A TRANSCRIPTOMIC APPROACH FOR THE FISH EMBRYO TEST WITH ZEBRAFISH AND MEDAKA TO IDENTIFY ENDOCRINE DISRUPTION

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Introduction

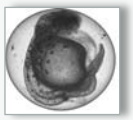
The current testing approach in regulatory ecotoxicology for endocrine disrupting chemicals (EDCs) relies on long-term reproductive, animal intensive studies. These labour intensive tests entail high costs and are ethically disputable. Thus, the need for alternative test strategies for EDCs is urgent, and zebrafish and medaka embryos have good potential as alternative test approaches for EDCs. Both species have their particular advantages in this context. The rapid development of zebrafish is beneficial for screening applications, whereas the longer embryogenesis of medaka may alleviate the prediction of chronic effects. Since the morphological evaluation does not elicit endocrine effects, our approach is based on transcriptomics. In order to show that endocrine effects become apparent in the transcriptome of fish embryos, we exposed zebrafish and medaka embryos to six endocrine disruptors and subsequently performed microarray analyses for zebrafish and quantitative PCR for selected steroidogenic genes for medaka. In the transcriptome, various pathways were affected by each substance, and also substance-sprecific response patterns could be identified. The medaka transcriptome data, on the other hand, provided complementary information about species specific and longer-term exposure response. We therefore propose zebrafish and medaka fish embryos as suitable tools for testing endocrine disruption.

Methods

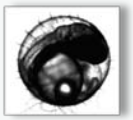
Fish Embryo Test

- Exposure: zebrafish 48 h; medaka 7d
- 96-well-plates, 200µl test solution/ well
- Assessment of non-lethal & lethal morphological endpoints
- EC10 and EC20 were deduced from concentration-response curve determined by probit-analyis and used for subsequent transcriptome analysis
- Four and three replicates were used for microarray and qPCR testing, respectively, each replicate containing 24 embryos

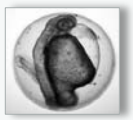
48 hpf zebrafish embryo after Genistein exposure showing head, tail deformation and oedema



7 dpf medaka embryo after Methyl-paraben exposure showing oedema



24 hpf zebrafish embryo after Bisphenol A exposure showing head, tail deformation and oedema



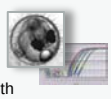
Test substances

- Genistein (phytoestrogen)
- Propanil (herbicide)
- Methylparaben (anti-fungal preservative, E218)
- Bisphenol A (BPA, plastic compontent)
- Prochloraz (fungicide)
- Linuron (herbicide)

Zebrafish Microarray

- 4 x 44K Zebrafish Oligo Microarray Kit (V2) one colour (Agilent® Technolgies)
- Data analysis: GeneSpring GX 11
- Functional analysis (FatiGO, babilomics 4[1]): GO Analysis, Interpro, KEGG pathway
- Data validation: qPCR

Medaka HPG-qPCR Array (Zhang et al 2008[2])



- SYBR ® Green based
- 18 of 36 genes associated with the hypothalamic-pituitary-gonadal axis were measured

Results

Propanil, Methylparaben, Genistein and BPA enhance steroid biosynthesis and estrogenic pathway in zebrafish embryo transcriptome

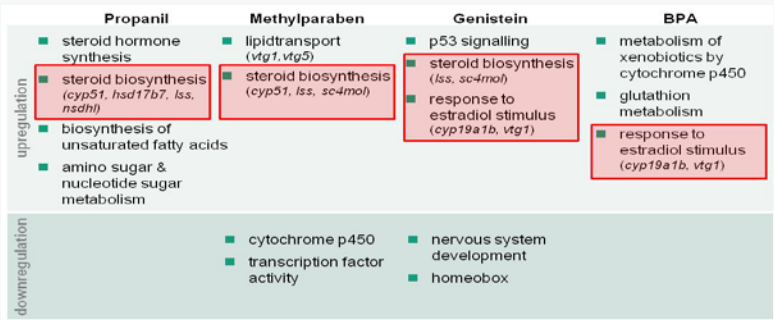


Figure 1 Functional analysis of zebrafish embryo microarray data for Propanil, Methylparaben, Genistein and BPA. Differentially expressed genes (p<0.01) were clustered by the self-organizing-map algorithm. Final clusters were then subjected to KEGG Pathway, Gene Ontology (GO) and InterPro analysis by testing for overrepresented genes against the rest of the zebrafish transcriptome applying the fisher's exact test, setting the cut-off p-value at 0.05. For each substance, upregulated and downregulated pathways are shown, the red boxes highlight the pathways affected by at least two substances.

Medaka: Methylparaben and Genistein elicit an estrogenic, Propanil and BPA an antisteroidogenic gene response

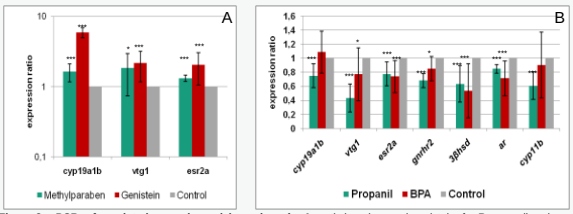


Figure 2 qPCR of regulated genes in medaka embryo for **A** methylparaben and genistein, for **B** propanil and bpa. Analysed genes were: aromatase b (*cyp19a1b*), vitellogenin 1 (*vtg1*), estrogen receptor 2a (*esr2a*), gonadotropin-hormone-releasing hormone receptor 2 (*gnrhr2*), steroid delta-isomerase (*3βhsd*), androgen receptor (*ar*), steroid 11beta-monooxygenase (*cyp11b*) and steroidogenic acute regulatory protein (*star*). Expression ratio was calculated using the delta-delta-Ct-method and normalized to *rpl7* and *16S* as reference genes

Linuron and prochloraz affect steroid binding in zebrafish and repress steroidogenic signalling pathways in medaka embryos

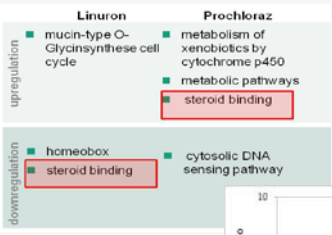
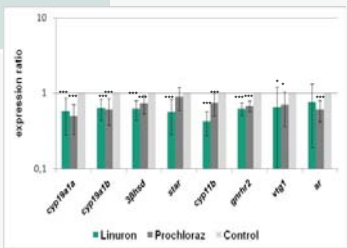


Figure 3 (left) Functional analysis of zebrafish microarray data for Linuron and Prochloraz (for details, see caption Fig.1), demonstrating steroid binding as affected pathway

Figure 4 (right) qPCR of regulated genes in medaka embryo for Linuron and Prochloraz, demonstrating downregulation of steroidogenic signalling pathways. Regulated genes were: aromatase a(*cyp19a1a*) aromatase b (*cyp19a1b*) vitellogenin 1 (*vtg1*), gonadotropin-hormone-releasing hormone receptor 2 (*gnrhr2*), steroid delta-isomerase (*3βhsd*), androgen receptor (*ar*), steroid 11-beta-monooxygenase (*cyp11b*) and steroidogenic acute regulatory protein (*star*) (details see Fig. 2)



Conclusion

- Functional analysis of microarray data showed interference of Methylparaben, Propanil, Genistein and BPA with steroid biosynthesis and an induction of an estrogenic response in the zebrafish transcriptome, confirming the endocrine potential of these substances
- Complementary information was gained from qPCR analysis of HPG-related genes in medaka, which showed that genistein exhibits its estrogenic effect through the estrogen receptor (*esr2a*). Induction of *cyp19a1b* and *vtg1* could indicate long-term estrogenic effects by Methylparaben
- For Propanil and BPA, opposite effects on gene expression were observed for medaka and zebrafish, suggesting species specific or exposure-time related effects.
- Linuron downregulated and Prochloraz upregulated steroid binding in zebrafish embryos, whereas in medaka, both substances indicated antisteroidogenic potential.

➡ Endocrine disruption is detectable in the fish embryo transcriptome
➡ Distinct response patterns were apparent
➡ Zebrafish embryos are beneficial for fast testing and indicate potential endocrine disrupting hazards
➡ Medaka FETs provide additional complementary information about species specific responses or longer-term effects

We propose the fish embryo transcriptome analysis as a suitable tool to (pre)screen for EDCs!

References

[1]babilomics4 platform:fatigo.org
[2]Zhang, X., Hecker, M., Park, J.-W.; Tompsett, A. R.; Newsted, J.; Nakayama, K.; Jones, P. D.; Au, D.; Kong, R.; Wu, R. S. S. & Giesy, J. P.
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