

Concentration-Response Analysis of Differential Gene Expression in the Zebrafish Embryotoxicity Test Following Flusilazole Exposure

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The zebrafish embryotoxicity test (ZET) is considered a promising alternative model in predictive toxicology. Currently, morphological assessment of the embryo is the main readout for this assay. However, implementation of transcriptomics may help to detect more subtle effects, which may increase the sensitivity and predictability of the test. In this study, we tested a concentration response of flusilazole in the ZET. After exposure for 24 h postfertilization, microarray analysis revealed a number of processes to be regulated in a concentration-dependent way. We identified development related processes, retinol metabolism and transcription, as well as processes corresponding to the antifungal mechanism of action, steroid biosynthesis, and fatty acid metabolism, to be differentially regulated. Retinol metabolism and transcription were already significantly altered at concentrations that were not inducing morphological effects. Differential expression of genes related to steroid biosynthesis and fatty acid metabolism showed a concentration response similar to morphological response. An increase in concentration was also positively associated with an increase in magnitude of expression for individual genes within functional processes. Our study shows that transcriptomics analysis in the ZET is a more sensitive readout of compound-induced effects than morphological assessment. However, the interpretation of differential gene expression in terms of predicting morphological effects is not straightforward and requires further study.

Key Words: zebrafish embryotoxicity test; toxicogenomics; concentration-response; alternative test; flusilazole; triazole.

Many alternative assays for developmental toxicity testing have been developed and studied over the last decades. The relatively high number of animals that is required for predictive developmental toxicity testing and the need to replace, reduce, and refine experimental animal use stimulates the design of alternative methods in this area. Alternative methods vary from cell-line-based tests to organ cultures and whole embryo

systems of rat or zebrafish (Embry *et al.*, 2010; Genschow *et al.*, 2004; Nagel, 2002; Piersma *et al.*, 2004; Spielmann *et al.*, 2006). In the zebrafish embryotoxicity test (ZET), yolk-dependent non-free-feeding embryos are used, which are not considered experimental animals under European legislation (EU, 2010; Strahle *et al.*, 2011). Compared with other *in vitro* alternatives, such as the embryonic stem cell test (EST) or rat whole embryo culture (WEC), the ZET has several advantages. In the ZET, compound exposure in zebrafish embryos is studied from fertilization until hatching of the embryo, which comprises the development of primary organs (Kimmel *et al.*, 1995). Furthermore, there is detailed background knowledge about zebrafish embryogenesis, its development is independent of maternal fish, and the transparency of the embryo makes monitoring easy (Fleming, 2007; Hill *et al.*, 2005; Kimmel *et al.*, 1995; Yang *et al.*, 2009).

The zebrafish embryo is considered to be a promising model for predicting developmental toxicity of compounds in vertebrates including humans. An overall predictability of the test model of 81–87% has been reported by several different research groups (Brannen *et al.*, 2010; McGrath and Li, 2008; Selderslaghs *et al.*, 2011). In addition, de Jong *et al.* (2011) showed that as to the relative potencies of six triazole compounds, the outcome of the ZET correlated well with developmental toxicity data from rat *in vivo* studies and has the highest correlation with the *in vivo* data compared with EST and WEC. These studies support the zebrafish embryo as a suitable model for the prediction of developmental toxicity. The ZET and similar zebrafish embryo test methods mostly rely on morphological evaluation of the embryos as the endpoint of the test (Brannen *et al.*, 2010; Hermesen *et al.*, 2011b; Nagel, 2002). Molecular techniques such as PCR or whole genome transcriptomics may enable the detection of more subtle effects and provide a more objective endpoint as compared with morphological assessment alone. This could help to increase the sensitivity and predictability of the assay. In addition,

transcriptomics analysis may provide further insight into the mechanisms of action of toxicants (Daston and Naciff, 2010).

We recently showed that two different chemical classes tested in ZET can be distinguished based on their gene expression patterns (Hermesen *et al.*, 2011a). The effects on gene expression were already detectable before the onset of morphological effects. Similarly, Yang *et al.* (2007) showed that in zebrafish embryos, compounds could be identified as embryotoxic based on the toxicogenomic response even at low concentrations without morphological effects. Most transcriptomics studies use only a single concentration of a toxicant, generally based on the classical morphological endpoint. Some studies have addressed the importance of testing multiple concentrations (Andersen *et al.*, 2008 Goetz and Dix, 2009a; Naciff *et al.*, 2005; van Dartel *et al.*, 2011; Yang *et al.*, 2007). Recently, it was shown that the morphological effect size and extent of gene expression changes in mouse embryos and embryonic stem cells were interrelated (Robinson *et al.*, 2010; van Dartel *et al.*, 2011). In both these studies, the concentration of the compound was related to the magnitude of gene expression and the number of significantly regulated genes. This suggests that compound concentration will influence the toxicity prediction by gene expression analysis.

Therefore, we investigated the effect of compound concentration on gene expression as compared with morphological effects in the ZET. Flusilazole (FLU), one of our previously studied compounds (Hermesen *et al.*, 2011a,b), was used as a model compound. FLU has also been studied in a concentration-response design in the EST using transcriptomics, and results showed concentration-dependent effects on gene expression (van Dartel *et al.*, 2011). This known developmental toxicant causes teratogenic effects, such as skeletal and craniofacial malformations in rodents and zebrafish embryos (Farag and Ibrahim, 2007; Hermesen *et al.*, 2011b; Menegola *et al.*, 2001). The main developmentally toxic mechanism of action has been postulated to involve the disruption of retinol metabolism by altering the gene expression of the metabolizing enzyme *cyp26al* (Marotta and Tiboni, 2010; Menegola *et al.*, 2006; Tiboni *et al.*, 2009). In the present study, a series of eight concentrations of FLU was tested in the zebrafish embryo. Microarray analysis of exposed zebrafish embryos was performed at 24 h postfertilization (hpf) to study early compound-induced gene expression changes potentially leading to morphological effects at a later stage. We compared the morphology and transcriptomics data, and results showed concentration-dependent gene expression changes after exposure to FLU, starting at concentrations that did not induce morphological effects.

MATERIALS AND METHODS

Maintenance and breeding of zebrafish. *Danio rerio* used in this study was originally obtained as commercially imported Singapore wild-type stock,

maintained, and bred in our facilities for at least 6 years (Hermesen *et al.*, 2011b).

Zebrafish embryotoxicity test. The ZET was performed as described previously (Hermesen *et al.*, 2011b). In short, fertilized batches of eggs with a fertilization rate of at least 90% were collected 30 min after spawning and rinsed several times in Dutch standard water (DSW; demineralized water supplemented with 100 mg/l NaHCO₃, 20 mg/l KHCO₃, 200 mg/l CaCl₂·2H₂O, and 180 mg/l MgSO₄·7H₂O and then aerated for 24 h at 27°C). Coagulated eggs or debris were removed, and the eggs were evenly distributed among the test concentrations. Subsequently, embryos within the 4- to 64-cell stage were selected and transferred to a 24-well plate. One embryo was transferred to one well containing 2 ml test medium. Embryos were kept in an incubator at 26.5°C ± 1°C with a photoperiod of 14-h light:10-h dark.

Embryos were microscopically evaluated at 24 and 72 hpf using the general morphology score (GMS) system to quantify developmental retardation and teratogenicity (Hermesen *et al.*, 2011b), whereas parallel cultured embryos at 24 hpf were used for microarray analyses.

The results of the scoring at 72 hpf were analyzed with Proast dose-response curve fitting software (version 19.10) for morphological validation, using a nested group of exponential models (Slob, 2002).

Test compounds and concentrations. Embryos were exposed to eight concentrations of flusilazole (FLU; Sigma-Aldrich, Zwijndrecht, The Netherlands, cat. no. 45753). On the basis of a previous concentration-response curve (Hermesen *et al.*, 2011b), the benchmark concentration at which the GMS was decreased with 20% (BMC_{GMS20}) was calculated, and this concentration was used as one of the eight FLU test concentrations (13.5 μM). We selected the other seven concentrations in such a way that the concentrations were equally spaced on a logarithmic scale and that the complete concentration-response range was covered (0.28, 0.6, 1.35, 2.8, 6, 13.5, 28, and 60 μM). FLU was dissolved in dimethyl sulfoxide (DMSO) and further diluted in DSW with a final DMSO concentration of 0.2% (vol/vol). The control group was exposed to 0.2% DMSO.

RNA isolation and processing. For microarray analysis, six independent biological replicates, each including 20 pooled zebrafish embryos, for each of the experimental test concentrations, and eight biological replicates for the controls were collected at 24 hpf. This was done in three independent series. Embryos were stored in RNAlater (Ambion, Austin, TX) to stabilize RNA. Embryos were kept in RNAlater for at least 1 day at 4°C. To begin RNA isolation, RNAlater was removed and embryos were homogenized using a pestle in an Eppendorf tube. RNA was isolated from the embryos with the RNeasy mini kit and an additional DNase treatment (RNase-free DNase set) according to the manufacturer's instructions (all from Qiagen, Venlo, The Netherlands). The concentration of RNA was measured on the NanoDrop spectrophotometer (ND-1000; NanoDrop Technologies Inc., Wilmington, DE), and RNA integrity was assessed by automated gel electrophoresis on the Bioanalyzer 2100 (Agilent Technologies, Amstelveen, The Netherlands) using the RNA 6000 Nano Chip kit (Agilent technologies). Preparation of the RNA samples for expression profiling was done as described previously (Hermesen *et al.*, 2011a), and samples were hybridized onto 4x180k *D. rerio* microarrays (Agilent Technologies). These microarrays contain 180,882 spots of which 4908 are the various controls. 175,974 spots are based on the zebrafish nucleic acid database sequence of which 38,915 are mapped to a current National Center for Biotechnology Information zebrafish gene. These correspond to 18,819 unique genes (based on Entrez Gene ID). For 62% of the genes, human homologs were identified. Quality control was performed on raw microarray data using a scatterplot and MA-plot as well as a normal probability plot to assess signal distribution. The positive and negative controls on the slides were solely used for quality control and therefore excluded from further analysis.

Data analysis. Microarray data were normalized and further analyzed using R statistical software (version 2.11.0). Normalization of the Cy3/Cy5 extracted signals was done in a three step approach as described by Janssen

et al. (2007). Microarray data are available at ArrayExpress (<http://www.ebi.ac.uk/arrayexpress/>) under accession number E-MTAB-832.

Principal component analyses (PCA) (Pearson, 1901) were done to detect nuisance effects of experimental variables “series,” “labeling,” “slide,” “array,” and “RNA isolation batch.” A clustering of samples was observed according to series. For this reason, we fitted a linear model (Cui and Churchill, 2003; Kerr *et al.*, 2000; Wolfinger *et al.*, 2001) with concentration and series as fixed factors as series did not have enough levels to effectively model as random. For this, we used the microarray analysis of variance (MAANOVA) package (Wu *et al.*, 2003) in R. The nuisance effect caused by series was removed from the data by analyzing per probe only the model coefficients per concentration and the probe specific mean signal, with residuals added per sample. Probes that were significantly differentially expressed between treatment groups were identified using one-way ANOVA with a Benjamini and Hochberg false discovery rate (FDR) (Benjamini and Hochberg, 1995) adjusted *p* value of < 0.05. The highest concentration group was not included in this analysis because overt toxicity at this concentration level was present.

From these probes, we selected those showing monotonous and biphasic concentration-dependent changes. Probes that had increasing fold ratios (FR) with increasing concentration or had decreasing FR with increasing concentration were selected. Furthermore, gene expression was allowed to have a biphasic response as well, which includes one maximum or minimum response. A margin of ln-transformed FR 0.01 was taken into account for which the genes could deviate from the defined monotonous or biphasic response. Again, the highest concentration was excluded from the selection of concentration-dependent responding genes. After selection, probes were pooled on their Entrez Gene ID to obtain a list of unique genes.

To visualize the concentration response, we performed PCA analysis on the ln-transformed data of the selected genes.

K-means clustering was conducted on the same selection of genes to identify different clusters or concentration-response relationships in terms of gene expression regulation. Per cluster, functional annotation and term enrichment were determined with tools on the DAVID website (<http://david.abcc.ncifcrf.gov/>) (Huang *et al.*, 2009). Enrichment was considered significant with an FDR corrected *p* value < 0.1. For the most significantly regulated pathway or biological process regulated for each k-means cluster, the absolute average fold change was calculated based on all genes of the term present in the list of monotonous/biphasic responding genes.

We performed a *t*-test on the selected genes between the control and each of the concentration groups to identify genes significantly differentially expressed from the control group (*p* < 0.05). With these genes, we performed the DAVID analyses again to determine the level of significance per concentration of the four already identified terms.

For a more detailed look at the processes, we determined the fold change of every gene present in that process for each of the concentrations.

Validation of gene expression with real-time PCR. Microarray data were validated using real-time PCR. RNA from all samples per experimental group was pooled and reverse transcribed with a high capacity complementary DNA reverse transcription kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed in duplicate with TaqMan gene expression assays (Applied Biosystems) on a 7500 Fast Real-Time PCR system using the manufacturer’s instructions. Run conditions were 95°C of 20 s for the first cycle, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. Six genes were selected for validation. Two genes, *cyp26a1* (Dr03086662_m1) and *aldh1a2* (Dr03131681_m1), were annotated to retinol metabolism. Two genes, *hoxb6a* (Dr03144219_m1) and *irf8* (Dr03140549_m1), were annotated to transcription. Furthermore, a gene annotated to steroid biosynthesis, *lss* (Dr03130609_m1), and a gene annotated to fatty acid metabolism, *acat2* (Dr03093281_m1), were chosen. *Hprt1* (Dr03095135_m1) was used as housekeeping reference gene for all genes. The gene expression was normalized to the value of the reference gene for each reaction according to the comparative Ct method, and relative expression compared with the DMSO control was determined. Data analysis included a Student’s *t*-test with a cutoff value of *p* < 0.05 for statistical significance.

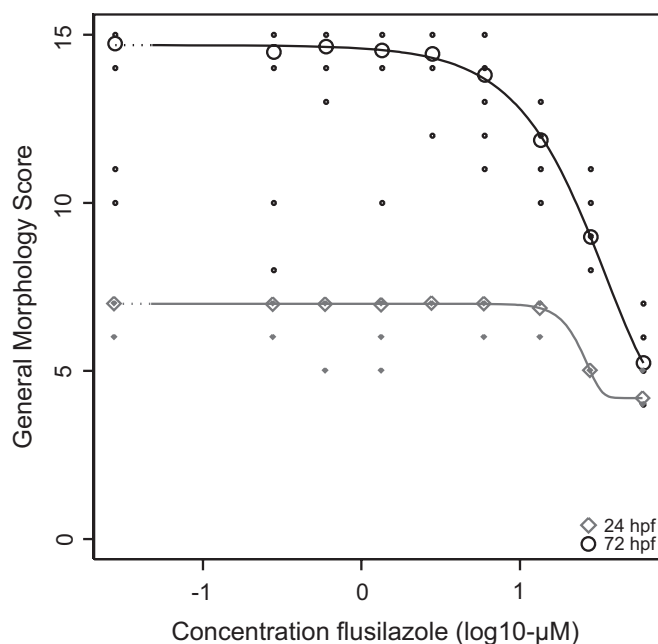


FIG. 1. Concentration-response curve of GMS in zebrafish embryos at 24 hpf (gray diamonds) and 72 hpf (black circles) exposed to flusilazole. Large symbols represent the mean of the scoring of single embryos (small symbols).

RESULTS

FLU-Induced Effects on Development

FLU caused a concentration-dependent developmental delay at 72 hpf as was measured by GMS (Fig. 1), and we observed a concomitant increase in teratogenic effects such as pericardial edema and malformations of head and heart (Fig. 2). In addition, embryos exposed to concentrations of 6 µM and higher were not hatched at 72 hpf. At 24 hpf, a delay in development was observed from 28 µM upward. Embryos exposed to 28 µM did not show signs of movement or a heartbeat yet which were present in the controls. In addition to these two endpoints, the embryos exposed to 60 µM did not have complete detachment of the tail (Fig. 2).

FLU-Induced Gene Expression Changes

We identified 4036 probes corresponding to 2950 significantly regulated genes with ANOVA (FDR < 0.05) for all the experimental groups, excluding the highest dose group. Genes were selected that had monotonously increasing FR with increasing concentration and monotonously decreasing FR with increasing concentration or had a biphasic response with one maximum or minimum response. A margin of ln-transformed FR 0.01 was taken into account for which the genes could deviate from the defined response. With these strict criteria, we identified 237 probes corresponding to 205 genes that were regulated monotonously or biphasically with FLU concentration (Supplementary table 1).

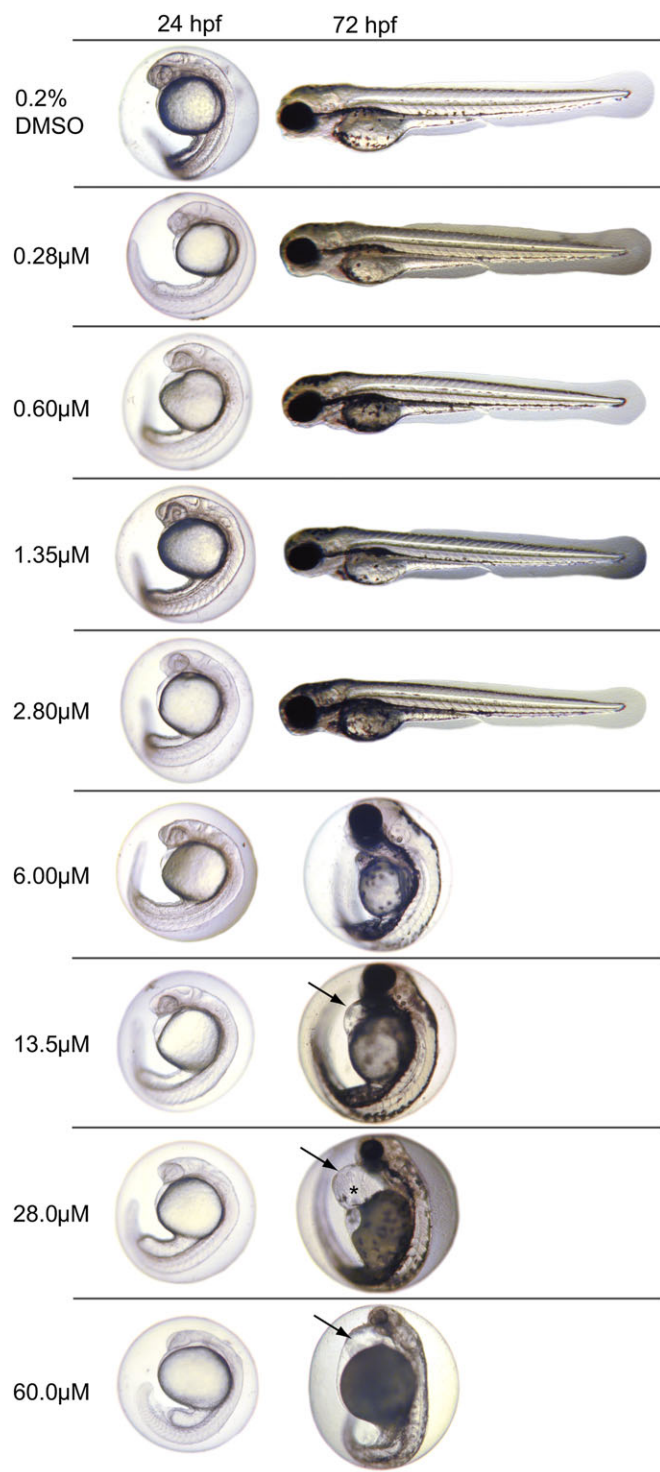


FIG. 2. Representative pictures of zebrafish embryos exposed to a concentration series of flusilazole for 24 and 72 h. Arrows indicate pericardial edema, and * indicates malformed heart.

PCA using these genes showed that already at the lowest concentration, a significant change from controls occurred followed by a concentration dependent further deviation from

the control in a different direction on the PCA plot (Fig. 3). The individual replicates per concentration tested were clustered together, demonstrating less variability within than between the experimental groups.

Process Enrichment

By conducting k-means clustering using the 205 genes, six clusters of concentration-response relationships were identified (Fig. 4). The clusters showed either a concentration-dependent upregulation or downregulation with differences in terms of magnitude of response, i.e., fold change. Two of these clusters (C and D) showed a response crossing the x-axis, demonstrating that the response of their genes in terms of directionality changed with concentration. Process enrichment for each cluster was determined to identify known and unknown processes affected by FLU exposure. The predominant terms and processes that were significantly enriched are depicted in Figure 4. Cluster A, consisting of seven genes, showed the greatest response in fold change and was enriched for retinol metabolism. Enrichment analysis for cluster B, also a cluster showing upregulation, revealed terms mostly related to development, such as transcription and homeobox. Steroid biosynthesis was identified to be the main enriched process for cluster C. One of the concentration-dependent downregulated clusters (cluster D) was mainly enriched for processes related to lipid metabolism and glycolysis/gluconeogenesis. Clusters E and F did not show significant term enrichment.

Gene Expression Responses Within Selected Terms

Four terms, the most significantly enriched pathways or biological processes from each cluster, were selected for further investigation: retinol metabolism, transcription, steroid biosynthesis, and fatty acid metabolism. The absolute average fold change of the genes present in these terms showed concentration-related effects at the level of the terms (Fig. 5) as did the average fold change at the individual gene level (Fig. 6). Retinol metabolism appeared to be most sensitive to FLU exposure showing a steep increase in absolute average fold change and a significant enrichment already at 2.8 μM (Fig. 5). At this concentration, no morphological effects were present at 72 hpf. No significant enrichment for retinol metabolism was evident anymore at the highest concentration, probably due to extensive gene expression scatter related to the high toxicity at this exposure level. Both up- and downregulated genes were responsible for the enrichment of this process (Fig. 6B, Supplementary table 1). Most of the genes positively related to retinol metabolism cause a decrease of available retinoic acid by either storage or degradation, whereas the downregulated gene is associated with an increase in retinoic acid (Fig. 6B) (Dobbs-McAuliffe *et al.*, 2004; Niederreither *et al.*, 1997). Transcription was also significantly enriched from 2.8 μM upward (Fig. 5B). On the individual gene level, both up and downregulated genes contributed to the enrichment of the process (Fig. 6A,

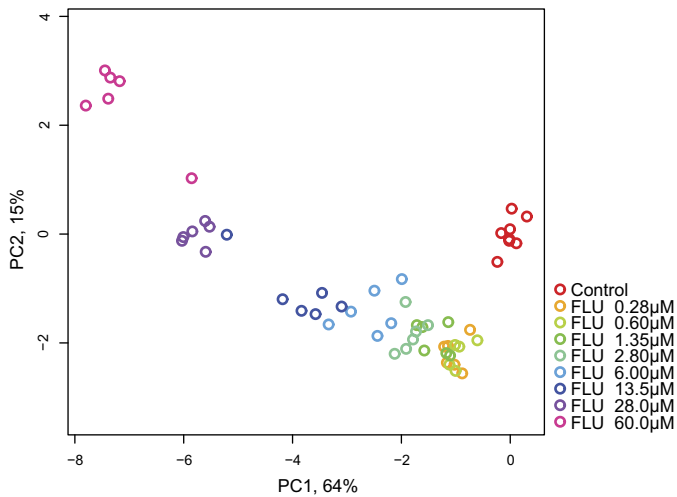


FIG. 3. PCA of zebrafish embryos at 24 hpf after exposure to FLU compared with control using 205 significantly monotonous/biphasic responding genes. Each symbol represents one experimental unit (20 embryos).

Supplementary table 1). The lowest concentration at which steroid biosynthesis is significantly enriched is 13.5 μ M, also the concentration at which morphological effects at 72 hpf were

observed. All genes in this process are upregulated with concentration (Fig. 6C, Supplementary table 1). Fatty acid metabolism, significantly enriched using the overall gene expression patterns as identified by k-means analysis, is not significantly enriched as a term for any of the concentrations, but it did show a high absolute average fold change at the two highest concentrations. All genes in this process were downregulated with concentration (Fig. 6D, Supplementary table 1) with the steepest decline in fold change from 13.5 μ M onward which corresponds to the increase in absolute average fold change seen in Figure 5A.

The number of genes significantly regulated per concentration after performing a *t*-test on the monotonously and biphasic responding genes appeared to be concentration-dependent as well (Fig. 5C).

Real-time PCR revealed similar concentration-response patterns in terms of fold change as was found in the microarray data (Supplementary figure 1). *Cyp26a1*, *hoxb6a*, and *lss* expression changes were already significant at the lowest concentration. The correlation r^2 between the microarray data and real-time PCR results for each gene ranged from 0.73 to 0.99 (Pearson correlation).

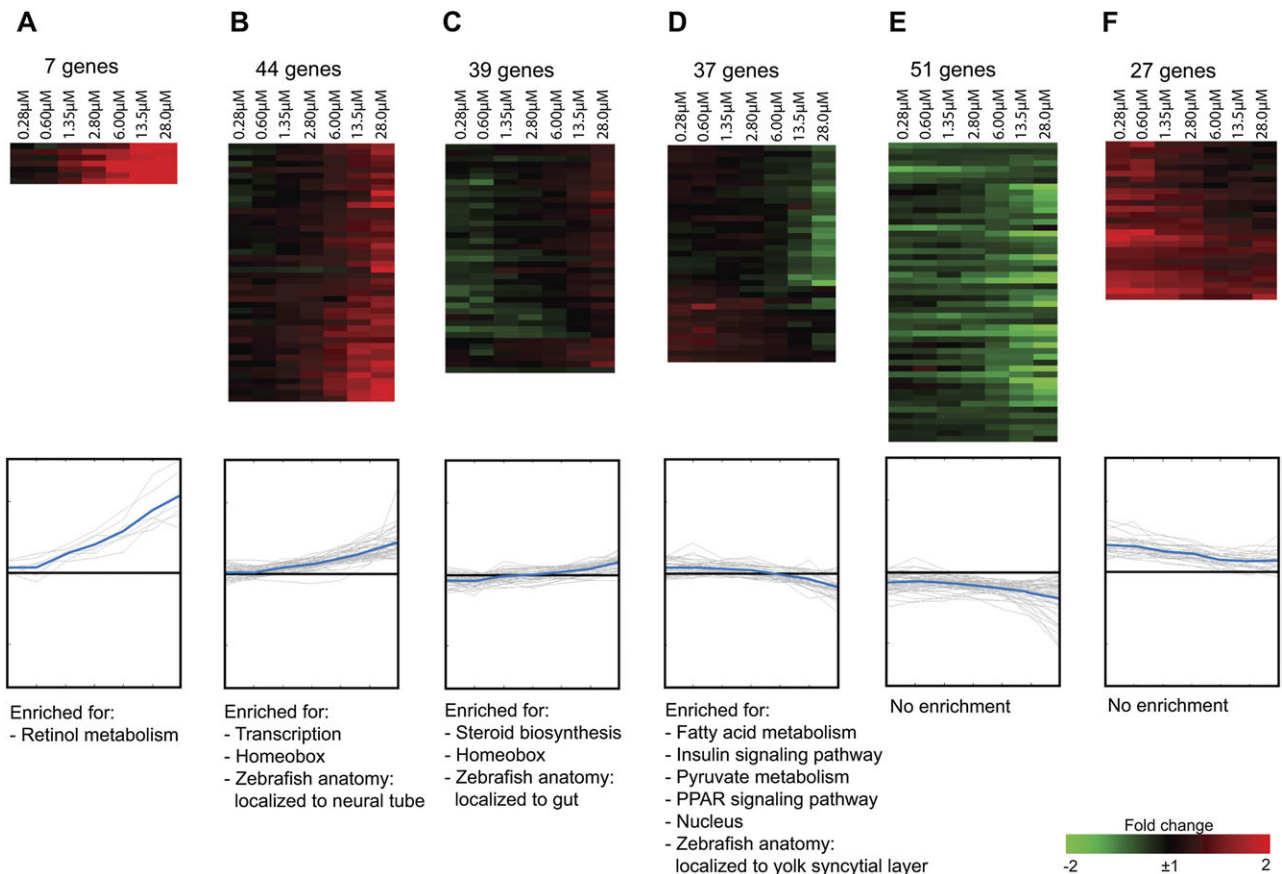


FIG. 4. K-means clustering of 205 significantly regulated monotonous/biphasic responding genes. Heat maps show the fold change per concentration per gene, and graphs show the average response of the cluster. Term or process enrichment was determined with DAVID and considered significant with an FDR-adjusted *p* value < 0.1.

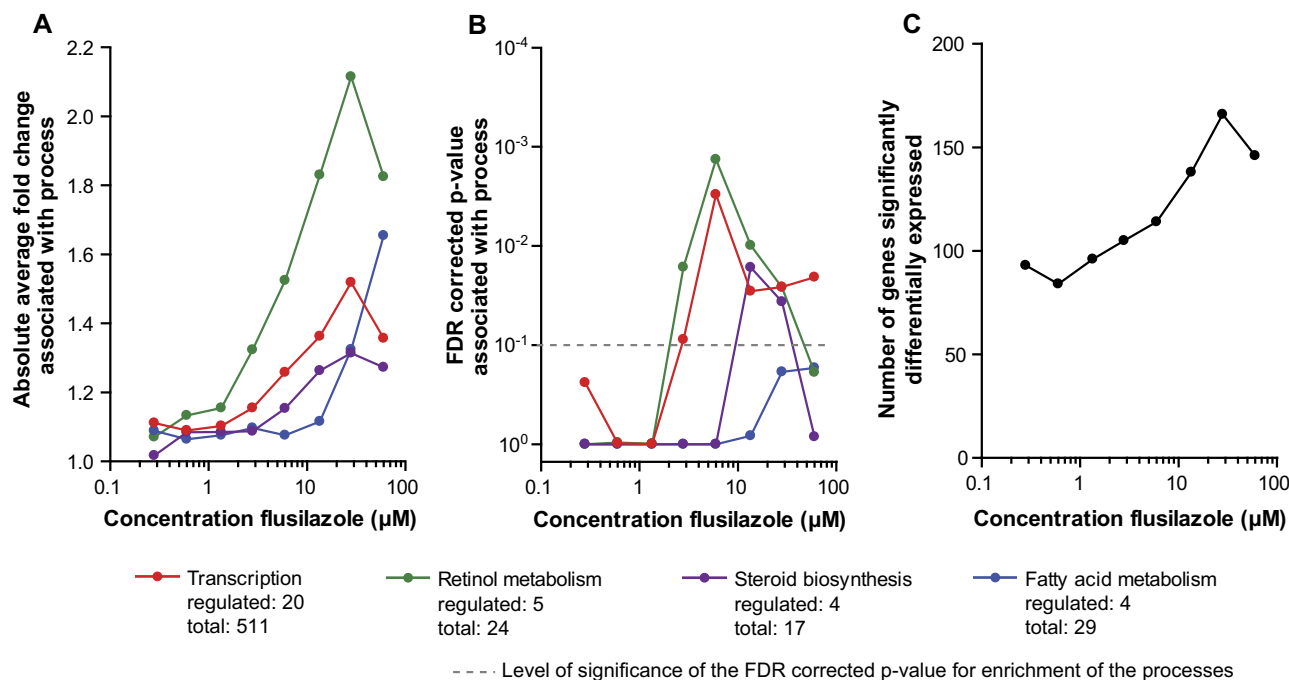


FIG. 5. (A) Concentration-related effect of FLU exposure in 24 hpf zebrafish embryos on absolute average fold change of a subset of enriched processes. Numbers indicate the number of genes significantly regulated and total number of genes in the process. (B) Concentration-related effect of FLU exposure in 24 hpf zebrafish embryos on FDR-corrected p value of a subset of processes indicating at which concentration a process is enriched. Level of significance is depicted by the dotted line. (C) Number of significantly regulated genes compared with control after t -test ($p < 0.05$), which were used for determination of level of significance of enrichment.

DISCUSSION

Transcriptomics analysis is a promising approach in predictive toxicology. In several different *in vitro* alternative models for developmental toxicity testing, transcriptomics has been applied. The implementation of transcriptomics in the ZET has already shown promising results (Hermesen *et al.*, 2011a; Yang *et al.*, 2007). Several studies in different model systems have shown that compound concentration may influence the gene expression pattern (Andersen *et al.* 2008 Goetz and Dix, 2009a; van Dartel *et al.*, 2011). Therefore, also the prediction of toxicity on the basis of transcriptomics should consider the impact of compound concentration. In this study, we investigated compound concentration-related gene expression patterns in the ZET and their relations to the morphological concentration-response curve.

FLU induced a concentration-dependent delay in development in the zebrafish embryos after 72 hpf. At this time point, morphological effects were observed already at a concentration of 6 μM, although these were only minor effects, such as delayed hatching. Embryos exposed to 28 and 60 μM FLU were severely affected and already showed a delay in development at 24 hpf. At 24 hpf, gene expression in the embryos was analyzed, and already at the lowest concentration of FLU, genes corresponding to the toxic mechanism of action, such as *cyp26a1*, were found to be significantly regulated (Fig. 6B).

Selection for genes that follow a logical concentration response, being either monotonously increasing/decreasing or biphasic with one minimum/maximum, yielded 205 genes that were significantly regulated. Comparing these 205 genes with our previous study, in which, we found 128 genes regulated by FLU 13.5 μM, 30 genes are present in both lists of genes with *cyp26a1* as the most significantly upregulated gene (Hermesen *et al.*, 2011a).

A PCA based on the 205 genes showed a nice concentration-response relationship. The concentration-related track deviated sharply at the lowest concentration, possibly indicating two types of responses. We speculate that given the absence of morphological effects, the lowest concentration may cause a primary physiological response in gene expression within homeostatic control. At higher concentrations, the observed morphological effects are accompanied by more profound gene expression changes indicating a toxicological response. Indeed, the number of regulated genes increased with concentration (Fig. 5C) as was also demonstrated in different model systems by van Dartel *et al.* (2011) Robinson *et al.* (2010). These findings indicate that not only the number of genes, which are affected change with compound concentration, but also that the degree of regulation of gene expression is highly dependent on the concentration that is tested. This corresponds to the morphological results because we observed increased delay in development at higher concentrations.

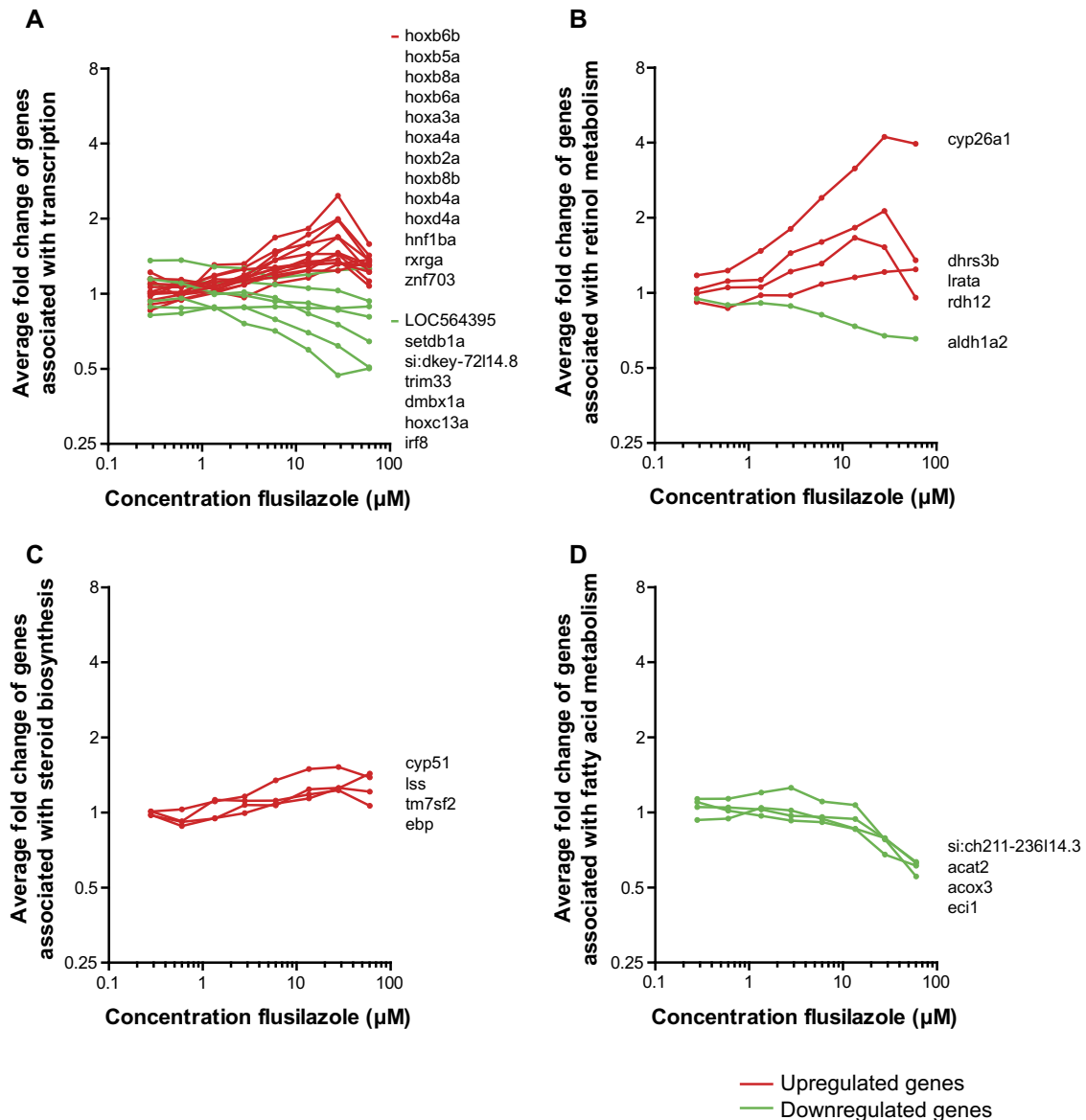


FIG. 6. Average fold change of individual genes from the 205 significantly regulated monotonously responding genes, which contributed to the significant enrichment of transcription (A), retinol metabolism (B), steroid biosynthesis (C), and fatty acid metabolism (D).

Process enrichment of k-means clusters revealed different terms as transcription, steroid biosynthesis, fatty acid metabolism, and retinol metabolism of which enrichment and absolute average fold change were positively associated with concentration. Similar processes were found to be enriched in our previous study, such as retinol metabolism, development related processes, and fatty acid metabolic processes (Hermesen *et al.*, 2011a), showing good reproducibility between the studies.

Transcription is one of the general terms that we found to be enriched after k-means clustering. However, 13 of the 20 genes present in this term were homeobox-related genes, and the term transcription is therefore likely strongly related to development and growth (Emoto *et al.*, 2005; Schilling and Knight, 2001). Within this term, genes showed upregulation as well as

downregulation. The absolute average fold change correlates nicely with concentration, indicating that the extent of gene expression regulation may correlate to the extent of developmental delay in the embryo (Figs. 1 and 2). This term was already enriched at 2.8 μM FLU, at concentrations lower than those causing delay in development observed at 72 hpf. These findings may indicate that development is disrupted at a lower concentration than we can observe morphologically. However, homeostatic control mechanisms may effectively protect the embryo from developing adverse effects at this concentration, leaving the embryo morphologically unaffected (Piersma *et al.*, 2011).

Retinol metabolism, essential for development, showed the highest absolute average fold change and is one of the terms

significantly enriched already at a low concentration. For retinol metabolism, our data show that this process is concentration-dependently regulated and is also found to be significantly regulated already at concentrations below those causing morphological anomalies at 24 hpf. It has been shown that triazoles can modify the expression of *cyp26a1* (Hermsen *et al.*, 2011a; Marotta and Tiboni, 2010; Menegola *et al.*, 2006; Tiboni *et al.*, 2009). In this study, we found a concentration-dependent increase in expression of *cyp26a1* (Fig. 6C, upper line), also confirmed by real-time PCR, which is consistent with results from studies using other *in vitro* and *in vivo* models (Papis *et al.*, 2007; Tiboni *et al.*, 2009). It has been hypothesized that triazoles inhibit the function of *cyp26a1* thereby causing an increase in retinoic acid. As a consequence, in order to restore the balance, *cyp26a1* messenger RNA is upregulated to enhance degradation of retinoic acid. This would also be in line with the upregulation of the other genes in this pathway (*rdh12*, *dhrs3b*, and *lrata*), which cause a decrease in retinoic acid by either storage or degradation (Feng *et al.*, 2010; Liden and Eriksson, 2006; O'Byrne *et al.*, 2005). Moreover, the one gene negatively correlated with concentration (Fig 6C, green line), *aldh1a2*, is normally associated with an increase in retinoic acid (Dobbs-McAuliffe *et al.*, 2004; Niederreither *et al.*, 1997), and this response is therefore also congruent with a homeostatic response. Furthermore, some of the genes within this pathway show a biphasic response, for example, *rdh12*. At 13.5 μ M, the peak expression of this gene is reached, and at higher concentrations, it "collapses." Remarkably, morphology at the 24 hpf time point shows effects upwards from 28 μ M. Therefore, the observed peak gene expression responses may perhaps be related to the threshold of adversity. At the maximum of differential gene expression, the limit of the homeostatic control mechanism may have been reached. Consequently, exceeding this limit by higher exposures may cause a collapse of homeostasis observed as reduced magnitudes of differential gene expression. Several studies have described similar phenomena in other systems (Daston, 2008; Daston and Naciff, 2005). Such gene expression patterns may perhaps be useful as markers for developmental toxicity.

The specific antifungal action of triazoles is mediated by the *cyp51* enzyme (lanosterol-14 α -demethylase). This enzyme is inhibited by triazoles, including FLU, thereby blocking ergosterol biosynthesis and steroid biosynthesis (Goetz *et al.*, 2009; Trzaskos and Henry, 1989). Toxicogenomics analysis of rat livers revealed that triazoles were affecting steroid biosynthesis and lipid metabolism-related pathways *in vivo* (Goetz and Dix, 2009b; Tully *et al.*, 2006). In this study, steroid biosynthesis was significantly regulated at 13.5 μ M and higher and showed a maximal 1.3-fold induction of absolute average fold change. All genes associated with steroid biosynthesis, including *cyp51*, showed a concentration-dependent upregulation. Possibly, the upregulation, we found is a compensatory response to the blockage of *cyp51* by FLU. The four related

genes (*cyp51*, *lss*, *ebp*, and *tm7sf2*) found differentially expressed in this study showed a concentration-dependent increase in expression, as was confirmed by real-time PCR for *lss*, and all contribute in a similar way to the steroid biosynthetic process. A similar increase of absolute average fold change was found in the EST for sterol biosynthesis, a related process, by van Dartel *et al.* (2011) (Goetz and Dix, 2009b; Hester *et al.*, 2006; Tully *et al.*, 2006; van Dartel *et al.*, 2011).

Fatty acid metabolism was also an enriched term in our k-means analysis. At the highest two concentrations, the absolute average fold change increased dramatically. The genes belonging to this process were all negatively correlated with FLU exposure and showed all more downregulation at the concentrations at which development and morphology are seriously impaired as well, as we also observed with real-time PCR. Because the embryos at 60 μ M were severely affected, the fatty acid metabolic process may indicate a sort of cytotoxic effect similar as the observation of a high lipid content in fungal cells after exposure to triazoles (Baird and DeLorenzo, 2010; Trzaskos and Henry, 1989). Lipid metabolism-related processes are found after exposure to other triazoles as well. Goetz and Dix (2009b) showed a common disturbance of fatty acid metabolism in rat *in vivo* and rat and human *in vitro* gene expression studies using multiple triazoles. van Dartel *et al.* (2011)-related processes to be enriched after FLU exposure in the EST.

Concentration-response transcriptomics studies have shown to be more sensitive in relation to the classical outcome of adverse morphological effects (Andersen *et al.*, 2008; Naciff *et al.*, 2005). Our results showed that for the interpretation of embryotoxicity with transcriptomics in the ZET, it is important to take into account compound concentration. However, to set a threshold of adversity on the basis of gene expression is still very difficult (Daston and Naciff, 2010). Genes or processes that show similar concentration-response characteristics to morphology may predict an adverse effect in the assay. For instance, fatty acid metabolism in our study showed a similar concentration response as the morphological effects because this process was enriched at the same concentration as morphological effects were present. Still, it remains to be seen whether these parameters are valid predictors for developmental toxicity of other compounds and for developmental toxicity in man. Furthermore, the peak response in biphasic responding genes may also be related to the threshold of adversity as discussed above. However, a general developmental toxicity threshold signature can only be made on the basis of testing multiple compounds and therefore requires further study.

In conclusion, the results of our study show that transcriptomics provide a more sensitive endpoint of the ZET with additional insights into mechanisms of action. This study shows that genes monotonously/biphasically expressed can be functionally anchored to development and mechanism of toxicity of the compound, demonstrating that the ZET shows specific responses informing about mechanism of action. This

confirms that transcriptomics in zebrafish can be an important mechanism-revealing methodology. It could also improve interspecies extrapolation given that the relevance of mechanisms can be compared between species. Furthermore, compound concentration affects gene expression, and therefore, the choice of test concentrations is an important aspect to consider in the design of transcriptomics experiments.

SUPPLEMENTARY DATA

Supplementary data are available online at <http://toxsci.oxfordjournals.org/>.

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