Studying the effects of genistein on gene expression of fish embryos as an alternative testing approach for endocrine disruption

Viktoria Schiller [a,*], Arne Wichmann [a,b], Ralf Kriehuber [c], Elke Muth-Köhne [a], John P. Giesy [d], Markus Hecker [d,e], Martina Fenske [a]

[a] Fraunhofer Institute for Molecular Biology and Applied Ecology, 52074 Aachen and 57392 Schmallenberg, Germany
[b] RWTH Aachen University, Institute for Environmental Research (BioV), 52074 Aachen, Germany
[c] Forschungszentrum Jülich GmbH, Department of Safety and Radiation Protection, 52425 Juelich, Germany
[d] University of Saskatchewan, Toxicology Centre, Saskatoon, Canada
[e] University of Saskatchewan, School of the Environment and Sustainability, Saskatoon, Canada

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ABSTRACT

Assessment of endocrine disruption currently relies on testing strategies involving adult vertebrates. In order to minimize the use of animal tests according to the 3Rs principle of replacement, reduction and refinement, we propose a transcriptomics and fish embryo based approach as an alternative to identify and analyze an estrogenic activity of environmental chemicals. For this purpose, the suitability of 48 h and 7 days post-fertilization zebrafish and medaka embryos to test for estrogenic disruption was evaluated. The embryos were exposed to the phytoestrogen genistein and subsequently analyzed by microarrays and quantitative real-time PCR. The functional analysis showed that the genes affected related to multiple metabolic and signaling pathways in the early fish embryo, which reflect the known components of genistein's mode of actions, like apoptosis, estrogenic response, *hox* gene expression and steroid hormone synthesis. Moreover, the transcriptomic data also suggested a thyroidal mode of action and disruption of the nervous system development. The parallel testing of two fish species provided complementary data on the effects of genistein at gene expression level and facilitated the separation of common from species-dependent effects. Overall, the study demonstrated that combining fish embryo testing with transcriptomics can deliver abundant information about the mechanistic effects of endocrine disrupting chemicals, rendering this strategy a promising alternative approach to test for endocrine disruption in a whole organism in-vitro scale system.

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1. Introduction

The endocrine system of vertebrates is susceptible to disruption by environmental chemicals such as anthropogenic industrial products and byproducts as well as natural compounds. One example is genistein, a plant-derived isoflavone that is categorized as a weak estrogen (OECD, 2006). Many studies have shown that genistein has estrogenic activity in humans, rodents and fish. For example, Kausch et al. (2008) reported increased vitellogenin gene expression in male zebrafish (*Danio rerio*) after 11 days of exposure to genistein, and similar results were observed in male Japanese ricefish (medaka, *Oryzias latipes*) (Zhang et al., 2002; Scholz et al., 2004). Gonadal effects, such as pre-ovulatory oocyte atresia and altered secondary sex characteristics in females as well as intersex gonads in males were observed in medaka after exposure to genistein for 100 days (Kiparissis et al., 2003). Although these studies provided important information about the effects of genistein on aquatic vertebrates, they required the use of numerous animals, as do most of the current testing strategies for endocrine disruption, proposed by regulatory authorities like the US EPA or as outlined in the conceptual framework for the testing and assessment of potential endocrine disrupters of the OECD (OECD, 2006; US EPA, 2009). Whilst there are considerable efforts within the EDC (endocrine disruptive chemicals) screening programs of US EPA and OECD to increasingly include *in vitro* and other animal test free methods in the risk assessment process, there is generally still great reliance on vertebrate test data. Therefore, reliable alternative

testing methods are urgently required to further minimize animal testing according to the 3Rs principle of replacement, reduction and refinement (Russell and Burch, 1959).

Bridging the underlying mechanism of endocrine disruption, e.g., by genistein, with the resulting apical effects is critical. This is, however, not provided by most current studies, which either focus on a particular molecular mechanism in defined tissues, *in vitro* test systems, or on the resulting adverse outcome in the whole organism. A first step towards the development of a testing approach that does not rely on morphological or reproductive endpoints in adult animals, is the design of short-term studies that enable the elucidation of the molecular mechanism of action in non-protected developmental stages.

Fish embryos offer several benefits in this context: First, fish embryos are almost fully developed by the end of the test, thus allowing effects to be assessed in a whole organism. Second, fish embryos within the chorion and early post-hatch are life-stages regarded as non-protected animals according to the revised European directive on the protection of animals used for scientific purposes (Directive 2010/63/EU) (EU, 2010). Finally, zebrafish and medaka are fecund all-year spawners with transparent eggs and a rapid embryogenesis (2 days in zebrafish, 7 days in medaka), favoring them for high-throughput and screening applications. Zebrafish embryos allow a fast 2-day testing procedure, whereas medaka embryos require longer exposure times. This, in turn, facilitates the investigation of long-term exposure effects, which are more relevant for endocrine disruption, but are impossible to assess in the zebrafish embryos. Moreover, sexual development is different in medaka and zebrafish: Zebrafish is an undifferentiated gonochorist without sex chromosome-related sex determination, and all zebrafish develop early ovaries prior to the differentiation of testes and mature ovaries (Takahashi, 1977; Maack and Segner, 2004). Medaka is a differentiated gonochorist, with a male heterogametic sex determination, and testes and ovaries develop directly from the undifferentiated gonad (Matsuda et al., 2002; Saito and Tanaka, 2009). These species-specific differences in developmental and sexual differentiation imply that medaka and zebrafish are complementary models for endocrine disruption testing. The obvious drawback of using embryos as alternative test models is that conventional endocrine disrupting effects, like gonadal malformations or sex ratio alterations, cannot be assessed in sexually undifferentiated developmental stages. However, this issue can be managed by using suborganismic test endpoints, which do not rely on sexual development and reproduction. Transcriptomics promise to be a suitable and sensitive approach to identify gene targets and regulative pathways affected by endocrine disrupting compounds (Wang et al., 2004; Moens et al., 2006; Scholz and Mayer, 2008; Wang et al., 2010), which can be used as markers or indicators of endocrine activity of chemicals. Therefore, we conducted microarray experiments with zebrafish embryos following a 48 h exposure to genistein, in order to analyze the transcriptomic response to this compound.

The primary aim of the study was to assess the suitability of fish embryos for the detection of estrogenic disruption. We further used two complementary fish species, zebrafish and medaka, to address differences in species-specific responses and to carve out distinctive advantages for EDC testing. We chose genistein as a test chemical, firstly, because of its well documented estrogenic mode of action for fish (Bennetau-Pelissero et al., 2001; Ng et al., 2006), what we anticipated to facilitate a correlation of fish embryo results with the estrogenic disrupting effects described for adult fish, and secondly, because genistein exhibits an interesting endocrine disrupting mode of action as it does not exclusively act estrogenic, but reportedly also show estrogen-independent effects (Shim et al., 2007; Kim et al., 2009; Sassi-Messai et al., 2009). By using a chemical, which is known for its estrogenic adverse

effects but of a broader endocrine mechanism of action, we intended to challenge our transcriptomics-based test approach in terms of its suitability and sensitivity to detect estrogenic disruption.

As a phytoestrogen, genistein can bind to estrogen receptors and activate estrogen receptor-dependent pathways (Henley and Korach, 2006; Sassi-Messai et al., 2009). It shows a high affinity to the estrogen receptor b in humans, but indicates no preferences in zebrafish since the receptor isoforms are more conserved in structure than their mammalian orthologs (Kuiper et al., 1998; Sassi-Messai et al., 2009). Besides, genistein gives evidence to also activate estrogen-independent pathways, including pro-apoptotic pathways (Shim et al., 2007; Kim et al., 2009; Sassi-Messai et al., 2009).

With the present study, we aimed at providing substantiating evidence for the suitability of a fish embryo-based transcriptomics approach to test for estrogenic disruption. From the results it can be anticipated that this approach, after a thorough validation with additional estrogenic substances has been provided, will be seen as a promising alternative for screening and testing in the context of endocrine disruptor hazard assessment. In summary, the functional transcriptome analysis identified different genistein-induced pathways related to processes like pro-apoptotic signaling and the disruption of brain and nervous system development. Interesting was also the downregulation of *hox* genes in zebrafish, since several *Hox* genes are known to be estrogen-dependent and to play an important role in embryogenesis and the differentiation of certain tissues (Daftary and Taylor, 2006). Above all, gene expression in both, zebrafish and medaka, clearly indicated an estrogenic response to genistein exposure. It is therefore suggested that our approach of combining the fish embryo test with transcriptomics is promising for the detection and analysis of endocrine disruption.

2. Materials and methods

2.1. Fish maintenance and exposure

Adult Japanese ricefish (medaka, *O. latipes*) and zebrafish (*D. rerio*) were maintained in 200 L glass aquaria under flow-through conditions, with a 14:10 h light:dark photoperiod. Fish were cultured in activated charcoal and particle-filtered, UV-sterilized tap water, entering the aquaria from a 400 L storage tank in which the water was pre-heated to 26 ± 2 °C and aerated. Zebrafish were kept in large spawning groups at a male to female ratio of approximately 2:1; medaka were separated into smaller spawning groups of six fish by tank dividers at a male to female ratio of 1:2. Zebrafish eggs were collected in square metal mesh-covered glass spawning trays and harvested from the aquaria 1 h after light onset. Medaka eggs were striped from the females between one and two hours after light onset. Before testing, the eggs were rinsed in ISO (International Organization for Standardization) medium, prepared according to the Annex 2 of the OECD Guideline 203 (ISO 6341-1982) (OECD, 1992) and diluted 1:5. Medaka eggs were freed from attachment filaments using tweezers.

Genistein powder (G6649, Sigma-Aldrich, Munich, Germany) was dissolved in acetone (ROTIPURAN®, purity ≥ 99.8%, p.a., Carl Roth, Karlsruhe, Germany) to make up 0.5 mg/L and 1 mg/mL stock solutions for the zebrafish and the medaka experiments, respectively. Appropriate volumes of the stock were diluted in 2 mL acetone to make test solutions for each concentration. The test solutions were used to coat 250 mL screw-cap glass bottles (SCHOTT, Mainz, Germany) and the acetone was allowed to evaporate completely. The bottles were then filled with 200 mL pre-aerated ISO water and stirred at 26 °C for several hours. A 200-μL aliquot of each test solution was then transferred to U-bottom 96-well plates

(Greiner Bio-One, Frickenhausen, Germany) and allowed to saturate the walls with genistein overnight. The solutions in the plates were renewed prior to the test start. Then, freshly fertilized zebrafish and medaka embryos were placed into the wells (eggs entered the test solutions no later than 2 h post-fertilization (hpf)) and incubated for 48 h and 7 days, respectively, at 26 °C ± 1 °C. The test solution was refreshed every 2 days in the medaka test series. In order to account for any potential effects of residual acetone after evaporation, a solvent control was included in all tests. The acetone of the solvent controls was evaporated in the same way as for all other test compound solutions.

A concentration response relationship for genistein was established for zebrafish based on lethal and non-lethal morphological defects (see section "Morphological assessments" and Appendix A for details) using nine concentrations between 0.97 and 5.22 mg/L, and data evaluated by probit analysis (ToxRat® Professional 2.10, for details, see Fig. 1). Since the microarrays were performed on embryos after 48 h, the concentration–response curve is provided for this time point only. For medaka, no concentration–response curve could be generated as the limit of solubility was reached at 10 mg/L of genistein, where only 20% of the embryos were affected. Therefore, the EC values for medaka were derived from empirical testing. For the microarray and qPCR tests, embryos were subsequently exposed to the effective concentrations EC_{10} and EC_{20}. Accordingly, zebrafish embryos were exposed to 2.2 mg/L and 2.4 mg/L, medaka embryos to 6 mg/L and 10 mg/L genistein. For the microarrays, four replicates, each comprising of 24 zebrafish embryos, were run in parallel for each treatment, i.e., either genistein, solvent or ISO-only. For the medaka qPCR test, the same design was applied as for zebrafish, but with three instead of four replicates per treatment. After exposure, each replicate group of 24 fish embryos was pooled together, snap-frozen over dry ice and stored at −80 °C.

2.2. Chemical analysis

To confirm the nominal test concentrations of genistein, stock solutions at the beginning of the test and test solutions (taken from test vessels at test start and from the 96-well exposure plates at test end) were chemically analyzed. The samples were subjected to GC–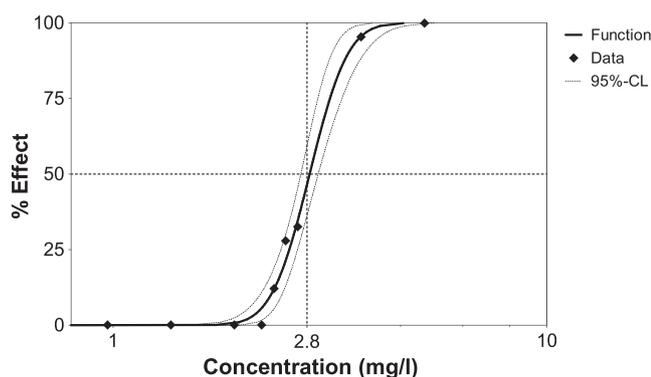MS/MS analysis (Varian 240MS-IonTrap), subsequent to extraction with *tert*-butylmethylether (BME) and derivatization by MSTFA (N-methy-N-(trimethylsilyl) trifluoroacetamide) (detailed protocol in Wenzel et al., 2001).

Chemical analysis could not be conducted on all test solutions used, therefore concentrations in the figures are depicted as nominal values.

2.3. Morphological assessments

The developmental status of the fish embryos (zebrafish and medaka) was assessed using the inverted microscope system AF6000 from Leica (Wetzlar, Germany). Morphological endpoints were recorded after 24 and 48 hpf for zebrafish and after 1, 2, 5 and 7 dpf for medaka. The endpoints assessed are described in the Background Paper on Fish Embryo Toxicity Assays (Braunbeck and Lammer, 2006) and comprised lethal effects, such as coagulation, no heartbeat or lack of somite development, and sublethal abnormalities, e.g. reduced blood flow, formation of edema and malformations of tail. A detailed list of all morphological endpoints is provided in Appendix A.

2.4. RNA extraction and reverse transcription

Total RNA was isolated from frozen, pooled embryos by homogenization in TRIreagent (Sigma-Aldrich, T9424) followed by extraction with chloroform and ethanol and final purification using RNeasy Mini Spin Columns (QIAGEN, Hilden, Germany) according to the manufacturers' protocols. RNA was then stored at −80 °C. RNA concentrations were measured by using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Schwerte, Germany). For cDNA synthesis, RNA was treated with DNAse (Sigma-Aldrich, AMP-D1) and reverse transcribed using SuperScript™ III Reverse Transcriptase (Invitrogen, Carlsbad, USA). A negative cDNA control sample, without added reverse transcriptase, was included in all qPCR measurements. For the microarray analysis, RNA quality was verified on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). All further processed RNA samples had RNA integrity numbers between 8.8 and 9.8 and a 260/280 absorbance between 1.8 and 2.2.

2.5. Real-time quantitative PCR

Zebrafish microarray data were validated by quantitative PCR using the iQ5 real-time PCR detection system (BioRad®, Hercules, CA, USA) and SYBR®GreenER qPCR SuperMix (Invitrogen, Carlsbad, CA, USA), with the primers listed in Appendix B. Only cDNA from the EC_{20} exposure were used for the validation. After incubating for 2 min at 50 °C and for 10 min at 95 °C, targets were amplified at 95 °C for 15 s, 59 °C for 30 s and 72 °C for 30 s (40 cycles), together with the two reference genes, *β-actin* and *rpl13*, used to normalize the expression values. Identical RNA samples were used for the microarrays and the qPCR.

For medaka, qPCR was applied as an alternative to microarrays. Three biological replicates (24 embryos per replicate) were used for these real-time qPCR experiments. The expression of 23 genes was analyzed in 7 day old medaka embryos using the Applied Biosystems 7300 Real-time PCR system with SYBR®Green qPCR Master Mix. The primers shown in Appendix C were selected from the medaka HPG PCR array collection developed by Zhang et al. (2008), which comprise a broad range of genes involved in the hypothalamic–pituitary–gonadal axis. Additional primer sets were designed using primer-BLAST (NCBI) for target genes found to be differentially expressed in zebrafish. After incubating for 10 min at 95 °C, target sequences were amplified at 95 °C for 15 s, 59 °C for 30 s and 72 °C for 30 s (40 cycles). The data was normalized using two reference genes, *16S* rRNA and *rpl7*.

Fig. 1. Concentration–response to 48 h genistein exposure in zebrafish embryos. Newly fertilized zebrafish embryos were exposed to 0.97; 1.36; 1.9; 2.2; 2.35; 2.5; 2.66; 3.73; and 5.22 mg/L of genistein for 48 h post-fertilization. The concentration x-axis is logarithmic. The graph shows data points (black rhombus) depicting the percentage number of occurring effects (lethal and non lethal) in each concentration, the calculated function (black full line) and the 95% confidence levels (dashed lines) (function and confidence levels were retrieved by probit analysis, ToxRat®). Further, the EC_{50} value is tagged at the concentration axis.

For both, medaka and zebrafish, 40 ng cDNA were used as starting template. Two technical replicates and a negative control were integrated in each experiment. Relative gene expression ratios were calculated by the comparative Ct method (ddCt) (Pfaffl, 2001). For zebrafish data, a student's test was used for testing significant differences between the EC_{20} conditions and the solvent control. As the medaka qPCR analysis comprised three conditions (control, EC_{10} and EC_{20}), one-way ANOVA with subsequent Dunnett's multiple comparison test was selected for the statistical evaluation.

2.6. Microarray analysis

Microarray experiments were carried out using Zebrafish (V2) Gene Expression Microarrays 4X44K (Agilent). Each array contained 43,604 oligonucleotide probes representing 16,865 different genes (partly in duplicates, and additional non-annotated genes) and was hybridized with 1.65 μg cRNA amplified from 500 ng of total RNA according to the Agilent one-color microarray hybridization protocol (One-Color Microarray-Based Gene Expression Analysis, version 5.7, Agilent). We carried out cDNA synthesis, cRNA Cy3 labeling, amplification and hybridization according to the manufacturer's protocols (Quick Amp Labeling Kit; Agilent). Microarrays were scanned with the Agilent Microarray Scanner (Agilent), and data were extracted using Agilent Feature Extraction Software (Agilent). The data were normalized using GeneSpring Software GX (Agilent) by setting the raw signal threshold to five, shifting the values of all probes to the 75th percentile and performing a baseline transformation to the median for each gene. Flags previously determined by the Agilent Feature Extraction software were then filtered. For statistical evaluation, one-way ANOVA was carried out for each gene, asymptotic p values were computed and the Benjamini–Hochberg False Discovery Rate was applied to correct for multiple testing. The corrected p value and the fold change cut-off were set to 0.01 and 2, respectively. Finally, Tukey Honestly Significant Difference (Tukey HSD) was chosen for post-hoc testing. Differentially expressed genes were clustered using the Self Organizing Map (SOM) algorithm, which uses the K-means calculation to create clusters of similarly-expressed genes and produce a two-dimensional grid to show the affinity between clusters. The Manhattan function was chosen for distance measurement. In the resulting clusters, entities lacking annotations were removed and excluded from further analysis. The final clusters were then matched to the KEGG Pathway, Gene Ontology (GO) and InterPro databases using FatiGO, a web-based tool for functional gene enrichment analysis, on the babelomics4 platform (http://fatigo.org). To this end, each cluster was tested for overrepresented genes compared to the rest of the zebrafish genome, applying Fisher's exact test and setting the p value cut-off to 0.05.

Microarray data have been deposited in the NCBI Gene Expression Omnibus GEO and can be accessed through GEO Series accession number GSE34616 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE34616).

3. Results

3.1. Chemical analysis

Chemical analysis of the test solutions from the zebrafish exposure showed that the genistein stock solution's actual concentration reached 104% of the nominal concentration (0.5 mg/mL). The exposure concentrations at test start were 82% and 104% of the nominal EC_{10} and EC_{20} concentrations (2.2 mg/L and 2.4 mg/L), respectively, and decreased to 76% and 83% of nominal within the 48 h until the end of the test.

The genistein stock solution concentration in the medaka test prior to test start was 102% of the nominal concentration (1 mg/mL). Test solutions at test start were not measured, but due to the average loss of 20% of nominal over 48 h in the zebrafish test, a decrease in genistein at a similar rate was implied for the first two days of the medaka test. After 7 days of exposure, the genistein exposure concentrations were analyzed and showed a sharp decrease to 19% and 18% (i.e., 1.12 mg/L and 1.4 mg/L) of the nominal 6 mg/L (EC_{10}) and 10 mg/L (EC_{20}). This drastic decrease of the concentration could be explained by microbial degradation and adsorption of genistein to the inner surface of the glass bottle. Since such high losses of genistein were unanticipated, the stock solutions, of which the EC_{10} and EC_{20} dilutions were prepared in both tests, were not renewed during the test period. For the 7 day medaka test, the replacement of the test solutions every other day consequently was ineffective in counteracting further losses of genistein (mainly through adsorption) in the 96-well test plates. As a result, medaka were exposed to a gradient of genistein concentrations ranging from 6 mg/L at test start to 1.12 mg/L at the test end for the EC_{10} value and, correspondingly, from 10 mg/L to 1.4 mg/L for the EC_{20} value. The concentrations in all figures are depicted as nominal.

3.2. Morphological defects

In zebrafish embryos, no morphological defects were detected after the 24 h exposure up to 2.6 mg/L of genistein. Higher concentrations resulted in edema, head and tail malformation and reduced spontaneous movement (Fig. 2). After the 48 h exposure up to 2.2 mg/L, we observed a reduced blood circulation as an additional effect. From the concentration response curve for the cumulative lethal and sublethal morphological effects (Fig. 1), we derived an EC_{10} of 2.2 mg/L, an EC_{20} of 2.4 mg/L and an EC_{50} of 2.8 mg/L.

In medaka embryos, the first morphological effects occurred after four days of exposure. At nominal 10 mg/L of genistein, 20% of exposed medaka embryos showed morphological defects, including reduced blood circulation, delayed blood vessel development and malformations of the notochord, tail and head (Fig. 3). It was not possible to determine the concentration–response relationship because the maximum solubility was reached at 10 mg/L.

3.3. Zebrafish microarray

Zebrafish microarray analysis resulted in the detection of 881 differentially expressed genes. All genes with a FDR-corrected p value of less than 0.01 were regarded significant. The subsequent Tukey HSD post-hoc test divided those genes into different groups representing the EC_{10} (55 genes) and the EC_{20} (881 genes) condition, when compared to the solvent and ISO-water controls. We also identified 36 genes that showed differential expression when compared to the solvent control and ISO-water control. A fold change cut-off of 2.0 reduced the number of differentially expressed genes in the EC_{10} and EC_{20} data sets to 20 and 662, respectively (Supplementary Tables S1 and S2). Nine genes were differentially expressed between the solvent and ISO-water controls (Supplementary data, Table S3).

SOM clustering identified six clusters in the EC_{10} group and eight in the EC_{20} group. Because each cluster in the EC_{10} group contained only 1–5 genes, these were merged into one cluster representing the upregulated genes and another representing the down-regulated genes. In the EC_{20} group eight separate clusters were identified (Fig. 4). Clusters I, IV and VIII contained relevant regulated endocrine genes, which could not be assigned to KEGG pathways, GO categories or InterPro data (Table 1). In addition, four distinct KEGG pathways were identified that were affected under EC_{20} conditions (Fig. 4). These included a strong induction of the p53 signaling pathway, a weaker but still significant upregulation of steroid
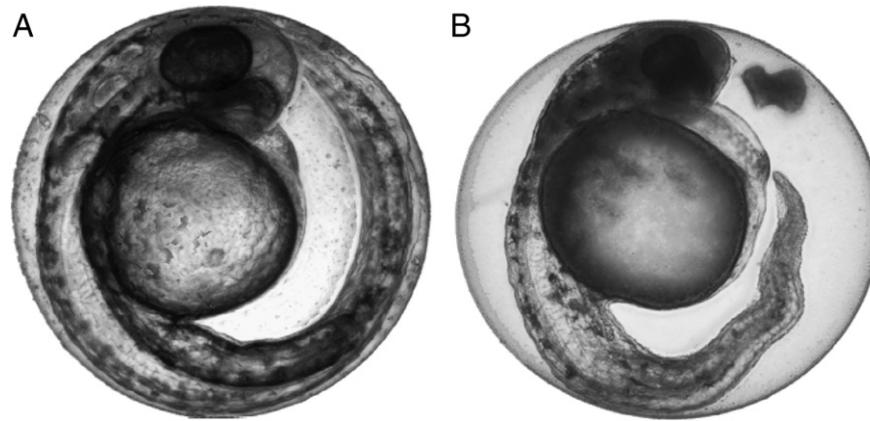
Fig. 2. 48 hpf zebrafish embryos A) of solvent control and B) genistein exposed (2.2 mg/L), showing edema as well as head and tail deformation.

biosynthesis and glycine/serine/threonine metabolism, plus the suppression of DNA replication. In the EC_{10} data set, GO analysis revealed upregulated genes involved in the response to estradiol as well as downregulated genes in the chromatin, nucleosome and chromosome function category. In the EC_{20} dataset, the most strongly upregulated genes grouped under the GO term 'apoptosis' and many downregulated genes grouped under GO terms relevant for general organ morphogenesis and nervous and endocrine system development. Further annotation provided by InterPro analysis associated genistein regulated genes with predicted protein domains which are mostly related to biological functions. No InterPro terms were retrieved from the EC_{10} data set, but several were retrieved from the EC_{20} data set, including homeobox family proteins and other embryogenesis-related proteins, such as Mab21-like 2 and transcription factor AP-2 in the downregulated cluster (Fig. 4).

In order to validate the microarray results for selected significantly changed genes represented in the above identified pathways (*vtg1, cyp19a1b, sc4mol, pax2a, nkx2.1, hoxa9a, hoxa10b* and *hoxa11b*), their regulation was confirmed by quantitative PCR. Corresponding to the microarray results, we found *vtg1, cyp19a1b* and *sc4mol* to be upregulated, whereas *pax2a, nkx2.1, hoxa9a, hoxa10b* and *hoxa11b* were downregulated (Fig. 5). Altered expression was

calculated by normalization to the reference genes *β-actin* and *rpl13*, which did not show any exposure-related changes, neither in the microarray nor in the qPCR analysis.

To corroborate the enhancement of apoptosis related genes, we additionally tested and confirmed a concentration dependent increase in apoptotic cells in the embryos by acridine orange staining. Methodology and results are appended to this paper in Appendix D.

3.4. Medaka quantitative PCR

There were significant changes in the expression of seven genes under the EC_{20} and one gene under the EC_{10} condition, respectively (Fig. 6). As in zebrafish, *cyp19a1b* and *vtg1* were upregulated under the EC_{20} condition. *Dio2* was repressed in the EC_{10} condition and for *pax2a* a trend towards downregulation could be observed, which corresponds to the zebrafish EC_{20} results. Differently to zebrafish, these two genes were upregulated in the EC_{20} group of medaka. Also differently regulated was *tp53*, which was induced in zebrafish and repressed in medaka. Expression of *nkx2.1* and of the homeobox genes *hoxa9a, hoxa10b* and *hoxa11b* remained unchanged after genistein exposure. Responses observed in medaka only involved the *estrogen receptor 2a* and *cyp19a1a*.
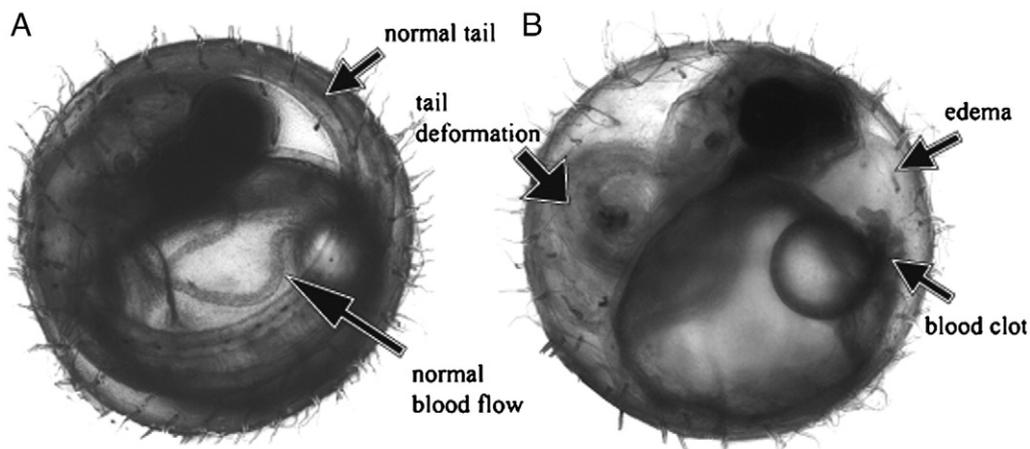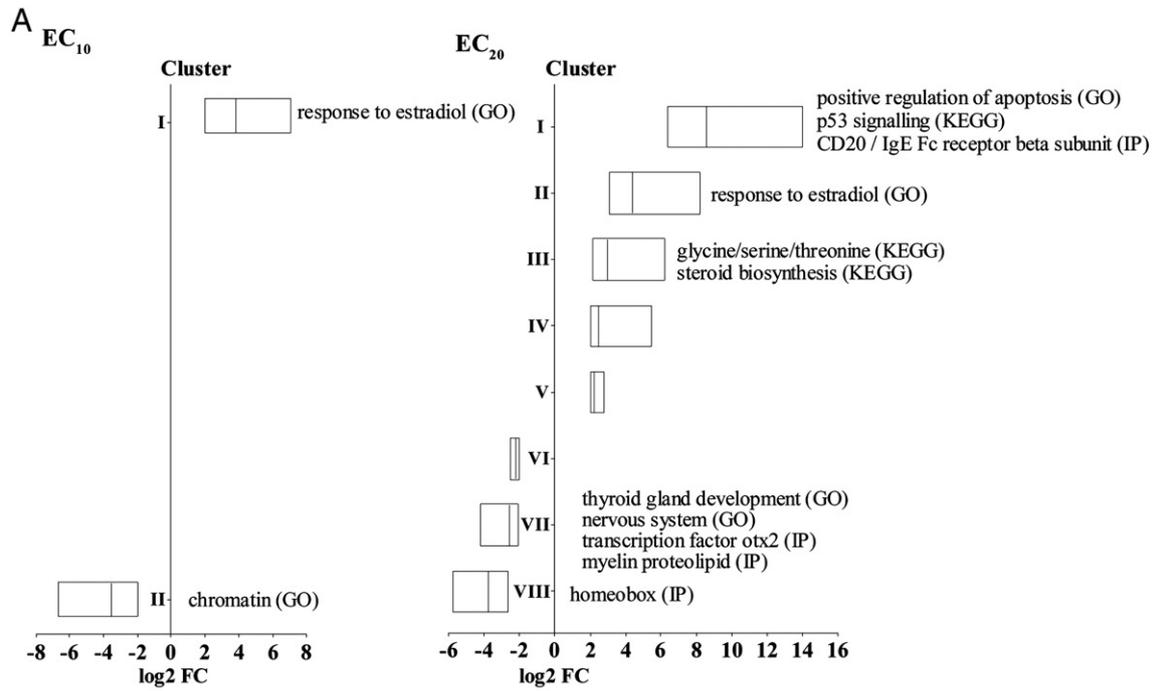

Fig. 3. 7 dpf medaka embryos of A) solvent control and B) genistein exposed (6 mg/L); black arrows pointing at tail deformations and cardiovascular defects in exposed embryos and the corresponding areas in control embryos.

Fig. 4. Gene clustering and functional analysis of the zebrafish microarray data. A) Graphical illustration of gene clusters for the EC_{10} and EC_{20} data set. Gene clusters (I–II for EC_{10}, left; I–VII for EC_{20}, right) are represented by bars and plotted on the y-axis according to the log2 fold change (x-axis) of the genes within each cluster. The vertical line in the bars shows the median log2 fold change of each cluster. The symbols within the bars show the results of the functional analysis, depicting the KEGG (rectangular shape), GO (round shape) and InterPro (oval shape) categories found to be overrepresented in each cluster (note that for the EC_{20} condition, the figure shows only a selection of categories, the complete set is listed in Tables S4–6 of the Supplementary data). B and C show the annotations of the functional categories and the affected genes in the clusters for EC_{10} (top) and EC_{20} (bottom). For clustering, all genes were included with a minimal log2 fold change of 2 and a maximal p-value of 0.01. For functional analysis, the p-value for significance was set on 0.05.

4. Discussion

This paper aimed at providing substantiating data for a fish embryo based alternative testing strategy suitable for the assessment of estrogenic effects. This study demonstrated that exposure to estrogens, such as genistein, interfered significantly with multiple molecular pathways in 2- and 7-day old zebrafish and medaka embryos, respectively, indicating the susceptibility

Table 1

Selected endocrine genes, differentially expressed in the EC_{20} zebrafish microarray data set (log 2 fold change > 2, p-value ≤ 0.01). These genes were not in the significant KEGG, GO or InterPro categories determined by functional analysis, but they exert important functions in the endocrine system.

Cluster	Gene symbol	Description	Log2 fold change EC_{20}	Accession	GO association
I	*lep a*	Leptin a	8.0 up	NM_001128576	GO:0005179 hormone activity
IV	*pth1a*	Parat-hormone 1a	3.4 up	NM_212950	GO:0005179 hormone activity
VIII	*dio2*	Deiodinase type II	3.1 down	NM_212789	GO:0042446 hormone biosynthetic process GO:0009790 embryo development

of fish embryo tests regarding the assessment of estrogenic chemicals.

The p53 signaling pathway was the most severely affected under the EC_{20} condition. The consequence of an activation of the tumor suppressor protein TP53 by various stress factors can be either cell cycle arrest caused by DNA replication blockage or apoptosis (Cox and Lane, 1995; Klein and King, 2007). Since the analysis of the GO categories identified the terms "positive regulation of apoptosis" and "positive regulation of caspase activity" in the same cluster, it was suggested that in the fish embryos the induction of *p53* may be predominantly pro-apoptotic. Since *bbc3* (BCL2 binding component 3) and *casp8* (caspase 8) are included in the same cluster, it is possible that genistein-dependent *p53* activation induces the release of cytochrome *c* by activation of *bbc3*, which in turn activates caspase 3 causing nuclear condensation and fragmentation, as
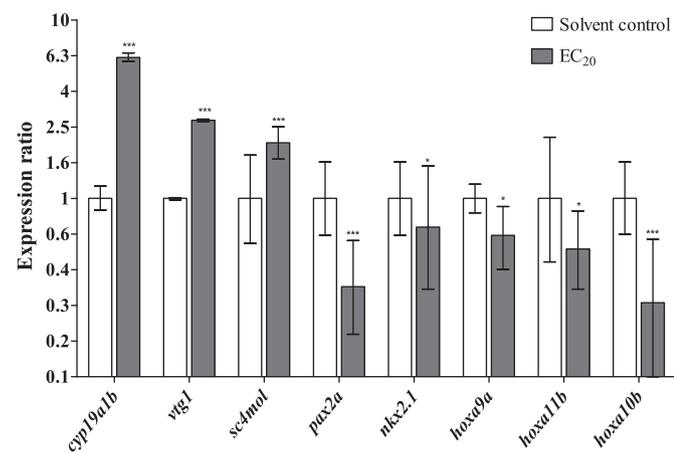

Fig. 5. Relative transcript levels of vitellogenin 1 (*vtg1*), aromatase b (*cyp19a1b*), sterol-C4-methyl oxidase-like (*sc4mol*), paired box gene 2 a (*pax2a*), NK2 homeobox 1 (*nkx2.1*), homeobox a9a (*hoxa9a*), homeobox a10b (*hoxa10b*) and homeobox a11b (*hoxa11b*) in zebrafish after a 48 h exposure to 2.4 mg/L genistein (EC_{20}), determined by quantitative PCR. Expression ratios (ER) were calculated according to the $2^{\Delta\Delta Ct}$ method (Pfaffl, 2001), p values determined by student's *t*-test are indicated by asterisks (* = <0.05,** = <0.01,*** <0.001).

previously described (Yoon et al., 2000; Ding et al., 2003; Reimertz et al., 2003; Xun, 2008). The transcriptional induction of *p53*, in turn, might be attributed to the inhibition of topoisomerase II, a crucial enzyme for DNA replication. This relationship has already been associated with the apoptotic impact of genistein in mammalian *in vitro* systems (Ye et al., 2000; López-Lazaro et al., 2007; Schmidt et al., 2008). Genistein has also been shown to promote early apoptosis in zebrafish embryos from 15 hpf, suggesting that it acts prior to the onset of somitogenesis (Kim et al., 2009; Sassi-Messai et al., 2009). Early apoptosis could induce mortality in the embryos, but mortality did not increase until 96 h (Sassi-Messai et al., 2009), which is in agreement with a life cycle study of our department (Wenzel et al., 2001) that showed a large increase in mortality only after 6 days. Although it remains unclear whether apoptosis in fish embryos and mortality in larvae can both be attributed to the p53 signaling pathway, the transcriptomic data of the present study provides some indication that there may be a link between larval mortality (Wenzel et al., 2001) and early genistein exposure. Additionally, GO analysis showed repression of genes necessary for the formation of nucleosomes at the EC_{10}, suggesting a pro-apoptotic activity even at low genistein concentrations. Sassi-Messai et al. (2009) detected apoptosis at concentrations from 0.68 mg/L via staining of apoptotic cells. Thus, in future experiments it would be interesting to determine the minimum concentration at which apoptosis-related induction is detectable in 48 hpf embryos at gene expression level. It has been shown that apoptosis can occur in the hindbrain and anterior spinal cord of zebrafish embryos following exposure to genistein (Kim et al., 2009; Sassi-Messai et al., 2009). This may be reflected by the GO and InterPro data of our study, which indicated a disruption of genes involved with the regulation of the nervous system development and the downregulation of Otx family transcription factors, which are required for brain morphogenesis (Acampora et al., 1999; Klein and Li, 1999). Furthermore, InterPro data revealed downregulation of genes responsible for the formation of the myelin proteolipid protein, the major component of myelin. All findings together suggest that the predominant effect of genistein was mediated by *p53*, inducing apoptotic effects in hindbrain and spinal cord. Acridine orange stainings of control and genistein-exposed zebrafish embryos verified these findings at the cellular level (Appendix D), showing apoptosis in the brain (data not shown) and in the spinal cord. Since these effects were also seen in exposures with estrogen receptor antagonists (Sassi-Messai et al., 2009), we hypothesize that genistein-mediated induction of *p53* signaling may be estrogen-receptor independent. Regulation of *p53* was also identified in medaka. However, contrary to zebrafish, *p53* was downregulated. As the exposure duration in medaka embryos was longer than in zebrafish, a negative feedback regulation may be the explanation for this opposing effect, as this was observed in fish in previous studies (Chen et al., 2005, 2009).

The next ranking cluster of upregulated genes in the zebrafish EC_{20} group was correlated to the GO categories "response to estradiol stimulus" and "response to steroid hormone stimulus", and included the genes *cyp19a1b* and *vtg1*. The upregulated cluster in the EC_{10} group showed also enrichment of genes of these categories, and accordingly, these genes were found to be upregulated in the medaka embryos. CYP19a1 (aromatase) catalyzes the conversion of testosterone to estradiol, and there are two functionally distinct aromatase gene isoforms *cyp19a1a* and *cyp19a1b* in zebrafish, which are expressed predominantly in the ovary and brain, respectively, at different rates (Kishida and Callard, 2001; Fenske and Segner, 2004). Both isoforms are crucial for gonadal development in fish and disruption of the aromatase function can cause severe effects on reproduction (Piferrer et al., 1993; Kitano et al., 2000; Fenske and Segner, 2004), but only the aromatase 1b is directly responsive to estradiol stimulus (Menuet et al., 2005; Diotel et al.,
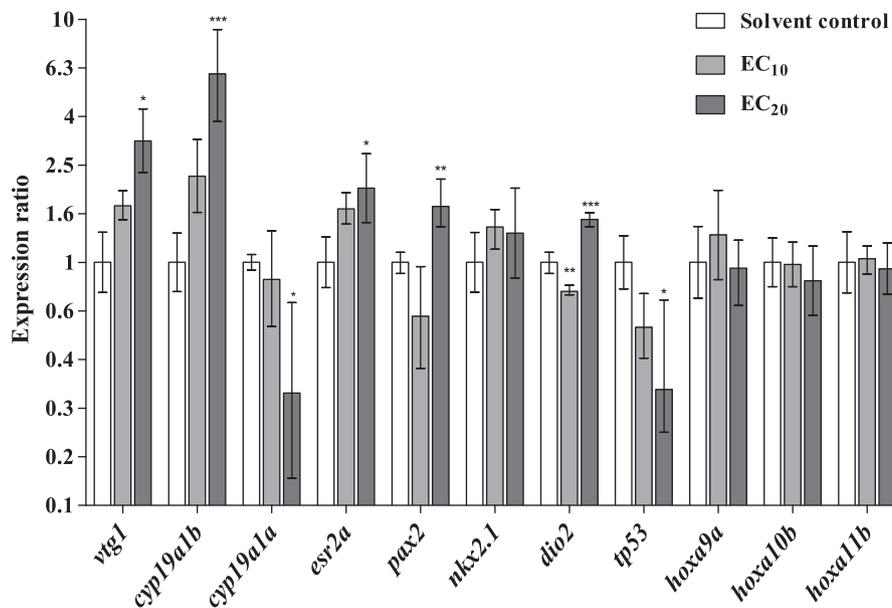
Fig. 6. Relative transcript levels of vitellogenin 1 (*vtg1*), aromatase b (*cyp19a1b*), estrogen receptor α (*erα*), estrogen receptor 2a (*esr2a*), aromatase a (*cyp19a1a*), paired box gene 2 a (*pax2a*), deiodinase 2 (*dio2*), NK2 homeobox 1 (*nkx2.1*), homeobox a9 (*hoxa9a*), tumor suppressor protein 53 (*tp53*), homeobox a10b (*hoxa10b*) and homeobox a11b (*hoxa11b*) in medaka after a 7 day exposure to 6 mg/L (EC$_{10}$) and 10 mg/L (EC$_{20}$) genistein, determined by quantitative PCR. Expression ratios (ER) were calculated according to the $2^{\Delta\Delta Ct}$ method (Pfaffl, 2001), p-values determined by Dunett's multiple comparison test are indicated by asterisks (* = <0.05,** = <0.01,***<0.001).

2010). For fish embryos, Kishida et al. (2001) suggested that *cyp19a1b* is necessary for central nervous system development and thus, represents a reliable marker for testing neurotoxic effects of xenoestrogens during embryogenesis. Accordingly, *cyp19a1b* transcription commences when the central nervous system begins to develop (Kishida and Callard, 2001) and is concentrated in radial glial cells, which are important for neurogenesis (Tong et al., 2009; Mouriec et al., 2009). In this context, the glial cells in the hypothalamus are the predominant area of *cyp19a1b* expression in the brain of 48 hpf embryos (Menuet et al., 2005). The synthetic estrogen analog ethinylestradiol has been shown to induce *cyp19a1b* expression additionally in the preoptic area, which has also been confirmed for genistein by in situ hybridization (Sassi-Messai et al., 2009; Chung et al., 2011; Vosges et al., 2012) and more recently in zebrafish embryos of a cyp19a1b transgenic line *in vivo* (Brion et al., 2012). This clearly demonstrates the estrogenic mode of action of genistein. The minimum concentrations reported so far to induce *cyp19a1b* expression ranged from approximately 0.5–0.7 mg/L for zebrafish (Sassi-Messai et al., 2009; Brion et al., 2012). The induction of aromatase by genistein and ethinylestradiol in the same celltype (glial cells) and brain area as well as the presence of estrogen response element (ERE) in the cyp19a1b gene reflect the estrogen-dependency of *cyp19a1b* regulation. Since it is known that *cyp19a1b* is essential for the regulation of sexual differentiation in fish, a dysregulation by estrogenic substances, such as genistein, may long-term have severe reproduction- and population-relevant effects. The first expression of *cyp19a1b* in response to estrogens (Kishida et al., 2001) coincides with the onset of estrogen receptor expression (Mouriec et al., 2009), and the expression of *cyp19a1b* then increases strongly between 24 and 48 hpf. As the estrogen receptor 1 (*esr1*) was not detected in the embryos before 48 hpf (Bardet et al., 2002), it is likely that the estrogen receptor 2a and/or estrogen receptor 2b (*esr2a* and *esr2b*, respectively, both expressed by 24 hpf) are responsible for regulating aromatase (Mouriec et al., 2009; Pikulkaew et al., 2010). Indeed, in medaka

we found *esr2a* being upregulated by genistein, implying this form as the active estrogen receptor at the embryonic stage, which was also described by Chakraborty et al. (2011). The identified regulation of *esr2*a in medaka only might be attributed to either the longer exposure time or a later developmental stage, compared to zebrafish. Additionally, sexual differentiation in medaka starts at a much earlier developmental time point than in zebrafish and thus, also the differentiation related regulation of gene expression (Maack and Segner, 2003; Kobayashi et al., 2004; Matsuda, 2008; Herpin and Schartl, 2009; Kondo et al., 2009). It could therefore be hypothesized that the estrogen receptors *esr2a* and *esr2b* are more susceptible in medaka than in zebrafish embryos. Although we did not detect altered estrogen receptor expression in zebrafish, an induction of *cyp19* by non-genomic estrogen receptor 2a signaling is still plausible, since this mechanism has already been reported to regulate neuronal gene expression particularly in the brain (Beyer and Raab, 1998; Beyer and Karolczak, 2000; Belcher and Zsarnovszky, 2001) The involvement of an estrogen receptor in estrogen signaling in fish embryos, in turn, indicates that other estrogen-receptor dependent pathways may also be affected by estrogenic compounds. In this context, gonadotropin-releasing-hormone (GnRH) neurons are important targets of steroid hormones and it has indeed been shown recently that the ontogeny of (GnRH) neurons can be modified in an estrogen-receptor dependent manner after EE2 exposure (Vosges et al., 2012). Since GnRH neurons are also essential regulators of gonadal development and sexual differentiation, their dysregulation may cause severe reproductive effects in adult fish.

Our data showed that the *cyp19a1a* isoform was downregulated in medaka, but not in zebrafish, which is in agreement with Kishida et al. (2001), who also reported a lack of estrogen-induced response of *cyp19a1a* in zebrafish embryos. This is also consistent with data from juvenile and adult zebrafish (Hinfray et al., 2006) and *in vitro* experiments in rainbow trout (Pelissero et al., 1996). Instead, the downregulation of *cyp19a1a*

by genistein in medaka was most likely estrogen-independent since there is no evidence yet that *cyp19a1a* may be estrogen responsive (Trant et al., 2001; Sawyer et al., 2006) and there is also no full ERE in its promoter region. Genistein rather inhibits the aromatase directly by interacting with its active site (Kao et al., 1998).

Vitellogenin is the yolk precursor protein predominantly expressed in the female liver, but it can also be induced in male fish in response to exogenous estrogens and thus, has become the best characterized biomarker for estrogenic disruption in fish (Sumpter and Jobling, 1995). Alterations in vitellogenin 1 (*vtg1*) expression have been associated with severe reproductive effects such as intersex and impaired gonadal growth (e.g. Jobling et al., 1998; Panter et al., 1998; Jobling et al., 2002). The induction of *vtg1* in fish by genistein has been reported before (Zhang et al., 2002; Scholz et al., 2004; Kausch et al., 2008), but the results indicate great variability and seem to depend on exposure time and concentration. In medaka, exposure to 10 μg/L of genistein for four weeks was sufficient to induce *vtg1* expression in male fish, whereas in adult male zebrafish an induction in liver tissue could only been detected at 5 mg/L after seven days. Our data confirm *vtg1* regulation in both effect concentrations for zebrafish and in the EC_{20} condition for medaka. Muncke and Eggen (2006) observed the induction of *vtg1* expression even already in zebrafish embryos from 24 hpf. According to previous studies vitellogenesis in zebrafish is mainly controlled via Esr1, but Esr2a may also be involved, as it is able to mediate the induction of the *esr1* promoter, and subsequently its expression (Menuet et al., 2005). However, Esr1 is most likely not involved in the fish embryo because, as stated above, it is not yet expressed at this early developmental stage. Instead, we believe that *vtg1* expression involves direct signaling through Esr2a, as implied by the increased *esr2a* expression in medaka. The absence of *esr2a* regulation in zebrafish suggests that basal estrogen receptor concentrations may already be present in the embryos and that only after depletion of these receptors, the estrogenic stimulus manifests in an upregulation of the corresponding estrogen receptor gene (Arukwe et al., 2001). The gene response would then be delayed and likely to be missed by the short 48 h-exposure of zebrafish. Another regulative pathway potentially responsible for the upregulation of *vtg1* in the zebrafish embryos is a calcium-dependent mechanism mediated by the *parathormone* (*pth*) gene, which was found strongly upregulated in our study. This correlation has previously been reported for estradiol by Bevelander et al. (2006). However, the exact mechanism by which genistein induced vitellogenin in the embryos remains to be clarified.

Overall, our results clearly demonstrate that an estrogenic response to genistein can be detected in fish embryos. Since the response to estradiol stimulus was the only biological process being upregulated at EC_{10} conditions, we conclude that the primary mode of action of genistein was estrogenic, besides its pro-apoptotic effect. This conclusion is substantiated by the results of another study (Sassi-Messai et al., 2009) that identified two different pathways, an estrogen receptor-independent induction of apoptosis and an estrogen receptor-dependent upregulation of *cyp19a1b* expression in the brain, through which genistein acts in zebrafish. The KEGG analysis confirmed the induction of steroid biosynthesis by genistein, mainly through the genes *lss* (encoding lanosterol synthase) and *sc4mol* (encoding a sterol-C4-methyl oxidase-like enzyme). This suggests that genistein may stimulate the synthesis of cholesterol, which is a component of the plasma membrane as well as a precursor of calcitriol (vitamin D), bile acid and steroid hormones. Cholesterol synthesis is generally known to be stimulated by estrogens (Messa et al., 2005), and the induction of steroid hormone synthesis by genistein in the ovaries of adult medaka *ex vivo* has also been documented (Zhang et al., 2003). Further,

since steroid hormones regulate the development and function of secondary sex characteristics in fish (Brantley et al., 1993), it has been proposed that the gonadal abnormalities observed in a medaka fish study after a 100-day exposure to 1 mg/L genistein were at least partly caused by disruption of steroid biosynthesis (Kiparissis et al., 2003). Thus, our microarray data clearly corroborate that genistein interferes with steroid biosynthesis, and longer-term this alteration of steroid biosynthesis genes is likely to affect normal gonadal development. Besides, steroids play a crucial role in the development and function of the central nervous system in fish (Arukwe et al., 2008), and further upregulation of steroidogenesis will therefore also impact the brain of the embryos. Since our data suggest induction of steroidogenesis occurs via cholesterol synthesis, we hypothesize that the brain of the embryos is particularly prone to a disruption of steroidogenesis due to the cholesterol-impermeability of the blood–brain-barrier, which requires de novo synthesis.

In addition, our microarray results revealed other pathways affected by genistein, including some regulated by homeobox or Hox genes, which are key regulators of animal development (Pearson et al., 2005). The downregulation of several developmentally-relevant *hox* genes (Fig. 4) fits in with the identified GOs indicating disruption of pattern formation and morphogenesis and the observed deformations of chorda, tail and head. The downregulation of *hox* genes in zebrafish embryos may be caused by the pro-apoptotic and anti-proliferating activities of genistein. Otherwise, certain *Hox* genes are also known to be regulated by estrogens and to control the development of female primary sex characteristics (Hewitt et al., 2005; Daftary and Taylor, 2006). It has been shown that *Hoxa9*, *Hoxa10* and *Hoxa11*, which belong to the same *HoxA* cluster, control the differentiation of the oviduct and the uterine and cervical anlagen in mammalian embryos (Taylor et al., 1997; Daftary and Taylor, 2006), and dysregulated expression during development causes reproductive defects and reduced fertility in adults (Hewitt et al., 2005). For example, exposure of neonatal mice to genistein causes uterine adenocarcinoma (Newbold et al., 2001; Daftary and Taylor, 2006) and it is assumed that this reflects the dysregulation of *Hoxa10* expression in the uterus (Akbas et al., 2007). Compared to mammals, teleosts have seven (or even eight in some species) instead of four *Hox* clusters, probably due to chromosome doubling following genome duplication (Amores et al., 1998; Kurosawa et al., 1999). Thus, genes of the mammalian *HoxA* cluster split into two clusters *hoxAa* and *hoxAb* in medaka and zebrafish. In agreement with the mammalian studies, we observed downregulation of *hoxa9a*, *hoxa11b* and in particular *hoxa10b* in genistein-treated zebrafish but failed to detect any *hox* gene regulation in medaka. This difference in the *hox* gene expression between both fish species may be a result of their evolutionary distance. In both fish species, *hoxa9*, *hoxa10* and *hoxa11* are expressed in the trunk and in the pectoral fin bud during embryonic development. Whereas the expression of *hoxAa* and *hoxAb* genes in the pectoral fin bud was shown to be comparable between medaka and zebrafish, the expression boundaries in the trunk was slightly shifted (Takamatsu et al., 2007; Ahn and Ho, 2008). This spatial shift could partially explain the differing expression patterns observed in medaka and zebrafish. We therefore hypothesize that the different responses of the hox genes in zebrafish and medaka indicate that it were mainly the trunk related hox genes that were susceptible to the estrogenic activity of genistein. Critically, slightly disaligned embryonic stages during analysis have to be taken into consideration as a possible cause of the species specific response in medaka at 7 dpf versus the zebrafish at 48 hpf, in particular due to the temporal regulation of the hox gene expression. However, if a correlation between hox genes and the sexual development could be confirmed by further investigations in

zebrafish, the regulation of hox may even be considered a useful marker in zebrafish embryos for the prediction of estrogenic reproductive effects in adult fish.

Finally, GO analysis also revealed the downregulation of genes involved in endocrine and thyroid development, such as *nkx2.1* and *pax2a* (encoding transcription factors) and *dio2* (encoding type II iodothyronine deiodinase, which converts thyroxine (T4) to triiodothyronine (T3)). For zebrafish, the knockout of *dio2* and the resulting lack of T3 have shown to disrupt embryonic development and pigmentation (Walpita et al., 2009). Interestingly, we could not detect pigmentation disorders in our treated zebrafish embryos. It can only be speculated whether the downregulation of *dio2* was, compared to the gene knockout, insufficient to disrupt pigmentation. The decrease of *pax2* and *dio2* transcription was consistently found both in zebrafish and medaka embryos and this is also consistent with other studies reporting thyroid-disrupting effects of genistein, such as the downregulation of deiodinase and thyroid peroxidase, reduced thyroid receptor activity or the disruption of iodide accumulation *in vitro* (Cody et al., 1986; Chang and Doerge, 2000; Schmutzler et al., 2007; Walpita et al., 2009). Contrary to this antithyroidal effect in medaka at the EC_{10} level, upregulation of *pax2* and *dio2* was detected at the EC_{20} level. This indicates a concentration-dependent effect for medaka and may reflect the apparently complex etiology of genistein in terms of thyroid disruption. Thyroidal effects of genistein seem ambiguous, and antithyroidal as well as thyroid receptor agonistic effects have been reported and long-term thyroidal effects were not always reproducible (Doerge and Chang, 2002; Schmutzler et al., 2007; Li et al., 2011). The cause of the disagreement in the response between zebrafish and medaka embryos cannot be clarified by the present data, but a thyroid disrupting impact of genistein at gene expression level seemed evident in both species. The key question for our testing approach, whether an estrogenic chemical generally impacts the thyroid activity (Schmutzler et al., 2007), however, cannot be resolved and requires investigations on further estrogenic chemicals.

5. Conclusions

The effects we observed on gene expression patterns in zebrafish and medaka were largely coherent with findings of other genistein studies (Kim et al., 2009; Sassi-Messai et al., 2009), which also demonstrated a primarily estrogenic response and the promotion of apoptosis. Despite the absence of characteristic apical endocrine disrupting effects in fish embryos, we were able to detect endocrine disrupting molecular effects of genistein even at low EC_{10} and EC_{20} exposure concentrations by using microarray analyses and quantitative RT-PCR. The combination of the two fish species zebrafish and medaka in the testing approach proved beneficial because the different exposure times elicited complementary toxicological effect information. For medaka, the downregulation of the aromatase isoform *cyp19a1a* was found and the *estrogen receptor 2a* was identified as the likely mediator of the estrogenic signaling and, whereas for zebrafish, a dysregulation of *hox* genes was evident. The species-dependent effects on the transcriptome identified in this study are presumably also related to the different modes of sexual differentiation of zebrafish (an undifferentiated gonochorist) and medaka (a differentiated gonochorist). Differences in exposure times and developmental stages, may, however, have also contributed to species-specific effects. Consequently, a two-species approach can provide the opportunity to derive key information on the molecular mechanisms underlying estrogenic disruption, caused by chemicals like genistein, which can affect sexual differentiation and reproduction differently in different fish species.

In summary, this study demonstrates that the combination of fish embryo tests with zebrafish and medaka and transcriptomics provides a promising approach, which can deliver a wealth of mechanistic information about endocrine disruption in fish. Moreover, if this approach is successfully validated for further estrogens or even chemicals of other endocrine activities, confirming endocrine disruption mode-specific patterns in the transcriptome response, then subsets of critical genes suitable for a more practical and cost-efficient PCR-based analysis could be extracted. Further validation of the assay should then be aimed at providing essential data on the sensitivity of gene expression endpoints to enable a comparison with existing screening methods for endocrine disruption. After successful validation, a transcriptomics-based assay as proposed by the current study, would then allow affordable routine measurements alongside the fish embryo test and could longer term be considered a very valuable alternative testing method for the assessment of environmental estrogens and considered a useful tool for EDC screening applications.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cbpc.2012.09.005.

Appendix A. Assessed morphological endpoints for 48 hpf zebrafish and 7 dpf medaka embryos (modified after Braunbeck and Lammer, 2006)

Lethal endpoints

- Coagulation
- Tail not detached
- No somite formation
- No heart-beat

Nonlethal endpoints

- Formation of somites
- Development of eyes
- Spontaneous movement
- Heart-beat/blood circulation
- Heart-beat frequency
- Pigmentation
- Formation of edemata
- Malformation of head
- Malformation of tail
- Malformation of heart
- Modified chorda structure
- Yolk deformation
- General growth retardation

Appendix B. Primers used in zebrafish quantitative PCR

Gene	Accession	Primer sense	Primer antisense
vtg1	NM_001044897	GTCATCAATGAGGATCCAAAGGCCA	GCCTCAGCGATCAGTGCACCAT
cyp19a1b	NM_131642	GCTCCAGACACGCTCTCCAT	CATCCTCCAGAGACTGCCTCA
pax2a	NM_131184.2	GCAGACCCCTACCTGACGTGGT	TTGGGCGTCGCCACTTTTGGTT
nkx2.1	NM_131589.1	GAACCCGGAGCCGAGATACCCA	CGTCGGGGTTAGATGGATCATGC
hoxa9a	NM_131532.2	CATCGAGGGGAAACCGGGCG	CCGGTCGCGCGTGAGGTAAG
hoxa10b	NM_131155.1	AAGGGGGTCCACTGGCACCG	TCGCCGTCAGCCAGTTAGCTG
hoxa11	NM_131147.1	GCGGCAGCAGCAATGGACAAAG	CGGTCAGTGAGGTTGAGCATTCTG
rpl13	NM_198143.1	TCTGGAGGACTGTAAGAGGTATGC	AGACGCACAATCTTGAGAGCAG
bactin	NM_131031.1	CGAGCAGGAGATGGGAACC	CAACGGAAACGCTCATTGC

Appendix C. Primers used in medaka quantitative PCR

Gene	Accession	Primer sense	Primer antisense
era*	D28954	GAGGAGGAGGAGGAGGAGGAG	GTGTACGGTCGGCTCAACTTC
erb*	AB070901	GCTGGAGGTGCTGATGATGG	CGAAGCCCTGGACACAACTG
errg1	EF544135	AAGAGGAGACGCAAGTCATG	TTATATGGCTTCTTGGGAGG
ara*	AB076399	ACCTGGCTCACTTCGGACAC	TCTGACGCCGTACTGCTCTG
cyp19a1a*	D82968	CTCTTCCTGGGTGTTCCTGTTG	GCTGCTGTCTTGTGCCTCTG
cyp19a1b*	AY319970	TCCTGATAACCCTGCTGTCTCG	GTTGGTCTGCCTGATGCTGTTC
vtg1*	AB064320	ACTCTGCTGCTGTGGCTGTAG	AAGGCGTGGGAGAGGAAAGTC
cyp3a*	AF105018	GAGATAGACGCCACCTTCC	ACCTCCACAGTTGCCTTG
star*	DQ988930	TGACAGGTTTGAGAAAGAATG	CAATGCGAGAACTTAGAAGG
cyp11b*	AB105880	CTAGACGACGTGGCGAAAGACT	CCTCTGCTCCTCTTCCTTCTCG
3bhsd*	EU159459	GGGCGGGACGAAACTCAG	GGAGGCGGTGTGGAAGAC
gnrhr1*	AB057675	TCCGACGAGCCGCATCTG	GATGAAGCCGACGACGATGAC
gnrhr2*	AB057674	GCAGCGGCACAGACATCATC	GGACAGCACAATGACCACAGAC
lhr*	EF535803	GTGCTACGAAGGCTACGAGATG	AGGTCAATGCGGCGGATTTC
npy*	EU047761	CTTCCACAGTCAAGTTACAAC	TGATCTGCAAGGACGAATG
anxa5*	Y11253	CTGATCGTGGCTCTGATGAC	CTGCTGAGGTGTTCTGGAAG
tp53	NM_001104742.1	CCTTATACCAAAAGGCAGAG	CTAAGCAAGATGGTGGTCAT
pax2	Z97020	CTTCTCCACCTGTAACCAGC	CTGCTGTGTGAAAGCATCTG
dio2	NM_001136521.1	CTGACTTCCTGCTGGTTTAC	AAAAGTGCTCAGTGAGTTGG
nkx2.1	AY568366	TTTCCGATATCTTGAGTCCC	TGCCGGTAAGAAGTGAGAG
hoxa9a	NM_001104666	CTCTGCACTACAACCCCTT	AGATACCGGTTAAAGTGGAA
hoxa10b	AB207987.1	GAGTCCAGACAAGCAAGAAA	AGTCTGGTGCTTGGTGTAAG
hoxa11b	AB207988	GGACAGACACTCAAGAACGA	AAGAAGAACTCCCTCTCCAG
16s*	S74868	CGATCAACGGACCGAGTTACC	AATAGCGGCTGCACCATTAGG
rpl7*	DQ118296	GTCGCCTCCCTCCACAAAG	AACTTCAAGCCTGCCAACAAC

*Primer sequences for genes labeled with asterisks were obtained from Zhang et al. (2008).

Appendix D. Acridine orange staining

D.1. Methods

Acridine orange (AO) was obtained from Sigma-Aldrich, dissolved in H_2O for storing as stock solution (2 mg/mL) and the diluted with ISO water to gain a working solution of 5 mg/L. For staining, vital zebrafish embryos were dechorionated, incubated in the AO working solution for 30 min in the dark and subsequently washed three times with ISO water. Three independent experiments were performed with each comprising five embryos per treatment. The staining was assessed using the DMI6000 module of the Leica microscope system AF6000. Stack images were retrieved and subsequently processed and analyzed using the ImageJ software 1.46 (U.S. National Institutes of Health, Bethesda, MD, USA). Z-stack images were focused with the plugin "Stack Focuser" to generate full-focus images (with extended depth of field) and the number of fluorescent particles, corresponding to apoptotic cells, was assessed for each embryo. For the purpose of best comparability and for the precision of measurement, we selected the area between the posterior limit of the yolk sac and the tail end for quantification of the apoptotic cells. The quantification was statistically analyzed by one-way ANOVA with subsequent post-hoc testing (Dunnett's multiple comparison test).

D.2. Results

Acridine orange staining showed a concentration-dependent enrichment of apoptotic cells in genistein-exposed zebrafish embryos. Predominantly, apoptosis occurred in the nervous system, as apoptotic particles were identified mainly in the brain (data not shown) and in the spinal cord.
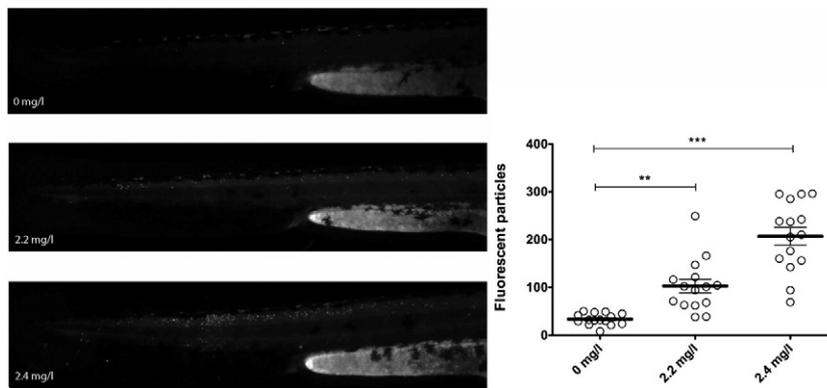
Figure. Apoptosis in 48 hpf zebrafish embryos after exposure to 2.2 mg/L (EC_{10}) and 2.4 mg/L (EC_{20}) genistein and solvent control (0.0 mg/L). Apoptotic cells were identified using acridine orange staining. The pictures on the left show the concentration-dependent increase of apoptotic cells in the spinal cord. The graph on the right provides the quantification of apoptotic particles as identified by the analysis of the particle number using ImageJ. Statistical analysis was performed using one-way ANOVA with subsequent Dunnett's multiple comparison test (asterisks illustrate the p-values: ** = <0.01, *** = <0.001).

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