Biomarkers for Exposure to Estrogenic Compounds: Gene Expression Analysis in Zebrafish (*Danio rerio*)

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ABSTRACT: Gene expression analyses in male zebrafish (*Danio rerio*) were carried out using microarray technique and quantitative polymerase chain reaction. Genes responding to the exposure to 17β-estradiol, bisphenol A and genistein were identified, among them genes involved in metabolism, reproductional and developmental processes. Threshold levels of 17β-estradiol (200 ng/L), bisphenol A (2000 μg/L), and genistein (5000 μg/L) for the upregulation of the *vtg1* gene in short-time exposures (11 days) were determined by qPCR. 14k microarrays were used to generate complete lists of genes regulated by these estrogenic compounds. For this purpose, liver samples from 10 exposed zebrafish and 10 controls were processed. In this case the expressions of 211 genes were significantly regulated by 17β-estradiol, 47 by bisphenol A and 231 by genistein. Furthermore, it is shown that fish exposed to 17β-estradiol and genistein have similarities in their gene expression patterns, whereas bisphenol A apparently affected gene expression in a different way. Only genes coding for egg-yolk precursor protein vitellogenin were found to be regulated by all three compounds, which shows that these genes are the only suitable markers for exposure to different estrogenic compounds. The regulated genes were assigned to gene ontology classes. All three estrogenic compounds regulated genes mainly involved in primary and cellular metabolism, but genistein regulated several genes involved in cell cycle-regulation and bisphenol A several genes involved in protein biosynthesis. Genistein also upregulated the expression of four eggshell proteins, which can be used as biomarkers for exposure to this chemical. Environ Toxicol 23: 15–24, 2008.
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INTRODUCTION

Estrogenic compounds as environmental pollutants have become a major issue since early reports on the feminization of wildlife (Shore et al., 1993; Purdom et al., 1994; Colborn, 1995). A wide variety of chemicals has been linked to estrogenic effects. In addition to natural and synthetic estrogens there is a large group of industrial compounds of non-steroidal character being known or under suspicion to mimic estrogens.

The egg-yolk precursor protein vitellogenin has frequently been used as a biomarker for exposure to estrogens and xenoestrogens (Sumpter and Jobling, 1995). Many studies have shown that male organisms of egg-laying vertebrates, exposed to estrogenic compounds, synthesize this protein in substantial amounts in contrast to unexposed controls (Lim et al., 1991; Bowman et al., 2000; Funkenstein et al., 2000; Lattier et al., 2001; Rey et al., 2005). This marker is frequently used in assays designed to indicate the endocrine potential of a sample. To specify observed effects and relate signals to individual estrogen groups, it is

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necessary to study the expression of the entire genome in response to representative estrogens.

Microarray experiments offer the opportunity to investigate estrogenic responses at the level of the entire transcriptome. Up to now, only a few groups have followed this approach to examine effects of environmental xenoestrogens. Custodia et al. (2001) investigated the effects of endocrine disruptors in groundwater on *C. elegans*, Kato et al. (2004) the effects of ethinylestradiol on rats. Furthermore, Mankame et al. (2004) and Wantanabe et al. (2004) examined the effects of endocrine disruptors on MCF-7 cell cultures and mice, respectively.

In this study we used zebrafish (*Danio rerio*) for transcriptome analysis. Exposure experiments to the chemicals 17β–estradiol (E2), bisphenol A (BPA), genistein, and ethinylestradiol (EE2) as a positive control were carried out because of their environmental relevance: E2 is excreted as a natural hormone by various vertebrates, EE2 is used in contraceptives and was already found in wastewater treatment plant effluents (Dyas et al., 1981; Lee et al., 2004), BPA occurs as softener in many plastic ware and is also used in epoxid resins, several phytoestrogens, such as genistein are found in many plants like soybean.

To obtain a more complete picture of the influence of estrogenic compounds on zebrafish and to find new marker-genes for the exposure to estrogenic compounds, we used two different approaches: (1) quantitative PCR (qPCR) to find the effective concentrations of different estrogenic compounds, using the expression levels of the gene coding for vitellogenin1 (*vtg1*), and (2) microarray experiments to investigate the changes of the whole-genome expression. In this way we have been able to relate gene expression to gene ontology (GO) classes.

MATERIALS AND METHODS

Exposure of Fish

Zebrafish were exposed to estrogenic compounds in 20 L tanks, being part of a flow-through system. Tap water was filtered using activated charcoal and collected in a 500 L tank where it was warmed up to 24°C prior to use. Exposure experiments were carried out in 20 L tanks, connected to the heating tank. Water flow and dosing with the chemicals were controlled by peristaltic precision pumps. A heater kept the water temperature at 24°C in each tank and air pumps circulated the water. The flow through rate was set to 21 mL/min. In this way, the tank volume was changed 1.5 times per 24 h. Further peristaltic pumps were used to adjust the concentrations. All estrogenic compounds were dissolved in ethanol stock solutions resulting in final ethanol concentrations <0.01% in the exposure tanks. Stock solutions were kept at 4°C and replaced by fresh solutions every other day. Up to 12 adult fish were kept in each tank. Zebrafish were acquired as wild types from Dehner (Munich, Germany) and used for experiments when they were fully grown. Only male fish were used for exposures. The fish were fed twice a day with Sera Vipan (Sera, Heinsberg, Germany). The daylight period was set to 12 h, controlled by a timer. The duration of an exposure experiment was 11 days.

The following concentrations were adjusted in the exposure tanks:

E2 (Sigma, Germany): 1, 10, 100, 200, 300, 400, and 500 ng/L.
BPA (Sigma, Germany): 0.1, 2, 20, 200, 400, 1000, and 2000 μg/L.
Genistein (LC Laboratories, USA): 1, 10, 100, 500, 1000, and 5000 μg/L.
EE2 (Sigma, Germany): 30 ng/L (as positive controls).

One tank was used for each concentration. For the negative controls, four tanks with 12 fish per tank were used to obtain sufficient negative controls for the microarray experiments. EE2 was only chosen as a positive control to ensure the correct setup of the flow-through system, not as a further group of microarray experiments.

Four adult female fish were kept in an additional tank without any estrogenic compounds. These were used as a calibrator for comparing different qPCR experiments. 17β-Estradiol concentrations in the tanks were measured by Enzyme Linked Receptor Assay (ELRA), which is similar to a competitive ELISA but uses the human estrogen receptor as binding protein (Seifert et al., 1999). The detection range for E2 is 70–500 ng/L. Water samples were taken once a day from the exposure tanks. This assay has been validated earlier by instrumental analysis.

After exposure, zebrafish were anesthetized on dry ice in a saturated CO_2 atmosphere and killed by cervical dislocation. Samples used for qPCR experiments were also used for microarray experiments.

Quantitative PCR

Liver tissues of exposed and control fish were dissected. Total RNA was extracted according to the TRI REAGENT RNA Isolation System protocol (Sigma, #T9424), based on phenol/chloroform extraction. 0.5 μL RNase Inhibitor (Protector, Roche, #3335399) was added. The RNA concentration was measured photometrically. Two microgram were reversely transcribed to cDNA according to the 1st Strand cDNA Synthesis Kit (AMV) protocol (Roche #1483188). The resulting cDNA was diluted 20-fold in DEPC-treated water. A master mix was prepared according to the protocol LightCycler-FastStart DNA MasterPLUS SYBR Green I (Roche #03515885) containing 0.5 μM primers. The following genes were examined in the qPCR experiments: *vtg1* coding for vitellogenin1 (Primers 5′-GCC AAA AAG CTG GGT AAA CA-3′ and 5′-AGT TCC GTC TGG ATT

GAT GG–3′), *ef1α* coding for elongation factor 1α (Primers 5′-CAG CTG ATC GTT GGA GTC AA-3′ and 5′-TCT TCC ATC CCT TGA ACC AG-3′) and *β-actin1* coding for beta-actin (Primers 5′-AAG CAG GAG TAC GAT GAG TCT G-3′ and 5′-GGT AAA CGC TTC TGG AAT GAC-3′). *ef1α* was used as reference gene for samples exposed to E2, *β-actin1* for the BPA and genistein samples, since earlier experiments showed that BPA can alter the gene expression of *ef1α*. To be able to compare the gene expression after EE2 exposure with the negative controls and further samples, both reference genes were measured in negative controls and EE2 samples.

Sixteen microliter of the master mix and 4 μL of diluted cDNA were combined in a glass capillary. Each sample was measured three times. The qPCR conditions for *vtg1*/*ef1α* were as follows: 1 cycle at 95°C (10 min); 40 cycles at 95°C (0 s), 56°C (5 s), and 72°C (9 s). Conditions for *vtg1*/*β-actin1* were as follows: 1 cycle at 95°C (10 min), 40 cycles at 95°C (0 s), 56°C (5 s), and 72°C (10 s). Crossing points (CPs) of the fluorescence curves were calculated by LightCycler Software 3.5 (Roche) with the second derivative maximum method (Operator Manual of the Light Cycler® Software 3.5.3, available at https://www.roche-applied-science.com/sis/rtpcr/lightcycler/index.jsp). CP describes the cycle number, at which the fluorescence signal gains in strength exponentially. Increasing template cDNA in a sample leads to decreasing CP. In addition, melting curves were established for the amplificates to control the purity, using LightCycler Software 3.5 (Roche). For comparison of gene expression levels, a calibrator-normalized relative quantification with efficiency correction was carried out. The pooled cDNA of four nonexposed female zebrafish served as calibrator and was used for normalization between qPCR runs. For relative quantification of differential expression between different samples, the CPs of the target gene *vtg1* were related to the reference genes, *ef1α* and *β-actin1*. Efficiency tests were performed to examine the quality of PCR reaction. Data analyses of CPs were carried out according to the method of Pfaffl et al. (2002) and the delta–delta method: ΔCP value = CP value (target gene) − CP value (reference gene). The ΔΔCP value considers the influence of differential expression caused by exposure: ΔΔCP value = ΔCP value (exposed sample) − ΔCP value (nonexposed control sample). The comparison of differential expression between exposed and control group is done by calculating the ratio: ratio = $2^{-\Delta\Delta CP}$. The delta–delta method assumes an identical efficiency of two for target and reference gene amplification. This leads to the equation of Pfaffl et al. (2002), which provides information about the relative level of expression in consideration of the actual efficiency:

$$R = \frac{\left(E_{\text{target}}\right)^{\text{CP}_{\text{target}}^{(\text{mean control}-\text{mean sample})}}}{\left(E_{\text{reference}}\right)^{\text{CP}_{\text{reference}}^{(\text{mean control}-\text{mean sample})}}}$$

whereas R = ratio, E = efficiency, reference = reference gene (*ef1α*/*β-actin1*), and target = target gene (*vtg1*).

Analysis of variance (one-way ANOVA) between different exposure conditions was performed followed by significance analysis, using the Tukey HSD (honestly significant difference) test. This *post hoc* test (multiple comparison test) can be used to determine the significant differences between group mean values in an analysis of variance setting. The comparison of the calculated HSD value with the differences of mean values between exposure conditions allows conclusions about significant differentiation in gene expression.

Microarrays

Ten male zebrafish from the following tanks were used for microarray experiments: E2 500 ng/L, BPA 1000 μg/L and genistein 5000 μg/L. The highest concentrations were chosen for E2 and genistein to obtain significant effects on the gene expression. The second highest concentration was chosen for BPA to avoid possible toxic effects. For competitive hybridizations on two-color microarrays, 30 male control zebrafish were taken from clean water tanks. RNA was extracted from these fish as described earlier. RNA concentrations were measured photometrically. Samples of 45 μg RNA were reversely transcribed to cDNA according to the CyScribe First-Strand cDNA Labeling System protocol (Amersham Biosciences #RPN6202X). cDNA was labeled with fluorescent CyDye (cy3 or cy5, respectively). Hybridization between labeled samples and DNA target sequences on 14k zebrafish arrays (Ocimum *Zebrafish 14K OciChip*™ #2260-000000) was carried out according to the array application guide of Ocimum Biosolutions. These arrays contain 14,067 oligonucleotides probes (50 mer) without replicates, representing consensus sequences to the zebrafish open reading frames (ORFs). These ORFs are part of zebrafish genes where the function is known (Expressed Transcripts, ETs) or which show homologies to genes, described in other animals (Tentative Consensus Sequences, TCs).

Ten competitive hybridizations of fluorescently labeled cDNA from exposed and control zebrafish, half of them with a color-flipped labeling, were performed for each of the estrogenic compounds. For each compound a dataset was processed using the statistical environment *R* (Ihaka and Gentleman, 1996; Gentleman et al., 2004). Expression values were calculated as difference between mean signal and mean background values and transformed to the log 2 scale. By doing so, NAs (not applicable) were generated for probes where the background exceeded the signal. For each dataset, statistics on the number of NAs per probe were carried out and all probes with more than four NAs were removed from the dataset. After this filtering the missing values were imputed, using the *transcan* algorithm (Little and Rubin, 1987).

Data normalization included nonlinear on-chip normalization and a linear normalization between all chips within a dataset. For on-chip normalization, Cy5 intensities of each chip were normalized against the corresponding Cy3 intensities by a nonlinear method based on the scatterplot smother *loess* (Cleveland, 1979). Between-chip normalization included scaling of all channels to equal mean and variance.

To detect differential expression between exposed and control zebrafish, *P*-values of Student's 1-sample *t*-test and mean fold changes between exposed and control samples were calculated. A combination of *P*-value and fold change thresholding ($P < 0.05$, fold change > 2) was used to select probes that responded to exposure. To cope with the multiple testing situation that arises in context with the simultaneous observation of more than 14,000 probes, we applied a permutation procedure that enabled us to estimate the number of false positives in our candidate lists. In detail, we carried out all $0.5 \times \binom{10}{5} = 126$ informative sample permutations of each dataset and calculated the numbers of probes selected by the above thresholds. Then, the false discovery rate (FDR) of the selection procedure was estimated as quotient of the mean number of probes that were selected in the permutated data (n_{exp}) and the observed number of responding probes (n_{obs}), $FDR \approx n_{exp}/n_{obs}$. In addition, the statistical significance of selecting n_{obs} or more sequences was estimated from the null distribution generated by the permutations.

The genes that are represented by the zebrafish array were assigned to GO categories according to the annotations of the ZFIN consortium (http://zfin.org). Annotations were completed, taking into account the graph structure of the GO: A gene was assigned to a category, whenever it was contained in one of its subcategories. The analysis included the number of genes in the different categories (from biological process, molecular function, and cellular component catalogs). We have calculated the number of differentially expressed genes for each of the three exposure experiments and in each category and looked for significant enrichments of differentially expressed genes in GO categories. A *P*-value was calculated by comparison with the hypergeometric distribution that served as a null model.

RESULTS

Quantitative PCR

No mortalities were observed among fish exposed to the applied concentrations. qPCR was performed in parallel to define threshold levels for estrogenic effects on the expression of the *vtg1* gene because its expression is regulated by all tested chemicals. These experiments were carried out with 4–11 exposed male fish for each concentration and 4 controls. The samples obtained after exposure to very low concentrations of all tested compounds were only measured in 4 qPCR experiments since no responses in the gene expression were expected. At higher concentrations, the number of experiments was increased to avoid missing possible changes in gene expression. Figure 1 shows the fluorescence curves of a typical qPCR run with genistein-exposed samples.

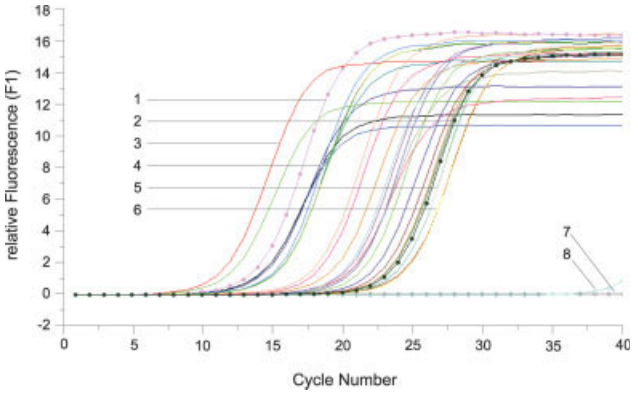

Fig. 1. A typical qPCR run with 32 samples, exposed to genistein and EE2. To keep this figure clear, only eight curves are labeled. (1) *vtg1* calibrator to be able to compare different runs; (2) *β-actin1* calibrator to be able to compare different runs; (3) sample exposed to 30 ng/L EE2 as a positive control, *vtg1* primer; (4) sample exposed to 5000 μg/L genistein, *vtg1* primer; (5) sample exposed to 30 μg/L EE2, *β-actin1* primer; (6) sample exposed to 5000 μg/L genistein, *β-actin1* primer; (7) *vtg1* primer, no sample added, negative control; (8) *β-actin1* primer, no sample added, negative control. The unlabeled curves are different samples, exposed to genistein and EE2. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Statistical analysis was performed with an one-way ANOVA and a Tukey HSD test. It showed an upregulation of the vitellogenin gene *vtg1* from male liver tissue starting at 200 ng/L E2, 2000 μg/L BPA, and 5000 μg/L genistein. The expression of *vtg1* in the nonexposed male fish was set to 1 to standardize the different expression ratios (Fig. 2).

The expression level of *vtg1* was 1,100 times higher in the E2 samples at 200 ng/L than in the control samples and reaches a maximum of 5000 at 400 ng/L. The results from the BPA samples were scattered except for exposures at 2000 μg/L. This means that no clear upregulation of *vtg1* is observable at lower concentrations. The genistein samples showed a concentration-dependent expression of *vtg1*. But even at the highest concentration the expression ratio did not reach the level of E2 exposures. The strongest *vtg1* expression was observed after EE2 exposure. It shows that this xenoestrogen has a stronger effect on the expression of *vtg1* than the natural hormone at comparable concentrations and can be used as a positive control.

Microarrays

Microarray experiments with liver samples from male zebrafish exposed to E2 (500 ng/L), BPA (1000 μg/L), and

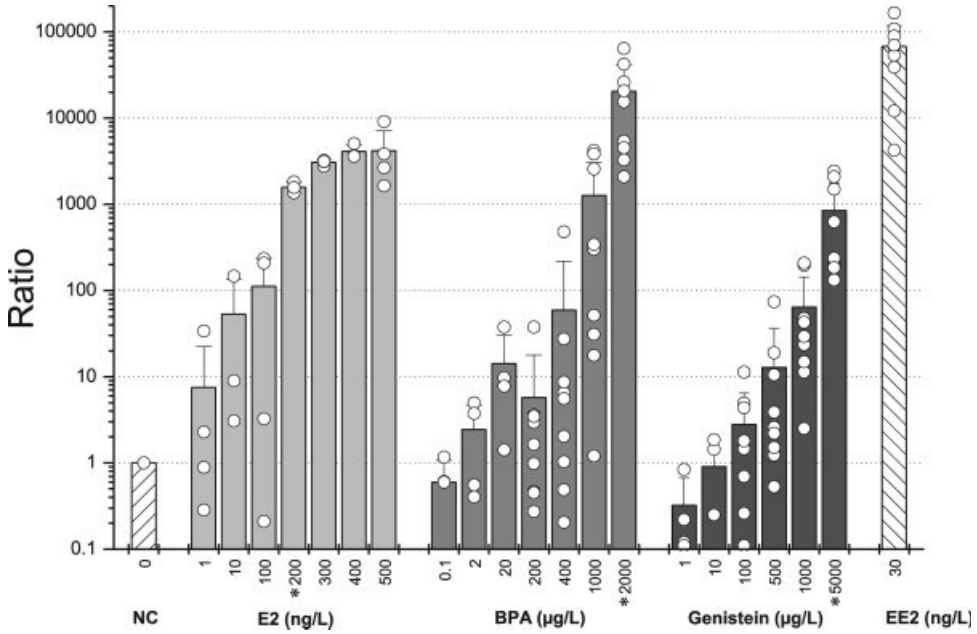

Fig. 2. Calculated ratios of the expression of the vtg1 gene, measured by qPCR. Each dot represents one sample. NC, nonexposed control. Asterisks indicate the concentrations with significant increases in the expression of vtg1.

genistein (5000 μg/L) were carried out to detect changes in gene expression using 14k microarrays. The design included 10 biological replicates (10 exposed and 10 control zebrafish) for exposure to each of the three chemicals. Lists of responding probes were generated by a combined threshold for *P*-values from *t*-statistics and mean fold changes ($P < 0.05$, fold change > 2). The number of false positives in the candidate lists was estimated by a permutation procedure that is described in the material and methods section. FDRs for the lists of probes were estimated as 11% (E2), 52% (BPA), and 14% (genistein).

The spotted probes were designed to detect sequences that are either expressed zebrafish transcripts (ETs) or TCs from other animals.

The expression of 211 sequences (100 ETs and 111 TCs) was regulated by E2, only 47 (29 ETs and 18 TCs) by BPA and 231 (108 ETs and 123 TCs) by genistein. A Venn diagram was used to indicate regulated ETs and TCs responding to one, two or three substances, respectively (Fig. 3).

BPA had the weakest effect on the gene expression pattern of all three tested chemicals. The only genes, regulated by all three chemicals, are the ones coding for five different vitellogenins (GenBank accession numbers: AY729644, AY729649, NM_001025189, XM_682549, and AF254638). The regulation of the expression of these genes also was the strongest observable regulation. Genistein upregulated the expression of 6 ETs, which code for eggshell proteins (GenBank accession nos. NM_131696, NM_131829, BC095596, NM_131827, BC097083, and BC097085) and downregulated the expression of *dmbx1* (AF398526) coding for an homeobox protein, which plays an important role in development. The expression of genes coding for homeobox proteins was regulated by all 3 chemicals: *hoxa3a* (NM_131534) by E2, *hoxa5a* (NM_131540) by BPA and *dmbx1* (AF398526) by genistein. The ETs that were downregulated strongest are coding for proteins involved in proteolysis, ion and protein transporter activity, alcohol

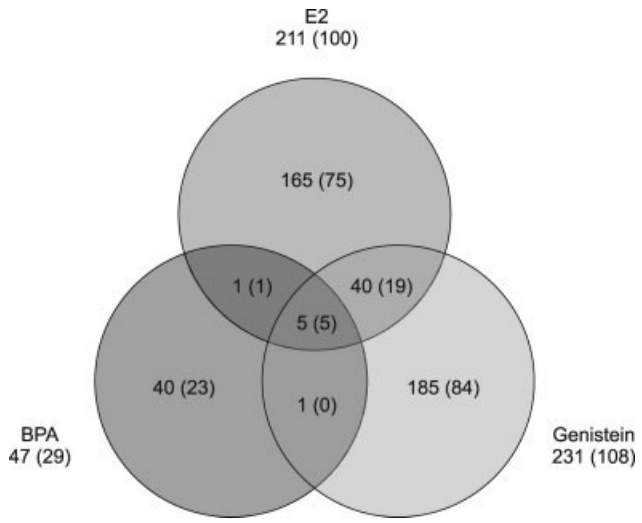

Fig. 3. Venn diagram of the up- and downregulated ETs and TCs. The first number indicates all regulated ETs and TCs. The number in brackets indicates the number of ETs. The overlapping areas represent the number of TCs and ETs that are regulated by two or all 3 chemicals.

TABLE I. Regulated ETs and TCs after E2 exposure, assigned to gene ontologies

Gene Ontology No.	Function	Regulated Gene	*P*-Value
GO:0005319	Lipid transporter activity	*vtg1, vtg3, vtg5*	0.002200
GO:0005200	Structural constituent of cytoskeleton	*acta1, bactin1, bactin2*	0.004593
GO:0005884	Actin filament	*acta1, bactin1, bactin2*	0.004593
GO:0006006	Glucose metabolism	*aldob, eno1, gapdh, gapdhs, hibadh*	0.007899
GO:0004887	Thyroid hormone receptor activity	*esr1, nr1d1*	0.008783
GO:0006869	Lipid transport	*vtg1, vtg3, vtg5*	0.009571
GO:0006096	Glycolysis	*aldob, eno1, gapdh, gapdhs*	0.017225
GO:0006066	Alcohol metabolism	*adh8a, aldob, eno1, gapdh, gapdhs, hibadh*	0.017339
GO:0006092	Main pathways of carbohydrate metabolism	*aldob, eno1, gapdh, gapdhs, hibadh*	0.021553
GO:0015629	Actin cytoskeleton	*acta1, bactin1, bactin2, myo6a*	0.029418
GO:0051287	NAD binding	*gapdh, gapdhs, hibadh*	0.035113
GO:0004386	Helicase activity	*chd1l, ercc3, smarca4, zgc:63517*	0.043104

metabolism, and neural tube formation. BPA on the other hand mainly regulated sequences, which code for proteins involved in metabolism. Furthermore, 45 common ETs and TCs can be found for E2 and genistein but only six for E2 and BPA and six for BPA and genistein. These are the five genes coding for vitellogenin and gapdh (BC095386) for E2 and BPA and a gene, coding for a so far unknown protein (AL954384) for BPA and genistein, respectively.

All regulated ETs and TCs were subsequently examined and assigned to GO classes according to the annotations of the ZFIN consortium (http://zfin.org). Tables I–III show the gene ontologies for up- and downregulated ETs and TCs of all three chemicals with a maximum *P*-value of 0.05.

The regulated sequences were assigned to 12 different GOs affected by E2, 20 by BPA, and 16 by genistein. The only two GOs regulated by all three chemicals are lipid transport (biological process, GO:0006869) and lipid transporter activity (molecular function, GO:0005319). The involved genes are *vtg1*, *vtg3*, and *vtg5*. E2 mainly regulated GOs that are involved in metabolism like glucose metabolism (GO:0006006) and alcohol metabolism (GO:0006066). BPA regulated many GOs that play a role

TABLE II. Regulated ETs and TCs after BPA exposure, assigned to gene ontologies

Gene Ontology No.	Function	Regulated Gene	*P*-Value
GO:0003735	Structural constituent of ribosome	*rpl13a, rpl18a, rpl24, rpl4, rpl7a, rps7, zgc:92237*	0.000003
GO:0005840	Ribosome	*rpl13a, rpl18a, rpl24, rpl4, rpl7a, rps7, zgc:92237*	0.000003
GO:0030529	Ribonucleoprotein complex	*rpl13a, rpl18a, rpl24, rpl4, rpl7a, rps7, zgc:103632, zgc:92237*	0.000007
GO:0006412	Protein biosynthesis	*eef2l, rpl13a, rpl18a, rpl24, rpl4, rpl7a, rps7, zgc:92237*	0.000173
GO:0005198	Structural molecule activity	*rpl13a, rpl18a, rpl24, rpl4, rpl7a, rps7, zgc:92237*	0.000989
GO:0008199	Ferric iron binding	*zgc:109934, zgc:92066*	0.001113
GO:0006826	Iron ion transport	*zgc:109934, zgc:92066*	0.001426
GO:0005319	Lipid transporter activity	*vtg3, vtg5*	0.002161
GO:0006879	Iron ion homeostasis	*zgc:109934, zgc:92066*	0.002161
GO:0000398	Nuclear mRNA splicing, via spliceosome	*sfrs1l, zgc:103632*	0.005215
GO:0006869	Lipid transport	*vtg3, vtg5*	0.005843
GO:0008380	RNA splicing	*sfrs1l, zgc:103632*	0.006505
GO:0044249	Cellular biosynthesis	*eef2l, rpl13a, rpl18a, rpl24, rpl4, rpl7a, rps7, zgc:92237*	0.007469
GO:0009058	Biosynthesis	*eef2l, rpl13a, rpl18a, rpl24, rpl4, rpl7a, rps7, zgc:92237*	0.015098
GO:0005737	Cytoplasm	*psmb5, rpl13a, rpl18a, rpl24, rpl4, rpl7a, rps7, slc25a5, tctp, zgc:92237*	0.018966
GO:0006096	Glycolysis	*gapdh, pgam1*	0.027270
GO:0043234	Protein complex	*psmb5, rpl13a, rpl18a, rpl24, rpl4, rpl7a, rps7, zgc:103632, zgc:92237*	0.034780
GO:0004239	Methionyl aminopeptidase activity	*pa2g4b*	0.038322
GO:0015934	Large ribosomal subunit	*rpl13a*	0.038322
GO:0006006	Glucose metabolism	*gapdh, pgam1*	0.041151

TABLE III. Regulated ETs and TCs after genistein exposure, assigned to gene ontologies

Gene Ontology No.	Function	Regulated Gene	*P*-Value
GO:0051301	Cell division	*ccna1, ccna2, ccnb1, ccnb2, cdc2, cdc20, smarca2*	0.000070
GO:0019031	Viral envelope	*zp2, zp2.2, zp2.4*	0.000189
GO:0005319	Lipid transporter activity	*vtg1, vtg3, vtg5*	0.002767
GO:0019439	Aromatic compound catabolism	*hgd, zgc:56326*	0.006961
GO:0007596	Blood coagulation	*f2, fgg, plg*	0.008502
GO:0043085	Positive regulation of enzyme activity	*cap1, chn1*	0.010257
GO:0006869	Lipid transport	*vtg1, vtg3, vtg5*	0.011908
GO:0006270	DNA replication initiation	*mcm2, mcm4*	0.023334
GO:0000074	Regulation of progression through cell cycle	*ccna1, ccna2, ccnb1, ccnb2, dvr1*	0.027547
GO:0005096	GTPase activator activity	*chn1, zgc:66165*	0.028654
GO:0006446	Regulation of translational initiation	*bzw1, zgc:56330*	0.028654
GO:0051258	Protein polymerization	*cyp11a1, fgg, tubb2c*	0.029243
GO:0000226	Microtubule cytoskeleton organization and biogenesis	*cyp11a1, tubgcp5*	0.034406
GO:0005083	Small GTPase regulator activity	*chn1, gdi2*	0.040564
GO:0009072	Aromatic amino acid family metabolism	*hgd, zgc:56326*	0.047101
GO:0043087	Regulation of GTPase activity	*chn1, gdi2*	0.047101

in protein biosynthesis like RNA splicing (GO:0008380) and structural constituent of ribosome (GO:0003735). Genistein on the other hand regulated GOs involved in the regulation of cell cycle. Three genes were assigned to blood coagulation (GO:0007596).

DISCUSSION

Microarray techniques belong to the most promising approaches to the large-scale analysis of gene expression (Schena et al., 1995; Duggan et al., 1999). Applications for zebrafish mainly focus on changes of gene expression during development (Linney et al., 2004; Mathavan et al., 2005).

In this study adult zebrafish (*Danio rerio*) were used to examine estrogenic effects on gene expression. Fish were exposed for 11 days in 20-L tanks to a range of concentrations of E2, BPA, genistein and EE2. The flow-through systems in these tanks, provided by peristaltic pumps, allowed stable conditions with constant concentrations of the tested compounds.

qPCR was used to determine threshold levels for the applied estrogenic compounds. Analyses are fast and inexpensive, but they are restricted to the expression of single genes. We used the *ef1α* gene as a reference for the E2 samples and *β-actin1* as a reference for the BPA and genistein samples. It was necessary to change the reference gene for the BPA and genistein samples, since we noticed that the expression of *ef1α* was regulated by BPA. This fact underlines the importance of testing the reference gene. A regulated reference gene interferes with statistical analysis.

The results showed an induction of *vtg1* at a concentration of 200 ng/L for E2, 2000 μg/L for BPA, and 5000 μg/L for genistein. For BPA a lowest observed effect concentration (LOEC) of 1000 μg/L was detected by Van den Belt et al. (2003), but under semistatic conditions for 3 weeks. The induction of *vtg1* at the determined, relatively high concentrations is caused by the fact that adult fish were examined at the transcription level. However, this approach allows the examination of the status quo of the tissue and avoids measuring accumulated proteins.

In our study an effect concentration of 5000 μg/L genistein was determined. In spite of this relatively high concentration, an estrogenic effect of genistein was verified at the level of transcription. The insensitive response of *vtg1* at genistein levels of 5000 μg/L points to minor environmental relevance. Environmental concentrations are usually below this concentration. But the experiments were performed to find additional biomarkers for exposure to different estrogenic compounds. Environmental relevance is expected when several estrogenic compounds occur in mixtures. Large differences in the expression of *vtg1* were observed (Fig. 2). At a concentration of 10 ng/L E2 some exposed fish show an expression ratio of 100 when compared with the negative controls. Comparable ratios could be found at 400 ng/L BPA and 1000 ng/L genistein. This shows that the three different substances have different estrogenic potentials and cause comparable expression ratios of *vtg1* at different concentrations.

Since the experiments were carried out with vertebrates, we intended to keep the number of fish used in the experiments as small as possible. However, the performed statistical tests allowed a good estimation of the LOECs and

TABLE IV. The influence of *P*-value and minimum foldchange on the FDR and amount of significantly regulated genes

	Compound	Regulated Genes	FDR[a]
$P^{b} < 0.05$; $fc^{c} \geq 2$	E2	211	11%
	BPA	47	52%
	Genistein	231	14%
$P < 0.01$	E2	274	19%
	BPA	87	71%
	Genistein	168	25%
$P < 0.005$	E2	186	13%
	BPA	44	71%
	Genistein	70	25%
$P < 0.001$	E2	86	5%
	BPA	7	79%
	Genistein	12	11%

[a] False discovery rate.
[b] *P*-value.
[c] Foldchange.

showed, which groups should be used for the microarray experiments.

To learn more about gene expression patterns depending on exposure with different estrogenic compounds, microarray experiments were carried out with 14k arrays. A major methodical difficulty of microarray experiments arises from the simultaneous analysis of thousands of sequences in presence of biological and technical noise. To cope with this difficulty, we worked with 10 biological replicates for each of the experiments. As a result, we were able to extract comprehensive sequence lists for the E2 and the genistein experiment and to keep the corresponding FDRs at rates around 10–15%. A stricter thresholding ($P > 0.01$, fold change > 2) would even have lowered the FDRs to about 5%. Table IV shows the FDRs for different settings.

Furthermore, 10 replicates are recommendable to keep the rate of true positives in the candidate list at probabilities of 90 or 95%. Our preliminary analyses showed that the number of candidate sequences as well as the true positive rate drop significantly, if the number of replicates is lowered to eight or less. In the 10-fold replicated BPA experiment, only 47 responding probes were identified at a high FDR of 52%. As all other conditions were the same, this must be due to less distinct response of the fish to BPA and/or a higher interindividual variability of the response. This matches with the highly individual response of the vitellogenin1 gene to BPA exposure that was observed in qPCR assays.

The microarray data confirmed that E2, BPA and genistein were upregulating the expression of the gene coding for vitellogenin. This effect seems to be common to all three substances, regardless of possibly different pathways. This underlines the use of vitellogenin as a suitable marker to detect estrogenic influences. After an exposure for eleven days the fold change of vitellogenin genes was up to 445.3 by E2, 12.7 by BPA, and 91.3 by genistein. No other genes showed such strong upregulations.

The upregulation of several zona pellucida (zp) proteins after genistein exposure is conspicuous. These results substantiate earlier findings of accumulating eggshell proteins after exposure to the estrogenic compound 4-nonylphenol (Arukwe et al., 1997). We interpret this upregulation as a protective measure of fish against the phytoestrogen genistein, since Brion et al. (2004) showed that exposure to estrogens during the early developmental stage has an irreversible effect on the sexual differentiation of zebrafish. Genistein as a natural substance can get into the water and has always been in the environment. It is not surprising when fish developed a mechanism to fortify the embryonic membrane and therefore protect early development. This could be of high importance, since genes coding for proteins that play a role in lipid transportation like apoa and molecule transport like rbp are downregulated. This could lead to harmful effects for the cell.

The highest fold change was found at the zona pellucida glycoprotein 3, *zp3b,* which was upregulated 8.15 times. *zp3b* could be a useful marker to detect the exposure to genistein since the other two tested compounds had no effect on this gene.

BPA apparently upregulates many genes, which code for proteins involved in protein processing, like folding or degradation. This is in contrast to genistein that up- and downregulates many genes involved in embryonic development. This difference in the gene expression regulating potential of both compounds suggests that they are using different pathways affecting the expression. It has been shown earlier that some estrogens are capable to control gene regulation by a non-receptor-mediated pathway (Das et al., 1997). The existence of several receptor- and non-receptor mediated pathways could provide an explanation for the difference in gene-regulation by a phytoestrogen and a xenoestrogen.

On the other hand, there are some similarities between E2 and genistein: 45 genes were regulated by both chemicals. This suggests that these two natural estrogens are using the same or a similar pathway affecting estrogenic responses. The xenoestrogen BPA is assumed to use a different pathway to upregulate the *vtg1* expression.

All three tested compounds regulate the expression of genes coding for homeobox proteins but in different ways. Homeobox proteins are known to play an important role in developmental stages. E2 upregulated the expression of *hoxa3a* 31 times the one of *hoxa2b* 2.68 times. Genistein upregulated the expression of a protein that is similar to NKX2-8 2.51 times and BPA down-regulated the expression of *hoxa5a* 2.56 times. This shows that all three estrogenic compounds can affect developmental processes but in different ways. This strengthens our assumption that they use different pathways.

The effects of BPA on the gene expression patterns of male zebrafish seem to be much weaker when compared

with E2 and genistein. Only 47 regulated genes were found and *vtg1* showed the weakest upregulation. A study by van den Belt et al. (2003), using an enzyme-linked immunosorbent assay to measure the amount of vitellogenin in zebrafish blood plasma, showed that there was a significant increase in vitellogenin in male zebrafish after an exposure to 1000 μg/L BPA for 3 weeks. Our study confirmed this result obtained after an exposure of eleven days.

It has already been shown that BPA can be found in the human amniotic fluid (Tsutsumi, 2005). This documents that humans can be exposed to this substance and that this chemical is able to alter human gene expression patterns was shown by Nishizawa et al. (2005).

Furthermore, there are hints for a carcinogenic potential of the tested chemicals. E2 upregulated the expression of *ctnnb2*, coding for β-catenin 2. Fritsch et al. (2006) showed recently that there is an increased expression of beta-catenins in germ cell tumors. BPA upregulated the expression of *oct1*. It has been shown that there is a strong expression of *oct1* in MCF7 cells (Jin et al., 1999). The expression of cyclin A was upregulated by genistein by the factor of 3. Cyclin A is used as a marker for human breast cancer (Husdal et al., 2006). This suggests that these substances can not only harm the reproductive ability of vertebrates, but might even be carcinogenic, depending on the concentration or duration of exposure.

This study shows that microarray experiments are useful to detect biomarkers for exposure to environmental compounds. But in the case of estrogenic substances it seems unlikely that in addition to vtg further biomarkers responding to all estrogenic compounds will be found. However, microarray experiments are expected to provide distinct gene expression patterns that can be related to specific toxic events.

The GO database offers a rich source of information on the processes, molecular functions and cellular components, in which a gene or its corresponding protein is involved. This database allowed a comparison of the different biological processes, cellular component and molecular functions regulated by the estrogenic compounds used in this study. It is conspicuous that most of the regulated genes play a role in primary (GO: 0044238) or cellular metabolism (GO: 0044237).

Not all of the regulated genes can be assigned to GOs yet, since the function of their products is not known. 50% of the 14.067 sequences that are immobilized on the microarray slides could be assigned to GOs. This limits the interpretation of the obtained data. The lists provided in Tables I–III show that the estrogenic compounds used in this study, especially genistein, are able to alter the gene expression patterns of zebrafish, however in different ways. Vitellogenin-coding sequences are the only ones that are upregulated by all tested chemicals. This is evidence that they interfere with the endocrine system but this seems to be the only obvious similarity.

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