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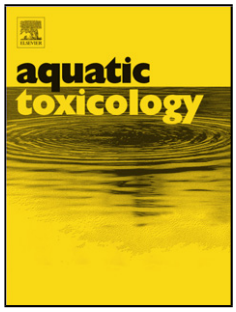

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Estrogen sensitive liver transgenic zebrafish (*Danio rerio*) line (*Tg(vtg1:mCherry)*) suitable for the direct detection of estrogenicity in environmental samples

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Highlights

However several transgenic fish lines have been developed in the last few years for studying and monitoring estrogenic substances, the transgenic zebrafish line (Tg(vtg1:mCherry)) we present here might be a useful tool in endocrine disruptor (EDC) research. The paper describes the following:

- The established transgenic zebrafish line Tg(vtg1:mCherry) expresses the reporter (mCherry) under the control of the promoter of the zebrafish vitellogenin1 gene.
- The incorporated 3357 bp promoter region carries 26 natural ERE sequences and is the longest natural promoter region among vitellogenin transgenic lines,
- The endogenous vitellogenin and the reporter are produced only in the left hepatic lobe of 5 dpf zebrafish embryos and show similar spatial expression pattern.

- Tg(vtg1:mCherry) embryos can detect 17-ß-estradiol from a concentration of 100 ng/L, 17-α-ethynilestradiol from 1 ng/L, zearalenone from 100 ng/L and bisphenol-A from 1 mg/L.
- Tg(vtg1:mCherry) adults appeared to be more sensitive, with detectable transgene activity from 5 ng/L 17-ß-estradiol concentration.
- Tg(vtg1:mCherry) was also suitable for the direct measurement of estrogenicity in wastewater samples without sample extraction.

Abstract

Environmental estrogens are a serious concern worldwide due to their ubiquity and adverse ecotoxicological and health effects. Chemical structure of these substances is highly diverse, therefore estrogenicity cannot be predicted on the basis of molecular structure. Furthermore, estimation of estrogenicity of environmental samples based on chemical analytics of suspects is difficult given the complex interaction of chemicals and the impact on estrogenicity. The full estrogenic impact of an environmental sample can thus only be revealed by a series of sensitive *in vitro* and *in vivo* ecotoxicological tests. Herein we describe a *vitellogenin* reporter transgenic zebrafish line (*Tg(vtg1:mCherry*)) that enables the detection of estrogenicity in the environmentally relevant, low concentration ranges in embryonic tests that are in accordance with 3Rs and relevant animal welfare regulations.

The transgene construct used for the development of *Tg(vtg1:mCherry)* carried a long (3.4 kbp) natural *vitellogenin-1* promoter sequence with a high number of ERE sites.

A test protocol was developed based on our finding that the endogenous *vitellogenin* and the reporter show similar spatial expression pattern and both endogenous and *vitellogenin* reporter is only produced in the left hepatic lobe of 5 dpf zebrafish embryos.

Seven generations of *Tg(vtg1:mCherry)* have been established, and the estrogen responsiveness was tested with different estrogenic substances and wastewater samples. Embryos were exposed from 3 to 5 days post fertilization (dpf). Fluorescence in embryos could be detected upon treatment with 17-ß-estradiol from a concentration of 100 ng/L, 17-α-ethynilestradiol from 1 ng/L, zearalenone from 100 ng/L and bisphenol-A from 1 mg/L. In the adult stage transgene activity appeared to be more sensitive to estrogen treatment, with detectable transgene activity from 5 ng/L 17-ß-estradiol concentration. The transgenic line *Tg(vtg1:mCherry)* was also suitable for the direct measurement of estrogenicity in wastewater

samples without sample extraction. The detection of estrogenic activity using the reporter line was confirmed by the bioluminescent yeast estrogen screen.

Keywords: transgenic, *vitellogenin*, endocrine disrupting chemicals, *in vivo*, YES

1 Introduction

Environmental estrogens are widely present in the aquatic environment. Due to their incomplete breakdown parent compounds and derivatives are detected in the effluents of wastewater treatment plants (WWTPs) and surface waters in pg/L to μg/L concentrations (Wright-Walters and Volz, 2009). Exposure to environmental estrogens may have serious consequences for wildlife populations including feminization of males, altered sex ratios, reduced fertility, other gonadal and reproductive abnormalities, immune system and cardiac disorders, or development of tumors (Sumpter 2005, Mills and Chichester 2005, Kavlock et al., 1996)

Chemical structure of estrogenic substances is highly diverse, therefore estrogenicity cannot be predicted solely on the basis of molecular structure. Furthermore, many components in environmental samples and their combined effects are unknown and hence deduction of estrogenic effects from chemical analysis and detection of know estrogenic compounds could be difficult. The full estrogenic impact of an environmental sample can thus only be revealed by a complex series of ecotoxicological tests on *in vitro* and *in vivo* model systems. Screening estrogenic substances in aquatic samples has become a major focus for researchers and regulatory authorities in the last two decades, however, due to the high cost and the lack of infrastructure these tests are still not part of routine water quality programs in most countries (WHO 2012).

The majority of screening protocols and guidelines proposed by OECD (revised in 2003) is based upon the measurement of estrogen receptor (ER) activation in *in vitro* model systems (e.g. Competitive Estrogen Receptor Binding Assay, OECD-TG-455, 457, 493) (OECD 2012) or *in vivo* animal models (such as fish), based on the protein level of estrogen sensitive genes (*estrogen receptor* (*ER*) and *vitellogenin* (*vtg*)) (OECD-TG-229 and 230) (OECD 2012) as biomarker. The gene product is usually measured by molecular biological tools and involves the sacrifice of the animal model. Most of these tests are using fish from protected life stages. As animal protection laws are becoming stricter, there is an increasing demand for alternative

test systems by which the number and suffering of experimental animals can be minimized or replaced. It is also possible to measure VTG level in the mucus of the epidermis with non-invasive procedures (Allner et al. 2016), however only in later developmental stages.
Zebrafish (*Danio rerio*) can be successfully used for investigating the mode of action of estrogenic substances and their reproductive and developmental effects (Segner, 2009). Toxicity including organ, tissue and cellular phenotypes can be monitored in the transparent, *ex utero* developing embryo. In addition, embryos of zebrafish up to the free feeding age at 5 days post fertilization (5 dpf) are considered as non-protected life stages by the EU Directive 2010/63/EU and relevant national directives and hence represent replacement method for testing of (adult) animals (Strähle et al. 2012).
Several zebrafish lines have been developed recently for the detection of estrogen toxicity. Most of these express reporters under the control of estrogen response elements (EREs) and are used to visualize the response of estrogen sensitive tissues, estrogen receptor subtype specific response and developmental dynamics of estrogen sensitive tissues (Legler et al., 2000, Gorelick and Halpern 2011, Lee et al., 2012).
Biosensor fish lines (medaka and zebrafish) and transient systems have also been developed with tissue specific promoters of biomarker genes (*cyp19a1b, vitellogenin, choriogenin H*). The most popular biomarker gene in the detection of estrogenicity is *vitellogenin*. (Wallace and Jared, 1968; Lazier and McKay, 1993; Tyler et al., 1999). Vitellogenin transgenic medaka (Zeng et al. 2005), zebrafish (*ere:zvtg1-gfp*) lines (Chen et al, 2010) and transient systems (Kim et al., 2009, Lee et al., 2012) have been generated previously, however, sensitivity was not tested for the detection of environmentally relevant estrogen concentrations. The *cyp19a1b-gfp transgenic* zebrafish line was shown to detect environmental estrogenicity in embryonic stages (Tong et al., 2009, Brion et al., 2012, Petersen et al., 2013, Fetter et al., 2014). However the fluorescent area represents a relatively small part of the brain and the gene is expressed at a basal level in controls too. Recently an estrogen sensitive choriogenin-H transgenic medaka line, sensitive to the lowest observable estrogen concentration causing physiological effects in medaka (15 ng/L 17-α-ethynilestradiol, EE2) has also been developed (Spirhanzlova et al., 2016). Besides these, zebrafish cell based *in vivo* assays are used and are capable of detecting well-known estrogenic ligands, but recent study showed that *in vivo* systems show better sensitivity than *in vitro* assays (Sonavane et al., 2016).

Routine estrogenicity assays demand the development of new, sensitive biosensor models, in which endogenous gene function is in accordance with fluorescent activity.

Environmental toxicology also needs models for detecting environmentally relevant estrogen concentrations and for studying the effects of environmental estrogens extensively. In this report our aim was to develop and characterize a sensitive *vitellogenin* reporter transgenic zebrafish line (*Tg(vtg1:mCherry)*) and to develop a test protocol for efficient detection of estrogenicity *in vivo* even in environmental samples.

2 Materials and methods

2.1 Fish maintenance

All experiments were performed on a laboratory-bred strain (AB) of zebrafish (*Danio rerio).* Prior to treatments fish were maintained in a special zebrafish recirculation system (ZebTEC, Tecniplast S.p.a.) under standard laboratory conditions (at 25.5±0.5°C with 14 hour light-10 hour dark cycle, oxygen level: ≥80% of saturation, pH: 7±0.2 conductivity 525±50 μS/m - all these parameters were automatically controlled by the ZebTEC system).

Adults were fed twice a day with complete fish food (zebrafish basic food, Special Diets Services /SDS/) supplemented with freshly hatched live *Artemia nauplii* twice a week. Larvae after the free swimming age were fed three times a day with fish food, supplemented with *Artemia* from 10 dpf (days post fertilization).

Fish were placed in breeding tanks (Tecniplast S.p.a.) in the afternoon the day before the experiment and spawning was triggered by the onset of the light cycle on the next morning. One hour after the onset of the light cycle eggs were harvested and normally developing embryos were selected under a microscope.

All embryo handling, injections and treatments of eggs and embryos were carried out in "system water" from the recirculation system.

Experiments were carried out in laboratories possessing required licences for animal experimentation (SF/6-8/2014, SF/6-6/2014, XIV-I-001/2303-4/2012)

2.2 Establishment of the vtg1-mCherry transgene construct

The putative transcription start site was identified upstream the *vtg-1* coding sequence by PROSCAN 1.7 (Prestridge, 1995). Then the upstream sequence was screened for elements needed for the estrogen sensitivity of the construct (EREs - Estrogen Response Elements) by DRAGON ERE Finder (Bajic et al., 2003), ALGEN PROMO (Messeguer et al., 2002, Farré

et al., 2003) and MATINSPECTOR (GenoMatix) (Quandt et al., 1995, Cartharius et al., 2005) softwares and manual sequence analysis.

Primers were designed for the amplification of a 3357 bp promoter sequence (vtg1 F: 5‘-TTTATCTTTCCCATGATGACAGCA-3‘, vtg1 R: 5‘-GGCTGGTGGTTTGTGAAGAAC-3‘), fragments for the gene construct were amplified and recombination sites (attachment sites, attB) (attB3: 5‘- GGGGACAAGTTTGTATAATAAAGTAGGCT-3‘, attB5: 5‘-ACCCAACTTTTGTATACAAAGTGGTCCCC-3‘) were incorporated to the 5’ ends of the fragments by PCR (10 x buffer, 0.198 pM forward and reverse primer, 25 nM dNTP, 4 nM $MgSO_4$, 1.00 µg DNA, 1 µL enzyme mix Taq DNA Polymerase (recombinant) (Thermo Scintific) in 25 µL final volume), reactions were run in an Applied Biosystems 2720 Thermal Cycler (94 ºC 2 min; 3x 94 ºC 1 min, 55 ºC 2 min, 70 ºC 9 min; 35x 94 ºC 2 min, 56 ºC 30 sec, 70 ºC 9 min; 70 ºC 5 min). The fragment harboring the putative promoter region upstream from the vtg-1 transcription start site was amplified from zebrafish genomic DNA, while for the amplification of the fluorescent protein encoding sequence, a pCS2 mCherry plasmid (modified Addgene plasmid) was used (mCherry F: 5‘-GCCGCCACCATGGTGAGCAAGGGCGA-3‘, mCherry R: 5‘-AGATCTCCGCGAATTAAAAAACCTCCCAC-3‘) and attBs sites were incorporated (attB1: 5‘-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3‘, attB2: 5‘-TACCCAGCTTTCTTGTACAAAGTGGTCCCC-3‘) (2x PCR buffer (containing 25mM $MgCl_2$), 0.25 nM dNTP, 0.5 pM forward and reverse primer, 1 µg DNA, 0.3 µL Taq polymerase; 97 ºC 2 min; 35x 94 ºC 20 sec, 60 ºC 20 sec, 72 ºC 3 min; 25x 94 ºC 20 sec, 62 ºC 20 sec, 72 ºC 3 min).

PCR products containing different recombination sites (*vtg-1* promoter – *attB3, attB5*, mCherryt – *attB1*, *attB2*) were cloned into donor plasmids (pDONR221-P1P2 and pDONR221-335) by Gateway recombination (*BP* reaction, Invitrogen, Thermo Fisher Sientific). Sequences were then cloned into the final vector (pSP17.2BSSPE-R3-R5-RFA-Venus Tol2LR) (Gehrig et al., 2009) harboring *Tol2* arms by *LR* cloning (Invitrogen, Thermo Fisher Sientific) (Roure, 2007). Plasmids were transformed and propagated in XL10 GOLD *Escherichia coli* competent cells (Agilent) and were pre-screened by colony PCR with M13 primers (M13F: TGTAAAACGACGGCCAGT, M13R: CAGGAAACAGCTATGAC) as recombination sites in donor plasmids are bonded by M13 primer sites (0.16 pM M13 forward and reverse primer, 2 mM $MgCl_2$, 2 mM dNTP, 1 µL Taq polymerase in 25 µL final volume; 3x 95 ºC 2 min, 45 ºC 1 min, 72 ºC 3 min; 41x 95 ºC 30 sec, 45 ºC 30 sec, 72 ºC 3 min; 72 ºC 5 min). Incorporation of the sequences was checked in purified plasmids by restriction digestion.

2.3 Establishment of the Tg(vtg1:mCherry) transgenic line

For efficient transposition, the construct was linearized. Transposase *mRNA* was synthetized from the encoding pCS2 plasmid by mMACHINE Kit (Ambion, Thermo Fisher Sientific). The construct (25 ng) was co-injected with *transposase mRNA* (25 ng) into the yolk of embryos by microinjection.

Transient expression of the reporter gene was examined in 5 dpf embryos following 100 ng/L 17-ß-estradiol (E2) treatment under a fluorescent microscope (Leica M205 FA, Leica DFC 425C camera, LAS V3.8 softer) using RFP filter.

Untreated embryos injected with the reporter gene construct were raised until fertility and crossed to wild-type zebrafish. The gene construct transmitting (so-called P0) founders were selected by fluorescent microscopy following 100 ng/L EE2 treatment (5 days of exposure, 30-70 embryos/treatment) and mCherry specific PCR of estrogen treated offspring (mCherry check F: 5'-GGGCGAGATCAAGCAGAGG-3', mCherry check R: 5'-CACAACGAGGACTACACCATCG-3', 10x TaqMan buffer, 0.4752 pM forward and reverse primer, 2.5 mM dNTP, 2 mM $MgCl_2$, 1 μL Taq polymerase; 95 ^oC 1 min; 35x 95 ^oC 15 sec, 56 ^oC 20 sec, 72 ^oC 1 min; 72 ^oC 3 min).

Offspring of founders found positive by the PCR were raised and used to establish the F1 generation of the *Tg(vtg1:mCherry)* line. F2 was generated by mating 6 selected F1 individuals (3 males and 3 females) with wild-type fish. F2 were then crossed again with wild-type individuals. F4 generation was established by intra- and intergenerational crossing of F2 and F3 "transgene carriers". The above mentioned microscopic examination was carried out in every generation while prior to the establishment of F4, PCR-based selection was also carried out to generate homozygous individuals. Homozygosity was confirmed by crossing these selected fish to wildtype, non-transgenic fish (test cross). Further generations (F5, F6 and F7) were generated by mating selected individuals of the previous generation *inter se.*

2.4 Evaluation of the fluorescent response in heterozygous and homozygous embryos

Homozygous individuals were crossed *inter se* and to wild-type fish to generate homozygous and heterozygous embryos. Larvae (from 3 dpf) were exposed to 100 μg/L E2 in 5 replicates containing 20 embryos / replicate, in static conditions. Embryos were examined under the microscope at 5 dpf from left lateral view. Number of fluorescent embryos and fluorescent signal were determined for both groups and results were compared to each other by the Mann-Whitney test.

2.5 Characterization of mCherry transcription

For the characterization of *mCherry* expression 500 F3 embryos were exposed to 200 ng/L 17-α-ethinyl-estradiol (EE2) from 0 dpf, and studied daily under a fluorescent microscope at different magnifications. Exposure solution was changed daily.

2.6 Testing mCherry inducibility by estrogenic substances

In F3 and F4 generations, fluorescent protein induction was tested with several concentrations of other estrogenic compounds. As it was found that the fluorescent signal was induced from 2-3 dpf and was strong from 3 dpf the exposure period was changed from 5 days (0 dpf-5 dpf) to 2 days (from 3 to 5dpf). The exposure was carried out in three replicates with 20 embryos/concentrations and solution was changed daily.

F4 embryos were exposed to E2 (200; 100; 50; 25; 12.5; 6.25; 0.1 µg/L), EE2 (200; 100; 50; 25; 12.5; 6.25; 3.125 ng/L), zearalenone (ZEA) (500; 250; 100; 50; 10; 5; 1; 0.1 µg/L), bisphenol-A (BPA) (20; 10; 8; 6; 5; 2.5; 1; 0.1; 0.01 mg/L), nonylphenol (NP) (300, 150, 75 µg/L) and atrazine (ATR) (10000; 1000; 100; 50; 25; 25; 12.5; 6.25 µg/L). All agents were purchased from Sigma-Aldrich. Treatments were carried out in petri-dishes, at 27.5 °C. The effects of solvents used for the stock solutions (ethanol (Reanal) for E2, EE2, ZEA and BPA and DMSO (Sigma-Aldrich) for ATR) were tested at the solvent concentration of the highest test concentration.

2.7 Imaging and analysis

Prior to microscopic analysis, embryos were anaesthetized in 0.02% MS-222 (Tricaine-methane-sulphonate, Sigma-Aldrich) solution, then larvae were oriented (to the left side) in 0.5% methyl-cellulose. Bright field and fluorescent images were captured from all embryos in 30x magnification (exposure time: 1s, Leica M205 FA, Leica DFC 425C camera, LAS V3.8 software).

Signal in the red range of the RGB (Red, Green, Blue) color range was evaluated by ImageJ software (Schneider et al., 2012). An elliptical area of the same size was selected on all images and moved to the area of the liver, then the signal strength and the size of the affected area was determined. From the two values an integrated value (Integrated density) was generated and the level of induction was calculated on the basis of comparison with controls. The estrogenicity (so-called relative estrogenic potency) of tested chemicals was determined by comparing the $EC_{50\ \text{integrated density}}$of compounds to the $EC_{50\ \text{integrated density}}$of E2.

2.8 Testing the mCherry induction in males

Adult (3-month-old) males from the F1 generation were exposed to 5, 10, 50 and 100 ng/L E2 for 4 days. Treatments were carried out in two replicates in 3 liter tanks containing 15 individuals each, in semi-static conditions (water exchange was carried out daily). Induction of the fluorescent signal was observed under a fluorescent microscope *in vivo* (through the body). Individuals were anaesthetized in 0.02% MS-222 (Sigma-Aldrich) prior to the examination.

2.9 Vitellogenin „whole mount" in situ hybridization

Expression of the endogenous *vtg* gene was examined in wild-type and transgenic embryos. Wild type embryos (50) and transgenic embryos (20) were exposed to 200 ng/L EE2 from 0 to 5 dpf. Exposure solution was changed daily.

In situ hybridization was carried out with a 545 bp probe designed for the *vtg mRNA* according to the protocol developed for zebrafish embryos by Thisse and Thisse (2008). Total RNA of estrogen treated embryos was reverse transcribed (Omniscript RT kit, Qiagen) and the probe sequence was amplified by PCR (vtg1 pr 6F: 5'-GTACTTGGCACACCATAGCG-3', vtg1 pr 6R: 5'-TCAAGGACCTCTGAGATGGC-3') (10x Finnzym buffer (containing $MgCl_2$, 0.198 pM forward and reverse primer, 0.5 mM dNTP, 0.12 µL Taq polymerase; 95 ^oC 1 min; 40x 95 ^oC 15 sec, 56 ^oC 40 sec, 72 ^oC 4 min; 72 ^oC 3 min). Purified PCR product was ligated to pCRII plasmid (Dual Promoter TA cloning Kit, Invitrogen, Thermo Fisher Sientific), plasmids were propagated in TOP10 *E. coli* competent cells (Thermo Fisher Sientific) then colonies were selected by colony PCR. Carrier plasmids were linearized and DIG-labelled antisense RNA probe was generated by *in vitro* transcription (DIG RNA Labeling Kit SP6/T7, Roche).

2.10 Testing wastewater samples

During the summer season, five effluent samples were collected from the activated sludge containing phase in a wastewater treatment plant in Gödöllő (Hungary), that uses UTB's Cyclator technology to treat local municipal wastewater (roughly 6500 PE).

2.10.1 Zebrafish wastewater exposure

Zebrafish embryos (F7) were exposed from 3 to 5 dpf directly to wastewater samples (100%) and dilutions (25; 37.5 and 50%) in three replicates (20 embryo/replicate), in static conditions. 100 ng/L E2 was used as positive control and system water as negative control. Treatments were carried out in petri-dishes, at 27.5 °C. Microscopy and evaluation was carried out as above.

2.10.2 Result verification by the yeast estrogen screening assay (BLYES)

For the BLYES test, wastewater samples were filtered through a membrane filter (0.45 µm). Samples were diluted with system water to 25; 37.5 and 50% sample final concentration and applied directly in the assays.

Final growth 1X yeast minimal medium (1X YMM $^{leu-,\ ura-}$) was applied for growing *Saccharomyces cerevisiae* BLYES strain (Routledge and Sumpter, 1996; Sanseverino et al., 2005) at 30 ºC and 170 rpm. The overnight culture of BLYES was centrifuged (4000 rpm, 4°C, 20 min) and the optical density was set with final growth 2X YMM $^{(leu-,\ ura-)}$ to OD_{600} value of 2.0. The measurement was carried out in 3 parallels using a 96-well microplate (Greiner Bio-one Gmbh, Germany). The serial dilution of 17-ß-estradiol served as positive control. The stock solution was made in system water. Negative controls (final growth 2X YMM and yeast cells) and solvent controls (final growth 2X YMM, yeast cells and system water) were also applied.

100 µl diluted wastewater samples were transferred to the wells of the microplate and 100 µl of the yeast cells were also added. Bioluminescence was measured after 5 hours of incubation at 30ºC by VictorX Multilabel Plate Reader (Perkin Elmer Inc.).

Data analysis involved the determination of the EC_{50} value of the positive control and the bioluminescence intensification values (Froehner et al., 2002) of the positive control and the wastewater sample.

$$Bioluminescence\ intensification\ (\%) = -[\frac{(C - S)}{C} * 100]$$

where

C is the mean bioluminescence (cps) value of the negative control

S is the mean bioluminescence (cps) value of the tested samples.

Samples were considered to be estrogenic if the bioluminescence intensification value was ≥ 20%.

2.11 Dose-response diagrams, statistical analysis

For the evaluation of fluorescence by image analysis and integrated density quantification, outliers were removed by the ROUT method (Motulsky and Brown 2006) and dose-response analysis was carried out following the log transformation and normalization of results. Sigmoidal dose-response diagrams were generated by non-linear regression and plotted with GraphPad Prism 4.0 (graphpad.com). EC_{10} and EC_{90} values were calculated according to the recommendations of GraphPad ($EC_F = (F/100\text{-}F)^{1/H} \times EC_{50}$) (F=effective concentration %, H=slope of the dose-response curve).

3 Results and discussion

3.1 Establishment of an estrogen sensitive biomarker zebrafish line

To develop a biomarker transgenic line, a *vitellogenin* (*vtg-1*) promoter region - anticipated to represent a sensitive promoter for the estrogen-dependent expression of the reporter (mCherry) - was selected on the basis of bioinformatical analysis. First, the putative transcription start site (TSS) was identified, then the region was examined for potential ERE sequences upstream to the TSS. On the 3357 bp promoter region, which is located on zebrafish chromosome 22 (positive strand, 24710551-24713908, GRCz10/danRer10, Sept. 2014, UCSC Genome Browser, previously Zv9/danRer7, July 2010, UCSC Genome Browser), 10 potential ERE sites were found with ALGEN PROMO, 5 half ERE sites with MATINSPECTOR and one full, previously undescribed sequence (5‘-CAGGGCAACCTAACCCA -3‘) with DRAGON ERE software. Additionally, based on sequences described in the literature (Klinge et al., 1997) 10 half ERE sites were found. The fragment carries 26 ERE sequences, while on the 1700 bp promoter sequence of a previously developed *vtg* transgenic zebrafish line (*ere:zvtg1-gfp*) (Chen et al., 2010), only 12 ERE sequences can be identified. These regions overlap, so the *vtg1-mCherry* construct harbors 17, while the *ere-zvtg1:gfp* line carries 8 ERE regions altogether (Figure 1). The full, previously undescribed ERE sequence identified on the vtg1 promoter sequence in the *vtg1-mCherry* transgene construct is part of the *ere:zvtg1-gfp* construct. The *Tg(vtg1:mCherry)* transgene construct was anticipated to mimic the expression of the native vtg gene, as it carries the longest natural promoter region used among vtg transgenic laboratory model fish lines and lacks artificial ERE sequences.

To test the expression of the construct (*vtg1:mCherry*), transient expression was analyzed in embryos following five days of exposure to 17-ß-estradiol under a fluorescent microscope.

The fluorescent signal appeared in 20% of embryos injected with the transgene construct. In addition to the fluorescence in the liver (Figure 2), the majority of embryos expressed strong transient fluorescent signal in the yolk, while in some embryos (<1%) weak expression was observed in the eye and kidney. Thus the functionality of the *vtg1::mCherry* construct was proven. Founders of the construct were identified and used for generation of a stable transgenic line.

In F1 embryos, fluorescent protein expression disappeared from the kidney and eye region, while it was still detected in the yolk in response to estrogen, suggesting that the signal is a result of transgene activity. Estrogen inducible fluorescent signal still appeared in the yolk of embryos in all subsequent generations, with a percentage of 5-100%. From F3, 3 distinct lines were generated from the offspring of 3 founder pairs. Although fluorescence in the yolk was relatively strong in case of one line, its offspring proved to be highly sensitive to estrogens. The other two lines showed no unspecific fluorescent activity. Fish were maintained as heterozygotes for the transgene until F3. In F3 and F4, homozygous individuals were generated by crossing siblings and verified by test crosses with non-transgenic wild type fish. From F4 the line has been maintained in homozygous form.

3.2 Comparison of the fluorescent signal in homozygous and heterozygous embryos of the (Tg(vtg1:mCherry)) line

It was supposed that fluorescent signal intensity in homozygous and heterozygous embryos are different, as homozygous individuals carry two transgene alleles. So homozygous and heterozygous larvae were compared on the basis of response to 100 μg/L E2. Significant difference was found in the number of fluorescent fish (P=0.0079), and also in integrated density (P=0,0110) (Figure 3), therefore from F3, only homozygous embryos were used in the experiments.

3.3 Pretesting the concentration dependency of the fluorescent signal in (Tg(vtg1:mCherry) F3 embryos

Concentration dependency of the fluorescent signal was first examined in F3 embryos following exposures to estrogenic substances from 3 to 5 dpf. Due to inhomogeneity, results showed a large variation. Exposures were carried out with E2, EE2 and ZEA, but significant signal intensity differences were only observed in case of EE2 (results not shown). There was no fluorescent signal in the solvent control in either case. All of the three lines proved to be suitable for the detection of estrogenicity, however subsequent experiments were performed on the line showing the highest level of induction.

3.4 Adult male (Tg(vtg1:mCherry)) exposure to 17-β-estradiol

Vtg is normally expressed in a high level in mature females, while in males the level is very low (Wallace and Jared, 1968; Lazier and McKay, 1993; Tyler et al., 1996, 1999a, b; Baumann et al., 2013). As in embryos, vitellogenin production in males can be triggered by exposure to estrogens too, and the effect exhibits concentration-dependency. In the liver of untreated females mCherry fluorescence can already be observed through the pigmented skin, while in males the fluorescent signal is undetectable (Figure 4) even after autopsy.

Inducibility of the transgene was demonstrated by E2 treatment of mature males. The reporter gene appeared to show a concentration-dependent response. *Tg(vtg1:mCherry)* males could detect E2 from 5 ng/L (Figure 5), that is lower than the E2 concentration detectable by the *ere:zvtg1-gfp* line (Chen et al., 2010). Moreover, fluorescence appeared at an earlier time point (at the second day of exposure) at higher concentrations (100 ng/L). Further concentrations were not tested on adults and hence, no EC_{50} concentrations were determined for adult fish. By the end of the exposure period, fluorescent activity was detectable in the liver of all treated individuals. *Tg(vtg1:mCherry)* is likely to detect lower concentrations too in longer exposure periods, as the *ere:zvtg1-gfp* line (Chen et al., 2010) gave a response to the lowest concentration only after 7 days of exposure.

It seems that mature *vtg1:mCherry* males are probably more sensitive to E2 than embryos. On the basis of literature, estrogen sensitivity of zebrafish is gradually increasing with the development of gonads, and the pattern of receptor paralogs and their expression also changes. In the liver of embryos, only the ß2 receptor is present. The liver of adult males express all three estrogen receptors (α, ß1, ß2) (Bardet et al., 2002) which could impact on the sensitivity and compound specificity if compared to embryos.

3.5 Examination of the expression of the fluorescent signal at different developmental stages

To establish an appropriate test protocol for the *Tg(vtg1:mCherry)* line, *mCherry* transcription was characterized in F3 homozygous embryos exposed to 17-α-ethinyl-estradiol (EE2).

Initially embryo exposures were carried out from 0 to 5 dpf. However liver development in zebrafish starts at 6 hpf, and the functional organ is formed at 50 hpf (Tao and Peng, 2009), results proved that *vitellogenin* production begins in later stages. The fluorescent signal was induced from 2-3 dpf and was strong from 3 dpf. The size of the fluorescent area increased gradually along with liver development and reached the maximum at 5 dpf in the

experimental period (Figure 6). This is in accordance with previous findings that the liver of the zebrafish embryo is able to produce *vtg* from 2-3 dpf (Chen et al., 2010). Therefore, the exposure period was changed from 5 days (0 dpf-5 dpf) to 2 days (from 3 to 5dpf) in further experiments.

3.6 Characterisation of endogenous vitellogenin expression in wild-type and transgenic embryos by Vtg in situ hybridisation

Regardless of the test compound or concentration, the reporter activity in the liver of embryos varied in all generations and lines exposed. The size of the expression area and strength of the fluorescent signal showed no correlation (data not shown). Therefore endogenous vitellogenin expression was characterized in wild-type and transgenic embryos to show if variability is also found for the native *vtg* expression.

Vitellogenin RNA in situ hybridization showed that in case of both wild-type and transgenic embryos the gene was not induced in all individuals, and embryos expressing *vtg* exhibit variability in the area of expression (Figure 7).

In situ hybridization images of transgenic embryos were compared to fluorescent images captured before fixation. Results clearly showed that the differences in endogenous *vtg* production overlap with the differences detected in *mCherry* expression (Figure 8).

Interestingly, although 5 dpf zebrafish embryos have developed both liver lobes (Tao and Peng, 2009; Chu and Sadler, 2009), the production of *vtg* at this stage was consistently observed only in the left lobe for both endogenous *vtg* and transgene, further confirming that the transgene is regulated similar to the endogenous *vtg* gene (Figure 9).

The number of fluorescent embryos was found to be substance and dose dependent in the *ere:zvtg1-gfp* (Chen et al., 2010) and the *3pERE-TATA-Gal4ff* (Lee et al., 2012) transgenic zebrafish lines too. This experiment showed that variability in the mCherry signal is associated with variability of the native *vtg* expression, which accounts for high biological variability. On the basis of these a minimum of 20 embryos per concentration in replicated experiments should be exposed to demonstrate concentration-dependent expression of the transgene. The source of variability is not known but could e.g. be associated with individual differences of concentrations at the target site or genetic factors and may be reduced by establishing inbred strains.

3.7 Sensitivity of the Tg(vtg1:mCherry) transgenic line to estrogenic substances

To test the sensitivity of *Tg(vtg1:mCherry)*, dose-response was studied with E2, EE2, ZEA, BPA, NP and ATR. Concentration dependent fluorescent response was detected in case of E2, EE2, ZEA and BPA (Figure 10). The lowest effective concentration (LOEC) of mCherry expression was 0.1 mg/L for E2, 6.25 ng/L for EE2, 0.1 µg/L for ZEA and 1.25 mg/L for BPA. LC values and estrogen equivalents are shown in Figure 11. In case of NP the fluorescent protein was expressed only in a few embryos, in a very small area of the liver. ATR in this developmental stage had no effect on *vtg* levels and there was no fluorescent signal in the solvent control in either case.

It is not easy to compare the sensitivity of the *Tg(vtg1:mCherry)* line to other estrogen detecting transgenic lines, as the exposure protocols and evaluation processes are different. Based on published data, the detectable estrogenic substance concentrations by *Tg(vtg1:mCherry)* embryos are similar to that of *zvtg1-gfp* (Chen et al., 2010), except for ZEA, however in the latter case exposure was carried out from 7 to 20 dpf, when the number of estrogen receptors is higher than at 5 dpf. EC_{50} levels calculated from dose-response curves of the aromatase biosensor line (Brion et al., 2012) are lower than the EC_{50} values of the new transgenic line. The difference in EC values is probably due to the different sensitivity of the genes and the different number and type of estrogen receptors expressed in the two organs.

NP triggered response only in a few embryos of the *Tg(vtg1:mCherry)* line, and the expression was restricted to a very small area of the liver. The *zvtg1-gfp* (Chen et al., 2010) line did not respond to NP, while the aromatase transgenic line (Brion et al., 2012) could detect the estrogenicity of a mixture of 4-nonyl-phenols with an EC_{50} of 406 nM.

On the basis of EC_{50} values of fluorescent protein induction, BPA proved to be approx. 3000x weaker, ZEA was nearly equivalent, while EE2 was an approx. 273x stronger estrogenic substance compared to natural estrogen (Figure 11). On the basis of literature EE2 seems to be about 16.48x more estrogenic than E2 (Rose et al., 2002). The most striking result was the almost similar estrogenicity of E2 and ZEA. According to data described so far the estrogenic effect of ZEA compared to E2 varies in a very large range, and is 5.4-1439x weaker than E2 (Schwartz et al., 2010; Arukwe et al., 1999; Le Guevel and Packdel, 2001; Olsen et al., 2005; Tollefsen et al., 2003). Variance might be due to different model systems and methods, however none have shown similar estrogenicity of these agents. Presumably embryos may have weaker biotransformation of the parent compound, or ZEA is diffusing

easily in the embryo, keeping internal concentrations of the parent compound higher than in an adult animal.

3.8 Testing estrogenicity of wastewater samples in Tg(vtg1:mCherry) embryos

To test if embryos are suitable for screening environmental estrogenicity, F7 *Tg(vtg1:mCherry)* embryos were exposed to undiluted and diluted effluent samples that were taken from the activated sludge containing phase. Embryos showed no fluorescent signal in four (WW1, WW2, WW3 and WW5) wastewater samples, while in the case of WW4, fluorescence was detected in the liver even at the lowest dilution (25%) (Figure 12A). The intensity of the signal increased gradually with concentration. The raw, undiluted sample was probably too concentrated for the fish and caused significant lethality and weaker fluorescence. These results were confirmed by BLYES test. According to the concentration-response curve of the positive control 17-ß-estradiol (data not shown), the EC_{50} value was 1.79 nM. This concentration caused 632% bioluminescence intensification in the BLYES test.

Similarly as observed for embryos the other four wastewater effluents (WW1, WW2, WW3 and WW5) did not induce bioluminescence (Figure 12B). This indicates that the *Tg(vtg1:mCherry)* line is suitable for testing wastewater and environmental samples.

4

5 Conclusions

In the present study we have established and characterized a transgenic zebrafish line (*Tg(vtg1:mCherry)*) that - in the presence of estrogenic substances - produces red fluorescent protein in the liver. The sensitivity was tested with different estrogenic substances in the fourth generation. According to liver development and the first appearance of the fluorescent signal during development, the exposure period was shortened from 5 to 2 days (3 to 5 dpf). Fluorescence in embryos could be detected upon treatment with 17-ß-estradiol from a concentration of 100 ng/L, 17-α-ethynilestradiol from 1 ng/L, zearalenone from 100 ng/L and bisphenol-A from 1 mg/L respectively. Transgene activity appeared to be more sensitive to treatment (E2) in the adult stage, with detectable transgene activity from 5 ng/L concentration.

The *Tg(vtg1:mCherry)* transgenic line could detect estrogenicity in wastewater samples following direct exposure with the same sensitivity as the commonly used yeast estrogen test, BLYES.

Results suggest that sensitivity of the *Tg(vtg1:mCherry)* line is in the range of environmentally relevant concentrations (detected in waters or effluents), and could detect estrogenicity in wastewater. On the basis of these, *Tg(vtg1:mCherry)* with the developed test protocol could be a sufficient tool in assessing environmental estrogenicity in the future. In addition to environmental toxicology tests this transgenic line may help in determining the onset of estrogen synthesis during sexual development and in investigating the molecular background of estrogenic effects and *vitellogenin* induction by morpholino injection or gene knockout. Females could also be subjects of examining androgens where the effect could be evaluated on the basis of the decrease in the strength of the fluorescent signal. Moreover, adverse effects of toxic substances on the liver may also be studied *in vivo*.

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Figure legends:

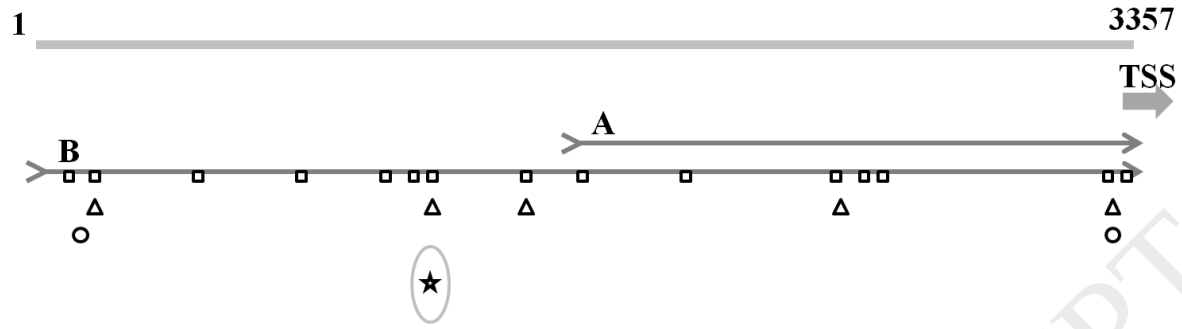

Figure 1. Half and full estrogen responsive elements on the 1700 bp fragment of the *vitellogenin-1* promoter of the *ere:zvtg1-gfp* line (Chen et al., 2010) (A) and on the 3357 bp *vitellogenin-1* promoter fragment of the *Tg(vtg1:mCherry)* line (B). Sites were identified on the basis of literature and by specific softwares (rectangles (sequences described in literature): TGACC (203-207, 1245-1249), CAGCTG (519-524, 1692-1697, 2601-2606), GGTCA (1512-1516, 2448-2452, 3290-3294), CAGTCA (2553-2558), TGACT (2563-2567), triangles (ALGEN PROMO): TGACCTTAA (202-210), TGACCTTAAA (202-211), TTGACCAG (201-208), TTGACCAG (1243-1251), TGACCAGAT (1244-1252), GCACGGTCA (1507-1515), ACGGTCAA (1509-1516), CAAGGGTCA (2443- 2451), AGGGTCAG (2445-2452), ACTTGGTCA (3285-3293), TTGGTCAG (3287-3294), circles (MATINSPECTOR):, TCCAGAGGTTAGGTTAACCTGGC (165-187), ATGACAGGCCAGGTTAACCTAAC (172-194), GGGGCAGGGCAACCTAACCCAAA (2163-2185), ATTAAGGGGCAGGGCAACCTAAC (2168-2190), TATACAGGTTAAAGTGACATTTA (3273-3295), star (DRAGON ERE FINDER): CAGGGCAACCTAACCCA (1240-1257). The only full, newly identified ERE sequence is highlighted with a circle. The putative transcription start site (TSS) is indicated by an arrow.

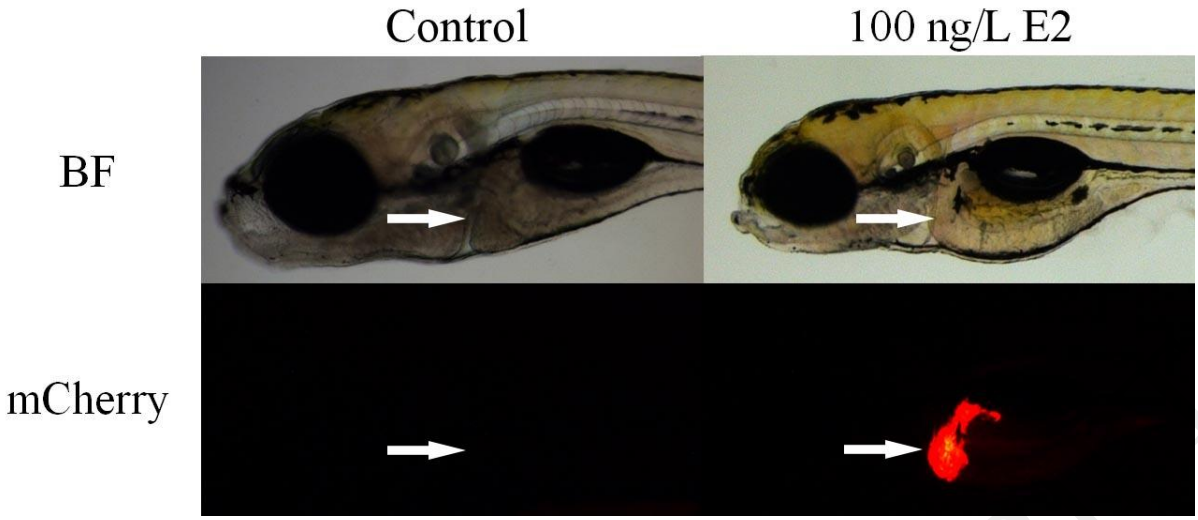

Figure 2. Red fluorescent signal in the liver of injected 5 dpf embryos, following E2 induction (right). In non-treated controls (left) no signal was detected. Liver is indicated by arrows. (BF: bright field, mCherry: red fluorescent filter view, single plain images)

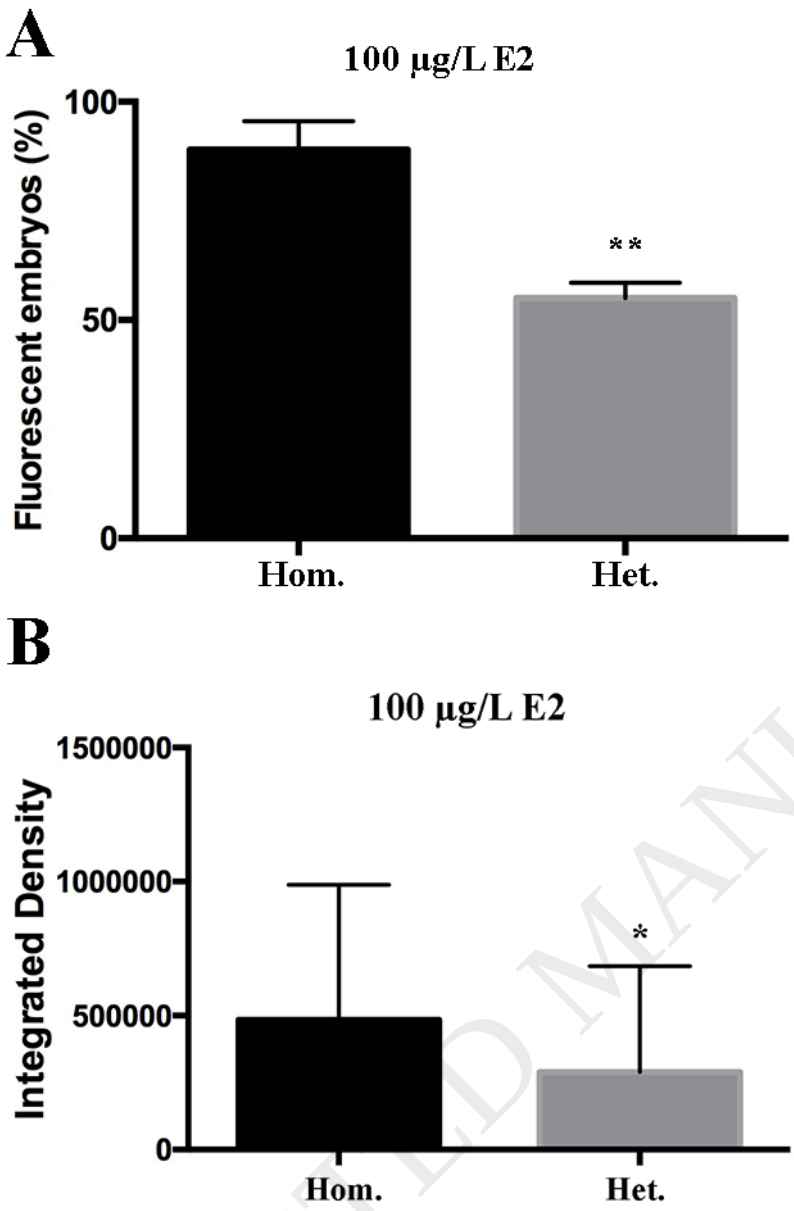

Figure 3. Fluorescence in homozygous and heterozygous embryos were compared under the microscope following exposure to 100 µg/L E2 from 3 to 5 dpf. Significant difference was detected both in the number of fluorescent fish (A) (P=0.0079, Mann-Whitney test) and the integrated density value (B) (P=0.0110, Mann-Whitney test) based on the intensity and spatial distribution of the fluorescent signal.

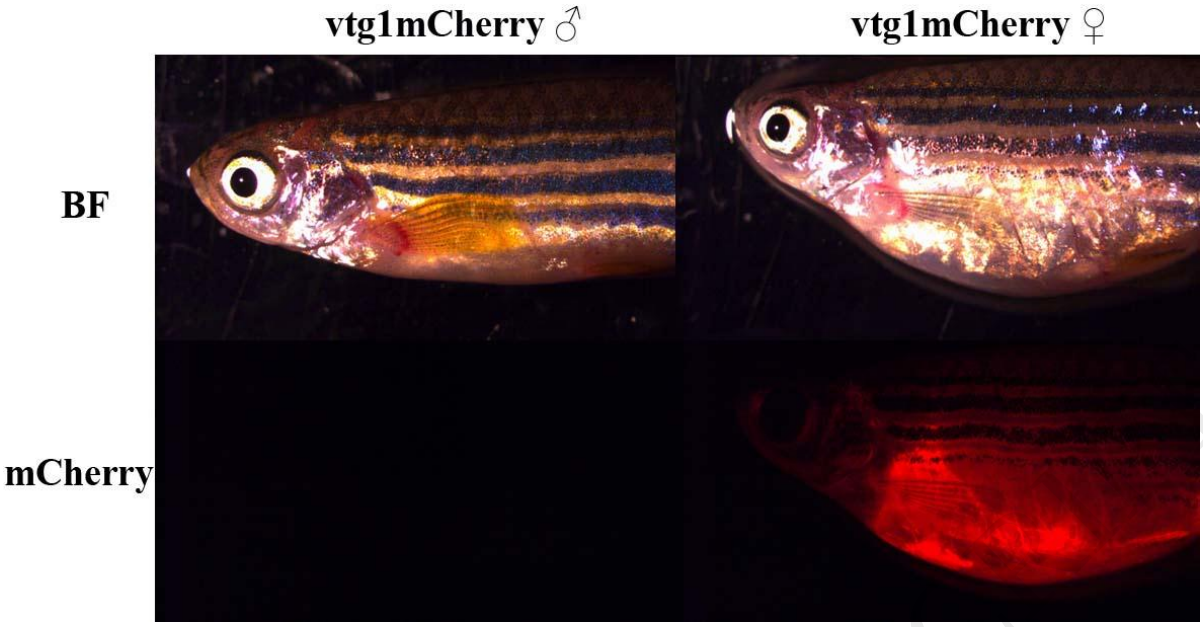

Figure 4. Fluorescence in untreated adult *vtg1:mCherry* transgenic males (9 months old) is undetectable while strong fluorescence of the liver in untreated females is visible even through the pigmented skin (BF: bright field, mCherry: red fluorescent filter view, single plain images).

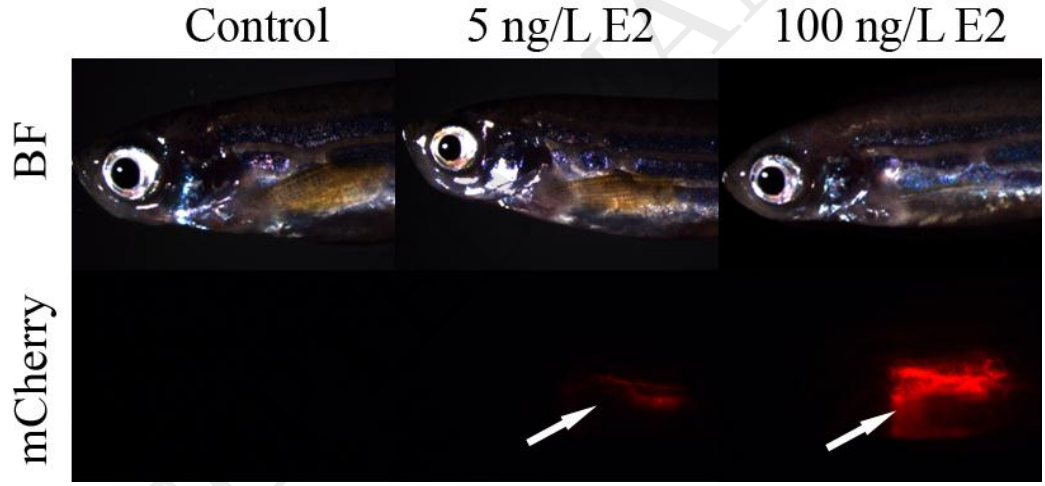

Figure 5. Fluorescent signal in a 9-month-old, untreated control male and 9-month-old 17-ß-estradiol treated males (E2: 17-ß-estradiol, BF: bright field, mCherry: red fluorescent filter view, single plain images, fluorescent signal is shown by arrows*)*

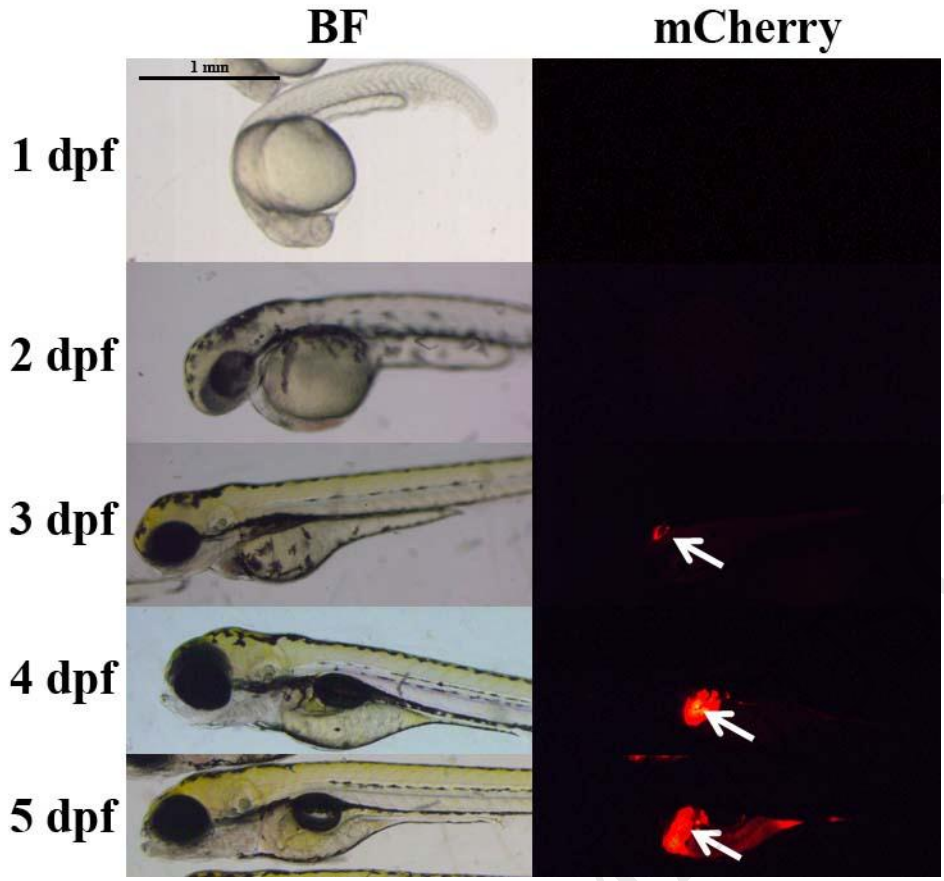

Figure 6. Fluorescent signal in developing transgenic embryos (*vtg1-mCherry*) exposed to EE2 (200 ng/L). Expression was detected in the liver (indicated by arrows) 3 dpf. (BF: bright field, mCherry: red fluorescent filter view, single plain images) Scale bar: 1mm.

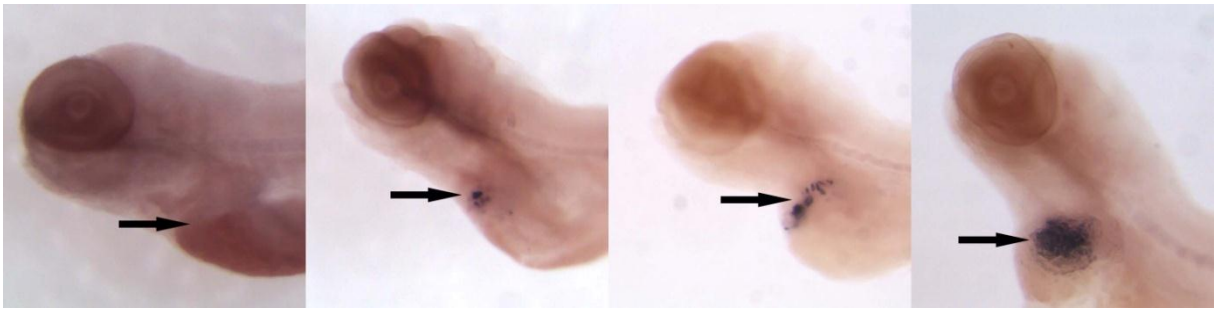

Figure 7. *Whole mount in situ hybridization* of 5 dpf, 17-α-ethynil-estradiol treated (200 ng/L EE2), wild-type embryos shown individual differences in *vtg-1* expression (liver is indicated by arrows on single plain images)

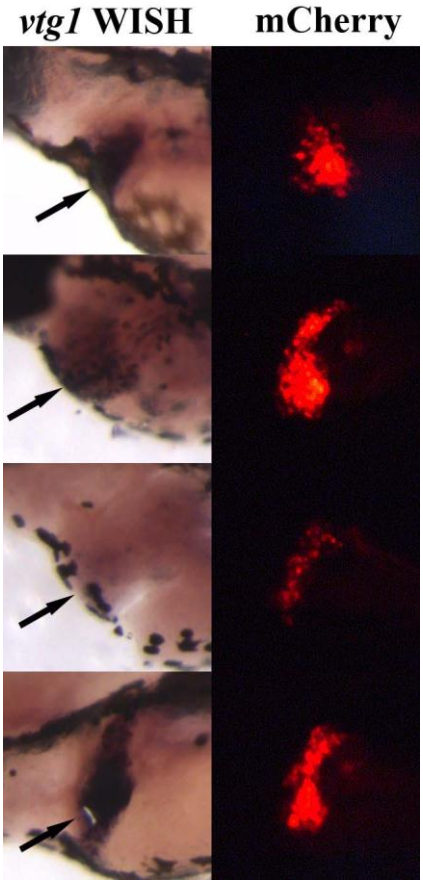

Figure 8. Similar biological variability of *vtg* and transgene response was clearly shown by comparing *in situ* hybridization images of 5 dpf transgenic embryos (*vtg1:mCherry*) to fluorescent images captured before fixation. Endogenous *vtg* production functioned similarly to the fluorescent protein, regarding spatial distribution and strength of the signal. Embryos were treated with 200 ng/L EE2. (*vtg-1* WISH: *vitellogenin-1* whole mount *in situ* hybridization, mCherry: red fluorescent filter view, single plain images, liver indicated by arrows)).

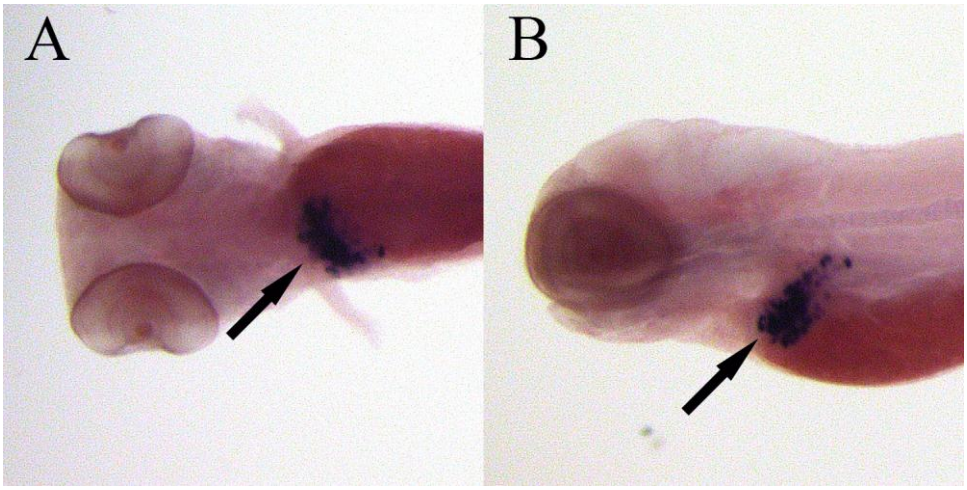

Figure 9. Expression of *vtg-1* was restricted to the left liver lobe (in 5 dpf wild-type larvae following 200 ng/L EE2 treatment) (dorsal (A) and left lateral (B) view, liver is indicated by arrows on single plain images).

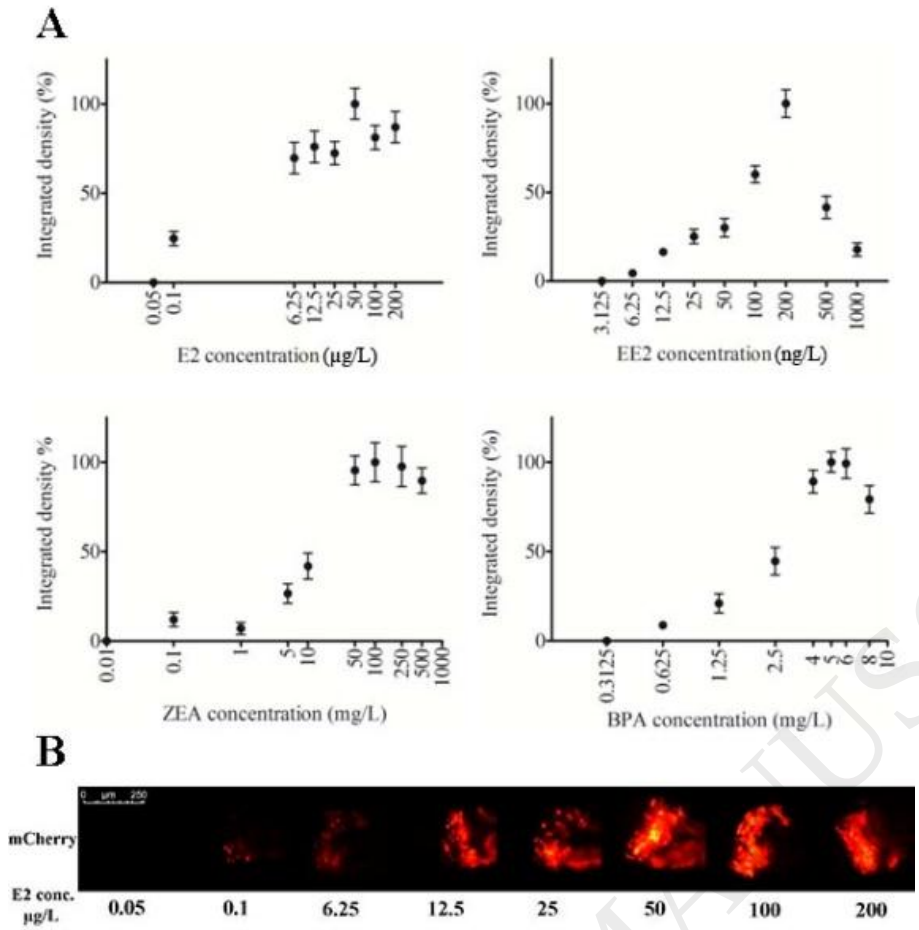

Figure 10. Dose-response diagrams for exposures to 17-ß-estradiol (E2), 17-α-ethynil-estradiol (EE2), bisphenol-A (BPA) and zearalenone (ZEA) in 5 dpf *vtg1-mCherry* larvae. Results are expressed as integrated density generated from signal strength and the size of the affected area (±SEM, n=.60) (A). Fluorescent images (mCherry) of the liver of E2 exposed larvae. Signal intensity increased gradually with concentration. (B) 100% refers to the observed maximum for each compound.

Substance	EC_{50}	EC_{10}	EC_{90}	relative estrogenicity
E2	8.304 µg/L	0.99 µg/L	69.67 µg/L	1
EE2	30.46 ng/L	11.80 ng/L	78.63 ng/L	272.62
ZEA	10.75 µg/L	3.18 µg/L	35.17 µg/L	0.78
BPA	2.601 mg/L	1.97 mg/L	3.43 mg/L	0,003

Figure 11. EC_{50}, EC_{10} and EC_{90} values and estrogenic potencies relative to 17-ß-estradiol for estrogenic substances based on the results of the *vtg1-mCherry* transgenic line (17-ß-estradiol (E2), 17-α-ethynil-estradiol (EE2), zearalenone (ZEA) and bisphenol-A (BPA))

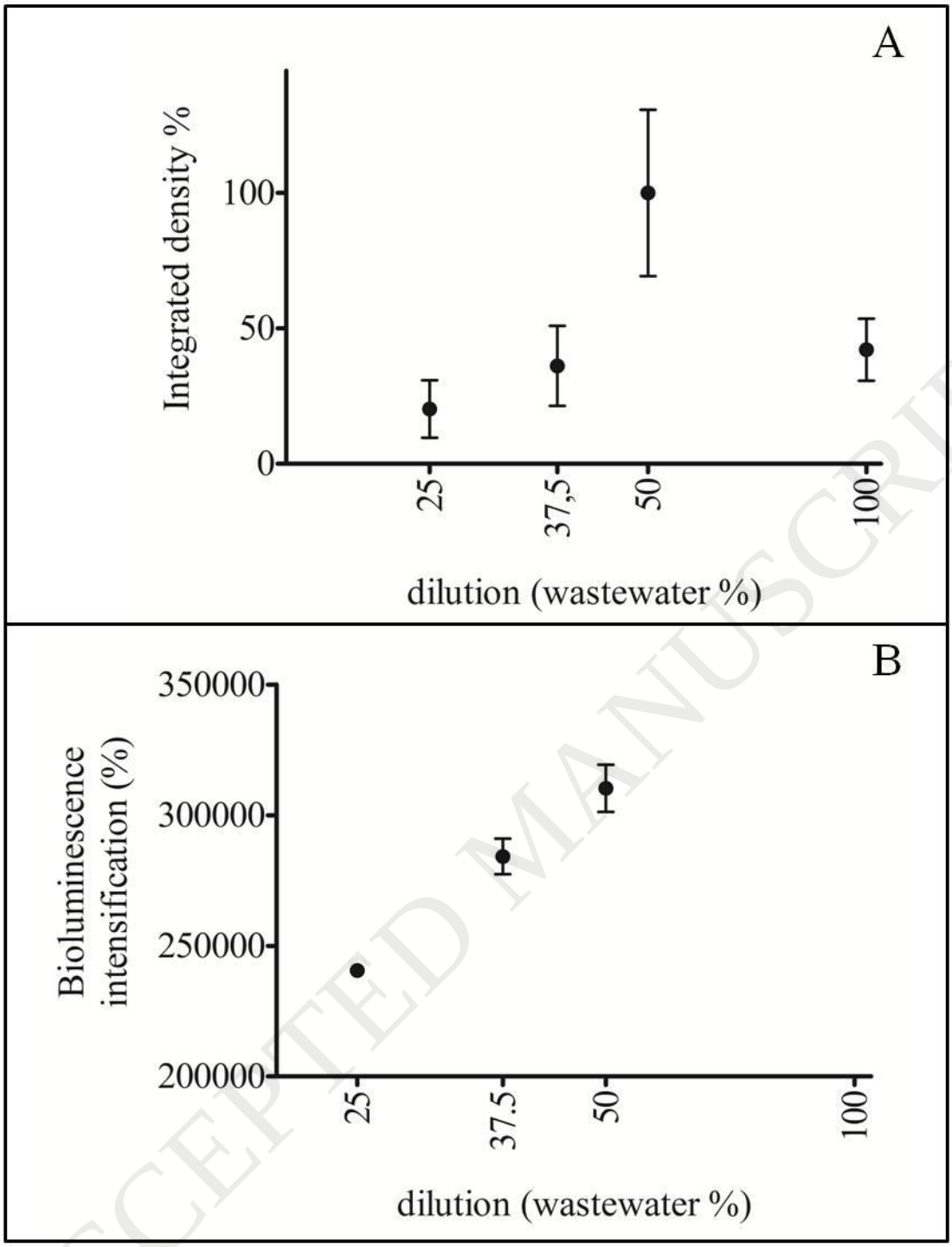

Fig. 12. Dose-response curves for wastewater (sample WW4) exposure in 5 dpf *vtg1-mCherry* larvae (A) and the BLYES test (B). Results are expressed as integrated density generated from signal strength and the size of the affected area (±SEM, n=.60) in case of zebrafish. On figure B, Y-axis indicates fold change bioluminescent intensification compared to the bioluminescence of the negative control. Undiluted samples could not be tested in the BLYES system.