Neuroendocrinology Letters Volume 43 No. 5 2022
ISSN: 0172-780X; ISSN-L: 0172-780X; Electronic/Online ISSN: 2354-4716
Web of Knowledge / Web of Science: Neuroendocrinol Lett
Pub Med / Medline: Neuro Endocrinol Lett

ORIGINAL ARTICLE

Use of all-male triploid population of zebrafish (*Danio rerio*) for evaluation of 17α-ethinylestradiol effects

Zuzana Weiserova[1], Viola Zentrichova[2], Denisa Medkova[1,4], Jana Blahova[1], Ales Franc[3], Michaela Garajova[3], Jan Mares[4], Eva Postulkova[4], Roman Franek[5], Martin Psenicka[5], Petr Marsalek[1], Zdenka Svobodova[1,5]

1 Department of Animal Protection and Welfare and Veterinary Public Health, University of Veterinary Sciences Brno, Czech Republic
2 Department of Animal Breeding, Animal Nutrition and Biochemistry, University of Veterinary Sciences Brno, Czech Republic
3 Department of Pharmaceutical Technology, Masaryk University, Brno, Czech Republic
4 Department of Zoology, Fisheries, Hydrobiology and Apiculture, Faculty of Agrosciences, Mendel University Brno, Czech Republic
5 South Bohemian Research Centre of Aquaculture and Biodiversity of Hydrocenoses, Faculty of Fisheries and Protection of Waters, University of South Bohemia in Ceske Budejovice, Vodnany, Czech Republic

Correspondence to: Zuzana Weiserová, MVDr.
Department of Animal Protection and Welfare and Veterinary Public Health, University of Veterinary Sciences Brno, Palackeho tr. 1946/1, 612 42 Brno, Czech Republic
TEL: +420 774 223 589, E-MAIL: KoutkovaZuzana@gmail.com

Submitted: 2022-07-04 Accepted: 2022-10-06 Published online: 2022-10-06

Key words: **EE2; feed contamination; oral intake; vitellogenin; xenoestrogen testing**

Neuroendocrinol Lett 2022; **43**(5):281–290 **PMID:** 36584404 NEL430522A07

Abstract

OBJECTIVES: We tested the toxicity of ethinylestradiol, a semisynthetic estrogen used in oral contraceptives, on all-male triploid zebrafish using commercial feeds and three different doses concentrations. We aimed to determine whether ethinylestradiol peroral administration resulted in vitellogenin production and whether all-male triploid zebrafish could serve as a model species for xenoestrogen testing.
METHODS: The actual concentrations of 17α-ethinylestradiol were 0.0035 (low); 0.0315 (medium) and 0.365 (high) μg/g. Positive control represented commercial feeds containing 0.0465 μg/g of β-estradiol. The experiment lasted 8 weeks.
RESULTS: Our results indicate that 17α-ethinylestradiol consumption does induce vitellogenin production in triploid zebrafish.
CONCLUSIONS: The simple presence of vitellogenin is a definite symptom indicative of the potential for such changes due to the action of estrogenic substances. As such, this experiment has shown that the use of all-male triploid zebrafish populations, rather than the mixed-sex populations of other species previously used, could serve as a suitable alternative model population for controlled testing of the effects of xenoestrogens on fish.

Abbreviations:

α	- angle of response
CH	- charge of particles (nC)
CI	- compressibility index
D_{10}	- % diameter of particles smaller than 10 μm
D_{50}	- % diameter of particles smaller than 50 μm
D_{90}	- % diameter of particles smaller than 90 μm
DB	- density bulk (g/ml)
DP	- density pycnometric (g/ml)
DT	- density tapped (g/ml)
DNA	- deoxyribonucleic acid
EE2	- 17α-ethinylestradiol
ELISA	- enzyme-linked immune sorbent assay
GM75	- Gemma micro 75
GM150	- Gemma micro 150
HR	- Hausner ratio
LC/MS	- liquid chromatography tandem mass spectrometry
M	- moisture content (%)
n.d.	- not detected
Ph. Eur.a	- content uniformity requirement according to the European Pharmacopoeia, article 2.9.6.
Pmax	- maximal deviation of individual sample from average (%)
RSD	- relative standard deviation
x_i	- average of content from 10 samples of each particular batch (%)

INTRODUCTION

Numerous chemical pollutants in the aquatic environment are suspected or known for their ability to affect the hormonal system of aquatic organisms. Many substances are used in human or veterinary medicine and enter surface waters through various pathways, especially municipal wastewaters (Sauer *et al.* 2018; Wang *et al.* 2018; Petrovici *et al.* 2020; Cerveny *et al.* 2021; Skocovska *et al.* 2021).

Ethinylestradiol (EE2) is a semisynthetic estrogen used in combined oral contraceptives or as a medical therapy for multiple diseases (National Center for Biotechnology Information, 2022). This substance attaches to estrogen receptors and has a more decisive action than natural β-estradiol (Piferrer & Donaldson, 1992). As EE2 resists degradation in the liver and is excreted via urine, EE2 and its derivatives can be detected in both wastewater and the environment (Scala-Benuzzi *et al.* 2018). Previous studies have reported on its ability to affect the endocrine system of aquatic organisms, including fish (Mortensen & Arukwe, 2007; Shved *et al.* 2008). EE2 can also alter gonadal development, resulting in delayed sexual maturity or developmental disorders in secondary sexual characteristics such as the presence of ovotestis (Länge *et al.* 2001; Andersen *et al.* 2003) or a reduced number of spermatogonia (Piferrer & Donaldson, 1992).

In addition to its specific effects on aquatic organisms, EE2 can also bioaccumulate in aquatic organisms (Länge *et al.* 2001; Pojana *et al.* 2007). Owing to its widespread use, EE2 has been registered in various environmental matrices worldwide (e.g. surface waters,

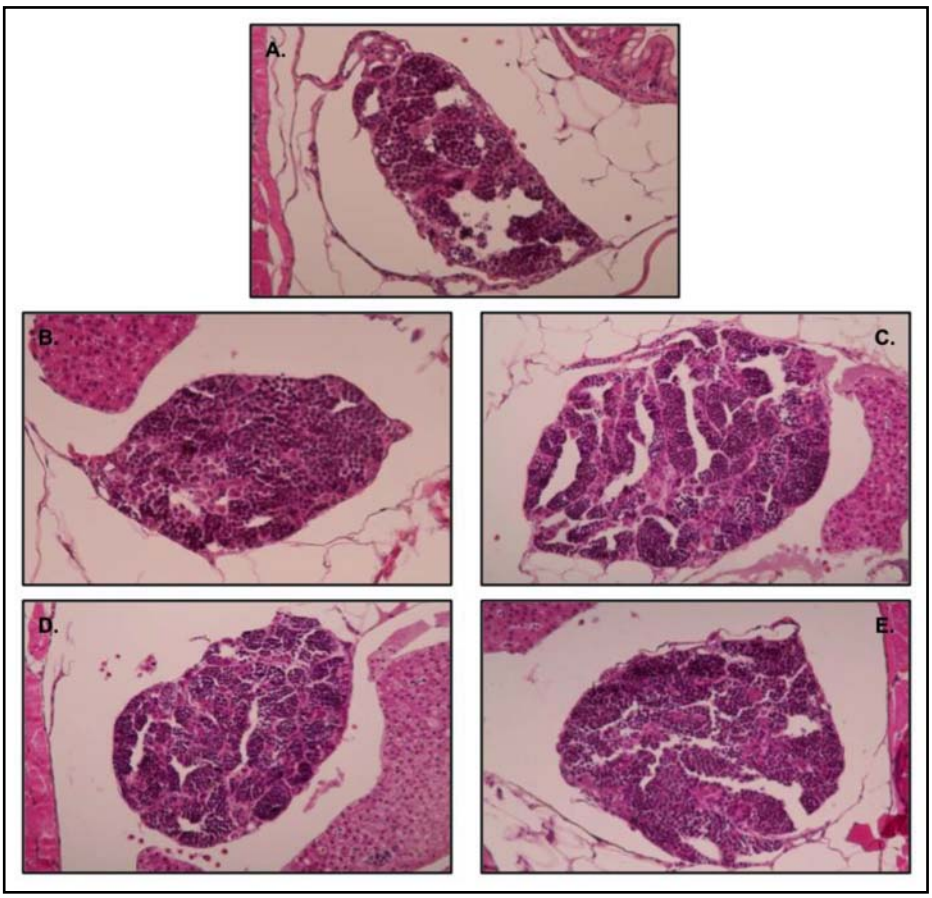

Fig. 1. Results of histopathological examination of testicular tissue in experimental groups.
No changes were observed among groups. A = control, B = positive control, C = low EE2 dose, D = medium EE2 dose, E = high EE2 dose. Magnification 200x.

wastewaters, sludge, sediment) (Barreiros *et al.* 2016) The most commonly analysed matrix is surface water, where EE2 content can reach concentrations of tens or hundreds of ng/l depending on the intensity of pollution. (Pojana *et al.* 2007; Zhang *et al.* 2011; Barreiros *et al.* 2016, Wang *et al.* 2018). Once in the aquatic system, EE2 can bind to sediments (Holthaus *et al.* 2002; Pojana *et al.* 2007; Zhang *et al.* 2011; Froehner *et al.* 2012) and may then bioaccumulate up the food chain (US EPA, 2000), from benthic feeders up to aquatic predators (Pojana *et al.* 2007; Zhang *et al.* 2011; Al-Ansari *et al.* 2013; Liu *et al.* 2015) and hypothetical even humans, where it may result in the disorders mentioned above. For this reason, we chose the peroral administration of the test substance.

Sex differentiation in zebrafish is specific and begins as early as the transition into juvenile ovaries at about 12 days post-fertilization. All zebrafish have a juvenile ovary; if it continues to develop, the fish will mature as a female, while elimination (apoptosis) of the immature ovary produces males (Slanchev *et al.* 2005; Delomas & Dabrowski, 2018). Sexual differentiation is completed within 40 days after hatching (Takahashi, 1977; Shang & Wu, 2004), with the outcome depending on both genetic and environmental factors (Santos *et al.* 2017). Zebrafish exposed to high water temperature (33°C), for example, showed significant increase in the incidence of male fish to a reference group (28°C) (Luzio *et al.* 2016). The papers (Andersen *et al.* 2003; Maack & Segner, 2004) postulated that aquatic contamination with endocrine disruptors is also likely to disrupt zebrafish gonadal development. Here, we use triploid zebrafish as a model species to test the effects of endocrine disruptor EE2 on gonadal development. Triploidy, where three complete haploid sets of homologous chromosomes are present, can be induced in many fish species through early intervention during embryonic development, causing retention of the second polar body (Piferrer *et al.* 2009). In the case of zebrafish, triploidy results in male-only populations (Kavumpurath & Pandian, 2008; Delomas & Dabrowski, 2018), making them ideal models for investigating whether exposure to estrogens affects the hormonal system (i.e. induces vitellogenin) in induced triploid male-only populations.

Determination of vitellogenin level tends to be the most commonly used for detecting sex disorder markers (Hansen *et al.* 1998; Li *et al.* 2018; Gupta & Verma, 2020). Vitellogenin is a phospholipoprotein molecule synthesized naturally in the adult female liver of nonmammalian vertebrates. As such, high levels are observed especially during yolk formation. Synthesis of vitellogenin takes place in the hypothalamus-hypophysis-gonadal axis. 17β-estradiol induces vitellogenin production in the liver and its release into the bloodstream, from where vitellogenin enters the oocytes through specific receptors and is incorporated into the egg yolk (Palmer *et al.* 1998; Wang *et al.* 2000; Reading *et al.* 2017). However, it has yet to be shown whether it is possible to induce vitellogenin induction in an all-male zebrafish population.

The main aim was to determine whether vitellogenin synthesis can be induced in all-male triploid zebrafish and used to test for xenoestrogen changes. Gonzalez *et al.* (2021) and Colli-Dula *et al.* (2014) are among the few who have investigated the effect of oral application of EE2 on fish. However, none of them tested the effect of EE2 in triploid zebrafish, the experiment did not focus on the effect in young fish and a sex-mixed or female-only populations were used. We hypothesize that all-male populations could provide a more straightforward, more conclusive, and comprehensive evaluation of the results of experiments focused on hormonal and histological changes in male fish. Use of all-male triploid zebrafish could replace sex-mixed populations to reduce the total number of experimental animals. If we use a diploid population, we must include a more significant number of individuals in the experiment because the experimental group contains both males and females, which we can distinguish only after the end of our experiment. Because triploid zebrafish are only males, fewer test individuals can be included in the experiment. To the best of our knowledge, this study is unique in its type of field. No toxicological study deals with use of all-male triploid zebrafish in toxicological tests for oral intake of xenoestrogens.

MATERIALS AND METHODS

Chemicals and Feed

EE2 (17α-ethinylestradiol; 17α-ethynyl-1,3,5(10)-estratriene-3,17β-diol) and β-estradiol (estradiol; 3,17β-dihydroxy-1,3,5(10)-estratriene) were obtained from Sigma-Aldrich (St. Louis, USA) with ≥ 98% active ingredient in both cases. Likewise, sodium bicarbonate, sodium hydroxide, dansyl chloride, and formic acid were purchased from Sigma-Aldrich (St. Louis, USA).

Fish were fed commercial feed (Skretting, Stavanger, Norway), Gemma micro 75 (GM75) complete feed provided for the first part of the experiment (fish < 1-month-old), and Gemma micro 150 (GM150) for the second part of the experiment (fish > 1-month-old). The only difference was particle size (GM75: 50–100 μm, GM150: 100–200 μm). The ingredients of both commercial feeds are following: protein 59%, fat 14%, fibre 0.2%, ash 14%, phosphorus 1.3%, calcium 1.5% and sodium 0.7%. Colloidal silicon dioxide (Aerosil® 200) was obtained from Evonik (Germany). Used as a sorbent in feed mixtures prevents particles from sticking during drying.

Feed preparation

Our study applied a new method for impregnating test substances into a feed based on a coating process. We prepared four batches of test feed, three with different concentrations of EE2 and one containing 0.1 μg/g of β-estradiol. The latter was used for a positive control

Tab. 1. Body weight of experimental fish at the end of the experiment (mg). We found no significant differences among the experimental groups ($p > 0.05$)

Group	Average	Standard deviation	Median	Maximum	Minimum
High EE2 dose	41.7	29.3	32.7	95.1	1.7
Medium EE2 dose	71.2	61.9	63.3	267.5	5.7
Low EE2 dose	53.2	43.0	36.8	145.8	9.9
Control	74.2	40.1	37.55	138.0	3.0
Positive control	68.9	42.3	56.3	158.1	16.1

group. Low EE2 dose (0.01 μg/g) was taken to represent typical environmental concentrations (Xu *et al.* 2008; Cabas *et al.* 2011; Blanco *et al.* 2016), while we prepared medium (0.1 μg/g) and high (1 μg/g) doses to evaluate any dose-response relationships. Since significant losses of the active substances often occur during the technological preparation of the feed, the contents of the tested substances were verified by liquid chromatography-mass spectrometry. The actual concentrations analysed are given in table 4.

First, we prepared a stock solution of 1 mg of EE2 in 1 ml 96% ethanol. Then used to prepare the three EE2 test solutions. Solution A (0.1 mg/10 ml), solution B (0.01 mg/10 ml) and solution C (0.001 mg/10 ml) used ethanol as dissolvent. At the same time, we prepared the positive control (solution D) using 0.01 mg/10 ml of β-estradiol. Next, 396.0 g of GM 75 and GM 150 feed was mixed with 4.0 g of Aerosil® 200 in a UMC 5 blender (Stephan, Germany) and mixed for 1 minute at 400 rpm. Aerosil® 200 (colloidal silicon dioxide) is commonly used in low concentrations as a physical sorbent to prepare dosage forms with a low content of solid (Dobsikova *et al.* 2012) or liquid/dissolved (Modra *et al.* 2018) active substances in toxicological studies. The result is uniform physical sorption of the drug or toxin in a mixture with a high content uniformity. This sorption is easily reversible, and in the digestive tract colloidal silicon dioxide forms a hydrogel, leading to a higher wettability of the active substance and a uniform release of poorly soluble drugs. Therefore, it increases dissolution rate and bioavailability (Friedrich *et al.* 2006). For this purpose, colloidal silicon dioxide has been widely used for more than fifty years and appears to be utterly safe without its absorption (Diab *et al.* 2017). Its use guarantees the creation of standard conditions for uniform absorption of hormones by fish.

These mixtures were then divided into four groups of 100 g each (4x GM 75 and 4x GM 150), placed in a 400 ml beaker and manually mixed with 10.0 ml of each following solutions: A (dedicated for EE2 1 μg/g); B (dedicated for EE2 0.1 μg/g); C (dedicated for EE2 0.01 μg/g); and solution D (dedicated for β-estradiol 0.1 μg/g). The feed mixtures were left in closed beakers for 24 hours to ensure homogeneity and then dried in open beakers in a HORO-048B hot-air dryer (HORO Dr. Hoffmann GmbH, Germany) for 5 hours at 40°C. Two control feeds of GM 75 and GM 150 were also prepared in the same way without hormones. As the extremely low hormone concentrations used in this experiment do not affect the mixture's physical properties, control feeds (Control - GM75 and Control - GM150) were also used to evaluate particle properties (Table 3).

Feed analysis

The feed preparations were tested to discern flow properties, density, electrostatic charge, moisture content, content uniformity, particle size and distribution. Flow properties, i.e. Hausner ratio (HR), compressibility index (CI) and angle of response (α), were evaluated according to the European Pharmacopoeia (European Pharmacopoeia, 2019); pycnometric density was assessed using a Pycnomatic-ATC helium pycnometer (Porotec GmbH, Germany), according to the European Pharmacopoeia (European Pharmacopoeia, 2019); the electrostatic charge was determined using a JCI 150 Faraday cage and JCI 178 charge measuring unit (Chilworth technology Ltd., UK), according to Svacinova *et al.* (2019); moisture content was determined using an HX204 halogen moisture analyser (Mettler Toledo, Switzerland); and particle size distribution was determined using an LA-960 laser scattering particle size distribution analyser (Horiba, Japan). The sample refraction index was set as 1.500.

Ten samples (0.5 g) were taken randomly from each feed concentration, and content uniformity was evaluated using liquid chromatography (atmospheric pressure chemical ionisation) tandem mass spectrometry (LC/MS), according to the European Pharmacopeia (European Pharmacopoeia, 2019).

Each 0.5 g sample was vortexed in a glass test tube with acetonitrile (3 ml) for 2 min and then extracted in an ultrasonic bath (Bandelin Electronic GmbH & Co., Berlin, Germany) for 15 min. After extraction, the samples were centrifuged at 800 g for 15 min at 20°C, after which the supernatant was collected in a glass test tube. The extraction was then repeated and both supernatants combined, with 0.5 ml of the mixed supernatant used for derivatization using dansyl chloride. The supernatant was evaporated until dryness at 55°C under

Tab. 2. Vitellogenin content in whole-body zebrafish homogenates after oral exposure to EE2 and β-estradiol

Group	Positive samples /no. samples	Median [ng/g]	Concentration range detected [ng/g]
High EE2 dose	13/13	23,376,111	14,545,022–48,337,917 [a]
Medium EE2 dose	10/12	11,210	2,367– 101,317 [ab]
Low EE2 dose	7/12	6,750	5,077 – 140,623 [b]
Control	0/9	n.d.	n.d.
Positive control	7/12	4,363	2,669 – 13,687 [b]

Significant differences ($p < 0.05$) indicated by alphabetic superscript (different letters represent a statistical difference); total n = 58 fish.

a gentle stream of nitrogen. A 250 µl volume of sodium bicarbonate buffer (100 mmol/l; pH = 10.5) and 250 µl of dansyl chloride solution in acetone (1 g/l) were then added to the dry residue in a glass test tube, which was then gently vortexed and incubated at 60°C for 10 min. Subsequently, the mixture was again evaporated until dryness at 55°C under a gentle stream of nitrogen, after which the dry residues were reconstituted in 500 µl of methanol and filtered through a 0.2 mm nylon filter (Millipore, USA) ready for LC/MS analysis.

A UHPLC Accela 1250 chromatographic pump was connected to a TSQ Quantum Access MAX Triple Quadrupole LC/MS (Thermo Scientific, USA) equipped with an atmospheric pressure chemical ionization probe. The solutions were then passed through a 2.1 mm × 100 mm, 1.7 µm Kinetex C18 column (Phenomenex, USA) at a constant flow rate of 300 µl/min. The mobile phase consisted of deionised water containing 0.1% formic acid (v/v) (solvent A) and acetonitrile (solvent B). The gradient used consisted of a 0–2.0 min linear gradient from 20 to 90% solvent B; 2.0–7.5 min held at 90% B; 7.5–8.0 min from 90 to 20% B and 8.0–8.5 min held at 20% B. All solvents used in the preparation were of residual analysis purity (Chromservis, Czech Republic). The full loop injection volume for the sample was set at 10 µl. Atmospheric pressure chemical ionization was conducted in the positive mode under the following conditions: capillary temperature 325°C; vaporize temperature 300°C; sheath gas pressure 35.0 psi; auxiliary (drying) gas 10 a.u. and discharge current 4.0 µA. The instrument was calibrated daily with multi-level matrix-matched calibration curves. The inter-day precision, expressed as relative standard deviation, was 3.6% for β-estradiol and 5.0% for EE2, while inter-day accuracy was 3.9% for β-estradiol and 5.4% for EE2. The detection limit was determined as 3:1, with signal versus noise values of 0.097 ng/g for β-estradiol and 0.155 ng/g for EE2.

Experimental fish

In zebrafish (AB strain from European Zebrafish Resource Center), triploidy was induced by heat shock treatment, as described in Franek *et al.* (2019), with a 2 min temperature shock (41.4°C) applied to the fertilized embryos two minutes post fertilization. Surviving swim-up larval stages, which was approximately 25%, were then assessed for ploidy using flow cytometry. Also, three individuals displaying completed sex differentiation were taken from each experimental treatment group (see below) after testing. Their caudal fin was used to test ploidy using the same method.

Whole larvae (euthanized by tricaine overdosing) and fin clips were processed using the CyStain UV Precise T nuclei staining kit (Sysmex Partec GmbH, Germany), according to the manufacturer's protocol. The relative DNA content was then determined using a CyFlow Ploidy Analyser (Sysmex Partec GmbH, Germany), with samples compared against diploid control groups. In all cases, triploidy was confirmed in experimental fish.

Experimental design

After transport to the laboratory, the larval fish were allowed to acclimatize for three days. They were fed with unsupplemented feed three times a day ad libitum (morning, noon, and evening) such that all food was consumed (i.e. none remained in the water). The experiment was approved by the Ethical Commission (MSMT-15371/2019-4) and started when the fish were nine days old.

Fifty fish were placed into each of ten 12 l aquariums to make four experimental groups (three EE2 concentrations and one β-estradiol) and one control group, each duplicate. We also prepared a single diploid male-female control group, which was not sampled for vitellogenin. Throughout the experiment, the aquariums were aerated, and lighting was controlled to give 13 hrs light and 11 hrs dark. The aquariums were cleaned every morning, with water samples taken for physicochemical examination before and after each cleaning. Water temperature during the experiment was maintained at 24.8 ± 0.6°C and oxygen concentration at 8.00 ± 0.18 mg/l. Physico-chemical examination indicated pH at 8.32 ± 0.14, total ammonium at 0.26 ± 0.14 mg/l, N-NO_2- at 0.11 ± 0.08 mg/l and Cl- at 128.12 ± 44.66 mg/l.

During the experiment, larval fish were fed with GM75 and, after 4 weeks, the diet was gradually shifted to one based on GM150. We assessed the presence of body deformities and behavior (interest in feed,

Tab. 3. Feed particle properties

Sample	CI	HR	α	DB	DT	DP	CH	M	D_{10}	D_{50}	D_{90}
Control - GM75	6.55	1.07	40°85´	0.49	0.52	0.94	-1.42	3.60	66.7	94.3	144.9
Control - GM150	6.66	1.07	43°40´	0.46	0.50	1.34	-1.48	4.52	84.0	174.5	286.1

aggressive and territorial behavior, activity) during the feeding period throughout the experiment.

Sampling

At the end of the 8-week experiment, fish were taken from aquariums and euthanized by placing a 250 mg/l MS-222 (tricaine methanesulphonate) solution in a beaker. After 10 minutes, the fish were removed, dried, and weighed. Zebrafish to be tested for vitellogenin concentration were euthanised and frozen at -80°C, while those taken for histological examination were fixed in formaldehyde. Each fish represented one sample.

Vitellogenin detection

Vitellogenin detection in triploid zebrafish was performed in whole-body homogenate, using the Zebrafish vitellogenin ELISA (enzyme-linked immune sorbent assay) kit (Biosense Laboratories AS, Norway). As vitellogenin is an unstable protein (Nilsen *et al.* 2004), all samples and standards were kept on ice during the whole procedure, and a protease inhibitor was added for better sample stability. According to the manufacturer's instructions, dilution buffers, washing buffers, and substrate solutions were all prepared.

Nine to twelve fish were examined from each test group (total n = 58 fish). Each fish was weighed, and 1:4 dilution buffer was added. The sample was then homogenized in TissueLyser II (Qiagen, USA). After performing two cycles, each at 25 Hz for 70 s, the samples were centrifuged at 18 000 x g for 30 min at 4°C using a Microfuge 22R centrifuge (Beckman Coulter, United States). The supernatant was removed, and three different dilutions of each sample were prepared at a ratio of 1:250, 1:2,500 and 1:25,000. For the high feed EE2 concentration group, dilutions were set at 1:25,000, 1: 250,000, and 1:2,500,000 as the vitellogenin levels were expected to be high. The samples were then applied to 96-well microtiter plates, sealed, and incubated at room temperature for 1 hr. After incubation, the plates were washed three times using washing buffer and the detecting antibody added, after which the plates were again sealed and incubated at room temperature. The plates were then washed three times with washing buffer, and the secondary antibody was added. After the incubation, the plates were washed five times with washing buffer, and the substrate solution added, following which they were incubated in the dark at room temperature for 30 min. The reaction was then stopped by adding 2 mol/l H_2SO_4 and, after five minutes, the absorbance was read with a Varioskan flash microplate reader (Thermo-Scientific, USA) at 492 nm. Calibration series and regression calculations were performed according to the manufacturer's recommendations.

Histological examination

Before a histological examination of the testicular tissue, the fish (total n = 29 fish) were euthanised using MS222, fixed in 3.6 % formaldehyde, dehydrated in ethanol–xylene series, embedded into paraffin blocks, and cut into 4 µm sections using a Leica RM2235 rotary microtome (Leica Biosystems, USA). According to standard procedures, the paraffin slides were stained with hematoxylin and eosin using a Tissue-Tek DRS 2000 staining machine (Sakura, Netherlands). Histological sections of the testicular tissue were photographed using a Nikon Eclipse Ci microscope (Nikon, USA) mounted with a Canon EOS 1000D camera (Canon, Japan).

Statistical analysis

Unistat for Excel 6.5 software package was used for statistical analysis. At first, all data were tested using the Shapiro-Wilk test. Both results of vitellogenin and weight did not meet normal distribution conditions. A nonparametric multi-sample median test (Tukey-HSD) was used to test all pairwise comparisons among means of all groups. Differences between groups were considered significant at $p < 0.05$ and $p < 0.01$.

RESULTS

Fish morphology and behavior

No morphological abnormalities and no behavioral changes were detected throughout the experiment. As all fish showed interest in the feed throughout the experiment, we assume that the test substances did not affect the acceptance of the feed. All fish were weighed at the end of the experiment, and a multi-sample median test indicated no statistically significant differences ($p > 0.05$) among the groups (Table 1).

The largest triploid fish from the groups were selected for histological examination (total n = 29 fish). All samples from the test fish displayed typical male characteristics, with both treated and control samples demonstrating testes in a normal developmental state. Histological examination did not reveal pathological lesions in both control and experimental groups (Figure 1).

Tab. 4. Hormone content uniformity and dose concentration in each test batch

Sample	Concentration µg/g	x_i %	P_{max} %	RSD	Ph. Eur.[a]
High EE2 dose - GM75	0.372 ± 0.016	37.23	7.62	2.65	+
High EE2 dose - GM150	0.359 ± 0.014	35.80	6.81	3.55	+
Medium EE2 dose - GM75	0.031 ± 0.002	30.65	10.27	5.07	+
Medium EE2 dose - GM150	0.032 ± 0.002	31.98	9.44	1.54	+
Low EE2 dose - GM75	0.003 ± 0.0002	26.70	8.80	4.62	+
Low EE2 dose - GM150	0.004 ± 0.0002	35.70	9.55	1.98	+
Positive control - GM75	0.047 ± 0.001	46.56	4.16	1.36	+
Positive control - GM150	0.046 ± 0.002	45.51	8.82	4.03	+

Abbreviations: Concentration = final feed dose concentration EE2/ β-estradiol (µg/g) ± SD; + = passed.

Vitellogenin detection

Detail results of vitellogenin analysis are shown in Table 2. Vitellogenin was not detected in control group. Half of the detection limit (50 ng/g) was applied for statistical analysis in cases where vitellogenin was below this limit. . The highest median vitellogenin concentration (23,376111 ng/g) was detected in the experimental group exposed to high EE2, where a positive vitellogenin finding was also observed in all analysed individuals. A statistically significant difference ($p < 0.05$) was found between this experimental group and experimental group exposed to low EE2, control and positive groups. No significant difference was found between control group and experimental groups exposed to low and medium EE2.

Feed evaluation

Particle property tests (Table 3) on control preparations indicated that the prepared feed mixtures showed suitable physicochemical properties that allowed uniform dosing (Shah *et al.* 2008). Overall, the test values obtained were good indicators that the experiment would not be affected by uneven dosing of the active substance throughout its duration.

Analysis of EE2 and β-estradiol in fish feed

LC/MS analysis confirmed content uniformity for each experimental feed concentration (low, medium, high) in both types of feed (GM75, GM150) and the β-estradiol control, with average concentrations in each EE2 batch ranging from 26.7–37.2 % of the intended level (β-estradiol control = 45.5–46.6 %; Table 4).

DISCUSSION

This experiment aimed to test the toxicity of the hormone EE2, a semisynthetic estrogen used in combined oral contraceptives, on all-male triploid zebrafish to determine whether EE2 peroral administration causes alterations in male gonadal development and whether vitellogenin detection can be used to test such xenoestrogen changes. The oral administration was chosen because of the ability of EE2 to accumulate in the food chain. We wanted to confirm our hypothesis that there could be a reduction in the number of experimental animals using only the all-male zebrafish population instead of the sex-mixed population.

Previous studies on xenoestrogenic substances in the aquatic environment, including EE2, have reported adverse effects on fish. The metabolism of synthetic estrogen EE2 proceeds through the same pathways as natural steroid hormones. EE2 is an agonist of estrogen receptors – ERα and ERβ. When the receptors bind EE2, they act as transcription factors and cause the expression of steroid hormone gene that modulate biological activities in target tissue in both females and males (Björnström and Sjöberg, 2005; Jeannine *et al.* 2005). For example, Sun *et al.* (2019) recorded fin regeneration inhibition, locomotor activity changes, and decreased immune-related genes in fish exposed to 10-100 ng/l. Others have reported skeletal distortions, uninflated swim bladders, lymphedema formation or cartilage deformities in the progeny of EE2 exposed parents, and increasing vitellogenin mRNA in zebrafish exposed to 2.5-10 ng/l (Sun *et al.* 2017; Valcarce *et al.* 2017). While our experiment confirmed the ability of oral administration of EE2 to induce vitellogenin synthesis in all-male triploid zebrafish (and is the first such experiment to do so), we observed no outward signs of physiological change and no visible histological changes to gonad tissue, suggesting that our high dosage level may have been too low to induce such changes, or that the reduction in active dose concentration (Table 4; also see above) reduced the hormone's potential to cause such physical effects. We assume that the absence of morphological changes was not caused by the oral administration of EE2 instead of water application. At the same time the transdermal, subcutaneous or intramuscular administration of estrogenes is known (Kuhl 2005). In a similar study, Delomas and Dabrowski (2018) exposed triploid zebrafish to 100 ng/l for 28 days

post fertilization and also failed to turn triploid males into females. However, it should be noted that they focused on histological changes only.

Our results confirmed that increasing doses of EE2 in feed lead to rising levels of vitellogenin production in all-male triploid zebrafish tissues, with levels reaching 14,545,022 – 48,337,917 ng/g in whole-body homogenate after 8 weeks feeding with feed containing 0.365 ± 0.016 μg/g EE2 (high dose level). While all zebrafish in the high dose group tested positive for vitellogenin synthesis, an increasing percentage of those on lower doses tested negative, with just seven of twelve fish testing positive at the lowest dose rate (Table 2), confirming a dose-response relationship. Surprisingly, there was no positive vitellogenin finding in all positive control fish (only 7 out of 12), which could be due to the low dose of 17β-estradiol used for testing. In the control group, vitellogenin levels were below detection limits (Table 2). The higher estrogenic potential of EE2 compared to 17β-estradiol was also observed in the study by Rose *et al.* (2002). Lower doses of EE2 in water caused similar induction of vitellogenin in the whole-body homogenate of zebrafish as higher doses of 17β-estradiol. As EE2 is detectable in both the aquatic environment and the food chain (Dussault *et al.* 2009; Zhang *et al.* 2011), studies need to focus on both these pathways when studying fish contamination. We chose to administer EE2 as part of a commercial feed mixture, with EE2 concentrations of 0.0035 (low); 0.0315 (medium) and 0.365 (high) μg/g and positive control with 17β-estradiol (0.0465 μg/g). As used in this study, we assumed that ultra-low concentrations of hormones would not affect the 'behavior' of preparations with different dose mixtures. Indeed, the batches prepared all safely passed content uniformity tests (Table 3). Despite this, testing showed that the feed preparations had relatively low doses (ca. 30–47%) of an active substance compared to the theoretical content (Table 4). These results appear most likely due to absorption of the hormone on the beaker walls, a phenomenon commonly reported in toxicological studies using ultra-low active substance concentrations (Matejova *et al.* 2017). By undertaking this important back-calculation step, we will correct dosing levels in future studies, ensuring more relevant results. Nevertheless, the use of triploid forms of zebrafish has been successful, in the way we intended, and has opened up many new possibilities for other toxicological studies.

CONCLUSIONS

Our study is the first to confirm the ability of EE2 to induce vitellogenin synthesis in all-male triploid zebrafish. Despite the lack of noticeable physiological or histological changes in our zebrafish, the simple presence of vitellogenin in an all-male zebrafish population, and its presence in body tissue, is a definite symptom indicative of the potential for such changes due to the action of estrogenic substances. Though we observed statistically significant differences in vitellogenin at our high dose level (14,545,022–48,337,917 ng/g of vitellogenin), this appeared insufficient to induce physiological or histological changes; thus, it will be necessary to undertake further tests to determine the exact level of EE2 that causes physical effects in triploid zebrafish. We also found no behavioral changes during the experiment. A detailed retrospective analysis of the feed confirmed a uniform distribution of an active substance through the whole volume. Furthermore, it revealed a reduction in the intended test dose (30-47%), allowing the conclusion of the experiment to be corrected. Nevertheless, this experiment has shown that using all-male triploid zebrafish populations, rather than the mixed-sex populations of other species previously used, could serve as a suitable alternative for controlled testing of the effects of xenoestrogens on male fish.

ACKNOWLEDGEMENTS

This scientific work was financially supported by grant IGA 224/2019/FVHE and projects PROFISH CZ.02.1.01/0.0/0.0/16_019/0000869, Biodiversity (CZ.02.1.01/0.0/0.0/16_025/0007370) and CENAKVA (LM2018099). The authors express their deep appreciation to Mgr. Kevin F. Roche BSc. CSc. for manuscript improvement and for language editing. The authors also thank Jana Vrablova for laboratory work and technical support during toxicity tests on zebrafish.

REFERENCES

1 Al-Ansari AM, Atkinson SK, Doyle JR, Trudeau VL, Blais JM (2013). Dynamics of uptake and elimination of 17α-ethinylestradiol in male goldfish *(Carassius auratus)*. Aquatic Toxicology. **132–133**: 134–140. doi:10.1016/j.aquatox.2013.02.006

2 Andersen L, Holbech H, Gessbo Å, Norrgren L, Petersen GI (2003). Effects of exposure to 17α-ethinylestradiol during early development on sexual differentiation and induction of vitellogenin in zebrafish *(Danio rerio)*. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology. **134**(3): 365–374. doi:10.1016/S1532-0456(03)00006-1

3 Barreiros L, Queiroz J, Magalhaes LM, Silva AMT, Segundo MA (2016). Analysis of 17-β-estradiol and 17-α-ethinylestradiol in biological and environmental matrices — A review. Microchemical Journal. **126**: 243–262.

4 Björnström L, Sjöberg M (2005). Mechanisms of Estrogen Receptor Signaling: Convergence of Genomic and Nongenomic Actions on Target Genes. Molecular Endocrinology. **19**: 833–842. doi:10.1210/me.2004-0486

5 Blanco M, Fernandes D, Medina P, Blázquez M, Porte C (2016). Drospirenone intake alters plasmatic steroid levels and cyp17a1 expression in gonads of juvenile sea bass. Environmental Pollution. **213**: 541–548. doi:10.1016/j.envpol.2016.03.007

6 Cabas I, Chaves-Pozo E, Alcázar AG, Meseguer J, Mulero V, García-Ayala A (2011). Dietary intake of 17α-ethinylestradiol promotes leukocytes infiltration in the gonad of the hermaphrodite gilthead seabream. Molecular Immunology. **48**(15–16): 2079–2086. doi:10.1016/j.molimm.2011.07.001

7 Cerveny D, Grabic R, Grabicova K, Randak T, Larsson DGJ, Johnson AC, et al. (2021). Neuroactive drugs and other pharmaceuticals found in blood plasma of wild European fish. Environment International. **146**: 106188. doi: 10.1016/j.envint.2020.106188

8 Colli-Dula RC, Martyniuk CJ, Kroll KJ, Prucha MS, Kozuch M, Barber DS, Denslow ND (2014). Dietary exposure of 17-alpha ethinylestradiol modulates physiological endpoints and gene signaling pathways in female largemouth bass *(Micropterus salmoides)*. Aquatic Toxicology. **156**: 148–160. doi:10.1016/j.aquatox.2014.08.008

9 Delomas T, Dabrowski K (2018). Why are triploid zebrafish all male? Molecular Reproduction and Development. **85**: 612–621. doi:10.1002/mrd.22998

10 Diab R, Canilho N, Pavel IA, Haffner FB, Girardon M, Pasc A (2017). Silica-based systems for oral delivery of drugs, macromolecules and cells. Advances in Colloid and Interface Science. **249**: 346–362. doi:10.1016/j.cis.2017.04.005

11 Dobsikova R, Blahova J, Franc A, Jakubik J, Mikulikova I, Modra H, et al. (2012). Effect of β-1.3/1.6-D-glucan derived from oyster mushroom Pleurotus ostreatus on biometrical, haematological, biochemical, and immunological indices in rainbow trout *(Oncorhynchus mykiss)*. Neuroendocrinology Letters. **33**(Suppl 3): 96–106.

12 Dussault ÈB, Balakrishnan VK, Borgmann U, Solomon KR, Sibley PK (2009). Bioaccumulation of the synthetic hormone 17α-ethinylestradiol in the benthic invertebrates Chironomus tentans and Hyalella azteca. Ecotoxicology and Environmental Safety. **72**(6): 1635–1641. doi:10.1016/j.ecoenv.2009.04.019

13 European Pharmacopoeia (2009). 10th ed.; Elsevier, Council of Europe.

14 Franek R, Tichopad T, Fucikova M, Steinbach C, Psenicka M (2019). Production and use of triploid zebrafish for surrogate reproduction. Theriogenology. **140**: 33–43. doi:10.1016/j.theriogenology.2019.08.016

15 Friedrich H, Fussnegger B, Kolter K, Bodmeier R (2006). Dissolution rate improvement of poorly water-soluble drugs obtained by adsorbing solutions of drugs in hydrophilic solvents onto high surface area carriers. European Journal of Pharmaceutics and Biopharmaceutics. **62**(2): 171–177. doi:10.1016/j.ejpb.2005.08.013

16 Froehner S, Machado KS, Stefan E, Bleninger T, Rosa EC da, de Castro Martins C (2012). Occurrence of selected estrogens in mangrove sediments. Marine Pollution Bulletin. **64**(1): 75–79. doi:10.1016/j.marpolbul.2011.10.021

17 Gonzalez JA, Histed AR, Nowak E, Lange D, Craig SE, Parker CG, Kaur A, Bhuvanagiri S, Kroll KJ, Martyniuk CJ, Denslow ND, Rosenfeld CS, Rhodes JS (2021). Impact of bisphenol-A and synthetic estradiol on brain, behavior, gonads and sex hormones in a sexually labile coral reef fish. Hormones and Behavior. **136**: 105043. doi:10.1016/j.yhbeh.2021.105043

18 Gupta P, Verma SK (2020). Impacts of herbicide pendimethalin on sex steroid level, plasma vitellogenin concentration and aromatase activity in teleost *Clarias batrachus (Linnaeus)*. Environmental Toxicology and Pharmacology. **75**: 103324. doi:10.1016/j.etap.2020.103324

19 Hansen PD, Dizer H, Hock B, Marx A, Sherry J, McMaster M et al. (1998). Vitellogenin – a biomarker for endocrine disruptors. TrAC Trends in Analytical Chemistry. **17**(7): 448–451. doi:10.1016/S0165-9936(98)00020-X

20 Holthaus KIE, Johnson AC, Jürgens MD, Williams RJ, Smith JJL, Carter JE (2002). The potential for estradiol and ethinylestradiol to sorb to suspended and bed sediments in some English rivers. Environmental Toxicology and Chemistry. **21**(12): 2526–2535. doi:10.1002/etc.5620211202

21 Jeannine SS (2005). Estrogens, Progestins, and SERMs. In: Craig CR Stitzel RE, editors. Modern Pharmacology with Clinical Applications. 5th ed. Lippincott Williams and Wilkins. p. 704–715.

22 Kavumpurath S, Pandian T (2008). Induction of triploidy in the zebrafish, *Brachydanio rerio* (Hamilton). Aquaculture Research. **21**: 299–306. doi:10.1111/j.1365-2109.1990.tb00468.x

23 Kuhl H (2005). Pharmacology of estrogens and progestogens: influence of different routes of administration. Climacteric. **8**: 3–63. doi:10.1080/13697130500148875

24 Länge R, Hutchinson TH, Croudace CP, Siegmund F, Schweinfurth H, Hampe P et al. (2001). Effects of the synthetic estrogen 17α-ethinylestradiol on the life-cycle of the fathead minnow *(Pimephales promelas)*. Environmental Toxicology and Chemistry. **20**(6): 1216–1227. doi:10.1002/etc.5620200610

25 Li Y, Wang J, Zheng M, Zhang Y, Ru S (2018). Development of ELISAs for the detection of vitellogenin in three marine fish from coastal areas of China. Marine Pollution Bulletin. **133**: 415–422. doi:10.1016/j.marpolbul.2018.06.001

26 Liu J, Lu G, Xie Z, Zhang Z, Li S, Yan Z (2015). Occurrence, bioaccumulation and risk assessment of lipophilic pharmaceutically active compounds in the downstream rivers of sewage treatment plants. Science of The Total Environment. **511**: 54–62. doi:10.1016/j.scitotenv.2014.12.033

27 Luzio A, Santos D, Fontaínhas-Fernandes AA, Monteiro SM, Coimbra AM (2016). Effects of 17α-ethinylestradiol at different water temperatures on zebrafish sex differentiation and gonad development. Aquatic Toxicology. **174**: 22–35. doi:10.1016/j.aquatox.2016.02.003

28 Maack G, Segner H (2004). Life-stage-dependent sensitivity of zebrafish *(Danio rerio)* to estrogen exposure. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology. **139**(1–3): 47–55. doi:10.1016/j.cca.2004.09.004

29 Matejova I, Faldyna M, Modra H, Blahova J, Palikova M, Markova Z et al. (2017). Effect of T-2 toxin-contaminated diet on common carp (*Cyprinus carpio* L.). Fish & Shellfish Immunology. **60**: 458–465. doi:10.1016/j.fsi.2016.11.032

30 Modra H, Sisperova E, Blahova J, Enevova V, Fictum P, Franc A et al. (2018). Elevated concentrations of T-2 toxin cause oxidative stress in the rainbow trout *(Oncorhynchus mykiss)*. Aquaculture Nutrition. **24**(2): 842–849. doi:10.1111/anu.12613

31 Mortensen AS, Arukwe A (2007). Effects of 17α-ethynylestradiol on hormonal responses and xenobiotic biotransformation system of Atlantic salmon *(Salmo salar)*. Aquatic Toxicology. **85**(2): 113–123. doi:10.1016/j.aquatox.2007.08.004

32 National Center for Biotechnology Information (2022). PubChem Annotation Record for Ethinylestradiol, Source: Hazardous Substances Data Bank (HSDB). Retrieved July 26, 2022 from https://pubchem.ncbi.nlm.nih.gov/source/hsdb/3587#section=Toxicity-Summary.

33 Nilsen BM, Berg K, Eidem JK, Kristiansen SI, Brion F, Porcher JM et al. (2004). Development of quantitative vitellogenin-ELISAs for fish test species used in endocrine disruptor screening. Analytical and Bioanalytical Chemistry. **378**(3): 621–633. doi:10.1007/s00216-003-2241-2

34 Palmer BD, Huth LK, Pieto DL, Selcer KW (1998). Vitellogenin as a biomarker for xenobiotic estrogens in an amphibian model system. Environmental Toxicology and Chemistry. **17**(1): 30–36. doi:10.1002/etc.5620170105

35 Petrovici A, Strungaru SA, Nicoara M, Robea MA, Solcan C, Faggio C (2020). Toxicity of Deltamethrin to Zebrafish Gonads Revealed by Cellular Biomarkers. Journal of Marine Science and Engineering. **8**(2): 73. doi:10.3390/jmse8020073

36 Piferrer F, Beaumont A, Falguière JC, Flajšhans M, Haffray P, Colombo L (2009). Polyploid fish and shellfish: Production, biology and applications to aquaculture for performance improvement and genetic containment. Aquaculture. **293**(3): 125–156. doi:10.1016/j.aquaculture.2009.04.036

37 Piferrer F, Donaldson EM (1992). The comparative effectiveness of the natural and a synthetic estrogen for the direct feminization of chinook salmon *(Oncorhynchus tshawytscha)*. Aquaculture. **106**(2): 183–193. doi:10.1016/0044-8486(92)90202-V

38 Pojana G, Gomiero A, Jonkers N, Marcomini A (2007). Natural and synthetic endocrine disrupting compounds (EDCs) in water, sediment and biota of a coastal lagoon. Environment International. **33**(7): 929–936. doi:10.1016/j.envint.2007.05.003

39 Reading BJ, Sullivan CV, Schilling J (2017). Vitellogenesis in Fishes. In: Reference Module in Life Sciences. Elsevier. 635–646. doi:10.1016/B978-0-12-809633-8.03076-4

40 Rose J, Holbech H, Lindholst C, Nørum U, Povlsen A, Korsgaard B et al. (2002). Vitellogenin induction by 17β-estradiol and 17α-ethinylestradiol in male zebrafish *(Danio rerio)*. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology. **131**(4): 531–539. doi:10.1016/S1532-0456(02)00035-2

41 Santos D, Luzio A, Coimbra AM (2017). Zebrafish sex differentiation and gonad development: A review on the impact of environmental factors. Aquatic Toxicology. **191**: 141–163. doi:10.1016/j.aquatox.2017.08.005

42 Scala-Benuzzi ML, Takara EA, Alderete M, Soler-Illia GJAA, Schneider RJ, Raba J, et al. (2018). Ethinylestradiol quantification in drinking water sources using a fluorescent paper based immunosensor. Microchemical Journal. **141**: 287–293. doi:10.1016/j.microc.2018.05.038

43 Shah RB, Tawakkul MA, Khan MA (2008). Comparative Evaluation of Flow for Pharmaceutical Powders and Granules. American Association of Pharmaceutical Scientists. **9**(1): 250–258. doi:10.1208/s12249-008-9046-8

44 Shang EHH, Wu RSS (2004). Aquatic Hypoxia Is a Teratogen and Affects Fish Embryonic Development. Environmental Science & Technology. **38**(18): 4763–4767. doi:10.1021/es0496423

45 Shved N, Berishvili G, Baroiller JF, Segner H, Reinecke M (2008). Environmentally Relevant Concentrations of 17α-Ethinylestradiol (EE2) Interfere With the Growth Hormone (GH)/Insulin-Like Growth Factor (IGF)-I System in Developing Bony Fish. Toxicological Sciences. **106**(1): 93–102. doi:10.1093/toxsci/kfn150

46 Skocovska M, Ferencik M, Svoboda M, Svobodova Z (2021). Residues of selected sulfonamides, non-steroidal anti-inflammatory drugs and analgesics-antipyretics in surface water of the Elbe river basin (Czech Republic). Veterinarni Medicina. **66**(5): 208–218. doi: 10.17221/180/2020-VETMED

47 Slanchev K, Stebler J, de la Cueva-Mendez G, Raz E (2005). Development without germ cells: The role of the germ line in zebrafish sex differentiation. Proceedings of the National Academy of Sciences. **102**(11): 4074–4079. doi:10.1073/pnas.0407475102

48 Sun L, Gu L, Tan H, Liu P, Gao G, Tian L et al. (2019). Effects of 17α-ethinylestradiol on caudal fin regeneration in zebrafish larvae. Science of The Total Environment. **653**: 10–22. doi:10.1016/j.scitotenv.2018.10.275

49 Svacinova P, Vranikova B, Dominik M, Elbl J, Pavlokova S, Kubalak R et al. (2019). Comprehensive study of co-processed excipients F- Melts®: Flow, viscoelastic and compacts properties. Powder Technology. **355**: 675–687. doi:10.1016/j.powtec.2019.07.048

50 Sauer P, Stara A, Golovko O, Valentova O, Borik A, Grabic R et al. (2018). Two synthetic progestins and natural progesterone are responsible for most of the progestagenic activities in municipal wastewater treatment plant effluents in the Czech and Slovak republics. Water Research. **137**: 64–71. doi:10.1016/j.watres.2018.02.065

51 Takahashi H (1977). Juvenile Hermaphroditism in the Zebrafish, *Brachydanio rerio*. Hokkaido University: Bulletin of the faculty of fisheries Hokkaido University; 57–65.

52 US EPA, United States Environmental Protection Agency (2000). Methods for measuring the toxicity and bioaccumulation of sediment – associated contaminants with fresh water in vertebrates. Washington, DC, USA: Office of Research and Development Mid-Continent Ecology Division U.S. Environmental Protection Agency, Duluth, Minnesota; Office of Science and Technology, Office of Water, U.S. Environmental Protection Agency, Washington, D.C.; 1–192.

53 Valcarce DG, Vuelta E, Robles V, Herráez MP (2017). Paternal exposure to environmental 17-alpha-ethinylestradiol concentrations modifies testicular transcription, affecting the sperm transcript content and the offspring performance in zebrafish. Aquatic Toxicology. **193**: 18–29. doi:10.1016/j.aquatox.2017.09.025

54 Wang H, Yan T, Tan JTT, Gong Z. A zebrafish vitellogenin gene (vg3) encodes a novel vitellogenin without a phosvitin domain and may represent a primitive vertebrate vitellogenin gene. Gene. 2000; **256**(1): 303–310. doi:10.1016/S0378-1119(00)00376-0

55 Wang S, Zhu Z, He J, Yue X, Pan J, Wang Z (2018). Steroidal and phenolic endocrine disrupting chemicals (EDCs) in surface water of Bahe River, China: Distribution, bioaccumulation, risk assessment and estrogenic effect on *Hemiculter leucisculus*. Environmental Pollution. **243**: 103–114. doi:10.1016/j.envpol.2018.08.063

56 Xu H, Yang J, Wang Y, Jiang Q, Chen H, Song H (2008). Exposure to 17α-ethynylestradiol impairs reproductive functions of both male and female zebrafish *(Danio rerio)*. Aquatic Toxicology. **88**(1): 1–8. doi:10.1016/j.aquatox.2008.01.020

57 Zhang X, Gao Y, Li Q, Li G, Guo Q, Yan C (2011). Estrogenic Compounds and Estrogenicity in Surface Water, Sediments, and Organisms from Yundang Lagoon in Xiamen, China. Arch Environ Contam Toxicol. **61**(1): 93–100. doi:10.1007/s00244-010-9588-0