Advancing the Zebrafish embryo test for endocrine disruptor screening using micro-injection : Ethinyl estradiol as a case study

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ABSTRACT

Fish (embryo) toxicity test guidelines are mostly based on aquatic exposures. However, in some cases, other exposure routes can be more practical and relevant. Micro-injection into the yolk of fish embryos could offer a particular advantage for administering hydrophobic compounds, such as many endocrine disruptors. Single dose micro-injection was compared to continuous aquatic exposure in terms of compound accumulation and biological responses. 17α-ethinyl estradiol (EE2) was used as a model compound. First, the optimal solvent and droplet size were optimized, and needle variation was assessed. Next, biological endpoints were evaluated. The accumulated internal dose of EE2 decreased over time in both exposure scenarios. Estrogen receptor (ER) activation was concentration/injected dose dependent, increased daily and was related to *esr2b* transcription. *Vtg1* and *cyp19a1b* transcription was induced in both scenarios, but the *cyp19a1b* transcription pattern differed between routes. Injection caused an increase of *cyp19a1b* transcripts from 48 hours post fertilization (hpf) onwards, while after aquatic exposure the main increase occurred between 96 and 120 hpf. Some malformations only occurred after injection, while others were present for both scenarios. We conclude that responses can differ between exposure routes and therefore micro-injection is not a direct substitute for, but can be complementary to aquatic exposure. Nevertheless, *vtg1* and *cyp19a1b* transcription and ER activation are suitable biomarkers for endocrine disruptor screening in both scenarios.

Keywords: zebrafish embryo, micro-injection, endocrine disruptors, aquatic toxicology, bioaccumulation, exposure route

1. INTRODUCTION

Aquatic exposure is the most commonly used exposure route in fish and fish embryo toxicity tests. Most internationally accepted test guidelines (TGs) and regulations are limited to applying this exposure route only (e.g., OECD TG 229 (Fish Short Term Reproduction Assay) (OECD 2012b), 234 (Fish Sexual Development Test) (OECD 2011) and 236 (Fish Embryo Acute Toxicity Test) (OECD 2013)). Such fish tests are required in the context of several legislative frameworks, such as the environmental risk assessment of pharmaceuticals (as required by e.g. the European Medicines Agency), the REACH regulation for chemicals as implemented by the European Chemicals Agency (ECHA), or the identification of endocrine disruptors (ECHA, the European Food Safety Authority, etc.). Only a few fish test guidelines are based on dietary exposure, e.g. OECD TG 305 (OECD 2012c) to characterize bioaccumulation. Exposure routes other than the aquatic route can however be highly relevant in some cases, for instance for poorly water soluble chemicals. Hormones as well as endocrine disrupting compounds are often hydrophobic, and therefore endocrine disruptor screening may benefit from the exploration of additional biologically relevant and practically feasible exposure routes.

To overcome the practical issues that are associated with hydrophobicity, such as sorption to the walls of the wells and volatilization, other exposure methods have been developed including passive dosing (Mayer et al. 1999), and intraperitoneal or intramuscular injections directly into the fish. In general however, aquatic exposure to hydrophobic compounds may be less relevant because these compounds mainly accumulate in the sediment, food and in tissues of aquatic organisms rather than in the water column (Soffker and Tyler 2012). When using embryos, compounds can also be injected directly into the yolk (Kim et al. 2015; Li et al. 2015), which could offer a particular advantage for administering hydrophobic compounds as the yolk contains large amounts of lipids (Holtta-Vuori et

al. 2010) that are gradually used as a source of energy during development (Lange 1981; Speake et al. 1998).

Micro-injection has been described previously as a way of administering toxicants to fish early life stages. Already in 1984, a trout embryo micro-injection assay was developed in the context of carcinogen detection (Black et al. 1985; Metcalfe and Sonstegard 1984). Later, after polychlorinated biphenyls (PCBs), dioxins and dibenzofurans were detected in eggs of lake trout in the Great Lakes, an egg injection method was developed to mimic maternal transfer of lipophilic halogenated aromatic hydrocarbons in rainbow trout and investigate potential consequences (Walker et al. 1992; Walker et al. 1991). From 2000 onwards, a medaka fertilized egg nanoinjection assay has been in use to assess the toxicity of a variety of compounds, often with the aim of mimicking deposition in eggs through maternal transfer (Colman and Ramsdell 2003; Edmunds et al. 1999; Nassef et al. 2010; Villalobos et al. 2000). More recently, studies applying micro-injection in zebrafish embryos to study toxicity of – among others – diethyl phthalate (DEP), perfluorooctanoic acid (PFOS), and triphenyltin have been published (Kim et al. 2015; Li et al. 2015; Liu et al. 2012; Schubert et al. 2014; Xiao et al. 2017).

For further establishing micro-injection as an exposure route for use in the zebrafish embryo test, characterization and standardization of several technical and methodological aspects is required. In the most recent studies using micro-injection in zebrafish embryos, different solvents have been used, including ethanol (Kim et al. 2015), dimethyl sulfoxide (DMSO) (Li et al. 2015), phosphate buffered saline (PBS) (Liu et al. 2012) and triolein (Xiao et al. 2017). In the studies of Maes et al. and Schubert et al., the suitability of different solvents for micro-injection in zebrafish embryos was compared. DMSO, acetone and triolein were examples of solvents found to cause limited toxicity. Schubert et al. noted high variability of the injection volume when using DMSO and methanol, while this was not the case for triolein, and concluded that this variability could complicate further

applications. In this study, we compared a subset of the solvents that were previously proposed, determined toxicity, selected the most appropriate injection volume and characterized intra- and inter-needle variation based on measurements of the actual internal doses of the injected compound immediately after injection.

Second, for micro-injection to be used as an exposure route in the zebrafish embryo test, for example for hazard identification or risk assessment purposes, it is critical to be able to interpret results relative to the traditional aquatic exposure route in terms of chemical accumulation and biological responses. There have only been a limited number of studies comparing both exposure routes (Kim et al. 2015; Li et al. 2015). Kim et al. used both injection and aquatic exposure to investigate the effects of DEP on zebrafish embryos. While the study was not specifically focused on comparing both exposure routes, they observed mortality and impaired development with skeletal malformations after both injection and aquatic exposure. Li et al. did specifically compare toxicity of PFOS to zebrafish embryos after injection and aquatic exposure. Based on a previously reported bioconcentration factor they estimated internal doses after aquatic exposure, and were able to conclude that the toxicity of PFOS was higher after injection compared to aquatic exposure.

It is clear that further characterization of the method itself, and of the comparability of micro-injection to aquatic exposure, is required to assess the feasibility and the biological relevance of micro-injection as an exposure route in the zebrafish embryo test. In the present study, we therefore explore the use of a micro-injection method into the yolk of zebrafish embryos for endocrine disruptor screening. We used the endocrine disruptor 17α-ethinyl estradiol (EE2) as a model compound. This estrogen receptor (ER) agonist is mainly used as a contraceptive (Caldwell et al. 2008). It is a well-known environmental contaminant and it has a Log *Kow* of 4.15 (Al-Ansari et al. 2013) which makes it a good candidate for both aquatic exposure and injection into the yolk. We characterized internal doses of EE2 for both exposure routes, including measurements immediately

after injection and subsequent measurements every 24 h. Additionally, a comparison of the toxicological processes involved in both exposure routes was performed at different levels of biological organization and at different time points. ER activation was evaluated using *5xERE:GFP* (see further) transgenic zebrafish (Gorelick and Halpern 2011), mRNA expression analysis of 14 genes related to steroid synthesis and biotransformation was carried out, and an extensive set of morphological abnormalities and physiological endpoints (e.g., swimming activity, heart rate) was evaluated.

2. MATERIALS AND METHODS

2.1 Ethics statement

According to EU Directive 2010/63/EU and the Commission Implementing Decision 2012/707/EU, fish are non-protected animals until the stage of free feeding. This limit was set at 120 hpf for zebrafish. Experiments of this study did not exceed 120 hpf, but were nevertheless part of a larger project for which approval by the Ethical Committee for Animals of the University of Antwerp was obtained (project number 2015-30). Fish husbandry and all experiments were carried out in strict accordance with the EU Directive on the protection of animals used for scientific purposes (2010/63/EU) (EC 2012).

2.2 Fish maintenance and egg production

Adult wild type zebrafish (*Danio rerio*) were obtained from an in house zebrafish line of the University of Antwerp and kept in an automated housing system (ZebTec standalone, Tecniplast, Buguggiate, Italy) with recirculating standard embryo medium in a temperature controlled room at a 14/10 h light/dark cycle. Transgenic zebrafish (*5xERE:GFP*), containing five estrogen responsive

elements upstream of a c-fos minimal promoter and green fluorescent protein (GFP), were obtained from the Carnegie Institution of Washington, USA (Gorelick and Halpern 2011). Standard embryo medium was prepared using reverse osmosis water to which Instant Ocean® salts and $NaHCO_3$ were added until a conductivity of 500 µS/cm and a pH of 7.5 were reached. The recirculating water with a total volume of 250 L was continuously filtered using a mechanical, biological and UV filter. The temperature (28 ± 0.2 °C), pH (7.5 ± 0.3) and conductivity (500 ± 15 µS) of the water were monitored and controlled continuously. The nitrate and nitrite concentrations were measured twice a week using Tetra tests (Tetra, Melle, Germany). Values were always below 0.3 mg/L for nitrite and the concentrations for nitrate did not exceed 25 mg/L. Ammonium was measured using a spectrophotometer (Hanna Instruments, Temse, Belgium) and never exceeded 0.15 mg/L. Fish were fed three times a day: two times with 0.5% of their mean wet weight of granulated food (Biogran medium, Prodac International, Cittadella, Italy) and one time with frozen feed (alternately *Artemia* nauplii, *Daphnia*, *Chironomidae* larvae and *Chaoborus* larvae). Breeding tanks inside the rack were used for reproduction. Fish were placed inside the breeding tanks in the evening, in a ratio of 2 males / 1 female. Males and females were separated overnight, and the divider was removed in the morning. Spawning and fertilization occurred immediately after the lights were switched on. After 20 to 30 minutes, the eggs were collected and washed to remove impurities. The eggs were placed in standard embryo medium for micro-injection (control, internal negative control and injected embryos) or test solutions (for the aquatic exposures) within 2 hours post fertilization (hpf) and examined for fertilization and cleanness of the chorion.

2.3 Overall experimental approach

An overview of the experimental approach including the different steps taken to optimize the technique of micro-injection and to study biological effects is graphically depicted in Figure S1.

2.3.1 Optimization and evaluation of technical aspects of the micro-injection method.

In a first step, the optimal droplet size was evaluated by injecting 0.5 nL and 1 nL droplets of the solvents dimethylsulfoxide (DMSO), canola oil and triolein (section 2.5 for the injection procedure). Mortality was scored to test which droplet size was the best (section 3.1.1). After identifying the best droplet size, we selected the most suitable solvent (section 3.1.2). Five different solvents were tested: canola oil, triolein, DMSO, DMSO (75%) + phosphate buffered saline (PBS) (25%) and acetone. Mortality, hatching, morphological abnormalities and physiological endpoints were evaluated (section 2.10). Phenol red dissolved in PBS was used as injection control to assess potential effects caused by the injection procedure. Inter- and intra-needle variation was assessed by measuring the internal dose of EE2 in embryos immediately after injection of EE2 using DMSO as the solvent (section 2.6). Three needles were compared in this analysis and three replicates of 10 larvae each were measured per needle.

2.3.2 Comparison of biological responses between exposure routes.

Embryos were exposed via water (45 ng/L to 900 µg/L EE2, Table S1) or via injection (0.05 ng EE2/embryo to 1.5 ng EE2/embryo, Table S1). Samples were taken for EE2 measurements in exposure medium, to verify medium concentrations and to investigate whether there was a difference in EE2 concentrations in the medium of wells with and without embryos in a time span of 24 h. Embryos were sampled for measuring the internal dose of EE2. Embryos were washed twice in standard embryo medium before measurement (section 2.6). ER activation was evaluated by measuring fluorescence of transgenic *5xERE:GFP* zebrafish (section 2.8). Samples were taken for mRNA expression analysis, snap frozen in liquid nitrogen and stored at -80 °C until analysis (section 2.9) and embryos were morphologically and physiologically evaluated (sections 2.4 and 2.10).

2.4 Aquatic exposure of zebrafish embryos

Embryos were exposed to EE2, dissolved in 0.01% DMSO, via water (EE2 ≥ 98%, Sigma-Aldrich, Diegem, Belgium) within the first 2 hpf and placed in 48-well plates (1 embryo per well in 1 mL of medium). Eight wells of each plate were used as an internal negative control (to exclude plate effects). For each aquatic experiment, one 48-well plate containing 3,4-dichloroaniline (98%, Sigma Aldrich; nominal concentrations 0.5 mg/L, 1 mg/L, 2 mg/L and 4 mg/L) was used as a positive control. Twelve embryos were exposed per 3,4-dichloroaniline concentration (OECD 2013). In addition to the internal negative control wells in each exposure plate, one negative control 48-well plate containing standard embryo medium and one solvent control 48-well plate containing 0.01% DMSO were included in the aquatic exposure experiments. All plates were covered with Parafilm, placed in an incubator (Panasonic, MIR-254-PE) at 28 ± 0.5 °C and subjected to a 14/10 h light/dark cycle and the medium was refreshed daily. Mortality was examined daily (coagulation, lack of somite formation, non-detachment of the tail, lack of heartbeat (OECD 2013)). Hatching was examined from 48 hpf onward and empty chorions were removed daily. Experiments were only considered valid if (1) ≤ 10% of the controls died (negative control and solvent control plate), (2) ≥80% of the control embryos hatched by the end of the test (5 dpf), and (3) at least 30% mortality was observed at 5 dpf after exposure to the highest concentration of the positive control (OECD 2013). After the final scoring, larvae were euthanized at 120 hpf using an overdose of tricaine methanesulfonate (MS-222, 1 g/L, pH 7.5).

2.5 Micro-injection of zebrafish embryos

Micro-injections were performed using a pneumatic picopump (PV820; World Precision Instruments, Sarasota, FL, USA) equipped with a micropipette holder and pulled microcapillary pipettes (TW120F-3, World Precision Instruments, Sarasota, FL, USA) which were loaded with EE2 (0.1 – 1.5 ng/per

embryo dissolved in 100% DMSO and a droplet size of 0.5 nL) or with pure DMSO (injection control) by capillary force. One negative control plate as well as internal negative control embryos, which were not injected, were included. Before injection**,** the loaded capillary was placed in the holder of the pneumatic picopump and calibrated with a micrometer (5008285, World Precision Instruments, Fl, USA). To increase the visibility of the droplets, the calibration was performed in halocarbon oil 27 (Sigma-Aldrich, Diegem, Belgium). Eggs were placed in an agarose plate (1% agarose) with cut-outs prepared using a mould (FM-600, IBI Scientific, IA, USA), after which they were injected by a single dose into the yolk within the first two hours post fertilization. Embryos were transferred to 48-well plates and the medium was refreshed daily. As a part of the technical optimization (section 2.3.1) experiments, canola oil (Sigma-Aldrich, Diegem, Belgium), triolein (Acros Organics, Geel, Belgium), DMSO (≥99.9%, Sigma-Aldrich, Diegem, Belgium), DMSO (75%) + PBS (25%, Thermo Fisher Scientific, Gent, Belgium) and acetone (Merck, Overijse, Belgium) were tested as a solvent. Due to the high viscosity of canola oil and triolein, the capillaries were not loaded by capillary force for these solvents. Instead, each of these solvents was injected into the capillaries using a needle filled with solvent and a syringe filled with air, thereby forcing the solvents into the capillaries.

2.6 Concentration/dose measurements of EE2

2.6.1 Extraction of water samples.

Solid-phase extraction (SPE) using Oasis® HLB cartridges (60 mg, 3 mL; Waters, Elstree, United Kingdom) was performed to enrich levels of EE2 in the medium samples (2 replicates). Sample extraction volume was 100 mL and 3 mL for the 90 ng/L and high 90 µg/L concentrations, respectively. Prior to SPE, EE2-D_4 ($C_{20}H_{20}D_4O_2$, Toronto Research Chemicals, Toronto, Canada) was used as internal standard (IS) and was spiked in the samples (90 ng/L samples: 25 ng/L IS; 90 µg/L samples: 6.25 µg/L IS). Samples were loaded on the SPE cartridges which were pre-conditioned with

methanol (3 mL) and ultrapure water (3 mL). After loading, the SPE cartridges were dried under vacuum for about 40 minutes and eluted with methanol (6 mL). The eluent was dried under a nitrogen stream (Reacti-Therm 3, Thermo Scientific, MA, USA) at 33 °C to dryness. The final extracts were reconstituted with a solution of ultrapure water (90%) in methanol (10%). The reconstitution volume was 100 µL and 750 µL for the 90 ng/L and 90 µg/L samples, respectively.

2.6.2 Extraction of biological samples.

The biological samples of injected or aquatically exposed embryos/larvae (3 replicates, pool of 10 embryos/larvae) were mixed together with small glass shards, methanol (680 µL) and EE2-D_4 (5 ng). The mixture was vortexed vigorously for 10 minutes followed by sonication (1 h). The mixture solution was filtered (Costar Spin-X 0.45 µm, VWR®). The filtrate was pre-concentrated under a pure nitrogen stream at 33 °C to dryness. The final extract was reconstituted (100 µL with a solution of ultrapure water (90%) in methanol (10%)). The filtered extracts were transferred for analysis to a glass vial with insert.

2.6.3 Instrumental analysis of EE2 in samples.

Analysis of EE2 and EE2-D_4 was performed using ultra high-pressure liquid-chromatography (Agilent 1290 Infinity) coupled to tandem mass spectrometry (Agilent 6460 Triple Quadrupole system) (UHPLC-MS/MS). Chromatographic separation of the target chemicals was conducted on a C18 (Gemini-NX, 150 X 2.0 mm, 5 µm, Phenomenex) analytical column with a mobile phase consisting of ultrapure water (A) and methanol (B), both with 0.03% ammonium hydroxide. More technical details on the procedure and quality control can be found in the supporting information (section S1.1 and S1.2)

2.7 Modelling of EE2 accumulation and biotransformation/elimination

In the injection route, the concentration of EE2 in the medium is initially zero. Accordingly, the internal EE2 concentration can only decrease with time either as a consequence of simple diffusion out of the embryo (until embryo/water partition equilibrium is attained) and/or intrinsic biotransformation/elimination processes. We estimated the amount of EE2 that would simply diffuse out of the embryo, and compared this value with the measured internal concentrations. This approach provides an estimate of the magnitude of the biotransformation/elimination processes. Details of the computations are given in the supporting information (section S1.3). Additional calculations of weight based doses are available in the supporting information (section S1.4).

2.8 Estrogen receptor activation using 5xERE:GFP embryos

The embryos were sedated using 100 mg/L MS-222 at pH 7.5 (Sigma-Aldrich, Diegem, Belgium) before measurement at 24, 48, 72, 96 or 120 hpf. When sedated, the embryos were pipetted (30 µL) into a black V-shaped 96-well plate (AB-0800/K, Thermo Scientific, MA, USA) and measured (± 3 min) immediately in a preheated (28.5 °C) spectrofluorometer (Tecan Infinite M200 Pro). Excitation and emission wavelengths were 484 nm and 518 nm respectively. Embryos were euthanized using MS-222 (1 g/L, pH 7.5) after every measurement, embryos were therefore never used for fluorescence measurement more than once.

2.9 mRNA expression analysis

Wildtype embryos and larvae were sampled at five time points (24, 48, 72, 96 and 120 hpf). For each time point and exposure condition, four biological replicates were analysed. Each biological replicate consisted of 7 pooled embryos. The embryo medium was removed and vials were snap frozen in

liquid nitrogen. RNA extraction was performed using a Nucleospin kit (Machery-Nagel, Düren, Germany). Purity and integrity of the RNA were analysed using a NanoDrop spectrophotometer and a Fragment Analyser (Advanced Analytical Technologies, Heidelberg, Germany). The RNA integrity number was at least 9.4, indicating intact RNA. The $A_{260\ nm}/A_{280\ nm}$ ratio was at least 1.94 and the $A_{260\ nm}/A_{230\ nm}$ ratio did not exceed 2.32. cDNA was synthesised using a Revert Aid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Gent, Belgium) which was then diluted to 70 ng/L. QPCR was performed for 19 genes (Table 1, see Table S2 for primer sequences and accession numbers) using a Mx3005P system (Agilent Technologies, Diegem, Belgium) and Brilliant II SYBR green Master Mix (Agilent Technologies, Diegem, Belgium). Primers were obtained from Eurogentec (Liège, Belgium). Standard curves were performed in duplicate in each run to determine primer efficiencies. The thermal cycling profiles started with a denaturation period of 10 min at 95 °C, followed by 40 cycles of 20 s at 95 °C, 40 s at 55 °C (58 °C for *vtg1* and *hsd17b1*) and 30 s at 72 °C. Melting curves were evaluated to confirm that only one amplification product had been formed. Five reference genes were evaluated (*ef1a*, *actb1*, *18s*, *hprt1* and *rpn2*, Table 1) for stability under our experimental conditions. The two most stable genes (Vandesompele et al. 2002) (*rpn2* and *18s*) were used to normalize the data. Normalisation was performed by dividing the expression of the target gene by the geometric mean of the expression values of the reference genes. The qBASE+ software (version 3.1, Biogazelle, Zwijnaarde, Belgium - www.qbaseplus.com) was used to calculate the most stable reference genes, to calculate relative expression values (relative to *rpn2* and *18s*) and to correct for inter-run variation.

Table 1: Target and reference genes with their abbreviation.

Abbreviation	Target gene
esr1	estrogen receptor 1
esr2a	estrogen receptor 2a
esr2b	estrogen receptor 2b
vtg1	vitellogenin 1
cyp11a1	cytochrome P450, family 11, subfamily A, polypeptide 1
cyp17a1	cytochrome P450, family 17, subfamily A, polypeptide 1
hsd3b1	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1
cyp19a1a	cytochrome P450, family 19, subfamily A, polypeptide 1a (ovarian aromatase)
cyp19a1b	cytochrome P450, family 19, subfamily A, polypeptide 1b (brain aromatase)
hsd17b1	hydroxysteroid (17-beta) dehydrogenase 1
hsd17b3	hydroxysteroid (17-beta) dehydrogenase 3
sult2st3	sulfotransferase family 2, cytosolic sulfotransferase 3
ugt1a1	UDP glucuronosyltransferase 1 family polypeptide a1
cyp3a65	cytochrome P450, family 3, subfamily A, polypeptide 65
Abbreviation	**Reference gene**
ef1a	eukaryotic translation elongation factor 1 alpha 1
actb1	beta actin 1
18s	18S ribosomal RNA
hprt1	hypoxanthine phosphoribosyltransferase1
rpn2	ribophorin 2

2.10 Scoring of morphological and physiological endpoints

Morphological and physiological endpoints were evaluated using an aquatic exposure range from 90 µg/L to 900 µg/L, and for injection the doses ranged from 0.05 ng to 1.5 ng per embryo. Heart rate

was determined (Leica APOS8 stereomicroscope) on a heating plate (31.5 °C) by visually counting the heartbeat (between 24 and 25 hpf). To avoid a time-dependent effect (i.e., age of larvae increases during the day of measurement), all heart rate measurements were performed within 1 hour starting at 24 hpf and measurements were alternated randomly among exposure conditions. Morphological scoring involved assessment of 28 binary parameters (Table S3, (Michiels et al. 2017)). All embryos were analysed for swimming activity at 72, 96 and 120 hpf in a behavioural tracking instrument (Zebrabox 3.0, Viewpoint, France) for 50 min under normal light conditions (100% light setting, 1160 lux). Travelled distance and duration of activity were determined using the ZebraLab software (version 3.20.5.104). At 54, 72, 96 and 120 hpf, larvae were photographed next to a reference mark using a Canon 600D (18 megapixels) mounted on a Leica APOS8 stereomicroscope. Standard length and swim bladder surface were determined using the Image J software (version 1.47v), using the photographed reference mark as a calibrator. Swim bladder surface was estimated at 96 and 120 hpf based on swim bladder length (l) and height (h) measurement using the formula for the surface area of an ellipse ($S = \pi . 0.5h . 0.5l$).

2.11 Statistical analysis

All statistical analyses were performed using Graphpad Prism 7 (GraphPad Software, San Diego, California, USA). The cut off for statistical significance was set to $p \leq 0.05$. For analysis of survival and hatching curves, pairwise log rank tests with a Bonferroni correction were carried out. Heart rate, swimming activity, swim bladder surface, growth, fluorescence and mRNA expression were compared between exposure conditions within a time point, or for a given exposure condition between different time points, using one-way ANOVA. To evaluate the swim bladder surface of the same treatment over the 2 time points, an unpaired t-test was performed. To test whether observed increases in either fluorescence or EE2 medium concentration were significant over time, linear regression analysis was used. Two-way ANOVAs (with time and exposure condition as the two

factors) were performed for growth and mRNA expression. Since morphological abnormalities were scored binary (i.e., absence or presence, represented by a value of either 0 or 1), chi square tests with a Bonferroni correction were performed. This test was performed as well to evaluate differences between different droplet sizes. Pearson's correlation coefficients were used to investigate potential relationships between swim bladder inflation and swimming distance, swim bladder surface and swimming distance, and mRNA expression of the ERs and fluorescent intensity. An unpaired t-test was performed to detect potential differences between EE2 concentrations in the medium of empty wells and wells containing 24h old embryos. To describe the needle variation, coefficients of variation were calculated and a one-way ANOVA test was performed. For the internal dose measurements, unpaired t-tests, which were corrected for multiple testing with a Bonferroni correction, were performed between a given time point and the next time point to detect where an observed change of the internal dose was significant.

3. RESULTS

3.1 Optimization and evaluation of technical aspects of the micro-injection method

3.1.1 Selection of suitable droplet size.

Canola oil, triolein and DMSO were used to find the most suitable droplet size for injection in zebrafish embryos. Droplets of 1 nL triolein and DMSO caused significantly higher mortality ($p \leq 0.01$) compared to 0.5 nL droplets (Table 2). All other experiments were therefore performed with injections of 0.5 nL.

Table 2: Mortality percentages after injection of zebrafish embryos with different droplet sizes (n ≥ 26). An asterisk indicates significant differences ($p \leq 0.05$) between droplets of 0.5 nL and 1 nL per solvent.

	0.5 nL	1 nL
Canola oil	9 %	15 %
Triolein	18 %	35 % *
DMSO	4 %	27 % *

3.1.2 Solvent selection and characterization of needle variation.

Acetone, DMSO (75%) + PBS (25%) and triolein caused significant mortality compared to the control (Figure 1A). Injection of triolein resulted in a decreased heart rate compared to controls and injection controls (controls: 114 ± 3.4 beats per minute (bpm) versus triolein: 109 ± 3.1 bpm, $p < 0.001$). DMSO and canola oil caused no morphological or physiological effects. Out of these two potential solvents, DMSO was selected as the optimal solvent for this study because the high viscosity of canola oil caused practical problems. In addition, DMSO has already been used in similar micro-injection studies using zebrafish (Li et al. 2015; Maes et al. 2012; Schubert et al. 2014).

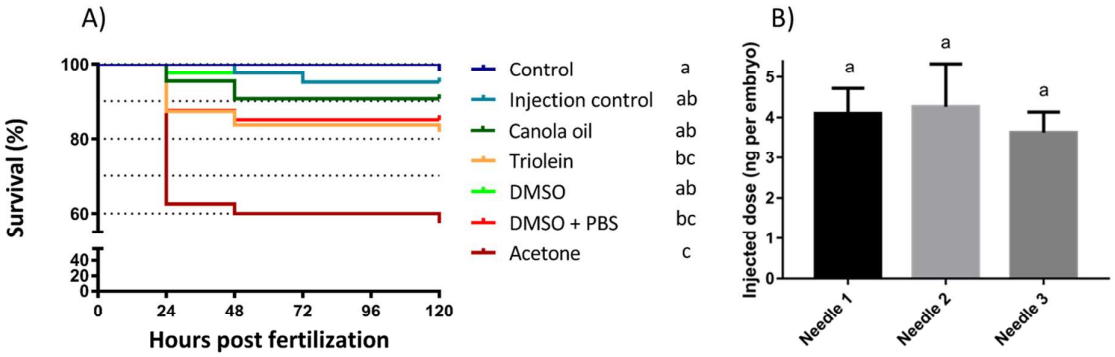

Figure 1: (A) Comparison of different solvent types. Survival curves of the embryos after exposure via injection to different types of solvents (n ≥ 40). Phenol red dissolved in PBS was used as injection control and the ratio DMSO/PBS was 75%/25%. Control embryos were not injected. (B) Needle variation without outlier. Histograms show mean ± standard deviation. Both graphs: Different letters in the legend indicate significant differences among treatments ($p \leq 0.05$).

The variation among three different injection needles and within a single injection needle (three replicates) is shown in Figure 1B. Except for one outlier, which has been removed from the analysis and may be explained by the fact that a part of the tip had broken off causing a higher injection volume, the results were highly consistent. There was no significant difference among the three needles ($p > 0.5$). After removing the outlier from the dataset, the within-needle coefficients of variation were 15, 25 and 14%.

3.2 Concentrations of EE2 in medium and in embryos/larvae

Despite daily renewal of the medium, EE2 concentrations in the external exposure solution decreased by 22-36% (Figure 2A) between 24 h and 120 h while the stock solution only decreased by 15% between 0 and 120 hpf, indicating additional loss from the medium in the wells. However, the rate of the decrease was not significantly different between both exposure concentrations (slopes: -0.30 for 90 ng/L and -0.28 for 90 µg/L, $p = 0.85$). A comparison between EE2 measurements in wells containing medium only, and wells containing 24 h old embryos which had been in that well for 24 h, showed that the presence of embryos in the wells significantly decreased EE2 concentrations in medium at high exposure concentrations (with embryo: 92.1 ± 2.0 µg/L, without embryo: 98.0 ± 1.3 µg/L, $p = 0.014$; Figure 2C). Internal negative controls did not contain EE2.

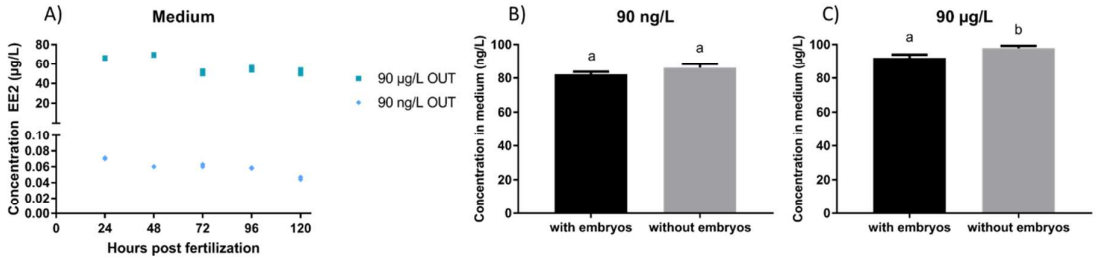

Figure 2: (A) EE2 concentrations in the medium which was removed from the wells every 24 h. (B,C) Effect of the presence of zebrafish embryos on EE2 concentration in medium with a nominal EE2 concentration of 90 ng/L or 90 µg/L after 24h (n = 3). The graphs show mean ± standard deviation. Letters indicate significant differences within exposure concentrations ($p \leq 0.05$).

The internal dose of EE2 was measured every 24 h for both exposure routes, as well as directly after injection. No EE2 was detected in the negative controls. The internal dose was detectable, but not quantifiable after aquatic exposure to 90 ng/L (the dose in a pooled sample of 10 larvae was below the estimated limit of quantification of 95 pg). For both exposure routes, we observed the highest internal dose at the earliest measured time point (injection: 0.87 ± 0.17 ng per embryo, t = 0 and aquatic: 2.13 ± 0.05 ng EE2 per embryo, t = 24 hpf), and a decrease afterwards (Figure 3). At 120 hpf, the EE2 dose in aquatically exposed larvae was on average 0.59 ± 0.02 ng/larva, and 0.0093 ± 0.00082 ng/larva in injected larvae.

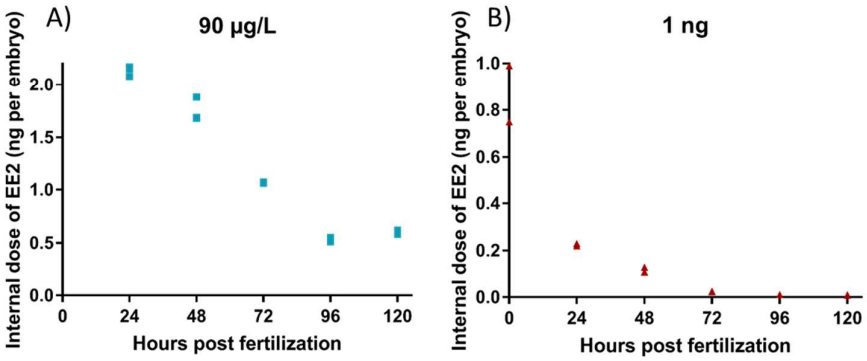

Figure 3: Internal EE2 dose in embryos after (A) aquatic continuous exposure to 90 µg/L EE2 or (B) single dose injection of 1 ng EE2. Three replicates of 10 embryos per replicate were measured for each time point and exposure condition.

The interpretation of the data must consider the inherently different nature of the exposure conditions. In the case of aquatic exposure, the temporal evolution of the internal concentrations is a consequence of the diffusive supply flux from the surrounding medium and the biotransformation/elimination capacity of the embryo. The EE2 concentration in the external medium is maintained approximately constant and there is no bulk depletion. The internal concentration therefore reflects the net outcome of concurrent uptake and biotransformation and/or elimination processes over the exposure time. Starting from $t = 0$, the internal concentration will initially increase, and may subsequently either reach a steady-state value (if the rate of uptake becomes equal to the rate of biotransformation/elimination) or pass through a maximum in the case where the rate of biotransformation/elimination becomes greater than the rate of uptake. Assuming that uptake of EE2 is unregulated, the results for the aquatic exposure indicate that the rate of biotransformation and/or elimination increases with exposure time, which may reflect the increased handling capability of the embryo with hpf. Furthermore, Figure 3A shows that the maximum in the internal concentration occurs no later than 24 hpf. In contrast, for the injection exposure, the concentration in the exposure medium is maintained at approximately zero, and thus the temporal evolution of the internal concentration (embryo+yolk) represents the biotransformation and/or elimination processes, because diffusion alone is not sufficient to explain the low concentrations at 24 hpf based on the equilibrium partitioning (see section S1.3 for a modelling approach of EE2 biotransformation/elimination after injection).

3.3 Comparison of biological responses between exposure routes

3.3.1 Estrogen receptor activation using 5xERE:GFP embryos.

The ability of EE2 to activate the estrogen receptors (ERα and ERβ2, (Gorelick et al. 2014)) resulting in a GFP signal was assessed during 5 days. In general, fluorescence increased as a function of time post fertilisation, and responded in a concentration/dose dependent manner within a time point (Figure 4). The fluorescent signal significantly increased compared to controls starting from 72 hpf and the response was fully established for all experimental conditions at 96 hpf. The order of magnitude of the signal, indicative of the number of receptors which are occupied, was similar between both exposure routes at the various time points (Figure 4 A-B). The ER activation fluorescent activities were more sensitive for the aquatic exposures: the response after aquatic exposure in the ng/L range was similar to that following an injection of 1 ng EE2.

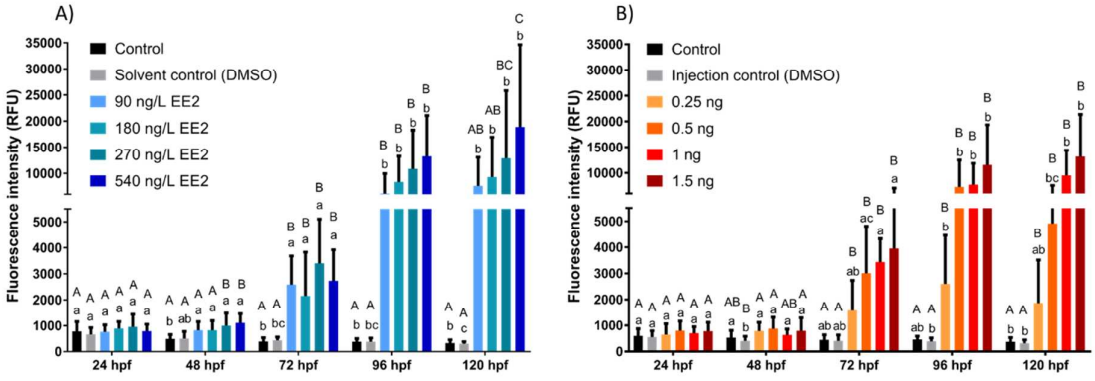

Figure 4: Estrogen receptor activation measured as fluorescent intensities in 5xERE:GFP embryos after (A) aquatic exposure, (B) exposure via injection. Comparisons are made within a treatment over time (small letters) or within a time point (capital letters). Error bars represent standard deviations.

3.3.2 mRNA expression.

3.3.2.1 Estrogen receptors and vitellogenin

A non-monotonic dose response was observed for the *esr1* mRNA expression at 96 and 120 hpf (Figure 5, only the intermediate aquatic concentration of 450 ng/L EE2 caused significant upregulation) and injection with 1 ng caused an increased signal at 96 hpf as well. The mRNA expression of the ER *esr2a* (Figure. 5) was not affected by EE2 treatment. The highest aquatic exposure concentration of EE2 (450 µg/L) caused a decrease in *esr2b* expression at 96 hpf and 450 ng/L caused an increase at 120 hpf. In addition, mRNA expression levels of *esr2b* in control embryos were also positively related to estrogen receptor activation (r = 0.98, p = 0.004, Figure S2). Exposure to EE2 also induced an increase in *vtg1* expression for all exposure concentrations and both exposure routes (Figure 5). The *vtg1* expression patterns of the embryos exposed to the two highest aquatic exposure concentrations and the injected embryos were similar over time, and again a non-monotonic response was observed.

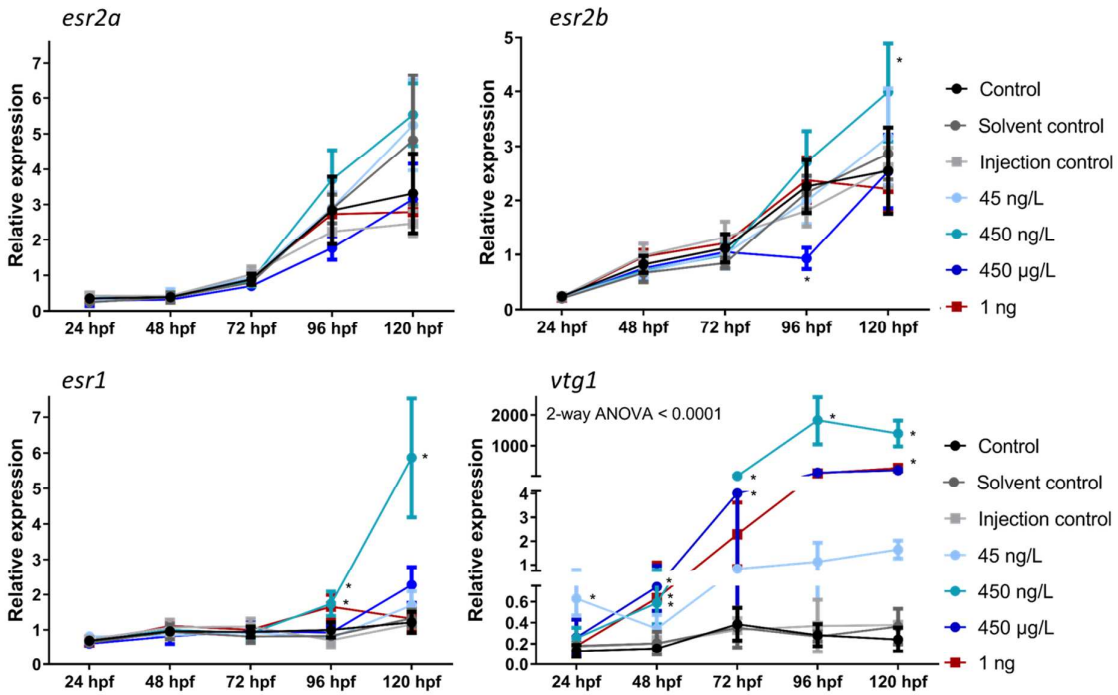

Figure 5: mRNA expression (relative to *rpn2* and *18s*) of the three nuclear estrogen receptors and vitellogenin 1 during the first five days of development after aquatic exposure to EE2 (blue) or after EE2 injection (red).

Asterisks indicate differences compared to the control within the same time point. Error bars represent standard deviations.

3.3.2.2 Steroid synthesis and biotransformation enzymes

The only enzyme of the steroid biosynthesis pathway, out of the seven that were included in our analysis, that changed after EE2 treatment was brain aromatase, *cyp19a1b* (Figure 6). All exposure conditions induced mRNA expression of *cyp19a1b*. The mRNA expression data indicate that *cyp19a1b* is more sensitive to estrogen exposure than *vtg1* as there is already a significant increase of mRNA expression of *cyp19a1b* after an aquatic exposure to 45 ng/L EE2. Interestingly, the expression pattern of brain aromatase differs between both exposure routes ($p < 0.0001$): injection caused a gradual increase from 48 hpf onwards, while the main increase after aquatic exposure occurred between 96 and 120 hpf. The mRNA expression patterns of ovarian aromatase (*cyp19a1a*) (Figure 6), *hsd17b3*, *cyp11a1*, *hsd17b1*, *cyp17a1* and *hsd3b1* (Figure. S3) were not affected by EE2 exposure.

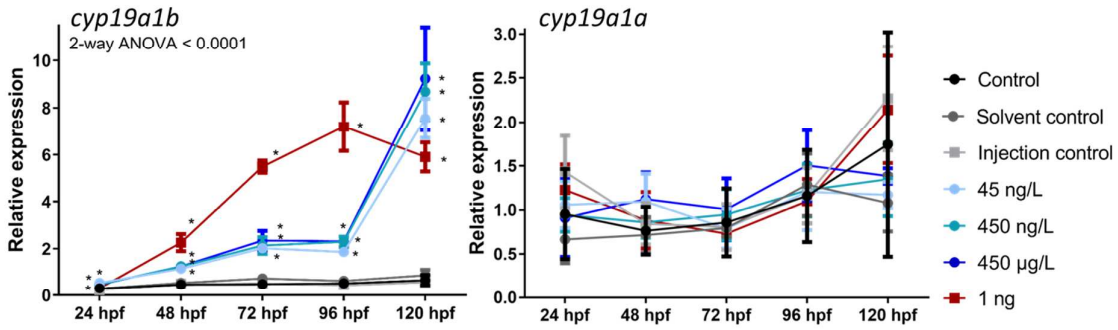

Figure 6: mRNA expression (relative to *rpn2* and *18s*) of brain (*cyp19a1b*) and gonadal (*cyp19a1a*) aromatase during the first five days of development after aquatic exposure to EE2 (blue) or after EE2 injection (red). Asterisks indicate differences compared to the control within the same time point. At 24 hpf there is a significant difference between the control and all aquatic exposures. For the other time points, all exposure

conditions could generate a significant difference compared to the control. Error bars represent standard deviations.

Although previous studies showed a response for all three metabolism related genes (*cyp3a65*, *ugt1a1* and *sult2st3*) that were tested after treatment with an estrogen receptor agonist (Hao et al. 2013; Xia et al. 2016), they showed no response to EE2 treatment in our study. The relative expression of *cyp3a65* and *ugt1a1* was low during the first 48 h, but increased afterwards for all exposure groups, including the controls. The relative expression of *sult2st3* was stable during the first 5 days of development (Figure S3).

3.3.3 Morphological abnormalities at 120 hpf.

Impaired swim bladder inflation was the only morphological effect observed after aquatic exposure (Figure 7). Injecting 1.25 ng EE2 caused impaired swim bladder inflation (Figure 7) and curved tails (13%, $p = 0.0002$) for a significantly larger proportion of larvae compared to controls. In addition, the occurrence of malformations of the head (6%), blood accumulations (9%), no blood circulation in the tail (9%) and oedemas (13%) was also significantly increased compared to controls after injecting 1.25 ng EE2. All injected larvae (1.25 ng EE2) that had a blood accumulation also showed lack of circulation in the tail.

The swim bladder surface significantly increased between 96 hpf and 120 hpf for each aquatic exposure condition ($p \leq 0.01$). At each time point however, larvae exposed to 450 µg/L showed a smaller swim bladder surface compared to the controls (Figure 7). After injection we observed a decreased swim bladder surface at 120 hpf for 1.25 ng EE2 compared to the injection controls. The swim bladder surface was not correlated with the swimming distance at 96 hpf or 120 hpf for neither exposure routes.

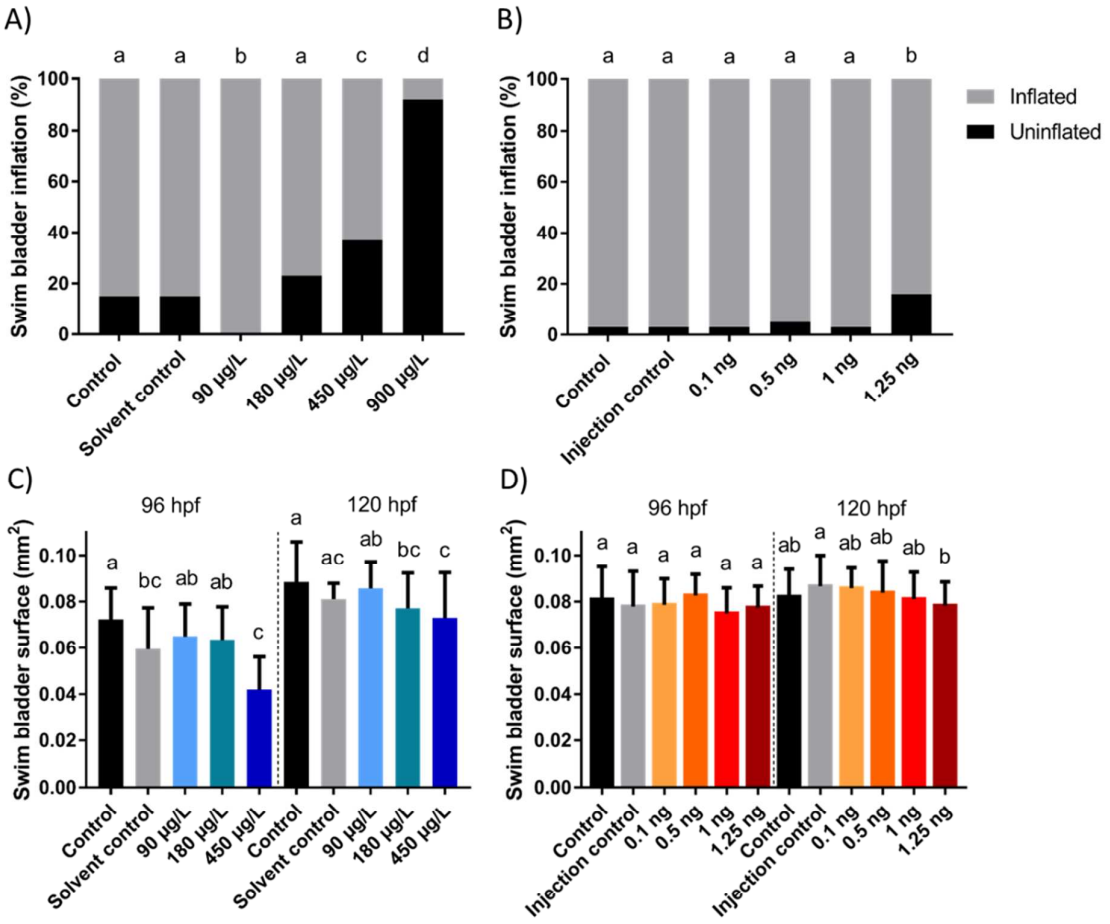

Figure 7: Swim bladder inflation after (A) aquatic exposure to EE2 or after (B) injection. Swim bladder surface at 96 and 120 hpf after (C) aquatic exposure or (D) injection. 92% of the larvae exposed to 900 µg/L didn't have an inflated swim bladder so no measurements were performed for this exposure group. Statistical comparisons were made within time points, letters indicate significant differences ($p \leq 0.05$). Error bars represent standard deviations.

3.3.4 Physiological endpoints.

3.3.4.1 Heart rate

For heart rate, injected doses of 0.05; 0.5; 1 and 1.5 ng EE2 were evaluated. At 24 hpf, the heart rate of embryos injected with a dose of 1 ng or 1.5 ng EE2 was significantly decreased ($p \leq 0.0001$) compared to the controls and injection controls. Aquatic exposure to EE2 did not affect heart rate.

3.3.4.2 Growth

Larvae injected with the highest dose (1.25 ng EE2) were significantly smaller at all time points compared to the controls (Figure. S4). Larval length significantly increased daily after aquatic exposure (90-900 µg/L EE2) as well as after injection (0.1-1.25 ng EE2) during the first four days (Figure. S4). Larval growth halted between days three and four after exposure to 900 µg/L EE2.

3.3.4.3 Swimming distance

Swimming distance of the aquatically exposed larvae was significantly lower compared to controls from 72 hpf onwards. At 120 hpf, the response was even concentration dependent (Figure. 8). The effect of injecting EE2 on swimming distance was less pronounced. After aquatic exposure larvae with a non-inflated swim bladder swam less distance at 96 hpf ($r = -0.94$, $p = 0.02$). For all physiological endpoints an aquatic exposure in the µg/L range was needed to result in effects comparable to the injected doses in the ng range, which was also observed for morphological endpoints.

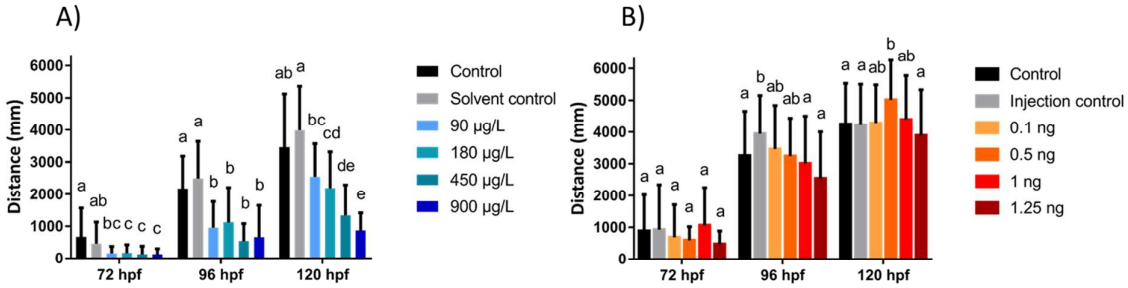

Figure 8: Swimming distance measured from the third day of development (72 hpf) until the fifth day (120 hpf) after aquatic exposure (A) or after injection (B). Statistical comparisons were made within time points, letters indicate significant differences. Error bars represent standard deviations.

4. DISCUSSION

4.1 Evaluation of technical aspects of the micro-injection method

In the first set of experiments a number of technical aspects of the micro-injection method were optimized and evaluated. Both canola oil and DMSO did not affect any of the toxicity endpoints. DMSO had already been used for the injection of zebrafish embryos in previous studies (Li et al. 2015; Maes et al. 2012; Schubert et al. 2014), but triolein is the more commonly used solvent in injection studies with fish embryos (Edmunds et al. 2000; Hano et al. 2005; Nassef et al. 2010; Schubert et al. 2014; Xiao et al. 2017). Our results however showed that the use of triolein can lead to effects on survival and heart rate. Our results further confirmed that droplet size is a very important factor determining embryo survival, as was previously shown by Schubert and colleagues (Schubert et al. 2014), and we selected an injection volume of 0.5 nl for injection in the yolk of zebrafish embryos. In our analysis, there was no significant inter-needle variation and intra-needle variation was limited (coefficient of variation: 14 – 25 %), to some extent addressing the issue on variability in injection volumes raised by Schubert et al. In general, we suggest to carry out a similar evaluation of the suitability of solvents and droplet size as part of the optimization of the micro-

injection technique for other fish species. As the size and structure of the eggs differ among fish species, the ideal droplet size could be species-specific. Droplet sizes between 1 and 3 nL were, for example, reported for medaka embryos (Edmunds et al. 2000).

4.2 Micro-injection versus aquatic exposure: compound accumulation and biological effects

4.2.1 Compound accumulation.

The internal dose of EE2 decreased as a function of time in both exposure scenarios (Figure 3). We hypothesize, for both routes, that both biotransformation and elimination are important. In general, a decrease of EE2 after injection was of course expected, since injection is only performed once and the embryo medium is clean, causing the injected EE2 to at least passively diffuse out of the embryo. However, the concentration at 24 hpf was much lower than expected, based solely on equilibrium partitioning (see section 3.2 and model described in section S1.3). Therefore, diffusion alone cannot explain the observations. In the aquatic setup, the exposure medium was renewed daily to keep exposure concentrations constant (Figure 2). However, the accumulated dose of EE2 in the larvae decreased as the larvae aged, indicating that the rate of biotransformation and/or elimination increases with exposure time (see section 3.2). The biotransformation genes – at least the set that we analysed – showed increased transcription as of 72 hpf, which coincides with the development and activation of the hepatopancreas (Wilkins and Pack 2013), the major organ responsible for biotransformation. At 72 hpf *in vitro* activity of some CYP's was observed as well in zebrafish embryos (Saad et al. 2017; Verbueken et al. 2017). A similar decreasing pattern of internal doses, after a maximum around 24 hpf, was also observed after aquatic exposure to benzocaine, phenacetin, metribuzin (Brox et al. 2016) and benz(a)anthracene (Kuhnert et al. 2013). In both studies, metabolites of the respective parent compounds were also detected which supports the hypothesis of biotransformation. In a recent study by Souder and Gorelick (Souder and Gorelick

2018), uptake of EE2 after aquatic exposure increased with increasing exposure concentration, exposure duration and developmental stage. First, the different response they observed (compared to our data) may be explained by the fact that Souder and Gorelick used different exposure windows. Our exposure lasted for 120 h, while they used shorter exposure durations (1 h to 24 h) targeting specific life stages. Second, Souder and Gorelick used a radioactively labelled form of EE2 and metabolites are therefore also expected to be radioactive. As a consequence they measured the sum of EE2 and its metabolites, while we used UHPLC-MS/MS for our measurements, specifically targeting EE2 only (i.e., the parent compound). This further supports the importance of biotransformation. Active elimination was likely a relevant process as well and could for example be mediated by the *abcb4* transporter (Fischer et al. 2013). A*bcb4* transcripts have been demonstrated to be high in the first hours post fertilization due to maternal transfer ((Fischer et al. 2013), Verbueken et al., unpublished data).

4.2.2 Receptor activation and molecular responses.

The internal dose at 120 hpf is approximately 100 times lower after injection of 1 ng compared to the aquatic exposure of 90 µg/L (Figure 3). However, aquatic exposure to 0.54 µg/L caused ER activation at 120 hpf that was twice as high as for 1 ng of injected EE2 (Figure 4). This indicates that estrogen receptors are activated more easily after aquatic exposure. Receptor activity for all injected and aquatically exposed *5xERE:GFP* larvae increased daily, even though the internal dose decreased over time. These observations imply that, for a given exposure condition, the synthesis of ERs increases over time. At a given time point, the receptor activity increased with increasing dose for both the aquatic and injection exposure modes. Our mRNA expression data indeed show an increased expression in unexposed larvae as they age, as well as an increased transcription of *esr1* after EE2 exposure (Figure 5). In addition, mRNA expression levels of *esr2b* in control embryos were indeed directly related to receptor activation in exposed larvae (Figure. S2 and section 3.3.2).

The most distinct difference between both exposure routes was the mRNA expression pattern of brain aromatase (*cyp19a1b*). Brain aromatase transcription levels were significantly higher from 48 hpf onwards after injection (Figure 6). Although *cyp19a1b* transcription responds relatively fast after targeted disruption (200 ng/L), meaning that embryos were only exposed during certain time frames of development, using EE2 in an aquatic setup (Periz-Stanacev et al., unpublished data), the response after injection in the current study was even faster. Possibly, this could be an indication of a difference in tissue compartmentalization between exposure routes, as the EE2 is taken up solely via the yolk after injection while both the yolk and the skin are involved in the aquatic exposure. Also, injection is more direct and could therefore trigger responses faster. Indeed, the immediate accumulation of a compound after injection in the yolk was confirmed by another study in which injected fluorescently labelled fatty acids were already present in the circulatory system of zebrafish larvae of 3 days old, 3 h after injection (Miyares et al. 2014). In the aquatic setup on the other hand, the diffusion of EE2 into the embryos is remarkably fast as well, as the highest dose is already reached before 24 hpf (Figure 3A). These larvae were exposed to a broad concentration range and while *cyp19a1b* is estrogen regulated (Kishida et al. 2001; Sawyer et al. 2006), there was no concentration dependency, as was also reported previously for this gene (Sawyer et al. 2006). However, *cyp19a1b-GFP* transgenic embryos responded in a concentration-dependent manner after EE2 exposure (Brion et al. 2012; Petersen et al. 2013). Based on the early induction of *vtg1* and *cyp19a1b* transcription (Figure 5-6) after EE2 treatment, and based on our internal dose measurements we confirmed the hypothesis that the chorion does not form a barrier for EE2 (Souder and Gorelick 2018) as there was already a significant response of both genes as well as measurable EE2 doses before hatching in the aquatic exposure.

Vitellogenin 1 has been recognized for many years as a biomarker for estrogen disruption, even for embryos – perhaps unexpectedly as vitellogenin is normally only produced by females (Hao et al.

2013; Muncke and Eggen 2006; Muncke et al. 2007). *Vtg1* transcription increased in a similar fashion during development for both exposure routes. The aquatic dose-response relationship was non-monotonic (Figure 5). In both scenarios, transcription was significantly increased at 48 hpf, which is in agreement with a previous study in which *vtg 1* induction was observed in a similar timeframe (Muncke and Eggen 2006). The increased *vtg1* mRNA expression that was observed around 72 hpf in control larvae has been previously described ((Muncke and Eggen 2006), Periz-Stanacev et al., unpublished data) and may be due to the interaction between the ERs and maternally transferred estrogen (Muncke and Eggen 2006). Finally, *vtg 1* appeared to be less sensitive to EE2 than brain aromatase. The response after injection of 1 ng EE2 was comparable to aquatic exposure of 450 ng/L for *vtg1*, but was already similar at 45 ng/L for *cyp19a1b*.

4.2.3 Morphological and physiological responses.

We observed different responses between the exposure routes for some of the morphological and physiological endpoints, suggesting that different exposure routes can result in different outcomes at the individual and perhaps also the population level. A significant number of larvae in the 1.25 ng EE2 injection group showed blood accumulations, lack of blood circulation in the tail, and oedemas. Decreased heart rate was observed for the two highest exposure groups (1 and 1.5 ng EE2) as well. These effects that were all absent after aquatic exposure, indicate that EE2 can impact the cardiovascular system after exposure via injection. This may be due to a difference in compartmentalization.

Swimming distance was decreased after EE2 exposure for both exposure routes. However, this effect was less pronounced after injection. As swimming activity is measured at the last days of the exposure period at which the internal dose of EE2 of the injected embryos is already metabolized and/or eliminated to a larger extent (the dose after aquatic exposure is ± 100 times higher than that

for the 1 ng injection at 120 hpf) compared to aquatic exposure, this is probably the cause of the difference between both exposure routes.

In general, we can conclude that effects at the morphological/physiological level are, in most cases, different between aquatic exposure (µg/L range) and injection of 1 ng EE2, although the internal dose was comparable at the start of the exposure (t = 0 hpf for injection and t = 24 hpf for the aquatic exposure). At the molecular level however (i.e., receptor activation and mRNA expression), zebrafish larvae appeared to be more sensitive after aquatic exposure.

5. POTENTIAL APPLICATION OF MICRO-INJECTION IN EDC TESTING

We explored the usefulness of a micro-injection method into the yolk of zebrafish embryos for endocrine disruptor screening using EE2 (a model compound). We identified two suitable, non-toxic solvents, and demonstrated that inter- and intra-needle variation was low. We further showed that one single injection within the first two hours post fertilization can be used to achieve relevant doses of EE2, leading to measurable biological responses until five days post fertilization (i.e., within the legal time frame (120 hpf) in which zebrafish embryos are not protected by the current EU legislation on the protection of animals used for scientific purposes (2010/63/EU) (EC 2012)). We determined that the internal dose in embryos after aquatic exposure in the µg/L range was comparable to injection in the ng range at the start of the exposure. Estrogen receptor activation and mRNA expression responded at lower doses after aquatic exposure. Effects at higher levels of biological organization were often not consistent between exposure routes, which could partially be caused by the differences in internal doses at the later time points.

Micro-injection could potentially be used as an alternative for, or complementary to, aquatic exposure when testing hydrophobic compounds. It could possibly also be applied as a way to mimic

maternal transfer of hydrophobic compounds, although the differences and similarities between maternal transfer and injection in terms of toxicokinetics and toxicodynamics have not yet been investigated. In order to use micro-injection in fish embryos for the routine identification of endocrine disrupting chemicals, further characterization and validation will be required, in addition to building case studies demonstrating the potential added value of this method. Our brain aromatase data provides the first and important example. The *cyp19a1b* mRNA expression response after injection occurred during early developmental stages, while the response after aquatic exposure may only be detectable at or after 120 hpf, thereby exceeding the limit set by EU legislation on the protection of animals used for scientific purposes (EC 2012). Based on these results, a further step would be to utilize *cyp19a1b-GFP* transgenic zebrafish embryos (Brion et al. 2012) for injection experiments, to establish whether this exposure route causes the GFP signals to be generated earlier in development. This strain is currently undergoing validation (OECD guidance document 150 (OECD 2012a)) after which it can be used for the identification of estrogen active chemicals. Adding micro-injection as a possible exposure route to such assay could aid in the screening of hydrophobic compounds. Addition of other transgenic reporter lines, such as the *5xERE:GFP* line used in our study, and other endpoints such as *vtg1* and *cyp19a1b* mRNA expression, would establish a more complete micro-injection based toolset for screening hydrophobic compounds for estrogen activity.

Supplemental Data

The Supplemental Data are available on the Wiley Online Library at DOI: xxx/yyy.

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Data Accessibility

Supporting data files are available through the Supplemental Data. Raw data are available on request from the corresponding author (dries.knapen@uantwerpen.be).

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