Analysis of Endocrine Disruption in Southern California Coastal Fish using an Aquatic Multi-Species Microarray

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ABSTRACT

BACKGROUND: Endocrine disruptors include plasticizers, pesticides, detergents and pharmaceuticals. Turbot and other flatfish are used to characterize the presence of chemicals in the marine environment. Unfortunately, there are relatively few genes of turbot and other flatfish in GenBank, which limits the use of molecular tools such as microarrays and qRT-PCR to study disruption of endocrine responses in sentinel fish captured by regulatory agencies.

OBJECTIVES: A multi-gene cross species microarray was fabricated as a diagnostic tool to screen the effects of environmental chemicals in fish, for which there is minimal genomic information. The array included genes that are involved in the actions of adrenal and sex steroids, thyroid hormone, and xenobiotic responses. This will provide a sensitive tool for screening for the presence of chemicals with adverse effects on endocrine responses in coastal fish species.

METHODS: A custom multi-species microarray was used to study gene expression in wild hornyhead turbot, collected from polluted and clean coastal waters and in laboratory male zebrafish following exposure to estradiol and 4-nonylphenol. Gene-specific expression in turbot liver was measured by qRT-PCR and correlated to microarray data.

RESULTS: Microarray and qRT-PCR analyses of livers from turbot collected near municipal wastewater discharge areas revealed altered gene expression profiles compared to those from reference areas.

CONCLUSIONS: The agreement between the array data and qRT-PCR analyses validates this multi-species microarray. The microarray measurement of gene expression in zebrafish, which are phylogenetically distant from turbot, indicates that this multi-species microarray will be useful for measuring endocrine responses in other fish.

INTRODUCTION

The European Community in 1996 defined endocrine disruptors as “an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse effects in an intact organism or its progeny or subpopulations”. Endocrine disruptors of concern include plasticizers such as phthalates and alkylphenols, pesticides, fungicides, detergents, dioxin, polychlorinated biphenyls and pharmaceuticals such as the synthetic estrogen 17α-ethynylestradiol. These xenobiotics are discharged into rivers, lakes and the ocean, where

they accumulate in aquatic species. Humans and wildlife are exposed to these compounds directly and through fish and shellfish consumption. In addition, humans are exposed to endocrine disruptors via polluted drinking water.

Some endocrine disruptors interfere with normal endocrine responses because the chemical has structural similarities to hormones such as steroids. As a result the endocrine disruptors bind to a hormone receptor or to an enzyme that catalyzes hormone synthesis or degradation (Atanasov et al. 2005, Baker 2001). Elevated concentrations of xenobiotics in the environment have raised awareness of their potential impact on human health (Tyler et al. 1998). Exposure of humans to endocrine disruptors may lead to increased rates of fetal death (Bell et al. 2001), intellectual impairment in children (Jacobson and Jacobson 1996), premature puberty in females (Herman-Giddens et al. 1997), and decreased reproductive ability in men (Sharpe and Skakkebaek 1993).

Amongst endocrine disruptors, alkylphenol ethoxylates, such as nonylphenol, have been widely studied owing to their wide diffusion in the environment through the use of alkylphenols in the plastics industry and in detergents, paints, herbicides and pesticides (Soto et al. 1991). It has been estimated that 60% of man-made alkylphenols enter the aquatic environment (Naylor et al. 1992), with most entering via sewage treatment works, where they are readily degraded to form relatively stable metabolites (Ahel et al. 1987). Nonylphenol is the predominant degradation product of the alkylphenols encountered in the aquatic environment (Giger at al. 1984). Exposures of cell cultures and laboratory animals to nonylphenol have demonstrated that it competes with estradiol for binding to the ER, but has only weak estrogenic activity. As a result there are concerns that exposure of humans and fish to nonylphenol will disrupt male and female reproduction and development.

It also is clear that xenobiotics affect other hormone pathways, such as thyroid hormone (Boas et al. 2006; Zhou et al. 2000), among others. Thus, there is a need for a tool that can screen many endocrine responses in fish taken from polluted water and monitor harmful effluents entering the ecosystem. Ideally suited for this purpose are microarrays, which can simultaneously measure the level of expression of hundreds of genes from a single tissue sample in each animal, collected from a polluted environment (Benson and Di Giulio 2008; Hardiman 2004; Hardiman and Carmen 2006; Marton et al. 1998). Microarray analysis of alterations – either up or down – in the levels of genes involved in physiological responses to estrogens,

androgens, glucocorticoids, thyroid hormone, and detoxification of chemicals provides a powerful tool for obtaining a more complete diagnosis of endocrine disruption in fish. A microarray profile of alterations in gene expression associated with a single compound represents a unique signature, which can be used to detect compounds with endocrine disrupting activity in the environment. Moreover, in addition to the practical use of microarrays for analysis of endocrine disruption in fish, microarrays can provide molecular information for elucidating the mechanism of action of nonylphenol, other xenobiotics and endogenous hormones such as estradiol.

The development of the microarray described here was motivated by the needs of the Los Angeles County Sanitation Districts (LACSD), the Orange County Sanitation District (OCSD), the City of Los Angeles Environmental Monitoring Division (CLAEMD), the City of San Diego Ocean Monitoring Program, the Southern California Coastal Water Research Project (SCCWRP) and university research groups in Long Beach, Riverside and San Diego. Together, these investigators monitor and study chemical and waste effluents discharged into the coastal marine environment and watersheds through biannual collection of sentinel fish at different sites from San Diego to Santa Barbara to assess accumulated levels and effects of environmental chemicals.

The sentinel fish, which are used to characterize the presence of chemicals in the marine environment, are hornyhead turbot and other flatfish because these fish are often bottom feeders and are at higher risk of exposure to chemicals that accumulate in sediments. These fish also live in a limited area, which allows one to localize the site of chemical pollution. The Orange County Sanitation District has supported several studies that captured turbot from different sites for the analyses of aberrant morphology in organs, for endocrine disruptors in liver tissue and blood and for vitellogenin, a biomarker for estrogen exposure (Deng et al. 2007; Rempel et al. 2006). Microarray and q-PCR analysis of turbot organs would provide enhanced sensitivity to xenobiotics in the marine environment, and facilitate control of toxic effluents. Unfortunately, few genes of turbot and other flatfish have been sequenced, which limits the use microarrays and qRT-PCR to study disruption of endocrine responses.

In this study we present data from a multi-gene cross species microarray, which was used to analyze gene expression in hornyhead turbot collected in the coastal waters of Orange County and Los Angeles County in California. Parallel experiments with qRT-PCR verify the microarray data. The multi-species microarray also was used to study gene expression in livers

of zebrafish exposed to estradiol and nonylphenol. The use of the multi-species microarray to study gene expression in hornyhead turbot and zebrafish, which are phylogenetically distant (Figure 1), suggests that this array will be a useful diagnostic screening tool to monitor responses to contaminants in *Perciformes*, *Pleuronectiformes* and fish from other taxa for which there is limited genomic sequence.

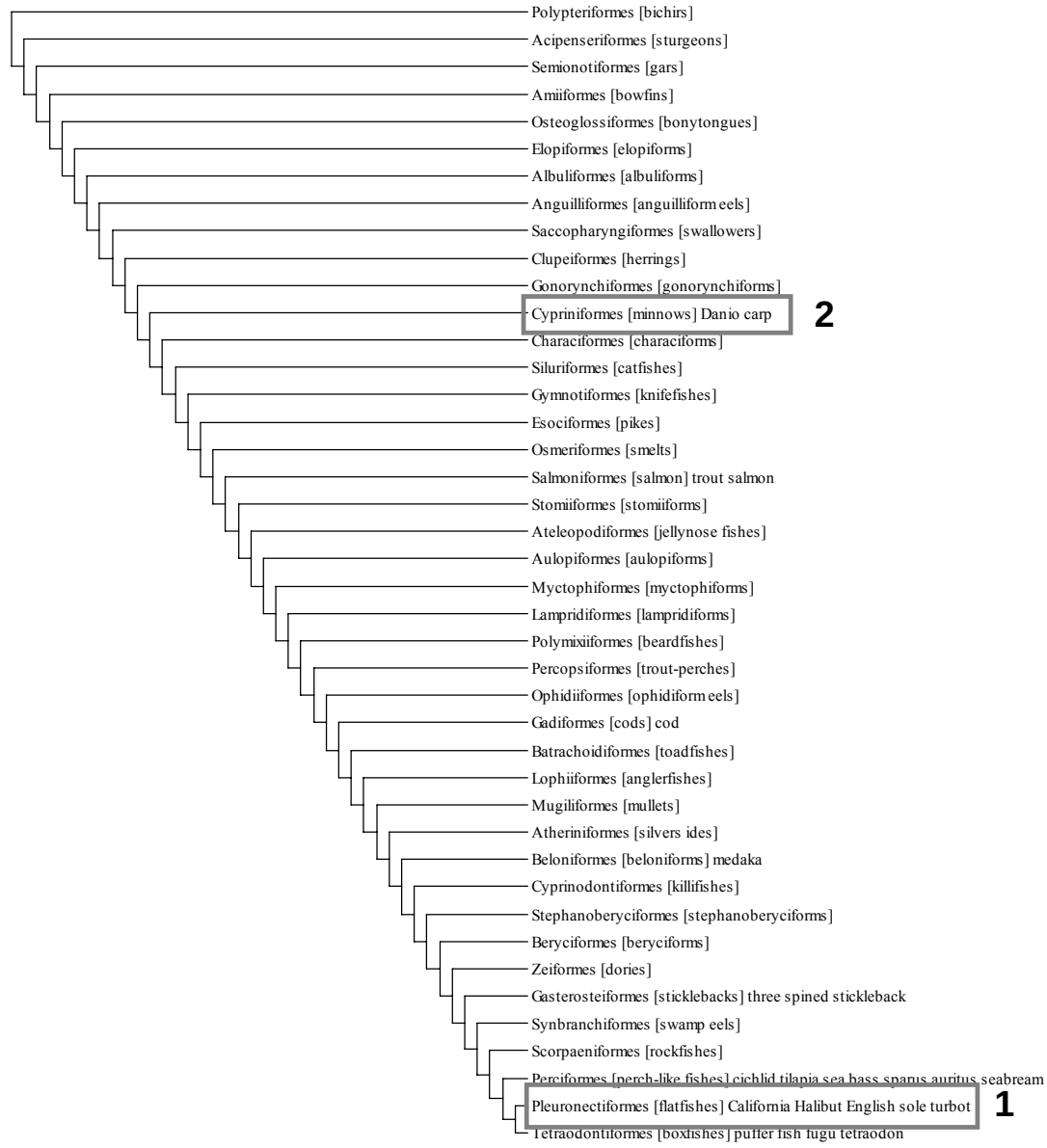

Figure 1. Flatfish (*Pleuronectiformes*) in an evolutionary context.
Adapted from the phylogeny at http://cichlidresearch.com/fish_html/cactinop.html (Nelson 2006). *Tetraodontiformes* (Fugu, Tetraodon) and *Perciformes* (cichlid, tilapia, sea bass, seabream, perch) are close phylogenetic relatives of *Pleuronectiformes* (turbot, halibut, sole), Box 1. *Cypriniformes* (zebrafish) are distant phylogenetic relatives, Box 2.

MATERIALS AND METHODS

Estradiol and 4-nonylphenol exposure studies in zebrafish.

Details of exposure of zebrafish to estradiol and 4-nonylphenol are described in the Supplemental Methods, See Supplemental Material.

Hornyhead Turbot vitellogenin and estradiol assay Details of the measurements of vitellogenin, estradiol, cortisol and testosterone (Kelley et al. 2001; Rempel et al. 2006) are described in the Supplemental Methods, See Supplemental Material.

Environmental hornyhead turbot sample collection

Male hornyhead turbot were collected off of the coast of Southern California as part of a Southern California regional marine monitoring study (Bight Field Sampling and Logistics Committee 2003). Livers from 3 individual fish from a station near Orange County Sanitation District outfall and four individuals from a monitoring station near Los Angeles County Sanitation District outfall were used for microarray analysis. These fish exhibited morphological abnormalities, high levels of vitellogenin and estradiol, low levels of cortisol, and histological abnormalities, such as the presence of immature oocytes (eggs) within the testis (Table 1).

Table 1. **Characteristics of hornyhead turbots sampled.** Individuals with morphological abnormalities induced by EDs were chosen for microarray experiments. Cortisol, estradiol, insulin like growth factor (IGF-1), thyroxine (T4), vitellogenin (VTG) levels are indicated. Morphological lesions (not caused during capture) and maturity stages are noted.

LOCATION	Station ID	Sample ID	Sex	Cortisol (ng/ml)	Estradiol (pg/ml)	IGF (ng/ml)	T (ng/ml)	VTG (ng/μg protein)	Morpholology Diagnosis	Lesion Grade	Maturity Stage
OCSD	4041	3	M	8.0	58.1a	16.0	0.7	0.1	Oocytes	Minimal	Stage 1
OCSD	4041	4	M	18.6	134.0a	20.2a	2.5a	0.2	Oocytes, Macrophage Aggregates	Minimal	Stage 1
OCSD	4041	6	M	89.4a	90.8a	17.1	0.5	0.4	None	NA	Stage 1
LACSD	4086	1	M	20.7a	2.2	16.3	0.4	1.5a	Fibrous Septa	Minimal	Stage 1
LACSD	4086	2	M	15.1	2.2	17.0	0.4	2.6a	Fibrous Septa	Moderate	Stage 2
LACSD	4086	3	M	1.1	21.3	15.9	0.4	1.0	None	NA	Stage 2
LACSD	4086	4	M	8.4	2.2	17.4	0.5	1.6	Oocytes, Fibrous Septa	Moderate	Stage 1

[a] denotes anomalies.

Control fish were obtained from a monitoring station in Dana Point, CA an area considered relatively non-impacted and maintained in a clean-water laboratory setting for four weeks. Measurement of vitellogenin in the control male turbot with an ELISA assay found 0.0037 ng/μg protein. This is from 27-fold to 700-fold lower than the vitellogenin levels in the turbot collected from polluted sites in Orange County and Los Angeles County (Table 1). This indicates that the control male turbot were not exposed to an estrogenic compound, which validates the use of their liver RNA as a control for the microarray and the qRT-PCR analyses.

Exposed animals were sacrificed immediately following capture and the livers were harvested and frozen in liquid nitrogen and stored at -70 °C. All the animals were treated humanely and with regard for alleviation of suffering.

Construction of the Multi-species Microarray

To overcome the scarcity of sequence data in GenBank for hornyhead turbot we constructed a 65mer oligonucleotide-based microarray containing conserved sequences from genes of interest. Oligo probes were designed by collecting available fish sequences in GenBank for a given gene (e.g. *ESR1/ Erα*, *Vtg*, *CYP3A* and *FXR*) using BLAST (Altschul et al. 1990). Sequences from *Tetraodoniformes* (Fugu, Tetraodon) and *Perciformes* (cichlid, tilapia, sea bass, seabream), which are close from a phylogenetic perspective to *Pleuronectiformes* (hornyhead turbot, California halibut) were selected (see Figure 1). Available sequences from medaka, stickleback and zebrafish also were used, in addition to some hornyhead turbot-specific cDNA sequences obtained by degenerate PCR cloning. Clustal X was used to construct multiple alignments to uncover conserved regions (Thompson et al. 1997) and nucleotide sequences within 1200 bases from the mRNA 3' end were identified and analyzed using Oligowiz (Nielsen et al. 2003; Wernersson and Nielsen 2005) to design 65-mer microarray probes. Each copy of an individual gene in several fish was subjected to a pair-wise BLAST comparison with the corresponding gene from other fish to insure that the DNA sequence was between 80% and 90% identical thereby increasing the likelihood that the homologous turbot sequence would contain at least 85% identity to one of the oligonucleotides. The gene names and corresponding accession numbers are provided in the Supplemental Methods, See Supplemental Table.

Oligonucleotides were synthesized to contain a 5' amine group and desalted by Operon Technologies (Alameda, CA) and Invitrogen (Carlsbad, CA), and used without further purification. Oligonucleotides were printed on amine-reactive silanized glass slides (Surmodics,

Inc., Eden Prairie, MN) as described previously at the UCSD BioMedical Genomics Microarray Facility (BIOGEM) (http://microarrays.ucsd.edu) (Hardiman et al. 2003).

RNA extraction, fluorescent target labeling and microarray hybridizations

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. 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.

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 and Skaletsky 2000) as outlined in the Supplemental Methods, See Supplemental Material. All the amplicons 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.

Microarray data analysis

Array data has been deposited in the EBI Array Express Database (accession number pending). Statistical analysis of the microarray experiment involved two steps: normalization of microarray data, and sorting of the genes according to interest. We normalized all samples simultaneously using a multiple-*loess* technique described previously (Šášik et al. 2004). In designing the interest statistic we borrowed ideas from the software package *Focus* (Cole et al. 2003). The interest statistic reflects a biologists understanding that a gene with a greater fold change (in absolute value) than other genes is potentially more interesting. Also, given two genes with the same fold changes, it is the gene with a higher expression level (and therefore higher

absolute change) that is more relevant. This approach is described in greater detail elsewhere (Ogawa et al. 2004).

Differential expression and signal intensity measurements.

To investigate alterations in gene expression of controls and exposed fish, we used two independent analytical methods, MA plots and q-q plots, as described in detail in Supplemental Methods, See Supplemental Material.

Quantitative real-time PCR analysis.

Relative turbot mRNA transcript levels were measured by real-time quantitative RT-PCR in a LightCycler 480. Total RNA was extracted from hepatic turbot samples as described above and reverse-transcribed using the Roche Transcriptor kit and 50 ng cDNA were quantified using LightCycler 480 SYBR Green Master kit. The 18S rRNA served as an internal control for normalization. Each sample was run in triplicate and mean values were reported. Normalized gene expression values were obtained using LightCycler Relative Quantification software. Relative gene copy numbers were derived using the formula $2^{\Delta CT}$ where ΔCT is the difference in amplification cycles required to detect amplification product from equal starting concentrations of turbot liver RNA.

RESULTS

Design of the multi-species endocrine microarray

Development of the multi-species microarray was motivated by the mission of the participating sanitation districts, university research groups and SCCWRP to monitor the endocrine status of turbot, halibut and other flatfish at different sites in coastal southern California. We wanted a broad measure of the effects of chemicals on a variety of endocrine responses in fish. Thus, the microarray included probes for genes encoding receptors for estradiol (*ESR1/ Erα; ESR2/ Erβ*), progesterone (*PR*), testosterone (*AR*), cortisol (*GR*), aldosterone (*MR*), thyroid hormone (*THRA/TRα;THRB/TRβ*), retinoids (*RAR, RXR*) and vitamin D (*VDR*), and other nuclear receptors: farnesoid X receptor (*FXR*), and pregnane X receptor (*PXR*), hydroxysteroid dehydrogenases and the enzymes involved in detoxification (*CYP1A1, CYP3A*) were represented (See Supplemental Material, Table 1). This provided a diagnostic tool for measuring altered expression of genes that are important in several endocrine pathways in fish, which increased the scope of detection for the presence of endocrine disruptors in coastal waters off of southern California.

A challenge in developing this platform was the paucity of available sequence information for hornyhead turbot in GenBank. To overcome this problem, we searched GenBank for sequences in other fish to find regions of sequence conservation that could used to construct a microarray slide that could detect altered gene expression in multiple fish species. Fortunately, the genomes of two *Tetraodontiformes*, Fugu and Tetraodon, have been sequenced. Moreover, many genes from various *Perciformes* were in GenBank. *Tetraodontiformes* and *Perciformes* are phylogenetically close to *Pleuronectiformes* as shown in Figure 1. Also of importance was the extensive catalog of sequenced genes from zebrafish, which is distant from of *Pleuronectiformes*. Sequences conserved in zebrafish, Fugu, Tetraodon and various *Perciformes* were likely to be conserved in turbot. A schematic representation of the design and application of the multi-species microarray to monitor xenobiotic exposure is shown in Figure 2.

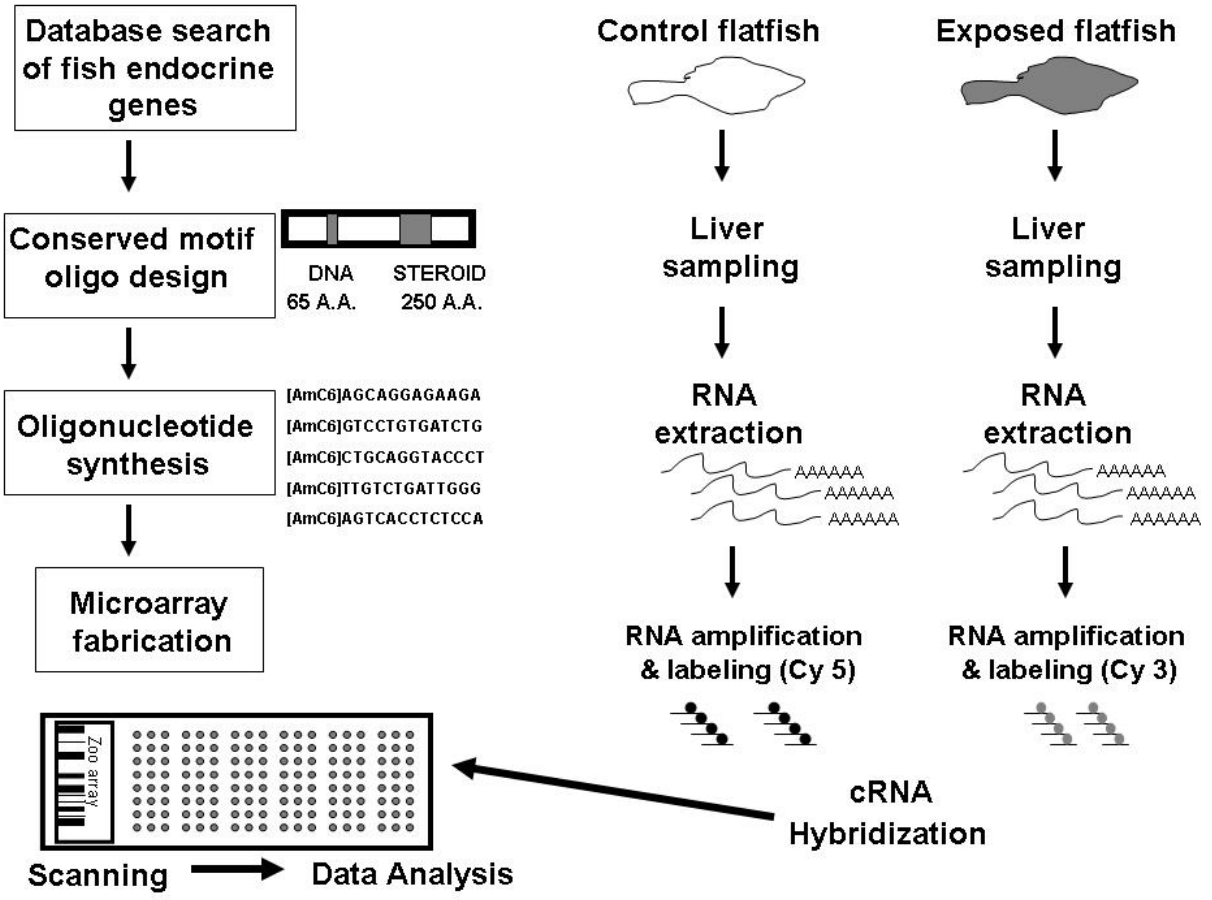

Figure 2. Development of a Multi-Species Endocrine Microarray for environmental monitoring. Schematic representation of the design and application of a microarray based test to monitor xenoestrogen exposure.

Differential expression and signal intensity measurements using the multi-species microarray

Male hornyhead turbots sampled at two California monitoring stations in Orange and Los Angeles Counties were assessed for exposure to xenoestrogens using the multi-species microarray. Figure 3 shows the measurements obtained using the multi-species microarray to examine control and exposed fish. Alterations in gene expression in hornyhead turbot liver relative to control fish were determined by using a threshold of log2 intensity ratio of 2 or greater. The MA plots revealed differential gene expression profiles between exposed and control turbot taken from sites in LACSD (panels A-D) and OCSD (panels E-G).

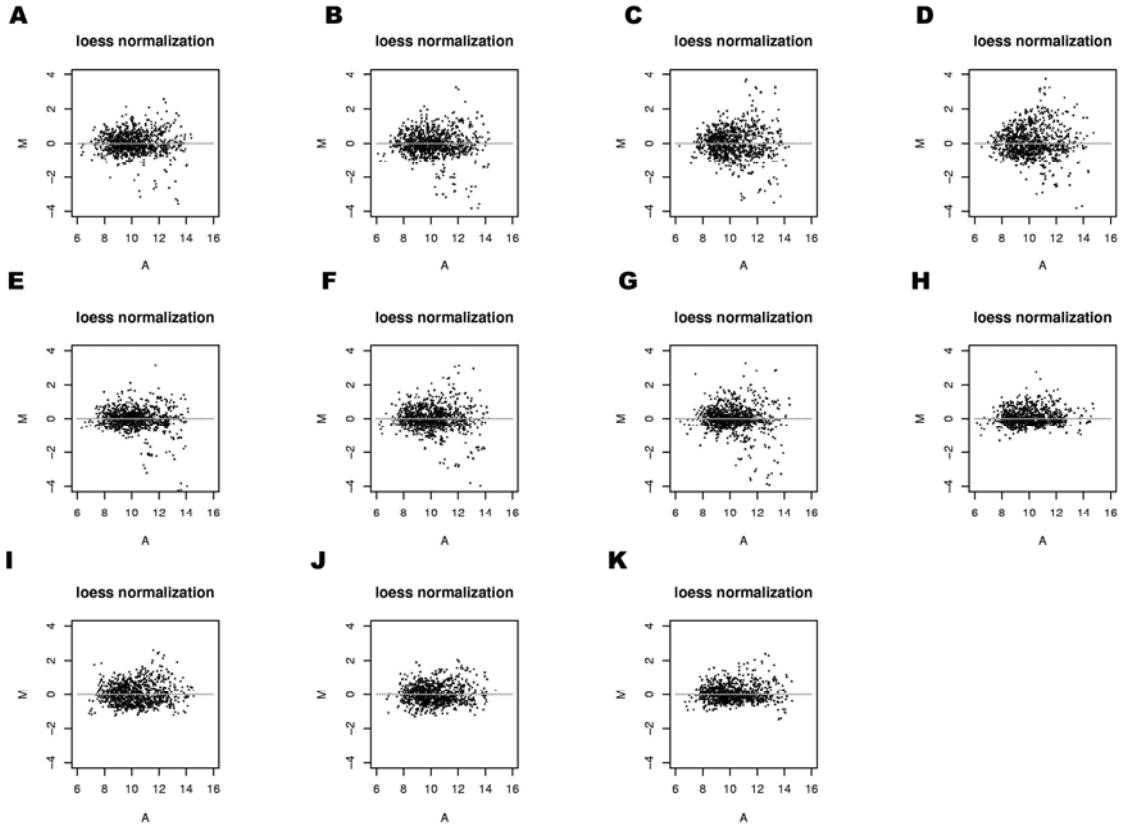

Figure 3. Differential expression and signal intensity measurements using the Multi-Species Endocrine Microarray for control and exposed fish. (*A-D*) Individual LACSD exposed fish versus pooled controls; (*E-G*) Individual OCSD exposed fish versus pooled controls; (*H*) Pooled controls versus pooled controls; (I-K) individual control fish versus pooled control. Each point represents data from a single 65-mer oligonucleotide probe. *M* is a measure of differential gene expression (log2 (exposed /control) in plots A-G) or absence of significant differential gene expression in the self-self plots (log2 (control / control intensity) in plots H-K. *A* is a measure of signal intensity (0.5 log2 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.

We employed normal quantile (q-q) plots to examine more closely differences in expression between the control and exposed fish (Figure 4). 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 panels H-K shows that the observed log2 ratio between control fish, both pooled and individual controls is reasonably close to a Gaussian. This distribution is due to individual variation in fish combined with unavoidable random experimental errors.

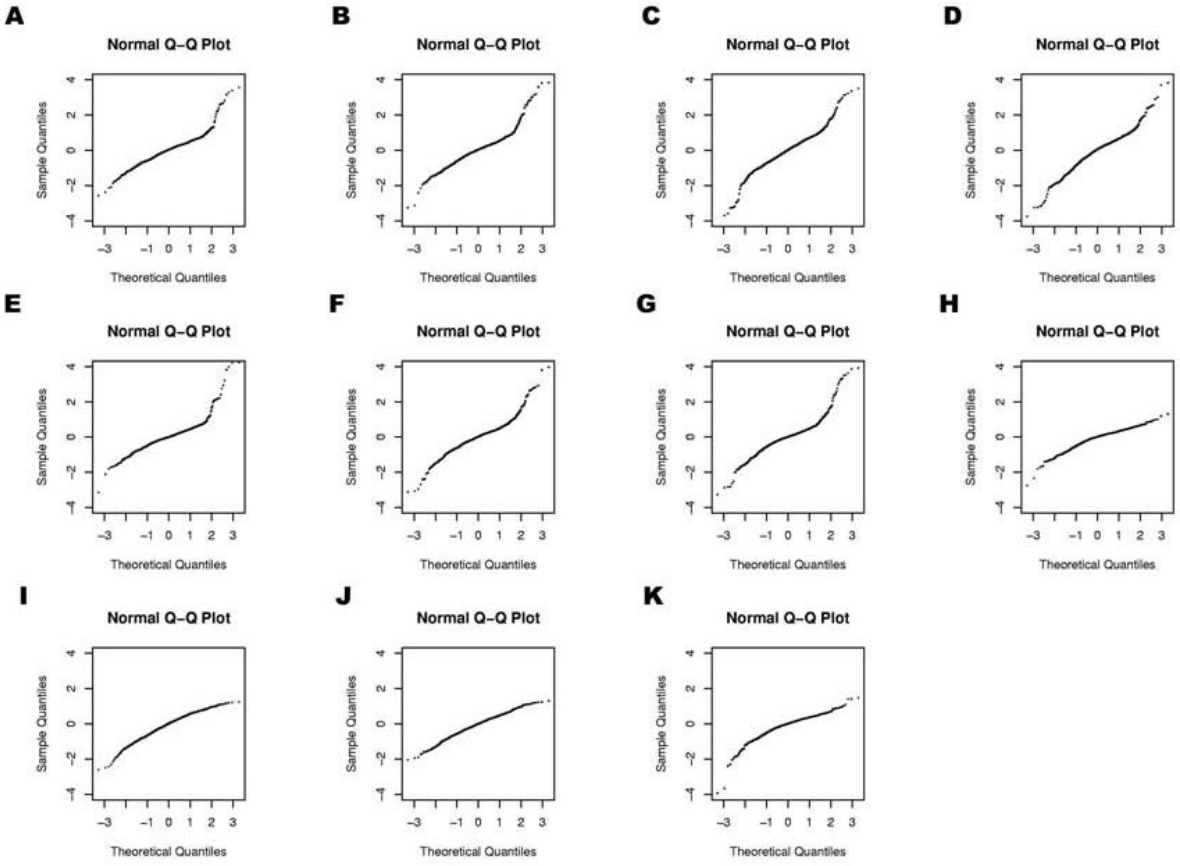

Figure 4. Normal-quantile-quantile (q-q) plots of Multi-Species Endocrine Microarray data. Q-Q plots were constructed to determine if control and exposed fish data sets derived from populations have a common distribution. (*A-D*) Individual LACSD exposed fish versus pooled controls; (*E-G*) Individual OCSD exposed fish versus pooled controls; (*H*) Pooled controls versus pooled controls; (I-K) individual control fish versus pooled control. The q-q plots examined the distribution of the log2 (exposed/control) fold changes and the deviation if any from a normal Gaussian distribution. A 45-degree reference line was plotted. When the two data sets derived from a population with the same distribution, the points fall approximately along this reference line, as is the case with the control samples data populations, both pooled

and individual (I-K). When the two data sets derived from populations with different distributions, the data deviated from this reference line (A-G). Exposed samples differ from the control, with a sharp rise observed in the quantile curve at log2 ratio values of 2, indicating the presence of large log2 ratios and differences in gene expression.

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 |log2 (exposed/control)| > 2 show significant regulation in the LACSD (panels A-D) and OCSD (panels E-G) exposed fish compared to controls, in agreement with the MA plots in Figure 3.

Gene expression patterns in male turbot from Southern California coastal regions.

A heat map of selected genes that were either strongly down-regulated or up-regulated in fish collected near the Orange County Sanitation District and Los Angeles County Sanitation District outfalls relative to controls is shown in Figure 5. Fish sampled at both impacted sites exhibited strong increases in expression of *CYP3A, RXR, ERβ, Vtg2* and *MR* relative to control fish. The *VDR* was up-regulated and transcripts encoding *FXR* and *PPARα* were down-regulated compared to control fish. Additionally, thyroid receptor α and β mRNAs were down-regulated.

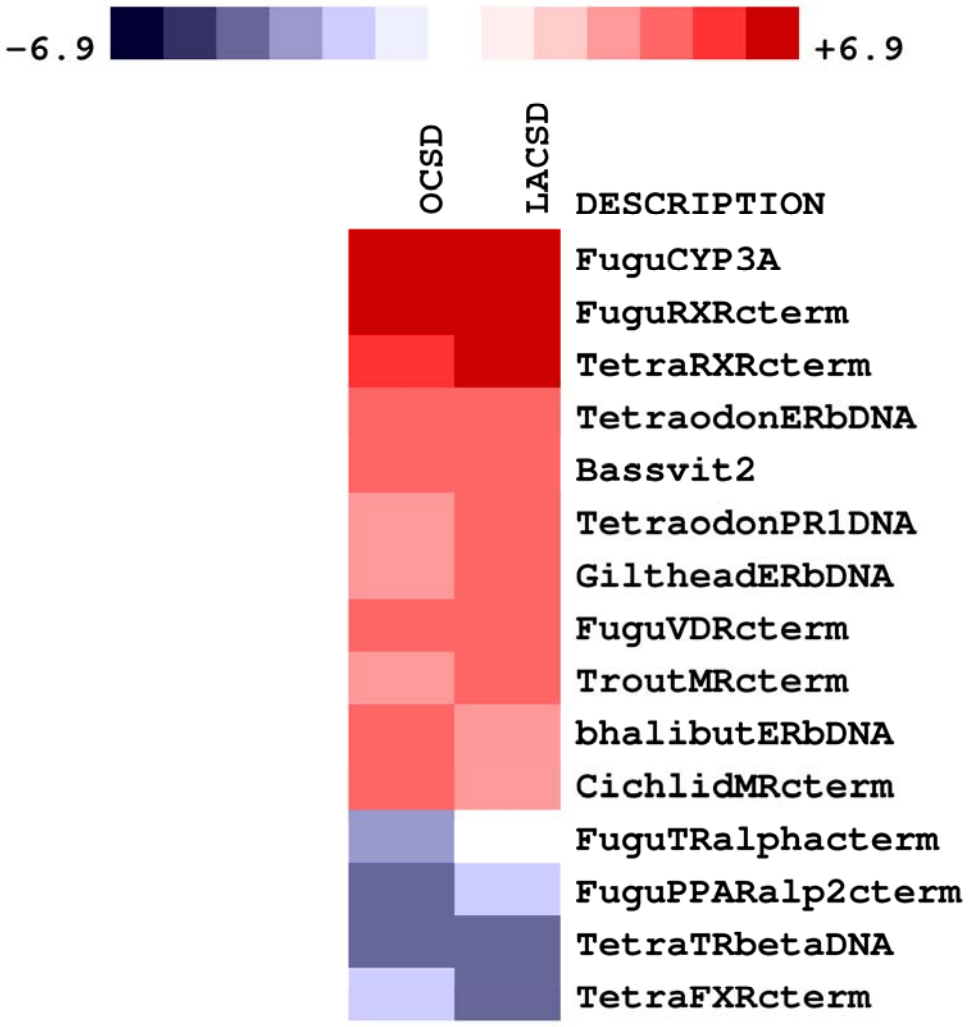

Figure 5. Gene expression profiling of turbots for exposure to endocrine disruptors.

Gene expression changes were investigated in male turbot liver collected in two coastal monitoring stations in Orange County (OCSD) and Los Angeles County (LACSD) in California that are considered contaminated. Control fish were obtained from a monitoring station in Dana Point a relatively non-impacted area and maintained in a clean-water laboratory for four weeks. Fold changes observed between exposed and control fish are depicted as a heat map. LACSD and OCSD data derived from four and three independent biological replicate microarray experiments respectively.

Quantitative RT-PCR analysis of turbot gene expression.

In order to use qRT-PCR to determine if the microarray data was accurately monitoring changes in hepatic gene expression in the turbot, we cloned, via reverse transcriptase PCR,

partial fragments corresponding to highly conserved regions in the 28S rRNA, *CYP3A, TRβ, Vtg1* and *Vtg2*. Identities of the fragments were confirmed by DNA sequencing and sequence data has been deposited in the NCBI Database (accession numbers pending). These short turbot-specific sequences were used for SYBR green quantitative PCR experiments on individual turbot from impacted regions. As shown in Figure 6, the greatest differences were observed with the *Vtg1* and *Vtg2* transcripts, with greater than a 15-fold induction observed in one turbot. TR*β* was down-regulated in two control fish examined. *CYP3A* was up-regulated in three fish. Thus, these qRT-PCR data validate the microarray analysis for these genes.

Moreover, vitellogenin 1 (Vtg 1) in control turbot is 0.0037 ng/μg protein, as determined by an ELISA assay. Thus control male turbot have from 27-fold to 700-fold lower vitellogenin levels that found turbot collected from polluted sites in Orange County and Los Angeles County (Table 1). The agreement between vitellogenin measured with the ELISA assay and with the qRT-PCR analysis (Figure 6) gives us confidence in qRT-PCR analyses of other genes in male turbot taken from polluted sites.

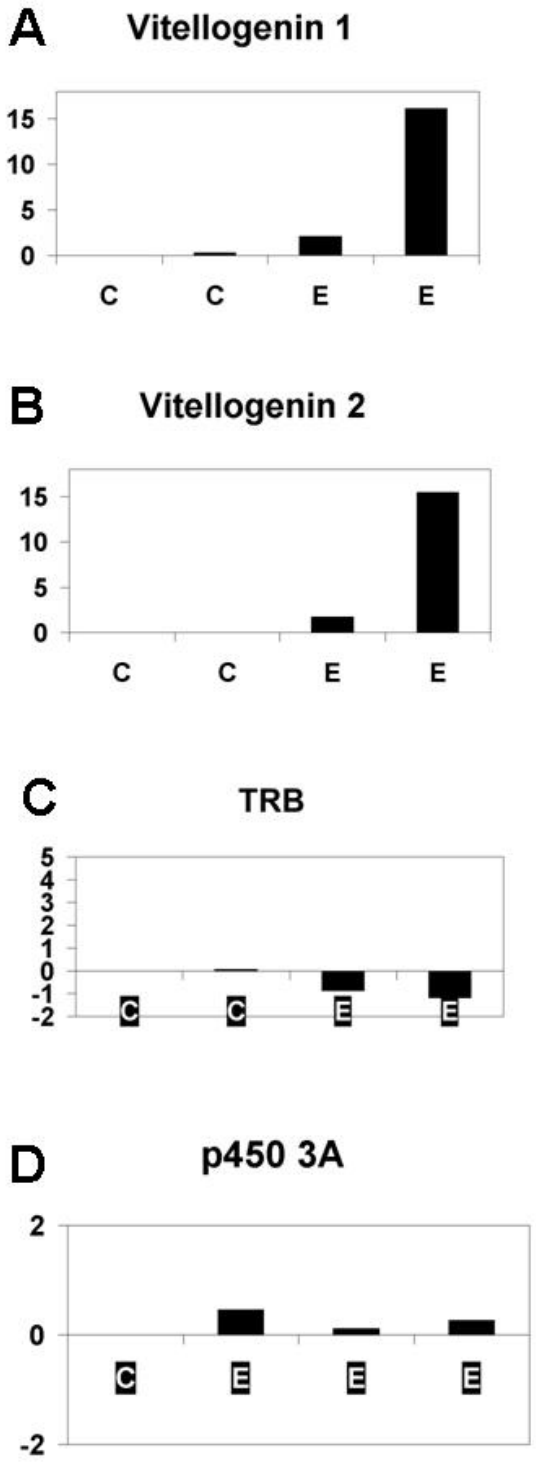

Figure 6. Validation of Multi-Species Endocrine Microarray

SYBR green qPCR for (**A**) Vitellogenin 1 (**B**) Vitellogenin 2 (**C**) Thyroid hormone receptor β (**D**) CYP3A specific transcripts in livers from control **C** and exposed **E** hornyhead turbot. The 18S rRNA served as an internal control for normalization. Mean values from triplicate measurements were plotted. Fold changes relative to control fish are plotted. Vitellogenin 1 and Vitellogenin 2 transcripts were strongly up-regulated (>15 fold) in one exposed fish. TRβ was down-regulated in two control fish examined. *CYP3A* was up-regulated in three fish and down-regulated in one fish.

Gene expression patterns in zebrafish exposed to estradiol and 4 nonylphenol

Alterations in gene expression in zebrafish liver were investigated using the multi-species microarray after exposing fish for two weeks to either the xenoestrogen 4-nonylphenol or estradiol at

10^{-7}M. These experiments were carried out to determine if probes designed from conserved sequences from *Tetraodoniformes* and *Perciformes* could hybridize to genes in zebrafish (*Cypriniformes*), which are distant relatives of *Pleuronectiformes* (Figure 1).

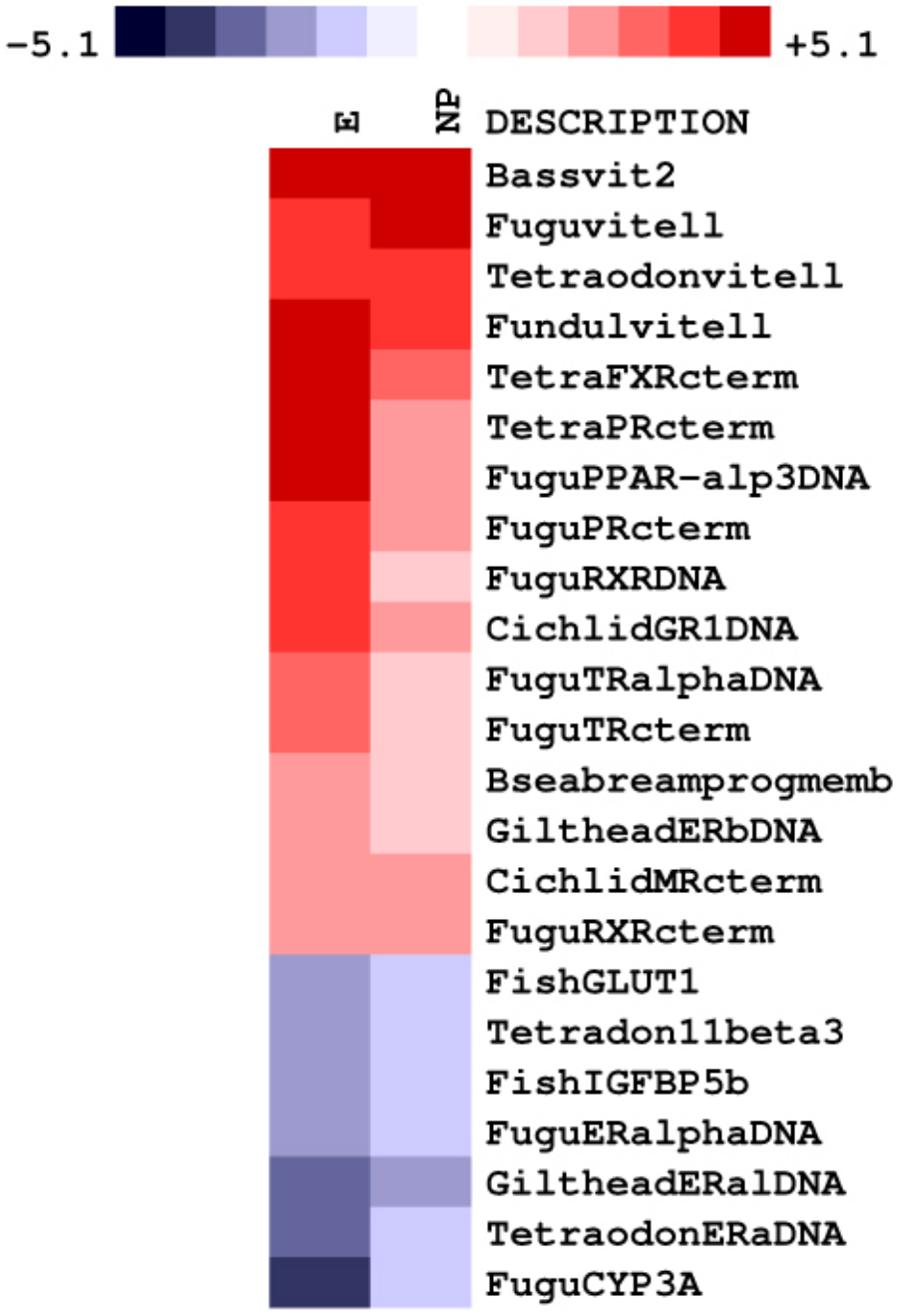

Figure 7. Cross species applicability of the Multi-Species Endocrine Microarray
Alterations in gene expression in zebrafish liver after a two week exposure to either 4-nonylphenol or estradiol using the multi-species endocrine microarray. Fold changes between exposed and

control fish are depicted as a heat map. Data derived from four independent biological replicate microarray experiments.

The data in the heat map in Figure 7 show that estradiol produced a broader and higher response than 4-nonylphenol. Both treatments induced strong up-regulation of vitellogenin, several nuclear receptors, proteins involved in oogenesis and in steroid metabolism. Amongst these, the *FXR* transcript was the most up-regulated. Other transcripts that were strongly up-regulated included the *PR*, the *MR*, *PPARα*, *PXR*, *RXRα*, *TRα* and *GR*. Other transcripts were down-regulated by treatment with estradiol and 4-nonylphenol including *ERα*, the glucose transporter (*GLUT*), an insulin-like growth factor binding protein (*IGFBP*) (Kelley et al. 2002) and the 11β-hydroxysteroid dehydrogenase-type3 (*11βHD3*) (Baker 2004). Estradiol also induced a strong decrease in both *CYP3A* and *ERα* expression. Although *CYP3A* expression was down-regulated in 4-nonylphenol treated fish, the response was muted compared to estradiol.

DISCUSSION

There is an increasing demand for robust bioassays that provide a comprehensive assessment of the effects of chemical contaminants in aquatic populations. Ideally suited for this purpose are microarrays because they can detect changes in many genes in a single tissue sample, providing snapshots of alterations in endocrine pathways in normal and contaminated fish. This knowledge identifies gene families and biochemical pathways that are affected, in addition to those identifying those that remain unaffected (Vilo and Kivinen 2001). A challenge in developing a microarray platform to study alterations in gene transcription in sentinel coastal species was the paucity of genomic sequence data. To overcome this obstacle, we constructed a 65mer oligonucleotide-based microarray containing conserved sequences from genes of interest. The novelty of this platform is that it used highly conserved probes from several fish species, permitting application of the array to studies involving turbot and zebrafish. We used 65-mers to accommodate sequence differences, polymorphic regions, and species specific codon usage. We reasoned that this approach would be successful, as it has been previously demonstrated that oligonucleotides 50 nucleotides in length can hybridize to RNA sequences that differ by 15% in their overall sequence (Li et al. 2005; Nielsen et al. 2003). The array focused on key targets with defined roles in endocrine pathways and processes, in addition to biomarkers for contaminant exposure.

The multi-species microarray assessed alterations in hepatic gene expression in male hornyhead turbots that were collected during a regional marine monitoring study at two monitoring stations in the Southern California Bight 2003 Survey that are considered impacted by pollution (Bight Field Sampling and Logistics Committee 2003). The assessed fish had been previously examined for exposure to xenoestrogens using the classical biomarker vitellogenin, measurements of plasma levels of testosterone and estradiol, as well as anomalies in gonadal morphology (Deng et al. 2007; Rempel et al. 2006).

Microarray analysis of turbot exposed to pollutants.

Microarray analysis detected differences in hepatic gene expression patterns in exposed turbot from the two monitoring areas compared to control individuals. Exposed turbot showed up-regulation of *CYP3A* and *RXR*. Interestingly, thyroid receptors α and β were down-regulated in fish from both monitoring areas indicating the presence of compounds that are able to interfere with the thyroid response.

Another example of the utility of microarray analysis of fish from polluted areas can be seen in a study of male flounder collected from an impacted site (Tyne) and a reference site (Alde) in the United Kingdom (Williams et al. 2003). Eleven transcripts were differentially expressed between male flounder collected from an impacted site relative to a reference site. Seven transcripts were more highly expressed in the Tyne male fish (*CYP1A1, UDPGT*, α-2HS-glycoprotein, dihydropyrimidine dehydrogenase, Cu/Zn SOD, aldehyde dehydrogenase and paraoxonase), while four transcripts (Elongation factor 1 (*EF1*), *EF2, Int-6* and complement component C3 mRNA) were found to be significantly less abundant in the Tyne male fish (Williams et al. 2003).

Microarray analysis of zebrafish exposed to estradiol and 4-nonylphenol

We observed differences between estradiol and 4-nonylphenol in the strength of the estrogen-like response, which we attribute to 4-nonylphenol having a low affinity for the ER (Kuiper et al. 1997). The data on gene expression in zebrafish provide another validation of the multi-species microarray and demonstrate its potential for investigating gene expression in *Pleuronectiformes* and *Perciformes*.

We found up-regulation of vitellogenin, *PR, RXR and ERβ* transcripts in male zebrafish exposed to both estradiol and 4-nonylphenol. Up-regulation of vitellogenin and *PR* are well-established responses to estrogens. And *RXR* has been shown to be upregulated by bisphenol A, an estrogenic chemical in murine embryos (Nishizawa et al. 2005). *ERβ* expression, has previously

been shown to be increased upon exposure to xenoestrogens in zebrafish (Islinger et al. 2003) and to 4-nonylphenol in many fish species (Soverchia et al. 2006). Up-regulation of *ERβ* was reported following exposure to alkylphenols in juvenile goldfish, medaka, rainbow trout and zebrafish (Inui et al. 2003; Islinger et al. 2003; Soverchia et al. 2006; Vetillard and Bailhache 2006) indicating that that it can be considered a biomarker for xenoestrogen exposure.

ERα expression was repressed with both estradiol and 4-nonylphenol treatments. Distinct patterns of expression for *ERα* and *ERβ* have been documented in fish (Choi and Habibi 2003), but differences in the interaction of xenoestrogens with the two subtypes of estrogen receptors have not been well characterized.

Following exposure to 4-nonylphenol a modest repression of *CYP3A* was observed in zebrafish. A strong repression of *CYP3A* mRNA however was observed following estradiol exposure. Similar results have been reported in trout (Pajor et al. 1990), suggesting an important role of the sex hormones in *CYP3A* expression, which is further supported by the sexually dimorphic expression of *CYP3A* genes reported in many fish species (Hasselberg et al. 2004; Hegelund and Celander 2003).

Interestingly, expression of the *PXR*, a sensor for xenobiotics (Moreau et al. 2008; Xie et al. 2000), was induced by both compounds. PXR mediates the effects of 4-nonylphenol on the activation of *CYP3A* genes in mouse, rat and humans (Masuyama et al. 2000; Masuyama et al. 2002). In juvenile Atlantic salmon, increases in *PXR* and *CYP3A* transcript levels were observed following 4-nonylphenol exposure suggesting a similar mechanism to that reported in the mammalian systems (Meucci and Arukwe 2006). Furthermore hepatic expression of *CYP3A* is induced by the organochlorine pesticide methoxychlor in male largemouth bass *Micropterus salmoides* (Blum et al. 2008).

We also found strong activation of *FXR, PPAR* and *RXRα* expression in zebrafish exposed to estradiol or 4-nonylphenol, which indicates xenoestrogens can affect a variety on physiological pathways. PPAR is involved in the regulation of lipid metabolism-related genes and its interaction with xenobiotic compounds is thought to be responsible for alterations in adipogenesis and diseases such as obesity in humans (Grun and Blumberg 2006). FXR is strongly activated by bile acids and serves as a central coordinator for bile acid biosynthesis metabolism and transport. Possible interaction of xenobiotics with FXR could lead to changes in bile acid homeostasis and hepatic toxicity. Our finding that that *FXR* was up-regulated in zebrafish exposed to estradiol and 4-

nonylphenol has not been reported previously in any fish. This finding demonstrates the utility of microarrays in uncovering the effects of hormones and chemicals, which can subsequently be used to construct a profile for exposure to a given chemical.

Of practical importance for the analysis of zebrafish exposed to estradiol and 4-nonylphenol is that zebrafish belongs to the order *Cypriniformes* and is phylogenetically distant from *Tetraodoniformes*, *Perciformes* and *Pleuronectiformes*, whose sequence information were used to guide the design of the array probes. Thus, the data obtained from the zebrafish experiments indicated that the multi-species microarray possesses cross-species utility.

CONCLUSIONS

The results obtained using the multi-species microarray to assess male hornyhead turbots in two coastal areas considered impacted by pollution revealed the presence of a mixture of endocrine disruptors containing xenoestrogens and most likely xenobiotics capable of interacting with the thyroid system. These results highlight the utility of the multi-species microarray as a diagnostic for the presence of endocrine disruptors in the aquatic environment. The broad use of the multi-species microarray to study the effects of environmental chemicals on fish was demonstrated in its application to zebrafish exposed to chemicals in a laboratory and a sentinel species *(Pleuronichthys verticalis)* collected near municipal wastewater discharge sites. The results presented here demonstrate the feasibility adding other genes of interest in fish physiology to a second generation multi-species microarray for assessing effects in exposed fish.

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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 17Î²-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/µg or greater was considered optimal for hybridization. 1 µg 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/µl 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 log2 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 (log2 [exposed/control] in plots A-G) or absence of significant differential gene expression in the self-self plots (log2 [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 log2 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 |log2 (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'- AGCCAAGCACATACACCAAA. 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 200mMTrisHCl/100mMKCl/100mM$(NH_4)_2SO_4$/20mMMgSO_4/1%TritonX/BSA/1mg/m), 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 Material Table 1

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

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

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

Supplemental References

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