skewed sex ratios, reduced fecundity and altered sex behaviours (e.g. Armstrong et al., 2016; Baumann et al., 2013; Colman et al., 2009). There have been some studies on the effects of oestrogen (including EE2) exposure in ovoviviparous fish (e.g. Jackson and Klerks, 2019; Kristensen et al., 2005; Volkova et al., 2012), but very little work has been published for viviparous species. Korsgaard and co-workers (2002) studied the effects of different concentrations of EE2 in pregnant eelpout over a period of three weeks and were able to demonstrate changes in the maternal-embryonal trophic relationship. This exposure also disrupted calcium concentrations in maternal plasma and the histotroph in *Z. viviparus*, possibly affecting growth and bone formation in the embryo-larvae (Korsgaard et al., 2002). Plasma levels of vitellogenin in the mother fish exposed to 5 ng/L EE2 (and higher) were found to be higher compared with in control fish (Korsgaard et al., 2002; Morthorst et al., 2016).

In this study, *X. eiseni* were exposed to EE2 at measured concentrations of 0.9 and 3.4 ng/L EE2 with a view to investigating whether environmentally relevant exposures (Desbrow et al., 1998; Tyler et al., 1998) affected sex partitioning, development and other possible health outcomes in the offspring. Female *X. eiseni* were initially housed with male fish before they were exposed individually for four weeks to EE2. Just prior to parturition, broods were dissected out of the mother fish and the live young transferred to tanks containing fresh water only where they were kept for a further four weeks. The offspring were terminated and analysed histologically to investigate their sex and stage of gonadal development. Measurements conducted on the adult females included for condition indices – condition factor, cardiosomatic index and hepato-somatic index, and the levels of hepatic VTG mRNA.

2 Materials and Methods

2.1 Fish

Xenotoca eiseni from stocks raised at the University of Exeter, UK were maintained in glass aquaria (50 L) with biological under gravel filters and a daily flow-through of 200 to 300 litres. The water was constantly aerated and kept at 26 ± 1 °C with a conductivity of 300 µS/cm and pH 6.8 to 7.2. The photoperiod was 12 h light and 12 h dark with artificial dawn and dusk transitions of 30 minutes.

Before the exposure, 144 naïve, seven months old adult fish were housed in six glass aquaria (50 L), each holding 12 males and 12 females. The fish were allowed to breed for 10 days. After this period, male fish were removed from the aquaria and the female fish kept in the same aquaria for another four days before they were transferred to the exposure aquaria. The timing of the gonadal sexual differentiation process has not been determined for *X. eiseni* and therefore we do not know if sexual differentiation had started in the developing offspring before the start of the EE2 exposure. However, this is likely to have been the case if we compare the ontogeny of sexual differentiation in this species (Tinguely et al., 2020) with the barred splitfin, another goodeid with a similar gestation period of 6-8 weeks (Guerrero-Estévez and Moreno-Mendoza, 2012).

2.2 Experimental Setup

2.2.1 Adult Exposure

Female fish were exposed to one of the four following treatments: Two EE2 treatments (nominal concentrations of 1 and 5 ng/L EE2), a solvent (ethanol) control or a water control. Every treatment consisted of 18 non-leaching 1 L plastic aquaria (888 Reptiles, Daventry, UK), each connected to a multi-channel peristaltic pump to deliver the respective exposure water from a reservoir glass tank (30 L) into the exposure tanks and providing a continuous flow-through system (Supplementary Figure S1). EE2 (Sigma Aldrich, Poole, UK) was dissolved at 50

mg/L in ethanol (analytical grade; Fisher Chemicals, Loughborough, UK) from which fresh weekly stock solutions of nominal 1 and 5 µg/L EE2 were prepared. The stock solution for the solvent control (100 µL ethanol/L) was also freshly made weekly. The final solvent concentration in the solvent control and both EE2 treatments was 0.00001%. The experimental system was conditioned for five days before the start of the exposure.

Two weeks after the onset of the breeding period and immediately prior to the start of the exposure, total body length and weight were recorded for each female. After this, females were transferred into the individual, pre-conditioned 1 L tanks, where they were exposed to one of the four treatments for four weeks, i.e. the remainder of the gestation period. Fish were fed to satiation with gamma-irradiated bloodworm (*Chironomidae*, Tropical Marine Centre, Chorleywood, UK) and TetraMin flake food (Tetra GmbH, Melle, Germany) and checked twice daily for any visible signs of ill health and feeding behaviour. Aquaria were siphoned every other day to remove food debris and faeces.

2.2.2 Offspring Maintenance

Just before parturition, embryos were dissected out and transferred to 1 L plastic aquaria. Broods were kept separately in groups of 9 to 10 individuals. If a group of fish remaining from one brood was less than 9 fish, they were excluded from the later developmental analysis as the density of fish with a tank can affect somatic growth rates in fish. Offspring were held for four weeks under continuous flow-through of fresh water only (approximately 10 L/day per tank) and fed daily with freshly hatched *Artemia* (cysts from ZM Ltd., Hampshire, UK). Aquaria were siphoned once a week.

2.3 Analysis of EE2 in Water Samples

For the measurement of exposure concentrations for EE2, duplicate 1 L water samples were taken weekly from the inlet (dosed reservoir aquaria; Supplementary Figure S1 (5)) for each treatment (including controls) on days 14, 21, 28, 35 and 42 of the overall experiment (Figure 1)

and from the collective water outlet for each treatment including also for the controls (Supplementary Figure S1 (7)) on days 21 and 35 of the overall experiment (what corresponds with the first and the third week of the exposure; Figure 1). The analysis of water from both, inlets and outlets was performed to assess for chemical loss caused by adherence to and/or biodegradation within the experimental system. No samples were taken and analysed for EE2 from the individual tanks.

The water samples were stabilised with glacial acetic acid (~100%, AnalaR; BHD Laboratory Supplies, Poole, UK) and methanol (HPLC grade; Fisher Chemicals) at final concentrations of 1% and 4%, respectively and spiked with deuterated 17α-ethinyloestradiol (EE2d4; Cambridge Isotope Labs., Andover, MA, USA) before solid phase extraction (SPE) onto Oasis HLB cartridges (Waters, Manchester, UK). Following extraction, EE2 concentrations were measured by HPLC-MS/MS using ionisation and fragmentation settings were as reported in Labadie et al. (2007). Detailed descriptions of SPE and HPLC-MS/MS are provided in the Supplementary Information.

2.4 Sampling

2.4.1 Female fish

At the end of the four week exposure period, females were euthanised by terminal anaesthesia with benzocaine (Sigma Aldrich; 50 mg/L, inhalative) followed by destruction of the brain, carried out ethically in accordance with the UK Code of practice for the humane killing of animals under Schedule 1 of the Animals (Scientific Procedures) Act, 1986. Wet weight and total body length were recorded for all fish to assess the condition factor ($K = 100 \times \text{weight (mg)} \times \text{length}^{-3}$ (mm)). The ovaries of gravid and non-gravid females were dissected out and weighed. Embryos were immediately dissected out of gravid ovaries, counted, and transferred to aquaria containing freshwater only, and the empty ovaries were weighed again to assess the brood weight. The broods were dissected out of the females for a number of reasons. Firstly, to ensure the embryos did not come into contact with EE2 other than via that maternally derived (i.e. avoiding any exposure directly via the tank water). Dissection of the broods prior to parturition

196 also prevented filial cannibalism that can occur in *X. eiseni* (our own observations). Also, due to
197 the protracted period of birthing in the study population, occurring over a period of 10 days,
198 allowing natural births to occur would have resulted in different exposure time periods for the
199 developing embryos.

200 To calculate the cardio- and the hepato-somatic indices (CSI and HSI, respectively), hearts
201 and livers were dissected out and weighed. Livers were immediately flash frozen in liquid
202 nitrogen and stored at -80 °C for subsequent RNA extraction. An overview of sampling is
203 presented in Figure 1.
204
205 *2.4.2 Offspring*
206 The offspring of exposed females were maintained in clean water with the aim of examining
207 their sexual development. After four weeks, offspring were sacrificed (terminal anaesthesia as
208 above), recorded for wet weight and total length, fixed *in toto* in Bouin's solution for two hours,
209 washed with, and stored in 70% methylated industrial spirit (Fisher Chemicals, UK) until
210 histological processing. An overview of the sampling regime is presented in Figure 1.
211
212 **2.5 Histology**
213 Fixed fish were dehydrated and embedded in Paraplast (Sigma Aldrich) using a Shandon
214 Citadel 2000 tissue processor. Three µm serial transverse sections were cut through the
215 abdominal region of the body, collected onto glass slides, and stained with haematoxylin and
216 eosin (both Shandon, Cheshire, UK) using a Varistain 24-4 slide stainer (Shandon). The slides
217 were mounted with Histomount (National Diagnostics, Hessle Hull, UK) and whole-body sections
218 examined for the presence of gonads. Using light microscopy, sex and stages of gonadal
219 development were identified. Staging of the gonadal development was followed as described
220 elsewhere (Tinguely et al., 2020). Briefly, in females, stage 0 represents the chromatin nucleolus
221 stage, stage 1 represents early primary growth oocytes and stage 2, mid primary growth oocytes.

In males, stage 0 describes an immature testis containing spermatogonia only and stage 1 is the onset of spermatogenesis.

2.6 *Vtg Gene Expression*

2.6.1 RNA Extraction and Reverse Transcription

Total RNA was extracted from liver tissue (n = 18) using the RNeasy Mini Kit (Qiagen, Crawley, UK) including an on-column DNase treatment according to the manufacturer's instruction and quantified using a NanoDrop (Thermo Scientific). The RNA quality was verified using the absorption ratios: $A_{260\ nm}/A_{280\ nm}$ and $A_{260\ nm}/A_{230\ nm}$.

One µg total RNA was reverse transcribed into cDNA using MMLV reverse transcriptase (Promega, Southampton, UK), 0.4 µM random hexamers and dNTPs (2 mM each) according to the manufacturer's protocol. The cDNA samples were then stored at -20 °C until further analysis.

2.6.2 Obtaining a partial X. eiseni 18S ribosomal RNA sequence

To obtain a housekeeping gene sequence for *X. eiseni*, partial 18s rRNA sequences from four Cyprinodontiformes were obtained from the NCBI GenBank database and aligned using ClustalW (Thompson et al., 1994). Based on the alignment (Supplementary Figure S2), primers (see Supplementary Table S1) were designed in conserved regions using the NCBI Primer-BLAST tool (Ye et al., 2012) to obtain overlapping sequence segments and these sequences were then amplified by PCR. A consensus sequence was established from the overlapping sequenced PCR products. Details of this approach are provided in the Supplementary Information.

2.6.3 Real-time Quantitative Polymerase Chain Reaction (RT-qPCR)

Hepatic expressions of *vtg* mRNA and 18S rRNA were determined by quantitative real-time PCR using target-specific SybrGreen assays on a CFX96 Real-time PCR Detection System (Bio-Rad Laboratories Inc., CA, USA). Sequences of *vtgA* (EU761161), *vtgB* (EU761162) and *vtgC* (EU761163) for *Xenotoca eiseni* were retrieved from the NCBI GenBank database. Specific primers for all three *vtg*s and 18S rRNA were designed with Beacon Designer 7.2 software

(Premier Biosoft International, Palo Alto, CA, USA) and purchased from Eurofins MWG Operon. RT-qPCR assays for each primer pair were optimised as described by Filby and Tyler (2005) and detailed in the Supplementary Information. Efficiency-corrected relative expression levels (RE) of *vtgA*, *vtgB* and *vtgC* were calculated according to Filby and Tyler (2005) by normalising to the housekeeping gene 18S rRNA.

2.7 Statistics

Unless stated otherwise, all data are presented as mean ± SEM. Water chemistry data, viability and the stage of gonadal sex cell development were analysed using one-way ANOVA followed by Bonferroni's multiple comparison test. CSI and HSI of the mother fish and weight, length and condition of offspring, and the number of offspring were analysed by one-way ANOVA followed by Tukey's multiple comparisons test. For transcript profiles, data points classified as outliers (using the ROUT method; Motulsky and Brown, 2006) were removed from the data set before being analysed by one-way ANOVA followed by Tukey's multiple comparisons test, and if necessary data were first log-transformed to meet the assumptions of normality. Results for weight, length and condition factor of mother fish were tested for differences across treatments using two-way (RM) ANOVA with Bonferroni's post-test. A Pearson's correlation analysis was performed to determine associations between brood size and average offspring weight. To analyse for relationships between treatment and offspring sex ratio, a chi-square test was applied. Results met the assumption of approximate normality and equality of variance and differences were considered statistically significant at $P \leq 0.05$. All data were analysed using GraphPad Prism (Graph Pad Software, Inc., San Diego, CA, USA).

3 Results

3.1 Water Chemistry

Concentrations of EE2 were consistent in the tank water in- and outlets across all treatments and across all sampling dates (Supplementary Figure S4) illustrating consistent exposure

concentrations with no significant loss of EE2 in the exposure tanks. Results were therefore combined for the individual tanks within treatments (Figure 2). The mean measured exposure concentrations were 0.9 ± 0.05 ng/L EE2 (n = 13) and 3.4 ± 0.1 ng/L EE2 (n = 14) for the 1 and 5 ng/L EE2 nominal concentrations, respectively. Occasionally, traces of EE2 were detected in both water control (0.16 ± 0.08 ng/L EE2; n = 12) and solvent control (0.07 ± 0.05 ng/L EE2; n = 12), but these levels were always at least 5.5-fold lower than for the lowest EE2 exposure. The nominal limit of detection (LOD) was < 0.1 ng/L EE2.

3.2 Adult Females

3.2.1 Growth and condition

Thirty-four of the 72 female fish were pregnant. Both, pregnant and non-pregnant females showed an increase in total body length during the exposure, except for the pregnant females of the water control. Throughout, there were no differences in growth and condition between water and solvent control fish (Supplementary Figure S5).

In non-pregnant females, there were no differences in total body length, weight and the condition factor between treatments before the exposure or between treatments after exposure. During the exposure period, non-pregnant females showed a significant increase ($P \leq 0.0001$) in weight except for the fish exposed to 0.9 ng/L EE2 and there was a significant loss ($P \leq 0.0001$) in condition during the exposure (Supplementary Figure S5).

In pregnant females, there were significant increases ($P \leq 0.01$) in weight during the experiment, except for weight in fish exposed to 0.9 ng/L EE2. After exposure, differences ($P \leq 0.01$) were seen for weight and condition of pregnant fish between the 0.9 ng/L EE2 treatment and all the other treatments (when including the brood weight). After subtraction of the brood weight, a significant decrease was observed for weight ($P \leq 0.001$) in pregnant fish exposed to 0.9 ng/L EE2 and, as occurred for non-pregnant fish, there was a significant loss in condition during the exposure (Supplementary Figure S5). Analysis of heart and liver weight, expressed as

CSI and HSI, showed no differences between fish in the different treatments except for the CSI between the water and solvent controls in non-pregnant fish (Supplementary Figure S6).

3.2.2 Vtg mRNA expression

Expression of the *X. eiseni* 18S rRNA gene was used as internal control to normalise the quantitative PCR data for *vtgA, vtgB* and vtg*C* mRNA expression. Overall, relative mRNA transcription levels for all three *vtg* genes were higher in non-pregnant females compared to pregnant females (Figure 3).

In non-pregnant control females, transcript levels were higher for *vtgA* and *vtgB* compared to *vtgC*. The average relative expression of any of the *vtg* genes did not differ across all treatments. However, there was an indication that *vtgC* in the exposures to EE2 might be lower in non-pregnant females, as expression levels were 4.5- and 2.6-times lower in fish exposed to 0.9 or 3.4 ng/L EE2, respectively, compared to fish in the solvent control, albeit this was not statistically significant (Figure 3A-C1).

In pregnant control females, transcript levels were at least 10 times higher for *vtgA* and *vtgB* compared to *vtgC*. Exposure to 0.9 ng/L EE2 did not affect the transcript levels for any of the three *vtg*s compared with solvent controls. However, exposure to 3.4 ng/L EE2 did show a tendency for a higher relative expression level in pregnant fish. The relative mRNA levels of *vtgA*, *vtgB* and *vtgC* were 3.4-, 6.9- and 2.0-times those of the solvent control fish, but not statistically different (Figure 3A-C2).

3.2.3 Brood sizes

There were five pregnant fish in the water controls, which collectively produced 142 embryos (Supplementary Table S3). Three of the offspring were still-born and another five died within 24 h after birth. In the solvent controls, there were nine pregnant females with a collective total of 183 embryos (two were still-born and seven died within 24 h of birth). In the 0.9 ng/L EE2 treatment, there were eleven pregnant females producing a total collective

offspring number of 214 (16 were still-born and 17 died within 24 h of birth). In the 3.4 ng/L EE2 treatment, there were eight pregnant females which carried 211 embryos (one was still-born and eleven died within 24 h of birth). A summary of the survivorship data is shown in Supplementary Figure S7.

Brood sizes did not vary across the different treatments (Figure 4B). There were significant differences in brood weight with broods of fish exposed to 0.9 ng/L EE2 weighing significantly less than broods of all the other treatments. The only statistically significant difference in brood weight amongst the other treatments occurred between the broods of the solvent control and the 3.4 ng/L EE2 treatment where broods of the 3.4 ng/L EE2 exposed females were comparatively heavier (Figure 4A). The differences in brood weight were reflected in the average individual offspring weight (Figure 4C). In the 0.9 ng/L EE2 treatment, offspring had a lower average weight compared with those in the solvent control and the 3.4 ng/L EE2 treatment. To calculate the average individual offspring weight, the brood weight was divided by the number of offspring. In both controls and the 0.9 ng/L EE2 treatment, there was a negative correlation between brood size and average offspring weight (water control: $r = -0.978$, $n = 5$, $p = 0.0039$; solvent control: $r = -0.932$, $n = 9$, $p = 0.0003$; 0.9 ng/L EE2: $r = -0.855$, $n = 11$, $p = 0.0008$; 3.4 ng/L EE2: $r = -0.312$, $n = 8$, $p = 0.4519$) (Figure 4D). One fish exposed to the nominal concentration of 0.9 ng/L EE2 had one offspring only, which is shown as an outlier in Figures 4A, B&C.

Some of the fish that were alive at birth but died within 24 hours were less developed than their siblings or they exhibited deformations (Supplementary Figure S8). The most common abnormality observed was a lower spinal abnormality resulting in a bent tail. This restricted the mobility of the young fish or even prevented any movement at all. There were five less developed young fish derived from females in the water control, corresponding to 3.6% of all water control offspring that were alive at birth. In the solvent treatment, there was only one fish with a bent tail (0.6%). Eight young in the 0.9 ng/L EE2 treatment were underdeveloped and two had bent tails, accounting for a total proportion of 5.0% deformities. In the 3.4 ng/L EE2 treatment, 5.2% of the offspring exhibited abnormalities – three fish were underdeveloped, and

eight fish had bent tails. Overall, there was a tendency for more deformities and developmental abnormalities in offspring from EE2-exposed females.

3.3 Offspring

3.3.1 Survival, Growth and Condition

Most of the offspring that lived for 24 hours after birth subsequently survived until their termination at four weeks (see Supplementary Table S3). Five died from the broods in both the water control and 3.4 ng/L EE2 treatments, four from the solvent control and eleven from the 0.9 ng/L EE2 treatment and those mortalities occurred within the first week after their birth. Four weeks after birth, a total of 129 offspring from the water control, 170 offspring from each the solvent control and the 0.9 ng/L EE2 treatment, and 194 offspring from the 3.4 ng/L EE2 treatment were examined for histological sex.

Not all broods could be divided into groups containing nine to ten fish and for those tanks containing a smaller number of fish, these were excluded from the analysis on the status of gonadal development and from growth comparisons. This is because growth is affected by the density of fish in a tank and somatic growth in turn can affect the rate of sexual development. Four weeks after birth, total body length and average body weight of broods from the 0.9 ng/L EE2 treatment were significantly lower than those of broods from the other treatments (Supplementary Figure S9). Condition factor of offspring from the 0.9 ng/L EE2 treatment was higher compared to both control treatments, but not the 3.4 ng/L EE2 treatment (Supplementary Figure S9). There were no differences in any of the body size or condition factors for the offspring of the 3.4 ng/L EE2 treatment compared with the water and the solvent control fish.

3.3.2 Sex Ratio and gonadal development

All broods were of mixed sex and sex ratios varied from 20%:80% to 68%:32% (male:female) (Supplementary Figure S10 and Supplementary Table S4). There were no differences in sex

385 outcome in the broods between the four treatments with overall sex proportions of 49%:51% in
386 the water control offspring, 46%:54% in the solvent control, 48%:52%, in the 0.9 ng/L EE2
387 treatment, and 52%:48% in the offspring of the 3.4 ng/L EE2 treatment (χ^2 (3, n = 8) = 1.2845, p
388 = 0.7328) (Figure 5).

389 In the offspring, male gonads were either at a stage before the onset of spermatogenesis
390 (stage 0) or undergoing spermatogenesis but prior to sperm packages (stage 1). Oocytes in
391 females were either at the onset of meiosis (stage 0) or early primary growth (stage 1). There
392 were no differences between the treatments for both sexes in the state of gonadal development
393 with the following average stages of development determined: water control, 0.66 ± 0.12
394 (males, n = 63) and 1.00 ± 0.00 (females, n = 66); solvent control, 0.71 ± 0.05 (males, n = 79) and
395 1.00 ± 0.00 (females, n= 91); 0.9 ng/L EE2, 0.62 ± 0.08 (males, n = 81) and 0.99 ± 0.01 (females,
396 n = 89); 3.4 ng/L EE2, 0.64 ± 0.08 (males, n = 101) and 0.99 ± 0.01 (females, n = 93).
397
398
399 **4 Discussion**

400 The effects of environmental contaminants on fish have been widely studied, but relatively
401 little is known on the extent to which contaminants are transferred from exposed females into,
402 and subsequent effects on, their developing offspring in viviparous species. Here, studying the
403 live bearing freshwater fish *X. eiseni,* we show that adult females respond to EE2 at the highest
404 concentration tested, a concentration found in some of the more polluted aquatic
405 environments, as measured by induction of hepatic *vtg* mRNA transcript levels, but we found no
406 discernible effects for this exposure on sex partitioning or sexual development in their offspring.
407 However, there was a tendency for a greater incidence of developmental abnormalities in the
408 offspring of females exposed to EE2, which would have fitness and survivorship impacts for
409 those offspring in natural environments.
410

411 **4.1 Water chemistry**

412 EE2 was found to be relatively stable throughout the exposure study, with average measured
413 concentrations of 0.9 ± 0.05 ng/L EE2 for the 1 ng/L nominal concentration (90% of nominal) and
414 3.4 ± 0.1 ng/L EE2 for the 5 ng/L nominal concentration (68% of nominal). EE2 is known to be
415 less soluble in water compared with natural steroidal oestrogens (Adeel et al., 2017) and readily
416 adsorbs to suspended solids (Lai et al., 2000) and tank surfaces and this is likely to have
417 contributed to the lower (versus nominal) measured concentrations seen for the nominal 5 ng/L
418 EE2 exposure.

419 We were not able to measure the level of EE2 in individual larvae due to their very small
420 body mass, making this impractical. It has been established, however, that other estrogenic
421 compounds including octylphenol (OP), phytosterol, and 17β-oestradiol (E2), can
422 bioconcentrate in the ovarian fluid of exposed viviparous female fish from where they may then
423 pass, via trophotaeniae, into (and potentially bioconcentrate in) the developing offspring
424 (Mattsson et al., 2001; Rasmussen et al., 2002). Future work might consider measurement of
425 the exposure toxicant in the developing offspring using pooled samples to provide sufficient
426 material for quantifying levels of chemical trophic transfer.

427

428 **4.2 Effects of EE2 exposure on adult females**

429 There were no differences in weight, length or condition of adult females between
430 treatments at the outset of the exposure (showing a non-biased distribution of fish between
431 treatments) and both pregnant and non-pregnant females increased in size (body length) in
432 most treatment groups. Both, non-pregnant and pregnant females gained weight during the
433 exposure, but in the case of the latter, this was attributed to the growing offspring and not any
434 increase in female somatic weight. In fact, for pregnant females exposed to 0.9 ng/L EE2 there
435 was a decrease in female somatic body weight. In non-pregnant fish, there was no effect of 0.9
436 or 3.4 ng/L EE2 on weight, length or condition indicating no obvious adverse effects on their
437 health. In both non-pregnant and pregnant fish (when discounting brood weight) there was a

loss in body condition over the trial, but this was not attributable to the EE2 treatments as this also occurred for the control fish. This may have been associated with stress of handling or the confinement of individuals in small tanks. The hepato-somatic and cardio-somatic indices did not differ between control and exposed fish.

Overall, we found higher levels of hepatic *vtgA, vtgB* and *vtgC* mRNA expression in non-pregnant females compared with pregnant females, an expected finding since these females were undergoing vitellogenesis during which time VTG is synthesised by the liver, secreted into and transported via the blood, taken up by growing oocytes and stored as yolk to serve as a food reserve for the developing embryos (Sumpter and Jobling, 1995). All three *vtg* gene transcripts measured have been shown previously to be expressed in the liver during vitellogenesis in female *X. eiseni* (Iida et al., 2019). Interestingly, the hepatic expression of all *vtg*s also occurs in pregnant females (Iida et al., 2019), indicating that VTG not only acts as a nutrient source for embryo development in this species (as for oviparous fish species), but may also play role(s) in the gestation process too (Iida et al., 2019). This aligns with studies on other goodeid fish species, where histotroph has been shown to be composed of a protein mixture similar to that in the maternal blood serum (Schindler, 2015) and therefore would also contain VTG. Supporting the hypothesis that VTG is a matrotrophic factor in the Goodeidae, in both the Chapultepec splitfin (*Girardinichthys viviparus*) and the Butterfly splitfin (*Ameca splendens*) hepatic VTG concentrations increase during gestation (Vega-López et al., 2007). Furthermore, recently, mother-to-embryo transfer of VTG during gestation has been shown in *X. eiseni* (Iida et al., 2019). This is not the case for all Goodeidae species, however, and in both the Blackfin Goodea (*Goodea atripinnis*) and the Bulldog Goodeid (*Alloophorus robustus*) VTG is not detected in the serum of gravid females (Hollenberg and Wourms, 1995).

There have been no published studies assessing hepatic *vtg* expression in *X. eiseni* in response to oestrogen exposure. We found that exposure of non-pregnant females to EE2 did not result in up-regulation of *vtg* mRNA expression, but this may have been because these females were actively synthesising VTG and therefore transcription levels in these females were

465 already relatively high. In pregnant, non-exposed females, the levels of hepatic *vtg* mRNA were
466 relatively low and this may account (in part) for the greater responsiveness of pregnant females
467 to EE2 exposure compared with in non-pregnant females; relative levels of hepatic *vtg*
468 expression in pregnant females were 186 and 152 times lower for *vtgA*, 36 and 31 times lower
469 for *vtgB* and 7 and 8 times lower for *vtgC* compared with non-pregnant females for water and
470 solvent controls, respectively. For pregnant females, there was a trend for higher levels of
471 hepatic *vtgA* and *vtgB* mRNA expression in fish exposed to 3.4 ng/L EE2 compared to solvent
472 control fish (with 3.4-, 6.9- and 2-fold induction of *vtgA*, *vtgB* and *vtgC*, respectively) but not for
473 0.9 ng/L EE2. The physiological roles of the different types of VTG are still not clear and not only
474 do their relative amounts in the bloodstream and in oocytes vary between different species, but
475 also within a species at different times of the reproductive cycle (Reading et al., 2017; Sullivan
476 and Yilmaz, 2018). In some fish species, the synthesis of VtgC appears to be less dependent on,
477 and less sensitive to, E2 induction compared to VtgA and VtgB (Reading et al., 2017). The findings
478 in our study indicate this may also be the case for VtgC in *X. eiseni* (here for EE2). In the marine
479 eelpout, exposure to OP, E2 and EE2, during pregnancy have all been shown to induce synthesis
480 of VTG, with effective EE2 exposure water concentrations of 10 ng/L and above (Korsgaard et
481 al., 2002; Rasmussen et al., 2002).

482 Non-pregnant fish in the 0.9 ng/L EE2 treatment showed only a slight weight increase during
483 the exposure, whereas non-pregnant fish in all the other treatments showed considerable
484 weight gain. Similarly for the pregnant fish exposed to 0.9 ng/L EE2 there was only a relatively
485 small increase in condition factor contrasting with that for pregnant fish in the other treatments
486 where increases in condition over the study period were considerable. Major weight gain that
487 results in a higher condition factor is expected for pregnant individuals. The relatively lower
488 weight of the pregnant fish in the 0.9 ng/L EE2 treatment compared with all other treatments
489 was explained by the lower brood weight; broods of these fish weighed less than all the other
490 treatment broods. The brood sizes amongst these treatments did not differ and thus this
491 difference in brood weight was due to a lower average offspring weight. The breeding period

492 we adopted (where males were placed in tanks together with females) extended over ten days
493 before the exposures and it is possible that gestation in the fish transferred to the 0.9 ng/L EE2
494 tanks were on average less advanced than in the fish transferred to the other tanks. In that case,
495 broods could have been less developed, thus explaining their lower weight. This could explain
496 the differences in viability and survivorship, which, again, differed in this treatment compared
497 to all the other treatments. In the natural environment, size of offspring in fish can have a
498 significant bearing on their health and survivorship potential (Anderson, 1988; Sogard, 1997). In
499 other studies, growth responses to the effects of EE2 on early life stages (albeit *ex utero*), for
500 comparable exposure concentrations, are variable. In some cases growth has reported to be
501 enhanced (e.g. zebrafish; Fenske et al., 2005), whereas in other cases inhibitory effects have
502 been reported, for instance for Nile tilapia (*Oreochromis niloticus*) and European grayling
503 (*Thymallus thymallus*) (Marques da Cunha et al., 2019; Shved et al., 2008). In some cases,
504 exposure to EE2 has been shown not to affect growth (e.g. medaka; Cleary et al., 2019). It should
505 be emphasised, however, that the effect of EE2 on *X. eiseni* in our study was not seen at the
506 higher (3.4 ng/L) EE2 exposure concentration.
507
508 **4.3 Effects of maternal EE2 exposure on offspring**
509 At four weeks of age, offspring of fish exposed to 0.9 ng/L EE2 weighed less than the
510 offspring in all other treatments. They also had a smaller total body length and a lower body
511 condition, which, again, in the natural environment, could have a significant bearing on their
512 health and survivorship potential (Anderson, 1988; Sogard, 1997). It is not possible to separate
513 out whether the smaller size of the four week old offspring in this treatment group was a
514 function of their smaller size at birth or if it was a consequence of the maternally derived
515 exposure to 0.9 ng/L EE2. In the latter case, however, an even more marked effect might have
516 been anticipated on the offspring of the fish exposed to 3.4 ng/L EE2, and this was not seen to
517 be the case. In the literature, reported effects of *ex utero* exposure to environmentally relevant
518 EE2 concentrations on early life larval growth are variable. In the European grayling, for instance,

519 exposure of developing embryos to 1 ng/L EE2 resulted in a reduced larval growth after hatching
520 (Marques da Cunha et al., 2019), whereas the same concentration in mummichog (*Fundulus*
521 *heteroclitus*) resulted in longer larvae at hatch compared to controls (Peters et al., 2010). Both
522 species are oviparous fish, but effects of the same EE2 concentration differ which could be
523 explained by the different times it takes these species to hatch (weeks vs days). These examples
524 highlight the difficulty in comparing data derived from species with different developmental
525 times let alone with a completely different reproductive system (oviparity vs viviparity).

526 We found no effect of EE2, at either exposure concentration on sex ratio or status of gonadal
527 development of the offspring in *X. eiseni*. The absence of an effect on sex outcome in the broods
528 might indicate that EE2 did not cross the maternal-embryonal barrier, but without measured
529 levels in the ovarian fluid or offspring we cannot state whether this was the case or not. We
530 suggest, however, that uptake of EE2 into the larvae was likely based on its lipophilic nature and
531 small molecular size. This is supported also by the fact that the EDCs, OP and E2 have both been
532 shown to pass into developing embryos via the mother in the viviparous eelpout (Rasmussen et
533 al., 2002). Furthermore, although environmentally relevant concentrations of EE2 have been
534 shown to alter the sex ratio (in favour of females) in various oviparous fish species for exposures
535 during sexual differentiation, (e.g. Baumann et al., 2013; Lange et al., 2009), findings in
536 viviparous fish species are similar to those of our own. In the eelpout, for instance, exposure of
537 developing embryos to OP via the mother at concentrations of oestrogenic equivalency to those
538 for EE2 in our study (measured 14 and 65 µg/L OP equating to 0.7 and 3.2 ng/L EE2 respectively,
539 assuming a 20 000 fold lower estrogenic potency of OP compared to EE2; see Caldwell et al.,
540 2012; Li et al., 2012; White et al., 1994) found no effects on offspring sex ratio (Rasmussen et
541 al., 2002). Similarly in the guppy (*Poecilia reticulata*), exposure of developing embryos via the
542 mother to 26 µg/L OP (equivalent to 1.2 ng/L EE2), 0.85 µg/L E2 (equivalent to 42.5 ng/L EE2,
543 assuming EE2 is 20-times more potent as an estrogen compared with E2; Caldwell et al., 2012),
544 and EE2 at 2 and 20 ng/L, did not affect sex ratio in the offspring compared with controls
545 (Kinnberg et al., 2003; Volkova et al., 2012).

546 *X. eiseni* offspring from mothers exposed to EE2 in our study showed higher proportions of
547 deformities and developmental abnormalities compared to control fish. The observations of
548 offspring being less developed and/or deformed (mainly with a bent tail) following maternal
549 exposure to EE2 is consistent with previous studies on eelpout where exposure (via the mother)
550 to measured 324-363 ng, 133 ng or 53.6 ng/L E2 (equivalent to 16.2-18.2 ng, 6.65 ng, and 2.68
551 ng/L EE2, respectively) induced abnormal development and malformations in embryos
552 (Morthorst et al., 2016, 2014; Rasmussen et al., 2002). Exposure to E2, OP or EE2 can cause
553 depletion of calcium levels in the ovarian fluid of pregnant females (Korsgaard et al., 2002;
554 Rasmussen et al., 2002) and a plausible explanation for the morphological abnormalities in the
555 EE2 treatment is a reduced calcium provisioning to the developing larvae (via the ovarian fluid),
556 in turn impacting on growth and bone formation (Morthorst et al., 2014). Skeletal abnormalities
557 have also been reported for developmental (but *ex utero*) exposure to EE2 in the estuarine
558 mummichog, albeit at concentrations exceeding environmentally relevant concentrations (up to
559 10 µg/L; Boudreau et al., 2004). In this case it was established that these abnormalities were
560 caused by estrogenic deregulation of the ossification process.
561
562 **4.4 Conclusion**
563 In conclusion, responses of the adult female *X. eiseni* to EE2, as measured via the hepatic *vtg*
564 transcript levels, suggest this species may not be as responsive to oestrogenic compounds
565 compared with some other fish species - where hepatic *vtg* transcripts responses have been
566 shown to be up to 1000-fold higher for a similar oestrogen exposure regime (Rehberger et al.,
567 2020; Uren Webster et al., 2015; von Siebenthal et al., 2018). We also found no effects of
568 maternally derived exposure to environmentally relevant concentrations of EE2 on sex or sexual
569 development in the offspring. We do not know, however, how much EE2 passed from the
570 mother into the developing offspring or when the window of sensitivity for the programming of
571 sex determination takes place, and this may have occurred earlier in the gestation than when
572 the EE2 exposure was initiated. *X. eiseni* however, offers a new and highly tractable model for

studies into effects of maternally derived drugs and pollutants, with the advantages of rapid development, high fecundity, ease of maintaining the species and a relatively short assay length of time. Further work is required to assess the sensitivity of this species to different toxicants and to better establish toxicant maternal transfer mechanisms. In this model it is possible also to dissect out embryos from females early in the gestation process and maintain them (under appropriate condition) *in vitro*, which opens up possibilities for studies to both define the timing of gonadal differentiation and enable investigations into the effect of chemicals on the timing of gonadal differentiation and to precisely define windows of developmental susceptibility for chemical effects.

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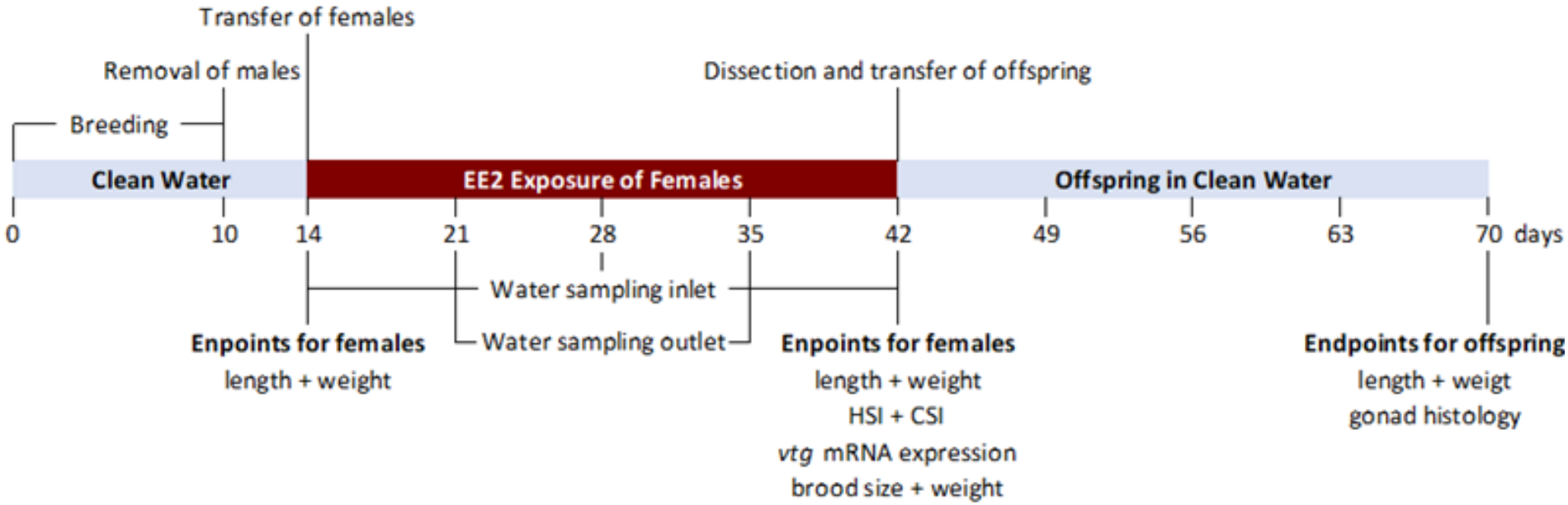


815

816 **Figure 1**: Overview of the experimental design and sampling timings. Key: HSI: hepato-somatic index; CSI: cardio-somatic index

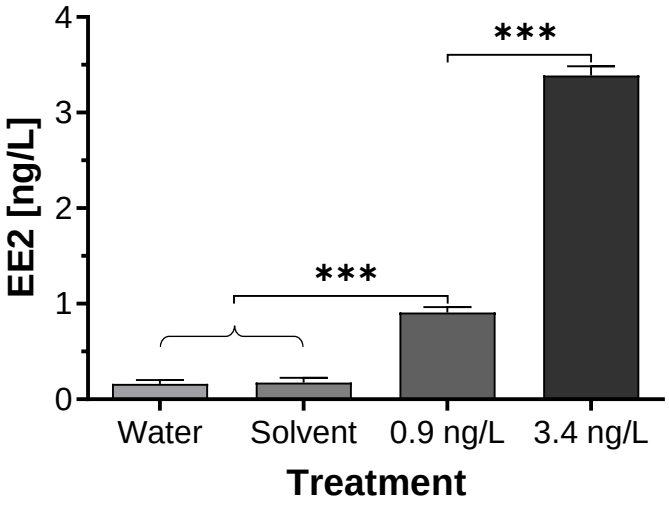
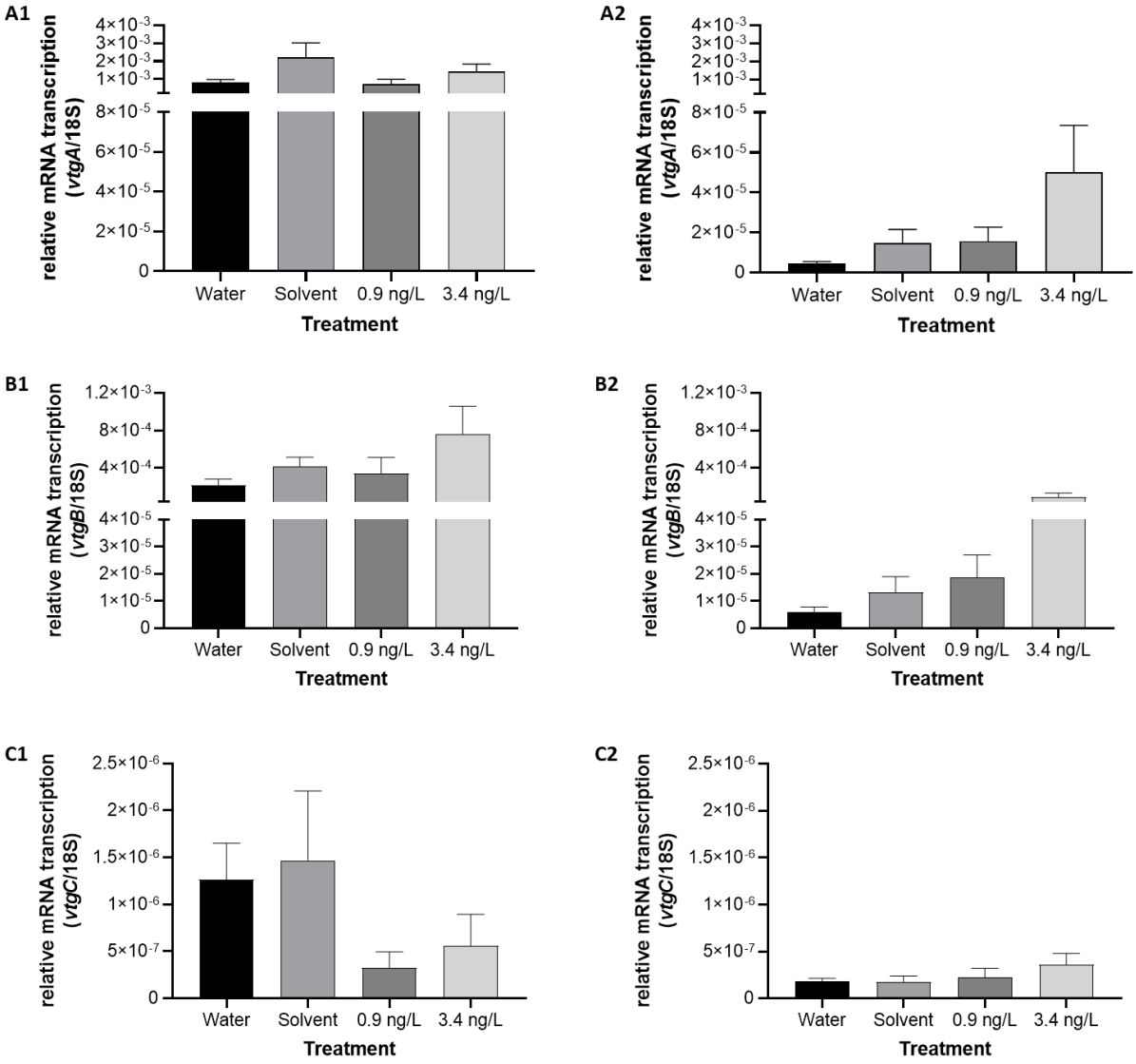


Figure 2: Water chemistry. Overall mean measured concentrations of EE2 in the exposure tanks, shown as mean + SEM. The mean measured exposure concentrations were 0.16 ± 0.08 ng/L EE2 (n = 12) for the water control, 0.07 ± 0.05 ng/L EE2 (n = 12) for solvent control, 0.9 ± 0.05 ng/L EE2 (n = 13) and 3.4 ± 0.1 ng/L EE2 (n = 14) for the 1 and 5 ng/L EE2 nominal concentrations, respectively. ******* = P ≤ 0.001. The measured concentrations of EE2 across sampling dates in exposure tanks are shown in Supplementary Figure S4.



827

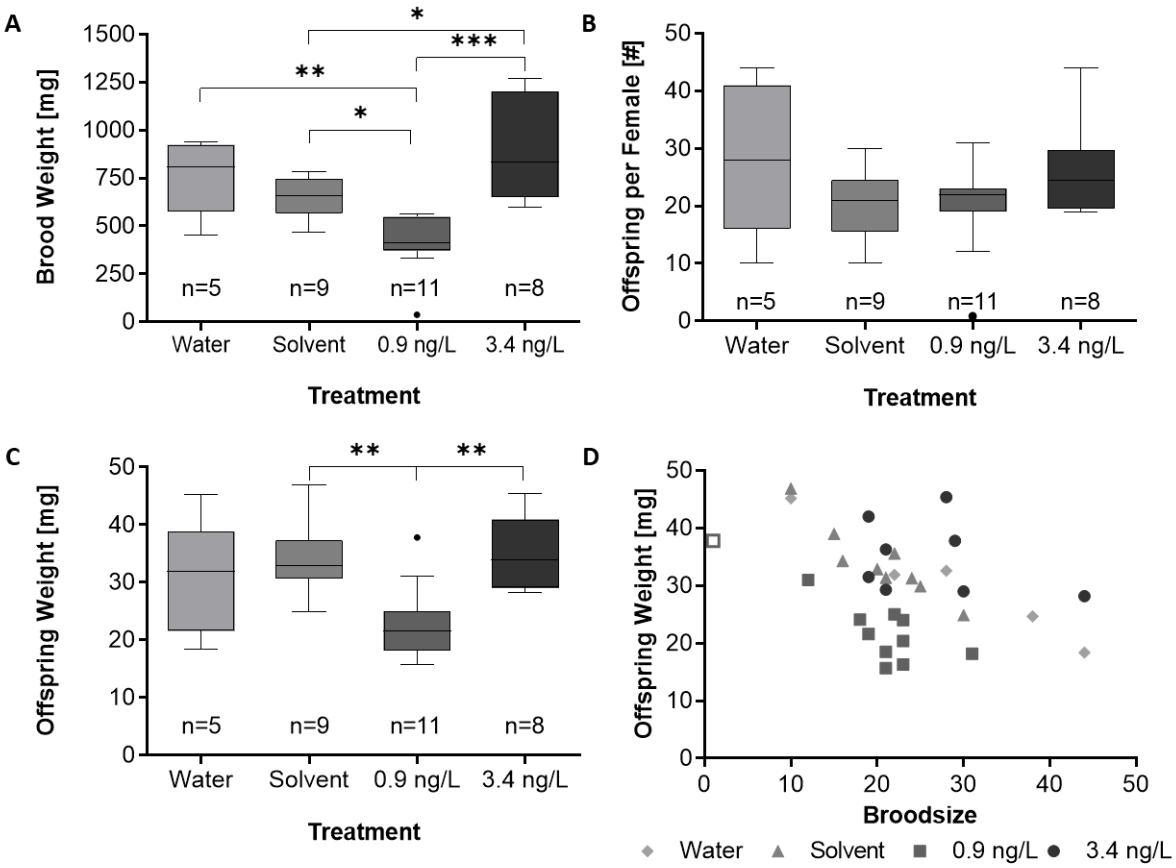
828 **Figure 3:** Hepatic relative transcript profiles (target gene transcription/*18S* transcription) of *vtgA*

829 (A), *vtgB* (B) and *vtgC* (C) in non-pregnant (A1, B1 & C1) and pregnant (A2, B2 & C2) *X. eiseni*

830 females exposed to EE2. Data are presented as mean ± SEM (n = 4-11). No statistically significant

831 differences were observed.

832



833

834 **Figure 4:** Offspring numbers and brood weights at birth. **A**: Total brood weight per female. **B**:
835 Total number of offspring per female. **C**: Average offspring weight calculated from brood weight
836 divided by offspring number. **D**: Correlation of offspring weight versus brood size. Offspring
837 numbers and weights are presented as box and whisker plots (min-max), where the box extends
838 from the 25th to 75th percentiles, the line in the middle of the box represents the median value
839 and whiskers represent the smallest and largest value. n = sample size (i.e. total number of
840 pregnant females per treatment), * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, ● = outlier.

841

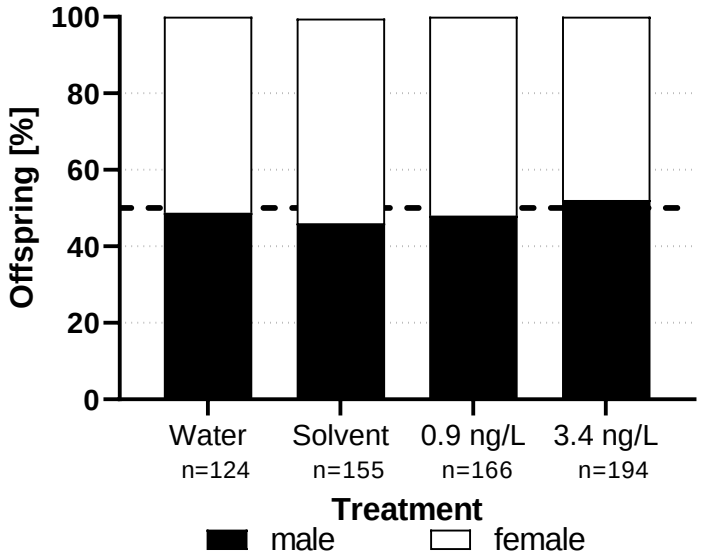


Figure 5: Sex ratio of offspring 4 weeks after birth, normalised to 100%. Overview on sex proportions for each treatment – water control: 49%:51% (m:f); solvent control: 46%:54%; 0.9 ng/L EE2 treatment: 48%:52%; 3.4 ng/L EE2 treatment: 52%:48%. There was no significant difference between treatments (χ^2 (3, n = 8) = 1.2845, p = 0.7328). n = sample size (total number of offspring per column); dashed line = 50% mark.

Tinguely et al_Highlights

- The viviparous redtail splitfin provides a novel freshwater ecotoxicological model.
- Effects are assessed of maternally derived EE2 on early developmental life stages.
- Non-pregnant females have higher *vtg* transcript levels compared to pregnant females.
- EE2 exposure appears to induce *vtg* transcript levels in pregnant females only.
- Sex ratio and gonadal development of offspring were unaffected by maternal exposure.

Tinguely et al_CRediT authorship contribution statement

Simone M. Tinguely: Conceptualization, Investigation, Methodology, Formal analysis, Visualization, Writing - original draft. **Arthur David:** Methodology, Investigation, Formal analysis, Writing - Review & Editing. **Anke Lange:** Methodology, Formal analysis, Writing - review & editing. **Charles R. Tyler:** Conceptualization, Supervision, Writing - review & editing.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Effects of maternal exposure to environmentally relevant concentrations of 17α-ethinyloestradiol in a live bearing freshwater fish, *Xenotoca eiseni* (Cyprinodontiformes, Goodeidae).

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Supplemental Material and Methods

Analysis of EE2 in Water Samples

Solid Phase extraction (SPE)

Within 24 hours, an internal standard of 10 ng EE2d4 was added to each 1 L stabilised water sample. All samples were then pre-filtered through glass wool and a filter paper (Whatman No. 1, Maidstone, UK) to remove particulates. Subsequently, the filtrates were loaded onto Oasis HLB (6 mL, 500 mg sorbent, 60 µm particle size) cartridges (Waters, Manchester, UK) pre-conditioned with 10 mL methanol and 10 mL distilled water. The maximum flow rate was set to 10 mL/min using a gentle vacuum. After the loading, the cartridges were washed with 10 mL distilled water, dried under vacuum and eluted with 10 mL methanol. The extracts were evaporated and reconstituted in 60 µL water/acetonitrile (7:3, v/v) and stored at -80 °C before analyses.

High-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS)

HPLC-MS/MS analyses were carried out using a Waters Alliance 2695 HPLC system coupled to a Quattro Premier triple quadrupole mass spectrometer from Micromass (Waters, Manchester, UK). Samples were separated using a reverse phase Kinetex C18 column (2.6 µm, 4.6 × 100 mm) (Phenomenex, Macclesfield, UK). The injection volume was 20 µL and mobile phase solvents were water (A) and acetonitrile (B) with 0.05% ammonium hydroxide in each. Separation was achieved at 20 °C using a flow rate of 0.25 mL/min with the following gradient: (A:B) 70:30 to 0:100 in 13 min; 0:100 for 5 min, then return to the initial condition at 19 min and equilibration for 6 min. Retention times were 13.05 min for EE2 and 13.01 min for EE2d4. Ionisation and fragmentation settings were as reported in Labadie et al. (2007). Mass calibration of the spectrometer was performed with sodium iodide. Data were acquired using MassLynx 4.1 and identification of analytes and surrogates was performed by comparing the retention times and the MS signals in the samples with those observed with standard solutions. Quantification was carried out by calculating the response factor of EE2 to EE2d4.

Concentrations were determined using a least-square linear regression analysis of the peak area ratio versus the concentration ratio of native to deuterated. A five-point calibration curve (R^2 > 0.99) covered the range from 50 to 2,000 pg (injected onto column) for EE2, which was within the linear range of the instrument.

Obtaining a partial X. eiseni 18S ribosomal RNA sequence

Using ClustalW (Thompson et al., 1994), the following partial 18s rRNA sequences from four Cyprinodontiformes were aligned: Eastern mosquitofish (*Gambusia holbrooki*; GenBank accession number FJ710842), mangrove killifish (*Kryptolebias marmoratus;* FJ438821), sheepshead minnow (*Cyprinodon variegatus*; EF431912) and mummichog (*Fundulus heteroclitus*; M91180). Based on the alignment (Supplementary Figure S2), primers were designed in conserved regions using the NCBI Primer-BLAST tool (Ye et al., 2012). Multiple primers (see Supplementary Table S1) were designed to gain overlapping sequence segments for the 18S sequence, from which a consensus sequence was established. Primers were purchased from Eurofins MWG Operon (Ebersberg, Germany).

PCR reactions were performed for all primer pairs using 20 µL reaction volumes containing 1 µL template cDNA, forward and reverse primers (0.2 µM each), dNTPs (0.2 mM each), 5xGoTaq reaction buffer (1.5 mM $MgCl_2$) and GoTaq Polymerase (0.5 u / 20 µL) (Promega,). An initial denaturation at 95 °C for 2 min was followed by 15 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 1 min and 20 cycles of denaturation at 95 °C for 30 s, annealing at 53 °C for 30 s, extension at 72 °C for 1 min. After the final extension at 72 °C for 5 min the reaction mix was allowed to soak at 4 °C for 30 min.

The PCR products were separated by agarose gel electrophoresis and DNA fragments of expected size (Table S1) purified using the NucleoSpin® Extract II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. The purified DNA was sequenced by Eurofins MWG Operon and verified by sequence alignment using BLASTn (Supplementary Figure S3).

Real-time Quantitative Polymerase Chain Reaction (RT-qPCR)

RT-qPCR assays were optimised first by optimising the annealing temperatures for each primer pair using cDNA on the following temperature-gradient program: 95 °C for 15 min followed by 40 cycles of 95 °C for 10 sec and annealing temperatures (Ta) for 20 sec, where Ta consisted of a temperature gradient between 55 °C and 62 °C. In order to validate the primer pairs, the detection range, the linearity and the amplification efficiency were determined using 5 dilutions of a 10-fold dilution series of cDNA that was run in triplicates under following qPCR conditions: 95 °C for 15 min followed by 40 cycles of 95 °C for 10 sec and Ta for 20 sec (where Ta were the optimised annealing temperatures) with a subsequent melt curve analysis to assess primer specificity. Standard curves were calculated by plotting the threshold cycle (C_t) against the logarithm of the cDNA dilution, whereby slopes of between -3.19 and -3.50 with linear correlations (R^2) of ≥ 0.997 were converted to efficiencies (*E*) of 1.93 to 2.06 (corresponding to 93.0 % - 106.0 %) with the following equation: $E = 10^{(-1/slope)}$ (Rasmussen, 2001). Details on primer sequences, Tas and efficiencies are provided in Supplementary Table S2.

Amplifications were performed in clear 96-well plates in triplicates using the optimised PCR conditions. In addition to a no reverse transcript control (NTC), a pooled cDNA sample was run on every plate to assess inter assay variability.

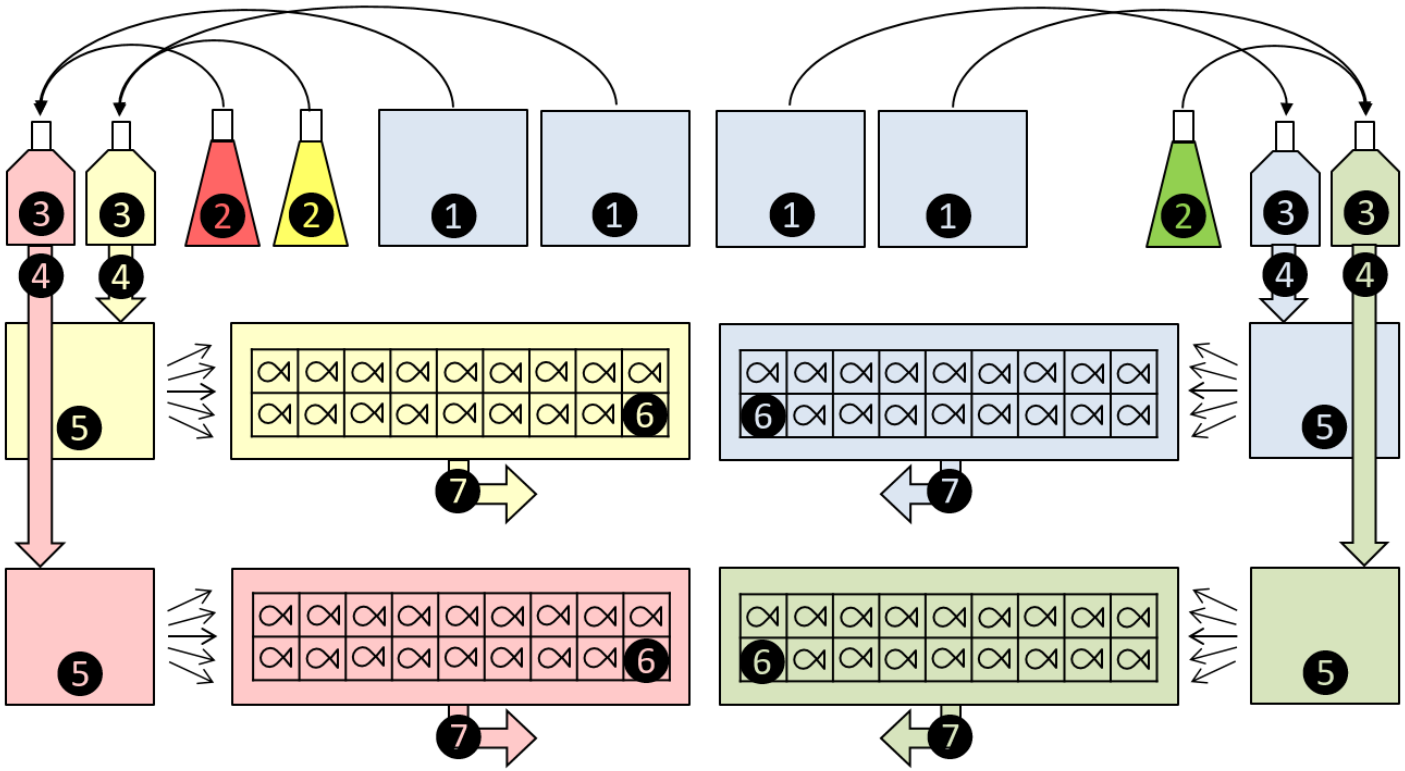

Figure S1: Schematic representation of the experimental setup. (1) Reservoir aquaria (30 L) with constant inflow of reconstituted water, (2) 5 µg/L (red) and 1 µg/L (yellow) EE2 nominal stock solutions as well as 0.01% ethanol (green) stock solution, (3) flasks with overflow (mixing one part stock solution with 1000 parts water; stock solution and water are pumped), (4) gravity feed into reservoir aquaria, (5) reservoir aquaria (30 L) with exposure water (nominal 1 ng/L EE2 and 5 ng/L EE2), solvent (nominal 0.00001% ethanol) and water control, (6) individual exposure aquaria (18 x 1 L per treatment) within a tray for the collection of overflowing water from the individual aquaria and (7) outlet to waste; pumps: (1→3) 240 L/d, (2→3) 240 mL/d, (5→6) 18 x 10 L/d. Water samples for chemistry were taken from (5) and (7).

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gi|126571066|gb|EF431912.1|      --TCTGGTTGATCCTGCCAGTAGCATATGCTTGTCTCAAAGATTAAGCCA 48
gi|213256|gb|M91180.1|FUNRG18S   --------------------AGCATATGCTTGTCTCAAAGATTAAGCCA 29
gi|224486514|gb|FJ710842.1|      ---------------------------------------------AAGCCA 6
                                                                        ******

gi|215261535|gb|FJ438821.1|      TGCACGTCTAAGTACACACGGCCGGTACAGTGAAACTGCGAATGGCTCAT 100
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                                 **** ** ******************   **********************
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gi|213256|gb|M91180.1|FUNRG18S   ----------------TCTGGGATGCGTGCATTTATCAGACCCAAAACCC 203
gi|224486514|gb|FJ710842.1|      CCTCGGGGCGGGGCCGCCGGGGATGCGTGCATTTATCAGACCCAAAACCC 206
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                                 * *****      *             **  * ******** ********

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                                 *************** * ***************    **********

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gi|213256|gb|M91180.1|FUNRG18S   TCATTCGAATGTCTGCCCTATCAACTTTCGATGGTACGCTACGTGCCTAC 343
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gi|224486514|gb|FJ710842.1|      CATGGTGACCACGGGTAACGGGGAATCAGGGTTCGATTCCGGAGAGGGAG 406
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gi|213256|gb|M91180.1|FUNRG18S   CCTGAGAAACGGCTACCACATCCAAGGAAGGCAGCAGGCGCGCAAATTAC 443
gi|224486514|gb|FJ710842.1|      CCTGAGAAACGGCTACCACATCCAAGGAAGGCAGCAGGCGCGCAAATTAC 456
                                 ********************************************************
                                          Cons_18S_F2 & R1

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                                                 Cons_18S_F3 & R2

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gi|213256|gb|M91180.1|FUNRG18S   CCGAAGCGTTTACTTTGAAAAAATTAGAGTGTTCAAAGCAGGCCCG-GTC 791
gi|224486514|gb|FJ710842.1|      TCGAAGCGTTTACTTTGAAAAAATTAGAGTGTTCAAAGCAGGCCCCCGTC 806
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gi|126571066|gb|EF431912.1|      GCCTGAATACCGCAGCTAGGAATAATGGAATAGGACTCCGGTTCTATTTT 865
gi|213256|gb|M91180.1|FUNRG18S   GCCTGAATACCGCAGCTAGGAATAATGGAATAGGACTCCGGTTCTATTTT 841
gi|224486514|gb|FJ710842.1|      GCCTGAATACCGCAGCTAGGAATAATGGAATAGGACTCCGGTTCTATTTT 856
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gi|215261535|gb|FJ438821.1|      GTGGGTCTTCC-----CTGAACTGGGGCCATGATTAAGAGGGACGGCCGG 905
gi|126571066|gb|EF431912.1|      GTGGG--TTCT-----CTGAACTGGGGCCATGATTAAGAGGGACGGCCGG 908
gi|213256|gb|M91180.1|FUNRG18S   GTGGGTTTTTCTC---CTGAACTGGGGCCATGATTAAGAGGGACGGCCGG 888
gi|224486514|gb|FJ710842.1|      GTGGGTTTTCTTCTCTCTGAACTGGGGCCATGATTAAGAGGGACGGCCGG 906
                                 *****  **       ***********************************

gi|215261535|gb|FJ438821.1|      GGGCATTCGTATTGTGCCGCTAGAGGTGAAATTCTTGGACCGGCGCAAGA 955
gi|126571066|gb|EF431912.1|      GGGCATTCGTATTGTGCCGCTAGAGGTGAAATTCTTGGACCGGCGCAAGA 958
gi|213256|gb|M91180.1|FUNRG18S   GGGCATTCGTATTGTGCCGCTAGAGGTGAAATTCTTGGACCGGCGCAAGA 938
gi|224486514|gb|FJ710842.1|      GGGCATTCGTATTGCGCCGCTAGAGGTGAAATTCTTGGACCGGCGCAAGA 956
                                 ************** ***********************************

gi|215261535|gb|FJ438821.1|      CGAACGAAAGCGAAAGCATTTGCCAAGAATGTTTTCATTAATCAAGAACG 1005
gi|126571066|gb|EF431912.1|      CGGACGAGAGCGAAAGCATTTGCCAAGAATGTTTTCATTAATCAAGAACG 1008
gi|213256|gb|M91180.1|FUNRG18S   CGGACGAAAGCGAAAGCATTTGCCAAGAATGTTTTCATTAATCAAGAGCG 988
gi|224486514|gb|FJ710842.1|      CGGACGAAAGCGAAAGCATTTGCCAAGAATGTTTTCATTAATCAAGAACG 1006
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gi|215261535|gb|FJ438821.1|      AAAGTCGGAGGTTCGAAGACGATCAGATACCGTCGTAGTTCCGACCATAA 1055
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gi|213256|gb|M91180.1|FUNRG18S   AAAGTCGGAGGTTCGAAGACGATCAGATACCGTCGTAGTTCCGACCATAA 1038
gi|224486514|gb|FJ710842.1|      AAAGTCGGAGGTTCGAAGACGATCAGATACCGTCGTAGTTCCGACCATAA 1056
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gi|215261535|gb|FJ438821.1|      ACGATGCCAACTAGCGATCCGGCGGCGTTATTCCCATGACCCGCCGGGCA 1105
gi|126571066|gb|EF431912.1|      ACGATGCCAACTAGCGATCCGGCGGCGTTATTCCCATGACCCGCCGGGCA 1108
gi|213256|gb|M91180.1|FUNRG18S   ACGATGCCAACTAGCGATCCGGCGGCGTTATTCCCATGACCCGCCGGGCA 1088
gi|224486514|gb|FJ710842.1|      ACGATGCCGACTAGCGATCCGGCGGCGTTATTCCCATGACCCGCCGGGCA 1106
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gi|126571066|gb|EF431912.1|      GCGTCCGGGAAACCAAAGTCTTTGGGTTCCGGGGGGAGTATGGTTGCAAA 1158
gi|213256|gb|M91180.1|FUNRG18S   GCGTCCGGGAAACCAAAGTCTTTGGGTTCCGGGGGGAGTATGGTTGCAAA 1138
gi|224486514|gb|FJ710842.1|      GCGTCCGGGAAACCAAAGTCTTTGGGTTCCGGGGGGAGTATGGTTGCAAA 1156
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gi|215261535|gb|FJ438821.1|      GCTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGTGGAGCCTG 1205
gi|126571066|gb|EF431912.1|      GCTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGTGGAGCCTG 1208
gi|213256|gb|M91180.1|FUNRG18S   GCTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGTGGAGCCTG 1188
gi|224486514|gb|FJ710842.1|      GCTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGTGGAGCCTG 1206
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gi|215261535|gb|FJ438821.1|      CGGCTTAATTTGACTCAACACGGGAAACCTCACCCGGCCCGGACACGGAA 1255
gi|126571066|gb|EF431912.1|      CGGCTTAATTTGACTCAACACGGGAAACCTCACCCGGCCCGGACACGGAA 1258
gi|213256|gb|M91180.1|FUNRG18S   CGGCTTAATTTGACTCAACACGGGAAACCTCACCCGGCCCGGACACGGAA 1238
gi|224486514|gb|FJ710842.1|      CGGCTTAATTTGACTCAACACGGGAAACCTCACCCGGCCCGGACACGGAA 1256
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                          Cons_18S_F4 & R3
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gi|213256|gb|M91180.1|FUNRG18S   AGGATTGACAGATTGATGGCTCTTTCTCGATTCTGTGGGTGGTGGTGCAT 1288
gi|224486514|gb|FJ710842.1|      AGGATTGACAGATTGATAGCTCTTTCTCGATTCTGTGGGTGGTGGTGCAT 1306
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gi|215261535|gb|FJ438821.1|      GGCCGTTCTTAGTTGGTGGAGCGATTTGTCTGGTTAATTCCGATAACGAA 1355
gi|126571066|gb|EF431912.1|      GGCCGTTCTTAGTTGGTGGAGCGATTTGTCTGGTTAATTCCGATAACGAA 1358
gi|213256|gb|M91180.1|FUNRG18S   GGCCGTTCTTAGTTGGTGGAGCGATTTGTCTGGTTAATTCCGATAACGAA 1338
gi|224486514|gb|FJ710842.1|      GGCCGTTCTTAGTTGGTGGAGCGATTTGTCTGGTTAATTCCGATAACGAA 1356
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gi|215261535|gb|FJ438821.1|      CGAGACTCCGACATGCTAACTAGTTACGCGGCCCC-GTGCGGTCGGCGTT 1404
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gi|213256|gb|M91180.1|FUNRG18S   CGAGACTCCGGCATGCTAACTAGTTACGCGGCCCCCGTGCGGTCGGCGGC 1388
gi|224486514|gb|FJ710842.1|      CGAGACTCCGGCATGCTAACTAGTTACGCGGCCCCCGTGCGGTCGGCGTC 1406
                                 ********** ************************ ************

gi|215261535|gb|FJ438821.1|      --CAACTTCTTAGAGGGACAAGTGGCGTTCAGCCACACGAGATTGAGCAA 1452
gi|126571066|gb|EF431912.1|      --CAACTTCTTAGAGGGACAAGTGGCGTTCAGCCACACGAGATTGAGCAA 1455
gi|213256|gb|M91180.1|FUNRG18S   GGTAACTTCTTAGAGGGACAAGTGGCGTTCAGCCACACGAGATTGAGCAA 1438
gi|224486514|gb|FJ710842.1|      --CAACTTCTTAGAGGGACAAGTGGCGTTCAGCCACACGAGATTGAGCAA 1454
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gi|215261535|gb|FJ438821.1|      TAACAGGTCTGTGATGCCCTTAGATGTCCGGGGCTGCACGCGCGCCACAC 1502
gi|126571066|gb|EF431912.1|      TAACAGGTCTGTGATGCCCTTAGATGTCCGGGGCTGCACGCGCGCCACAC 1505
gi|213256|gb|M91180.1|FUNRG18S   TAACAGGTCTGTGATGCCCTTAGATGTCCGGGGCTGCACGCGCGCCACAC 1488
gi|224486514|gb|FJ710842.1|      TAACAGGTCTGTGATGCCCTTAGATGTCCGGGGCTGCACGCGCGCCACAC 1504
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gi|215261535|gb|FJ438821.1|      TGAGTGGATCAGCGTGTGTCTACCCTTCGCCGAAAGGCGCGGGTAACCCG 1552
gi|126571066|gb|EF431912.1|      TGAGTGGATCAGCGTGTGTCTACCCTTCGCCGAGAGGCGTGGGTAACCCG 1555
gi|213256|gb|M91180.1|FUNRG18S   TGAGTGGATCAGCGTGTGTCTACCCTTCGCCGAGAGGTGTGGGTAACCCG 1538
gi|224486514|gb|FJ710842.1|      TGAGTGGATCAGCGTGTGTCTACCCTTCGCCGAGAGGCGCGGGTAACCCG 1554
                                 ********************************** *** * **********
                                       Cons_18S_R4

gi|215261535|gb|FJ438821.1|      TTGAACCCCACTCGTGATGGGGATTGGGGATTGCAATTATTCCCCATGAA 1602
gi|126571066|gb|EF431912.1|      CTGAACCCCACTCGTGATAGGGATTGGGGATTGCAATTATTTCCCATGAA 1605
gi|213256|gb|M91180.1|FUNRG18S   CTGAACCCCACTCGTGACAGGGATTGGGGGTTGCAATTATTCCCCATCAA 1588
gi|224486514|gb|FJ710842.1|      CTGAACCCCACTCGTGATAGGGATTGGGGATTGCAATTGTTTCCCATCAA 1604
                                  ****************  ********** ******** ** ***** **

gi|215261535|gb|FJ438821.1|      CGAGGAATTCCCAGTAAGCGCGGGTCATAAGCTCGCGTTGATTAAGTCCC 1652
gi|126571066|gb|EF431912.1|      CGAGGAATTCCCAGTAAGCGCGGGTCATAAGCTCGCGTTGATTAAGTCCC 1655
gi|213256|gb|M91180.1|FUNRG18S   CGAGGAATTCCCAGTAAGCGCGGGTCATAAGCTCGCGTTGATTAAGTCCC 1638
gi|224486514|gb|FJ710842.1|      CGAGGAATTCCCAGTAAGCGCGGGTCACAAGCTCGCGTTGATTAAGTCCC 1654
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gi|215261535|gb|FJ438821.1|      TGCCCTTTGTACACACCGCCCGTCGCTACTACCGATTGGATGGTTTAGTG 1702
gi|126571066|gb|EF431912.1|      TGCCCTTTGTACACACCGCCCGTCGCTACTACCGATTGGATGGTTTAGTG 1705
gi|213256|gb|M91180.1|FUNRG18S   TGCCCTTTGTACACACCGCCCGTCGCTACTACCGATTGGATGGTTTGGTC 1688
gi|224486514|gb|FJ710842.1|      TGCCCTTTGTACACACCGCCCGTCGCTACTACCGATTGGATGGTTTAGTG 1704
                                 ********************************************** **

gi|215261535|gb|FJ438821.1|      AGGTCCTCGGATCGGCCCCGCT--GGGGTCGGCCACGGCCCCGGCGGAGC 1750
gi|126571066|gb|EF431912.1|      AGGTCCTCGGATCGGCCCCGCC--GGGGTCGGCAACGGCCCTCGCGGAGC 1753
gi|213256|gb|M91180.1|FUNRG18S   AGGTCCTCGGATCGGCCCTGCC--GGTGTCGGTCACGGCCCTGGCGGAGC 1736
gi|224486514|gb|FJ710842.1|      AGGTCCTCGGATCGGCCCCGCCCGGGGGTCGGCAACGGCCCTGGCGGAGC 1754
                                 ****************** **   ** *****  *******  *******

gi|215261535|gb|FJ438821.1|      GCCGAGAAGACGATCAAACTTGACTATCTAGAGGAAGTAAAAGTCGTAAC 1800
gi|126571066|gb|EF431912.1|      GCCGAGAAGACGATCAAACTTGACTATCTAGAGGAAGTAAAAGTCGTAAC 1803
gi|213256|gb|M91180.1|FUNRG18S   GCCGAGAAGGCGATCGAACTTGACTGTCTAGAGGAAGTAAAAGTCGTAAC 1786
gi|224486514|gb|FJ710842.1|      GCCGAGAAGACGATCAAACTTGACTATCTAGAGGAAGTAAAAGT------ 1798
                                 ********* ***** ********* ******************

gi|215261535|gb|FJ438821.1|      AAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTA 1836
gi|126571066|gb|EF431912.1|      AAGGTTTCCGTAGGTGAACCTGCAGA---------- 1829
gi|213256|gb|M91180.1|FUNRG18S   AAGGTTTTC-------------------------- 1795
gi|224486514|gb|FJ710842.1|      ------------------------------------
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Figure S2: Sequence alignment of 18S sequences from four Cyprinodontiformes species; *Gambusia holbrooki* (GenBank accession number FJ710842), *Kryptolebias marmoratus* (FJ438821), *Cyprinodon variegatus* (EF431912) and *Fundulus heteroclitus* (M91180). Based on this alignment, PCR primers (indicated by boxes; for details see Table S1) were designed in order to obtain a partial 18S sequence for *X. eiseni*. Multiple primers (F1-F4; sense and R1-R4; antisense) were designed to gain overlapping sequence segments for 18S sequences genes. From the obtained and sequenced PCR products, a consensus sequence was established, and *X. eiseni* RT-qPCR primers designed.

Table S1: Overview of primers used to establish a partial 18S sequence for *X. eiseni*.

Primer	Primer sequence (5'-3')	Position *	Expected size (bp)
Cons_18S_F1	CACACGGSCGGTACAGTGAA **	21-40	
Cons_18S_R1	CCTTGGATGTGGTAGCCGTT	414-433	413
Cons_18S_F2	AACGGCTACCACATCCAAGG	414-433	
Cons_18S_R2	CAGCTAAGAGCATCGAGGGG	721-740	327
Cons_18S_F3	CCCCTCGATGCTCTTAGCTG	721-740	
Cons_18S_R3	CAAATTAAGCCGCAGGCTCC	1199-1218	498
Cons_18S_F4	GGAGCCTGCGGCTTAATTTG	1199-1218	
Cons_18S_R4	GACACACGCTGATCCACTCA	1505-1524	326
Further primer combinations tested			
Cons_18S_F1			
Cons_18S_R2			720
Cons_18S_F2			
Cons_18S_R3			805
Cons_18S_F3			
Cons_18S_R4			804

* Positions refer to the *Gambusia holbrooki* sequence (GenBank accession number FJ710842) in the sequence alignment shown in Figure S2. ** Degenerate primer where S denotes a mixture of G or C in the primer. Please note, primers F3, F4, R3 and R4 did not yield any PCR products for *X. eiseni* when tested under the described PCR condidtions.

Xenotoca eiseni 18S mRNA, partial sequence
ATCAGTTATGGTTCCTTTGATCGCTCTACCGTTACTTGGATAACTGTGGCAATTCTAGAGCTAATACA
TGCAAACGAGCGCTGACCTTCGGGGATGCGTGCATTTATCAGACCCAAAACCCATGCGGGGTGCTCCC
CGTGGGCGCCCCGGCCGCTTTGGTGACTCTAGATAACCTCGAGCCGATCGCTGGCCCTCCGTGGCGGC
GACGTCTCATTCGAATGTCTGCCCTATCAACTTTCGATGGTACGCTACGTGCCTACCATGGTGACCAC
GGGTAACGGGGAATCAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAGGAAG
GCAGCAGGCGCGCAAATTACCCACTCCCGACTCGGGGAGGTAGTGACGAAAAATAACAATACAGGACT
CTTTCGAGGCCCTGTAATTGGAATGAGTACACTTTAAATCCTTTAACGAGGATCCATTGGAGGGCAAG
TCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATCTTAAAGTTGCTGCAGTTAAAAAGC
TCGTAGTTGGATCTCGGGATCGAGCTGACGGTCCGCCGCGAGGCGAGCTACCGTCGCC

Figure S3: Partial sequence of 18S mRNA of *Xenotoca eiseni*. This 602 bp sequence was submitted to NCBI GenBank (Accession number: MT747184).

Table S2: Overview of RT-qPCR parameters

Gene	Accession no	Primer direction	Primer sequence (5'-3')	Position	Product size	Ta (°C)	Efficiency (%)
18S	MT747184	sense	TGTCTGCCCTATCAACTTTCG	220	114	61.5	93.0
		antisense	GGATGTGGTAGCCGTTTCTC	333			
vtgA	EU761161	sense	GAGATGGAGGTTAAGGTTGGAG	3,112	146	61.0	95.0
		antisense	GAAGATGAGGAGCGGTTGC	3,257			
vtgB	EU761162	sense	CCTGATGTCGGGTTACTCTTTG	2,316	132	61.0	99.8
		antisense	ATGGATGCGGCTGTCACG	2,447			
vtgC	EU761163	sense	ACCCTCACAGCGTTCAG	1,583	122	60.5	106.0
		antisense	GGACACAAGAGCCATCG	1,704			

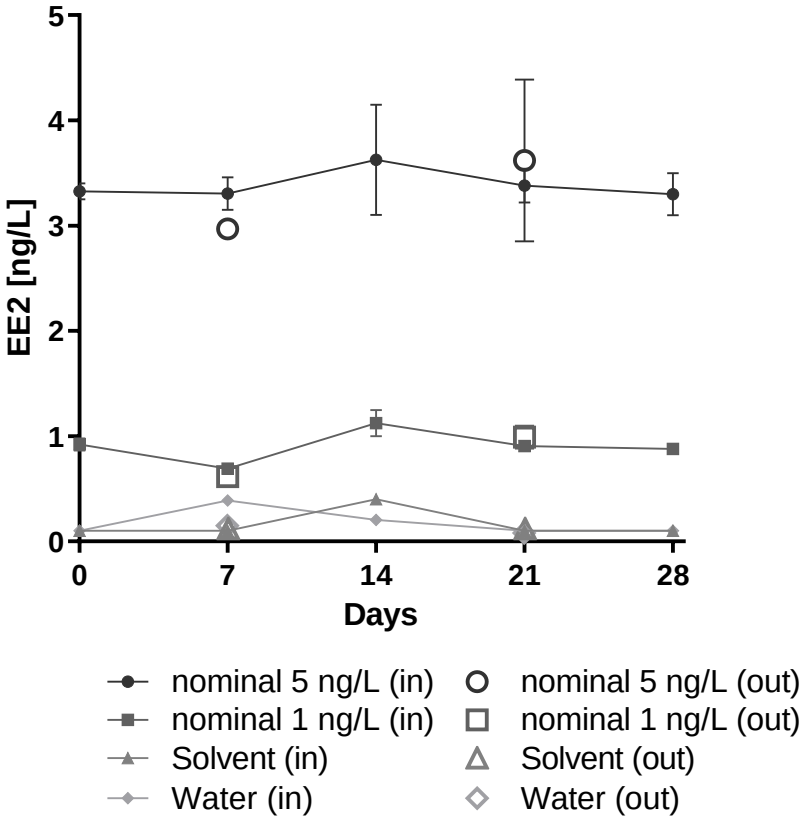

Figure S4: Water chemistry. Measured concentrations of EE2 across sampling dates in exposure tanks, shown as mean of duplicate measurements ± SD.

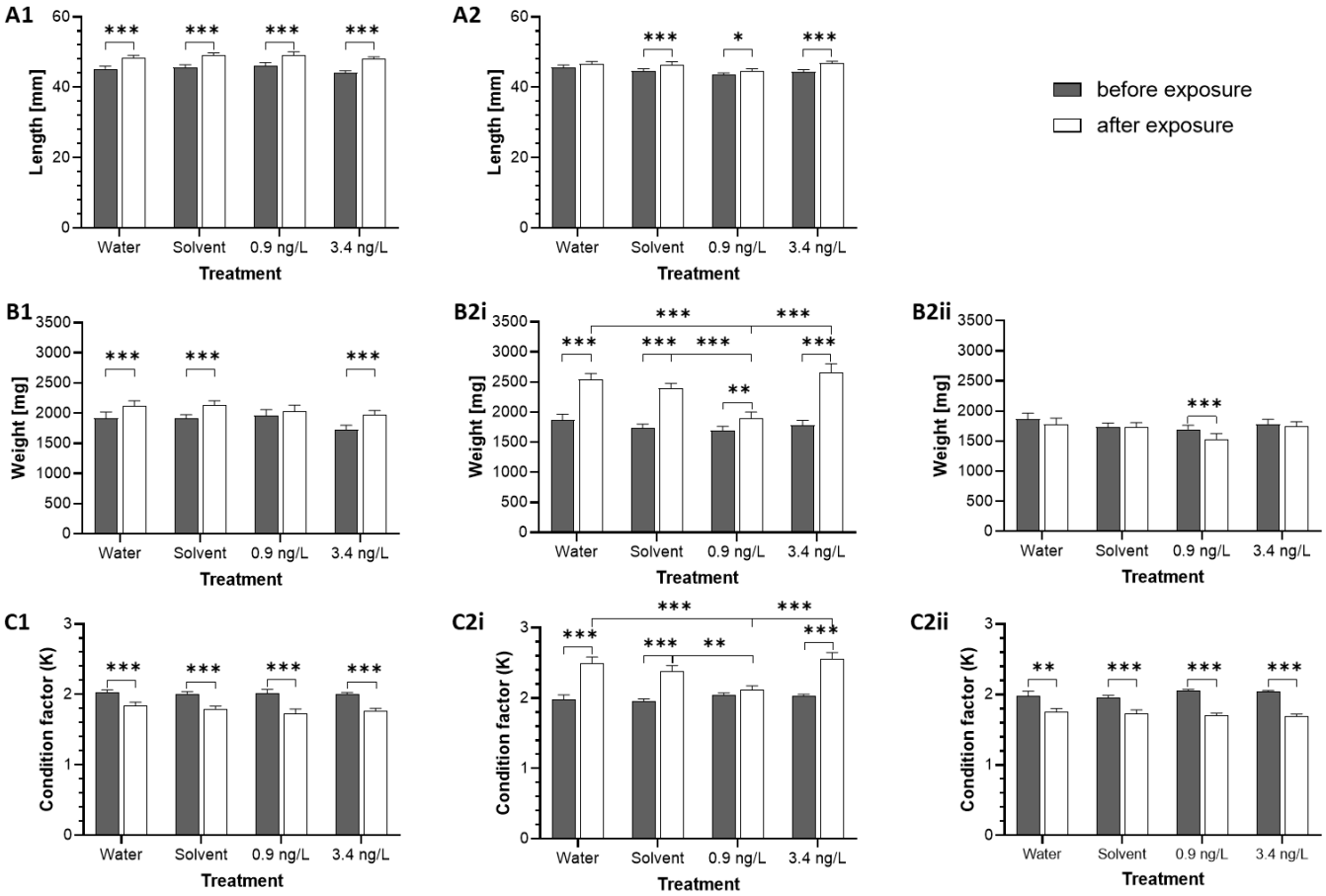

Figure S5: Growth and condition factor (K) of non-pregnant (A1, B1, C1; n = 6-13) and pregnant females (A2, B2, C2) at the start (grey bars) and end (white bars) of the exposure. For pregnant fish, weight and K are presented including (B2i & C2i; n = 5-12) and excluding the brood weight (B2ii & C2ii; n = 5-11). Data are presented as mean ± SEM; * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

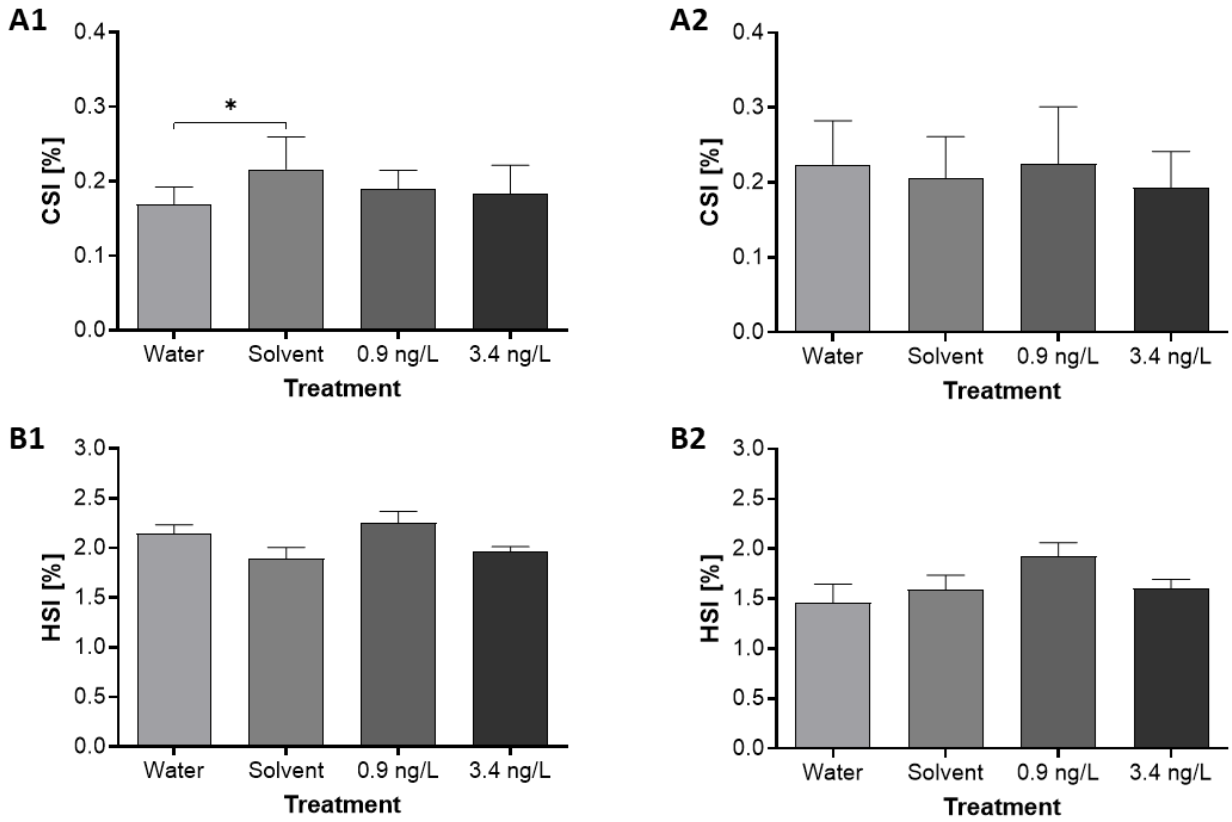

Figure S6: Cardio- (CSI) and hepato-somatic (HSI) indices of non-pregnant (A1 & B1; n = 6-13) and pregnant females (A2 & B2; n = 5-12) after exposure to EE2. Data are presented as mean ± SEM; * $P \leq 0.05$.

Table S3: Overview of pregnant females and offspring numbers for all treatments.

Treatment	Pregnant Females	Total offspring	Born dead	Dead within 24 h	Transferred to clean water	Dead within first week	Examined for sex ratio	Examined for growth and sexual development
Water	5	142	3	5	134	5	129	124
Solvent	9	183	2	7	174	4	170	155
0.9 ng/L EE2	11	214	16	17	181	11	170	166
3.4 ng/L EE2	8	211	1	11	199	5	194	194
Total	**33**	**750**	**22**	**40**	**688**	**25**	**663**	**639**

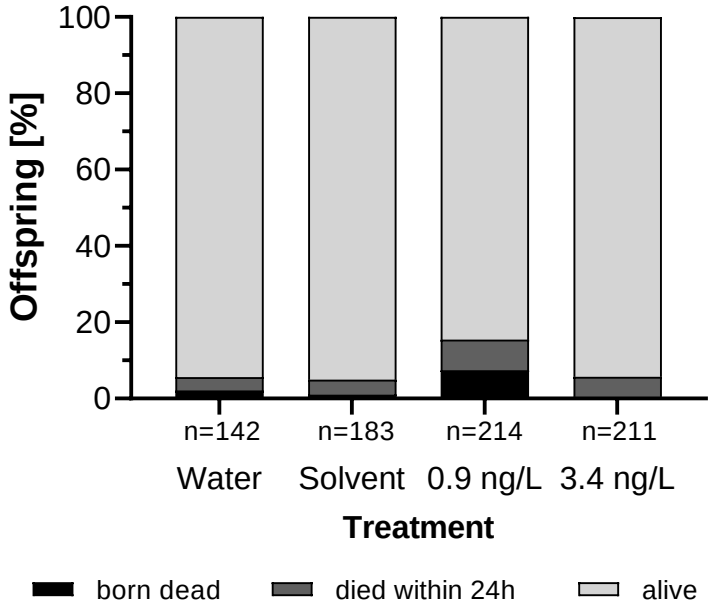

Figure S7: Viability and survivorship of the offspring within the first 24 hours normalised to 100%. n = total number of offspring per treatment. See also Supplementary Table S3.

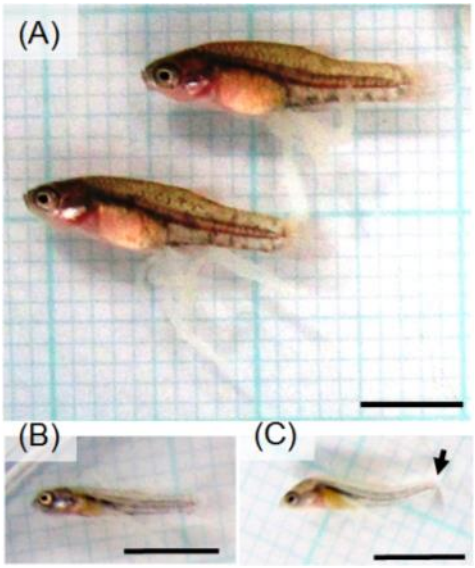

Figure S8: Images of offspring within 24 h after birth. **A**: Normal sized fish. **B**: Less developed fish. **C**: Less developed fish with bent tail (arrow). Bars: 5 mm.

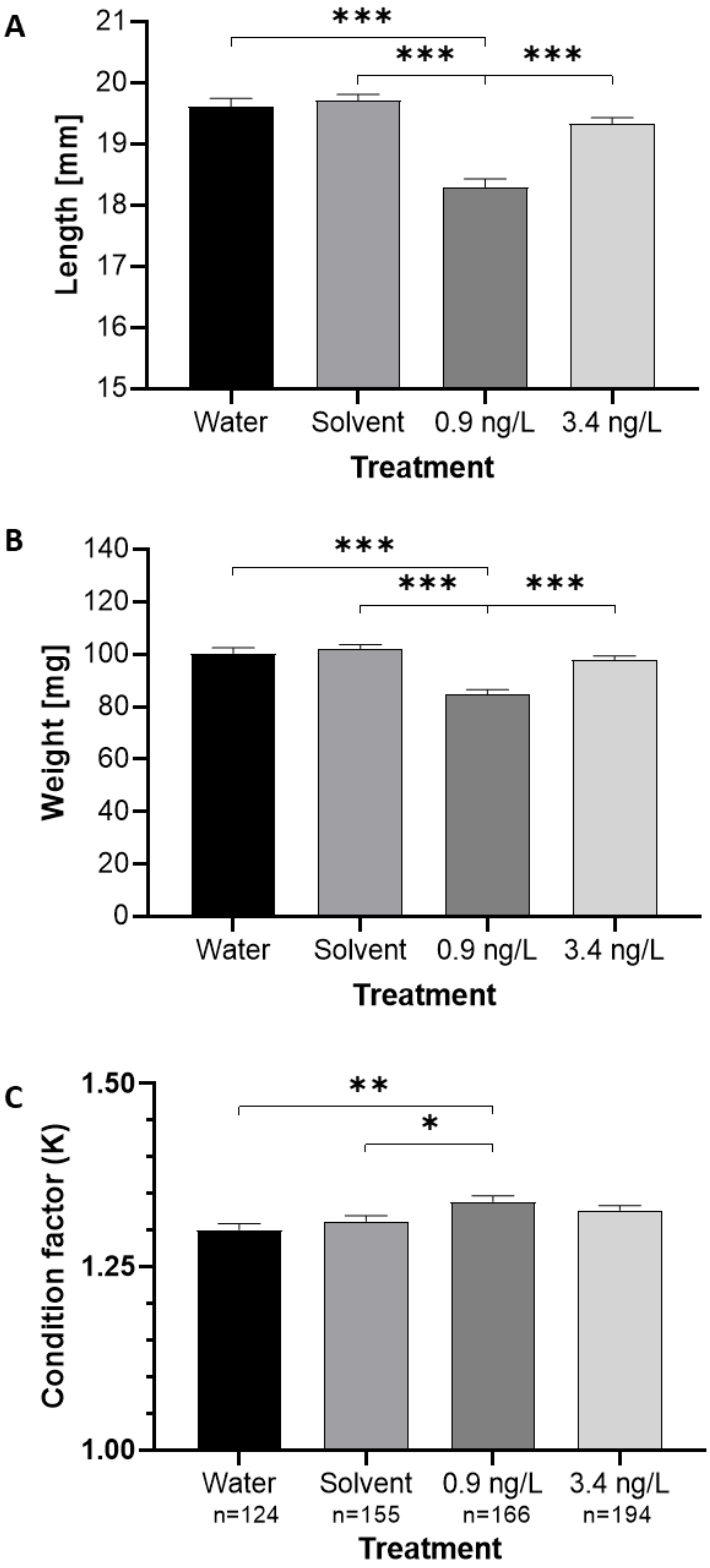

Figure S9: Condition of offspring 4 weeks after birth. **A**: Total body length. **B**: Weight. **C**: Condition factor (K). Data are presented as mean ± SEM; sample sizes (n) for all endpoints are given in Figure 9C; * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. To allow visibility of error bars for length and condition factor, the y-axis is starting at 15 mm and 1, respectively rather than 0.

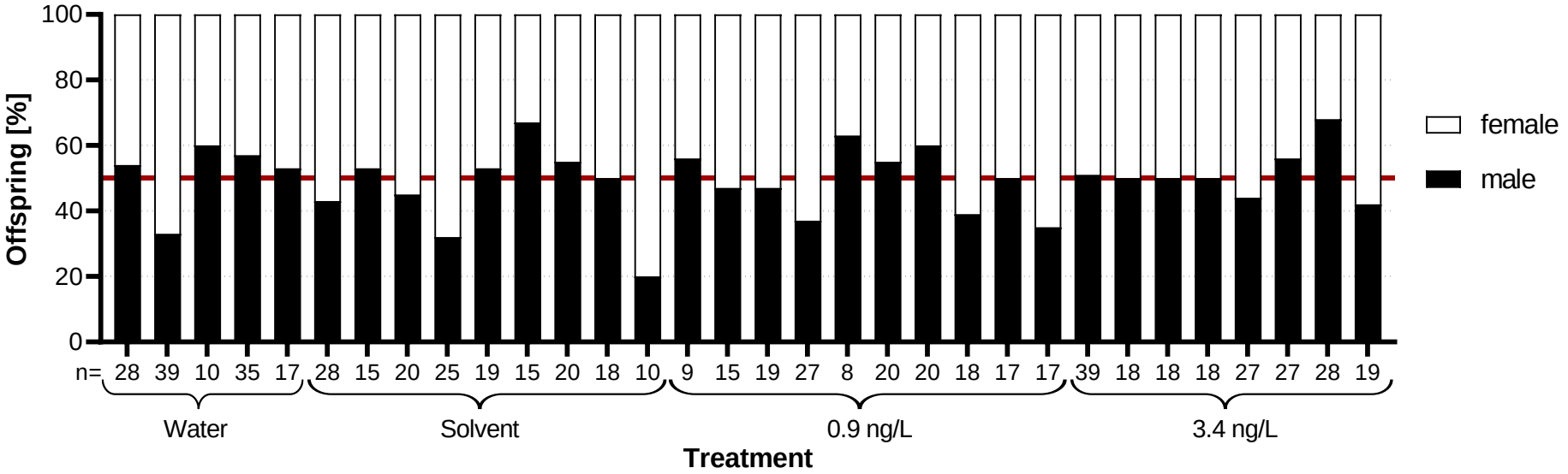

Figure S10: Sex ratio of offspring 4 weeks after birth, separated for each female and normalised to 100%. n = total number of offspring per column; red line = 50%

Detailed information for the sex ratio of each brood is provided in Supplementary Table S4.

Table S4: Sex ratios of offspring 4 weeks after birth for each brood.

Treatment		Brood 1	Brood 2	Brood 3	Brood 4	Brood 5	Brood 6	Brood 7	Brood 8	Brood 9	Brood 10
Water	males	15	13	6	20	9					
	females	13	26	4	15	8					
	total	28	39	10	35	17					
Solvent	males	12	8	9	8	10	10	11	9	2	
	females	16	7	11	17	9	5	9	9	8	
	total	28	15	20	25	19	15	20	18	10	
0.9 ng/L EE2	males	5	7	9	10	5	11	12	7	9	6
	females	4	8	10	17	3	9	8	11	8	11
	total	9	15	19	27	8	20	20	18	17	17
3.4 ng/L EE2	males	20	9	9	9	12	15	19	8		
	females	19	9	9	9	15	12	9	11		
	total	39	18	18	18	27	27	28	19		

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