

Supplemental Material

Methods

Estradiol and 4-nonylphenol exposure studies in zebrafish.

Approximately eighty male zebrafish were divided evenly between three 80 L glass aquaria. Each aquarium was individually heated to maintain a temperature of 26 - 29 °C with a light-dark cycle ratio of 14:10 h. Fish were acclimated for one week prior to commencing the experiment. One tank contained only tap water (negative control) and the others contained water with either 10^{-7} M estradiol or 4-nonylphenol. All exposures utilized a continuous flow-through system, to maintain constant concentrations of the chemicals during the two-week experiment, after which the fish were anaesthetized with 3-aminobenzoic acid ethyl ester (10 g/L) and liver samples harvested, frozen in liquid nitrogen and stored at -70 °C.

Hornyhead Turbot vitellogenin assay

Wells were coated with 100µl of 0.8µg/ml California Halibut vtg (provided by Amanda Palumbo of UC Davis) in 50mM carbonate buffer. Non-specific binding wells were coated with 1% non-fat milk in 50 mM carbonate buffer. Plates were then incubated at 37C for 2h. Wells were washed three times with 10mM Tris-phosphate buffer saline (TPBS), then blocked with 200µl of 2% non-fat milk in TPBS for 45min at 37C. The wells were then washed again three times with TPBS. Standards (purified Halibut vtg) and samples were diluted in TPBS. Primary antibody (rabbit anti-Turbot vtg

purchased from Cayman Chemical, Ann Arbor, MI) diluted in TPBS was added to standards and samples at a ratio of 1:1, for a final concentration of antibody of 1:1000. These solutions were then incubated for 2h at 37C. One hundred microliters of each solution was then added in triplicate to the wells and incubated again for 2h at 37C. The wells were then washed three times with TPBS. The secondary antibody (goat anti-rabbit labeled with alkaline phosphatase purchased from Biorad in Hercules, CA) was diluted to 1:2000 in TPBS then added to the wells and incubated for 45 min at 37C. The wells were washed twice with TPBS and once with PBS. The substrate *p*-nitrophenylphosphate diluted in diethanolamine buffer was added to each well at volume of 100µl. The plate was then incubated for about 1h in dark. The absorbance was measured with a microplate reader at a wavelength of 405nm.

Measurement of plasma concentrations of steroid hormones

Plasma concentrations of $^{17}\beta$ -estradiol, testosterone, and cortisol were measured by specific radioimmunoassays using ^{125}I -labeled steroid and polyclonal rabbit antisera obtained from DSL/Beckman Coulter (Webster, TX). Separation of free and bound antigen was achieved using a double antibody system (goat anti-rabbit gamma globulin serum) and polyethylene glycol as a precipitating aid. Counts per minute (cpm) of antibody-bound ^{125}I -steroid were measured in a Perkin-Elmer Cobra II gamma counter (Packard Instruments Co., Boston, MA). The standard curves were utilized to calculate concentrations of hormone in the unknowns using SigmaPlot 8.0 software (Four-Parameter Logistic Curve Function, SPSS Inc., Chicago, IL). Estimated coefficients of variation are between 6.1-7.5 (intra-assay) and 8.0-9.4 (inter-assay) for both assays

RNA extraction, fluorescent target labeling and microarray hybridizations

Isolation of total RNA from liver samples was performed using TRIzol reagent (Invitrogen) and the extracted RNA were further purified using the RNeasy Mini kit (Qiagen, Valencia, CA). The concentrations were determined by absorbance readings (OD) at 260nm using an ND-1000 (Nanodrop, Wilmington, DE). RNA was further assessed for integrity with the 6000 Nano LabChip assay from Agilent, (Palo Alto, CA). Microarray experiments were carried out at The UCSD Biomedical Genomics Facility (BIOGEM). 500 ng of total RNA were converted into fluorescently labeled Cy 3 or 5 cRNA using the Low RNA Input Fluorescent Linear Amplification Kit (Agilent). Fluorescent targets were purified to remove unincorporated nucleotides using RNeasy (Qiagen). Absorbance (OD) at 260nm was used to quantify the cRNA concentrations, and absorbance at 550nm and 650nm was used to measure the efficiency of Cy3 and Cy5 dye incorporation. An incorporation efficiency of 9 pmol/ μ g or greater was considered optimal for hybridization. 1 μ g of fragmented cRNA for each sample, control and exposed samples, were added to the slides in a hybridization solution containing 35% formamide, 5x SSC, 0.1% SDS and 0.1 mg/ μ l calf thymus DNA.

Hybridization was carried out for 18 hours at 42°C in a shaking incubator at 100 rpm. The microarrays were washed with 1x SSC/0.2% SDS for 5 min at room temperature followed by two 5 min washes with 0.1x SSC/0.2% SDS at room temperature. The microarray was rinsed briefly with water and dried by centrifugation at 800 rpm for 5 minutes. Technical replicate hybridizations were carried out with each sample.

Slides were scanned using an Axon 4000A scanner (Molecular Devices, Sunnyvale, CA) at the photomultiplier tube (pmt) settings of 500 for Cy3 and 600 for Cy5. Isolated extremal pixels were removed from the image by applying a pair of gray-scale thinning and thickening filters (Soille, 1998). The image was then subtracted from its morphological opening thereby removing the background, whether uniform or non-uniform. Since the background was removed and only instrument noise remained, the signal for the spot was extracted without further correction. Spot finding and flagging of low intensity features were accomplished using GenePix Pro software, version 6.0 (Molecular Devices, Sunnyvale, CA). Plotting of the data was carried using the R programming language and software environment.

Differential expression and signal intensity measurements.

We employed graphical and statistical methods to examine gene expression in control and exposed fish. Alterations in gene expression in turbot liver were investigated and differentially expressed mRNAs relative to control fish were determined by using a threshold of \log_2 intensity ratio of 2 or greater (Figure 3). We constructed MA plots to examine gene expression in control and exposed fish, where M is a measure of normalized differential gene expression (\log_2 [exposed/control] in plots A-G) or absence of significant differential gene expression in the self-self plots (\log_2 [control/control] in plots H-K). Positive M values indicate higher normalized signal intensity in the exposed RNA sample and negative M values indicate higher intensity in the control. M values of zero indicate equal intensity, namely genes whose expression remains unaltered between control and exposed fish. A is a measure of absolute signal intensity ($0.5 \log_2$ exposed

intensity + 0.5 log₂ control intensity) in plots A-G or (0.5 log₂ control intensity + 0.5 log₂ control intensity) in H-K. Higher A values indicate stronger signals and abundant transcripts.

Control RNA consisted of a pool of equal aliquots of total RNA from three control turbot. To determine if this protocol was valid, we constructed a self-self plot of pooled control RNA [panel H in Figure 3] and plots of individual control fish compared with the pooled control [panels I-K in Figure 3]. These plots are below the threshold of log₂ intensity ratio of 2, which validates the use of the pooled control RNA for comparison with RNA from turbot taken from polluted samples.

The q-q plots in Figure 4 examined the distribution of the log₂ (exposed/control) fold changes and the deviation from a normal Gaussian distribution. When a data set is derived from the Gaussian distribution, the normal-quantile plot is a straight line. The nature of plots in Figure 4 panels H-K shows that the observed log₂ ratio between control fish, both pooled and individual is reasonably close to a Gaussian. This distribution is due to individual variation in fish combined with unavoidable random experimental errors. When the log₂ ratio is taken between exposed and control fish (panels A-G), the curved ends of the q-q line indicate the presence of heavy tails in the distribution of log₂ (exposed/control). The exposed samples clearly differ from the control samples. Specifically, the sharp increase in the quantile curve at log₂ ratio of about 2 suggests that genes with $|\log_2(\text{exposed/control})| > 2$ show significant regulation in the LACSD (panels A-D) and OCSD (panels E-G) exposed fish compared to controls.

Amplification and sequencing of hornyhead turbot mRNAs

We amplified partial turbot transcripts using conserved sequences from other fish species to guide the choice of primer design. Gene-specific primers were designed using Primer3 software (Rozen *et al.*, 2000). A 120 bp sequence was amplified from the 18S rRNA using the following primers; forward 5'-GGGTTTAGACCGTCGTGAGA and reverse 5'-AGCCAAGCACATACACCAA. A 105 bp sequence was amplified from *cyp4503A* using the following primers; 5'-CCAGCACAGCCTTCAGTGTA and reverse 5'-AGAGAGGGTTGAAAAGGTCA. A 94 bp sequence was amplified from *TRβ* using the following primers; forward 5'-AGGAGGAGTGGGACCTCATC and reverse 5'-CTCAGGAATTTCCGCTTCTG. A 117 bp sequence was amplified from the *Vit1* transcript using the following primers; forward 5'-ATGAAGGGACAGACCTGTGG and reverse 5'-AACCCAGGAATGAGCATAGC. A 127 bp sequence was amplified from *Vit2* transcript using the following primers; forward 5'-ACTGGATGAGAGGCCAGACTT and reverse 5'-GGTAGAACCCAGGAATGAGC.

For amplification and sequencing, oligonucleotide primers were obtained from Invitrogen. PCR was carried out in 50µl volume, 5µl (10X reaction buffer 200mM TrisHCl/100mM KCl/100mM (NH₄)₂SO₄/20mM MgSO₄/1% TritonX/BSA/1mg/ml), 0.1mM dNTPs, 2.5 units of *Pfu*, 1µl 1µM forward primers, 1µl 1µM reverse primers, 200ng turbot liver cDNA. After a denaturing step for 10 min at 96°C, touchdown amplification was performed with 35 cycles of 45s at 96°C, 45s at 55° to 50°C, in one degree increments and 1 min at 72°C. All reactions were evaluated on a 1% agarose gel stained with ethidium bromide to validate the reaction and the products were directly sequenced using the respective forward and reverse PCR primers. All of the sequencing

reads were subjected to a series of quality control measures, including a phred quality score >20 , and manual trace inspection. The identity of each sequence was confirmed by performing BLAST searches of GenBank.

Supplemental Table 1

Sources of sequences for the 65 mer probes for the multispecies endocrine microarray.

Supplemental References

Soille, P: Morphological Image Analysis. New York: Springer, 1998.

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Sources of sequences for the 65 mer probes for the multispecies endocrine microarray.

Gene	Species Represented (Accession)
Hydroxysteroid Dehydrogenase Genes	
<i>17beta-HSD1</i>	(Zebrafish AAI63707), (Tilapia AAV74182), (Tetraodon CAG02816)
<i>11beta-HSD2</i>	(Zebrafish AAH65613), (Eel BAC67576), (Trout BAC76709), (Medaka ABQ09266), (Tetraodon CAG00815)
<i>11beta-HSD3</i>	(Zebrafish AAH71452), (Medaka AAS89258), (Stickleback AAS89257), (Carp ABI20737), (Tetraodon CAG01365)
Nuclear Receptor Genes	
Steroid Receptor Genes	
<i>Androgen receptor</i>	(Seabream BAA33451), (Cichlid AAL92878), (Medaka BAC98301), (Tilapia BAB20082), (Human AAA51770), (Tetraodon CAG02975)
<i>Estrogen receptor-alpha</i>	(Seabream AAD31032), (Halibut BAB85622), (Medaka P50241), (Fugu SINFRUP62437) (Tetradon CAG03596)
<i>Estrogen receptor-beta</i>	(Seabream Q9W6M2), (Halibut BAB85623), (Medaka BAB79705), (Fugu SINFRUP67205), (Tetraodon CAG03763)
<i>Glucocorticoid Receptor</i>	(Seabream Q9W6M2), (Halibut AB013444), (Cichlid AAM27888), (Fugu SINFRUP52715), (Tetradon CAG11713)
<i>Mineralocorticoid receptor</i>	(Cichlid AAM27890), (Fugu SINFRUP65506), (Tetraodon CAG11072), (Trout AAS75842)
<i>Progesterone receptor</i>	(Human AAA60081), (Zebrafish AAY85275), (Eel BAA89539), (Tetraodon CAG12799), (Fugu SINFRUP81329)
Other Nuclear Receptor Genes	
<i>Thyroid hormone receptor-alpha</i>	(Halibut Q91241), (Flounder AAV66919), (Fugu AAL06723), (Tetraodon CAG02086)
<i>Thyroid hormone receptor-beta</i>	(Seabream AAO86517), (Halibut Q91279), (Medaka BAD11773)
<i>Retinoic acid receptor</i>	(Salmon ABW77511), (Zebrafish AAB32277), (Tetraodon CAG07392), (Fugu ABF22438)
<i>Retinoid X receptor</i>	(Zebrafish A2T929), (Medaka BAB83838), (Tetraodon CAAE01008554)
<i>Farnesoid X receptor</i>	(Zebrafish AAH92785), (Fugu SINFRUP72134), (Tetradon CAG03422Z)
<i>Liver X receptor</i>	(Zebrafish AAH92160), (Fugu SINFRUP70795), (Tetradon CAF99925)
<i>Pregnane X receptor</i>	(Trout ABP38412), (Killifish ABR21208), (Fugu ABV29342), (Tetraodon CAG05861)
<i>Peroxisome proliferator activated receptor-alpha</i>	(Zebrafish CAI11869), (Seabream AAT85613), (Flounder CAD62447), (Fugu BAF52668), (Tetraodon CAF95270)
<i>Peroxisome proliferator activated receptor-gamma</i>	(Zebrafish ABI30002), (Seabream AAT85618), (Flounder CAD62449), (Fugu BAF52670), (Tetraodon CAG07050)
<i>Vitamin D receptor</i>	(Halibut BAA95015), (Zebrafish Q1L673), (Medaka ACB38279), (Fugu SINFRUP64850), (Tetraodon CAF94134)
Cytochrome P450 genes	
<i>Cytochrome P450 1A</i>	(Seabream AAB64297), (Halibut ABO38813), (Seabass AAB36951), (Trout AAD45966), (Tetraodon CAG03127)
<i>Cytochrome P450 3A</i>	(Zebrafish AAI09441), (Killifish Q9PVE8), (Seabass ABB90404), (Medaka AAK37960), (Trout AAK58569), (Tetradon CAF91666)
<i>Cytochrome P450 19</i>	(Seabream AAL27699), (Zebrafish AAK00643), (Halibut BAA74777), (Fugu BAF93506), (Tetraodon CAF99837)
Vitellogenin Genes	
<i>Vitellogenin 1</i>	(Seabream BAE43871), (Trout CAA63421), (Halibut ABQ58114), (Flounder BAD93696)
<i>Vitellogenin 2</i>	(Zebrafish CAK11092), (Carp AAD23878), (Seabream AAG25918), (Killifish Q98893)

SINFRUP accessions for Fugu are from Ensembl. All other accessions are from GenBank.