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# DEVELOPMENT AND VALIDATION OF A 2,000-GENE MICROARRAY FOR THE FATHEAD MINNOW (*PIMEPHALES PROMELAS*)

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**Abstract**—Gene microarrays provide the field of ecotoxicology new tools to identify mechanisms of action of chemicals and chemical mixtures. Herein we describe the development and application of a 2,000-gene oligonucleotide microarray for the fathead minnow *Pimephales promelas*, a species commonly used in ecological risk assessments in North America. The microarrays were developed from various cDNA and subtraction libraries that we constructed. Consistency and reproducibility of the microarrays were documented by examining multiple technical replicates. To test application of the fathead minnow microarrays, gene expression profiles of fish exposed to 17β-estradiol, a well-characterized estrogen receptor (ER) agonist, were examined. For these experiments, adult male fathead minnows were exposed for 24 h to waterborne 17β-estradiol (40 or 100 ng/L) in a flow-through system, and gene expression in liver samples was characterized. Seventy-one genes were identified as differentially regulated by estradiol exposure. Examination of the gene ontology designations of these genes revealed patterns consistent with estradiol's expected mechanisms of action and also provided novel insights as to molecular effects of the estrogen. Our studies indicate the feasibility and utility of microarrays as a basis for understanding biological responses to chemical exposure in a model ecotoxicology test species.

**Keywords**—Ecotoxicology Estradiol Fathead minnow Gene expression Microarray

## INTRODUCTION

A variety of in vivo assays are used for prospective and diagnostic ecological risk assessments. These assays typically measure apical responses such as survival, growth, fecundity, fertility, and hatching success. Although these whole animal bioassays have value in terms of predicting ecological effects, they do not point to specific toxic mechanisms of action of chemicals or toxic chemical mixtures. Knowledge of the mechanism of action is critical to extrapolation of chemical effects across species and compound structure and from the laboratory to the field [1,2].

A new technology that shows promise in terms of understanding a compound's mechanism of action is the gene microarray. Microarrays are research tools made by spotting or synthesizing thousands of gene sequences specific to an organism onto a solid support matrix. Microarrays have been utilized since the 1990s in the medical field [3] and are becoming more widely used in the field of ecotoxicology [4].

Use of gene arrays to examine environmental contaminants began in early 2000 [5–8]. Although a number of microarrays have been developed for fish, only a few contain more than 1,000+ genes. These include microarrays for zebrafish [9–11], rainbow trout, Atlantic salmon [12–14], and medaka [15,16].

The purpose of this study was to develop and validate a large-scale (1,000+) gene microarray for the fathead minnow (*Pimephales promelas*). We saw the need to develop this microarray because the fathead minnow has been a widely utilized species in ecotoxicology research and regulation in North America and elsewhere since the 1960s [17]. To date, only a small (200 gene) macroarray had been available for this species [18,19].

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## MATERIALS AND METHODS

### *Gene libraries*

Due to the paucity of available gene sequence information in the fathead minnow, in 2002 we undertook a comprehensive sequencing effort to obtain a large number of gene sequences. A variety of cDNA and subtraction libraries were constructed from different tissue types (e.g., brain, liver, gonad, and other tissues) from male and female fathead minnows. Subtraction libraries were developed using the Clontech (Palo Alto, CA, USA) suppressive subtractive hybridization kit with some modifications. For each subtractive hybridization, poly-A mRNA was isolated from two different tissues using the Oligotex mRNA Midi Kit (Qiagen, Valencia, CA, USA) and converted to cDNA. The cDNA pool that contains differentially expressed transcripts is referred to as the tester and the reference cDNA is referred to as the driver. Subtractions were performed in both directions (i.e., in one experiment, tissue A served as the tester and tissue B served as the driver; whereas in the second experiment, tissue A was the driver and tissue B was the tester) in order to obtain both upregulated and downregulated genes in the tissue types being compared. For each

subtraction experiment, the tester and driver cDNAs were digested with Rsa I, and the tester cDNA pool was divided into two portions, each of which was ligated with a different cDNA adapter sequence. Two sequential hybridizations then were performed. For the first hybridization, an excess of driver was added to each of the tester pools and the samples were heat denatured and then allowed to anneal to each other, resulting in the generation of several different hybrid sequences of cDNA. For the second hybridization, the two different tester pools were mixed together in the presence of an excess of driver without denaturing, and new hybrids were formed. The ends of the differentially expressed cDNA sequences were completed with DNA polymerase, and two rounds of polymerase chain reaction (PCR) were performed under the following conditions: The first PCR was one cycle at 94°C (25 s) and then 31 cycles at 94°C (10 s), 65°C (30 s), and 72°C (90 s). The second PCR was 16 cycles at 94°C (10 s), 68°C (30 s), and 72°C (90 s). Following the PCR reactions, the resultant pool of cDNA clones were ligated into pGEM T-Easy cloning vector (Promega, Madison, WI, USA) and transformed into DH5α cells, which were plated onto Luria-Bertani agar plates containing ampicillin and oxacillin (100 μg/ml each). Recombinant colonies then were picked from the plates and sequenced.

Unsubtracted, complementary DNA (cDNA) libraries also were used to identify potential genes for the microarray. The cDNA libraries were constructed by Invitrogen (Carlsbad, CA, USA) using their Gateway® entry vector system. One library was derived from pooled liver samples from adult male fathead minnows, and the second cDNA library was from pooled gonad samples from adult male and female fathead minnows.

### *Sequence assembly*

Sequences from the libraries were first quality screened using a number of criteria: Base calls of Q20 or higher were retained (Q20 indicates good confidence in the assignment of a base); cloning vector sequences were removed; intrinsic repeats were masked; and *Escherichia coli*, mitochondrial, chimera, and short sequences were removed. The sequences then were clustered and assembled in Transcript Assembler (Paracel, Pasadena, CA, USA) using the default parameters. From these efforts, a unique set of 2,000 sequences was identified. These sequences were fully annotated by comparing each sequence to known sequences in publicly available databases (e.g., the protein nonredundant database and the nucleotide database) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) using several basic local alignment search tools. We used an expectation value (e-value) of $1E^{-5}$ or lower to signify a positive match between our sequences and those in the databases. Genes were named based on the best match (lowest e-value) of the clone to a gene or protein sequence in fish. If there was not a hit in fish with an e-value less than $1E^{-5}$, genes were named based on mammalian sequences, or, if no other matches were found, invertebrate sequences. Sequences without a positive match are referred to as unknown genes. These sequences also were spotted onto the microarrays.

### *Microarray construction*

The fathead minnow microarrays were developed at EcoArray (Alachua, FL, USA) and printed by Agilent Technologies (Palo Alto, CA, USA). The microarrays were documented based on minimum information about microarray experiment guidelines [20]. Briefly, probes for each gene were designed using Agilent's probe design software. Each of the 60 nucleotide long probes was synthesized in situ on 1-inch × 3-inch (24.2 mm × 76.2 mm) treated glass slides using SurePrint inkjet deposition technology (http://www.chem.agilent.com). All probes were synthesized in duplicate at random locations on the slide surface. Because we could not confirm the direction of genes with an e-value greater than $1E^{-5}$, probes were designed and synthesized from both strands (forward and reverse complement) for these sequences. To facilitate evaluations of the microarray, multiple probes that spanned the vitellogenin (vtg) 1 gene also were synthesized on the chips. Probe sequences for each of the genes are available (under license agreement) from EcoArray. Probes for specific genes on the microarrays were assigned a unique probe identification number, similar in concept to accession numbers that are assigned to sequences in National Center for Biotechnology Information databases. This number (EA_Pp_###) allows for the cross-referencing of sequences from one experiment to another.

### *Fathead minnow exposure*

To provide samples for an assessment of microarray performance, adult fathead minnows were exposed to 17β-estradiol under experimental conditions similar to those described by Villeneuve et al. [21]. Briefly, adult (~6 months old) male fathead minnows from an on-site culture at the U.S. Environmental Protection Agency laboratory in Duluth, Minnesota, were paired with mature female fathead minnows (tissues from the females were not analyzed in this study), placed in tanks with a breeding substrate, and allowed to acclimate under spawning conditions (25°C, 16:8 light:dark photoperiod, fed adult brine shrimp twice daily) over a period of at least one week. Exposures were initiated by transferring randomly selected pairs of fish to exposure tanks supplied with Lake Superior water (control; no detectable estradiol) or estradiol dissolved in Lake Superior water (41 ± 25 or 107 ± 38 ng/L, measured concentrations). No carrier solvent was used. Water from each exposure tank was sampled prior to addition of fish (0 h) and approximately 1 h before sampling the animals (23 h). Estradiol concentrations in the water were quantified by radioimmunoassay using methods described elsewhere [22]. Four replicate tanks per treatment had two pairs of fish (separated by a mesh divider) per tank. General water quality characteristics during the experiment were: Mean (± standard deviation [SD]) temperature, 25.5 °C ± 0.3; pH, 7.33 ± 0.95; and dissolved oxygen, 5.92 ± 0.95 mg/L. At the conclusion of the exposures (24 h ± 90 min), fish were anesthetized with tricaine methanesulfonate (100 mg/L) buffered with $NaHCO_3$ (200 mg/L). Whole liver was removed and transferred directly to preweighed vials containing RNAlater® (Sigma, St. Louis, MO, USA). Prior to removal of the liver, plasma was sampled from the animals using a heparinized microhematocrit tube [22]. Plasma concentrations of vtg protein were measured in the males using an enzyme-linked immunosorbent assay [23]. For vtg protein statistical comparisons, analysis of variance was performed on log(base 10)–transformed data with a Tukey honestly significant difference post hoc analysis. Differences were considered significant at $p \leq 0.05$.

### *Hybridization of chips*

Total RNA was isolated from livers using TRI Reagent lysis solution (Molecular Research Center, Cincinnati, OH, USA)

following the manufacturer's protocol. The resulting RNA pellets were resuspended in RNA*secure*® resuspension solution (Ambion, Austin, TX, USA). The samples were treated with DNase (DNA-*Free*™, Ambion) to remove any trace amounts of DNA that were in the samples. The quality of the total RNA was determined by running denaturing-agarose gel electrophoresis with ethidium bromide staining. The purity of all of the RNA samples was between 1.8 and 2.0 (optical density 260/280 ratio).

The RNA samples were labeled using Agilent's low-input fluorescent linear amplification kit. Briefly, poly-A RNA was reverse transcribed from 500 ng of total RNA using a poly dT-T7 primer at 40°C for 2 h in 20 μl of master mix (final concentration: 1× first strand buffer, 0.5 mM dNTP mix, 0.01 M DDT, 10 U MMLV-RT, and 2 U RNaseOUT). The resulting cRNA was denatured and immediately used for one round of amplification by T7 in a transcription reaction solution (final concentration: 1× transcription buffer, 7.5 mM DTT, 1 mM dNTP-mix, 2.25 U RnaseOUT, 4% PEG 1.5 U inorganic pyrophosphatase, T7 RNA polymerase) in the presence of 0.3 mM Cyanine 3-CTP or Cyanine 5-CTP (PerkinElmer, Wellesley, MA, USA) at 40°C for 2 h. The amplified and labeled cRNA probes then were purified with RNeasy purification columns (Qiagen, Valencia, CA, USA). The amplification yield and dye incorporation efficiency of the cRNA probes were verified using a NanoDrop spectrophotometer (Agilent). The cRNA concentration was measured at 260 nm, Cyanine 5 incorporation was determined at 650 nm, and Cyanine 3 incorporation measured at 550 nm. For each Cyanine dye, samples that contained ≥10 pmol of incorporated dye were fragmented and resuspended with 2× hybridization solution (Agilent 60-mer oligo microarray processing protocol) for a final volume of 204 μl.

To eliminate dye bias, samples were run using a reference sample design, where all of the control and treated samples were labeled with Cyanine 5 and the reference samples were labeled with Cyanine 3. For all the experiments, the same reference sample was used (aliquots were made from one labeling reaction and stored at −80°C until used). The reference sample consisted of pooled liver/brain/gonad sample from adult male and female fathead minnows. In the reference design, each sample is compared to a common RNA reference sample, serving as a common denominator between different microarray hybridizations. This design decreases variability by normalizing signal output among different microarrays. This experimental design has been used to study diversity in cell lines and patterns of gene expression, allowing classification of breast and lung carcinoma samples, among other experiments [24]. An ideal reference RNA sample for normalizing gene expression data should provide positive hybridization signal at each probe element on the microarray. Pooling RNA from a mixture of cell lines or tissues achieves this. Our research effort conducted experiments using samples from the liver, brain, and gonad of fathead minnows (the present study and Villeneuve et al. [21]), which is why we pooled RNA samples from these tissues.

The microarrays were hybridized in Agilent hybridization chambers and incubated overnight (17 h) in a hybridization oven (model G2545A, Agilent) at 60°C. The following day, slides were washed for 1 min in a wash solution (Agilent 60-mer oligo microarray processing protocol), 0.005% *N*-laurosylsarcosine; washed for 1 min in 0.06× SSPE, 0.005% *N*-laurosylsarcosine; and then dried by dipping in a stabilization and drying solution for 30 s. Slides were scanned with an Agilent DNA microarray scanner, and raw images processed using Agilent's Feature Extraction Software, Version 8.1. The resultant data were analyzed using statistical approaches developed in Microsoft Excel® (http://www.microsoft.com/) and the AnalyzeIt statistical package (University of Florida, Gainesville, FL, USA).

*Normalization of microarray data and statistical analysis*

Data first were filtered to remove flagged features (e.g., saturated, nonuniform spots). Following filtering, data from each spot were local background subtracted, Lowess normalized, and log transformed (base 10). Lowess normalization is a method used to normalize a two-color gene expression dataset to compensate for nonlinear dye bias. The values from the two duplicate spots for each gene then were averaged. The mean of each gene across chips ($n = 3$ for each tissue/test concentration) then was determined and an analysis of variance was performed with a Tukey honestly significant difference post hoc analysis. Genes were considered differentially regulated if they had a $p$-value of ≤0.01 or lower. The relationship of various vtg 1 probes to their location along the vtg 1 gene was evaluated in Excel using the Pearson product moment correlation function.

*Real-time PCR.* To help validate microarray results, primers were designed for vtg 1, estrogen receptor (ER) α, keratin 18, reticulocalbin 3, and 18S rRNA using Primer Express software (Applied Biosystems, Foster City, CA, USA). Each primer set was evaluated for specificity and efficiency by running dissociation and standard curves, respectively. Standard curves included data from a minimum of four serially diluted cDNA samples. The efficiency of amplification was >94% for each primer set, including 18S rRNA, which was used as a normalizing gene. Primer sequences for each of the genes are available under license agreement (contact EcoArray for details).

DNase-treated total RNA was reverse transcribed to single-strand cDNA using random primers and Multiscribe reverse transcriptase according to the manufacturer's instructions (Applied Biosystems, 4322171). The following cycle parameters were used for this reaction: 25°C for 10 min, 37°C for 120 min, and 4°C to end the cycle and hold. The cDNA was stored at −20°C if not used immediately. Real-time PCR reactions were identical for all of the genes (except 18S) and consisted of 25-μl reactions that contained 50 ng cDNA, 12.5 μl SYBR Green Master Mix (Applied Biosystems 4309155; which included SYBR Green, buffer, Taq polymerase, and dNTPS), and 50 nM each of the forward and reverse primers. The PCR reactions for 18S rRNA primers (Applied Biosystems 4308329) differed slightly in that they used 0.5 ng of cDNA. For all of the samples, controls where no reverse transcriptase was added were run to ensure the removal of all contaminating DNA. An Applied Biosystems 7500 thermocycler was used for the PCR reactions with the following cycle parameters: One cycle of 50°C for 2 min, one cycle of 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 60 s. Each sample was run in duplicate and the averaged value was normalized to measured 18S rRNA values for each sample using a ΔΔCt method of analysis. The 18S rRNA values did not fluctuate significantly ($p = 0.83$) between treatment groups and thus were appropriate for normalizations. Analysis of variance was performed with a Tukey honestly significant difference post

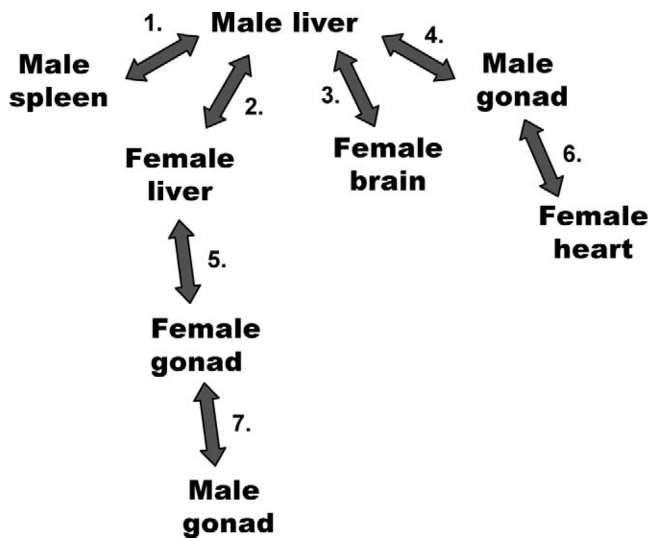


Fig. 1. Subtraction library strategy. The arrows represent the various tissues that were subtracted against each other. Seven subtractions were conducted for these experiments.

hoc analysis. Genes were considered differentially regulated if they had a *p* value of ≤0.01 or lower.

## RESULTS

### *Gene discovery*

To help identify genes for the microarray, seven subtraction libraries were constructed from various tissues from adult male and female fathead minnows. For the libraries, a tissue subtraction strategy was employed where one common tissue, which was obtained from a group of pooled fish, was subtracted against several other tissues obtained from the same group of pooled fish (Fig. 1). The rationale for using this approach was to obtain as many unique genes as possible, while at the same time minimizing the number of redundant clones sequenced. From these libraries, a total of 2,700 cDNA clones were sequenced. To add additional genes for the microarray, we sequenced approximately 2,400 clones from several cDNA libraries. The cDNA clones obtained from the subtraction and cDNA libraries, as well as clones from other sources (e.g., 288 sequences from A. Kolok, University of Nebraska; 288 sequences from R. Klaper, University of Wisconsin; and 2,264 sequences from another sequencing effort [25]) were assembled and annotated. From all this we identified approximately 2,000 unique genes based on the pairwise alignments done using Paracel transcript assembler. Approximately 90% of the 2,000 unique genes positively matched a clone with an e-value $<1E^{-5}$ or lower in the nonredundant or nucleotide databases available through the National Center for Biotechnology Information.

These 2,000 genes were used to construct the microarray. To evaluate performance of the microarrays, we examined gene expression in liver samples of male fathead minnows exposed to 40 or 100 ng estradiol/L for 24 h. Estradiol was chosen as a model compound for the microarray work because it has a well-known mechanism of action that is of environmental concern [26]. As a first step to validating the microarrays, we conducted several technical replicate experiments. In these experiments, reproducibility of the microarrays was documented by hybridizing a pooled sample of labeled cRNA onto two separate microarrays (Fig. 2). The data points in the graph cluster along a slope of one ($r^2 = 0.94$) for all of the spots, including both the low- and high-expressed genes. We documented the consistency of the labeling procedure by labeling aliquots of RNA in parallel and hybridizing the samples onto two separate microarrays ($r^2 = 0.93$). We also evaluated the microarrays by examining consistency in the probe design process. For these experiments, we compared the performance of 26 different probes that were designed along the vtg 1 gene (Fig. 3). A moderate but significant correlation coefficient was observed in the fold change values of various probes, with the location of the probes along the vtg 1 gene ($r = 0.61$ for the 40 ng/L group and $r = 0.67$ for the 100 ng/L treatment). Higher fold changes were observed for probes closer to the 3′ end of the vtg 1 gene. The average vtg fold change for the probes for the 40 ng/L treatment was 13.6 (SD = 8.6) and the average change for the 100 ng/L treatment was 76.7 (SD = 47.0).

We also measured vtg in the fish at the protein level and, as expected, exposure to estradiol caused a significant dose-

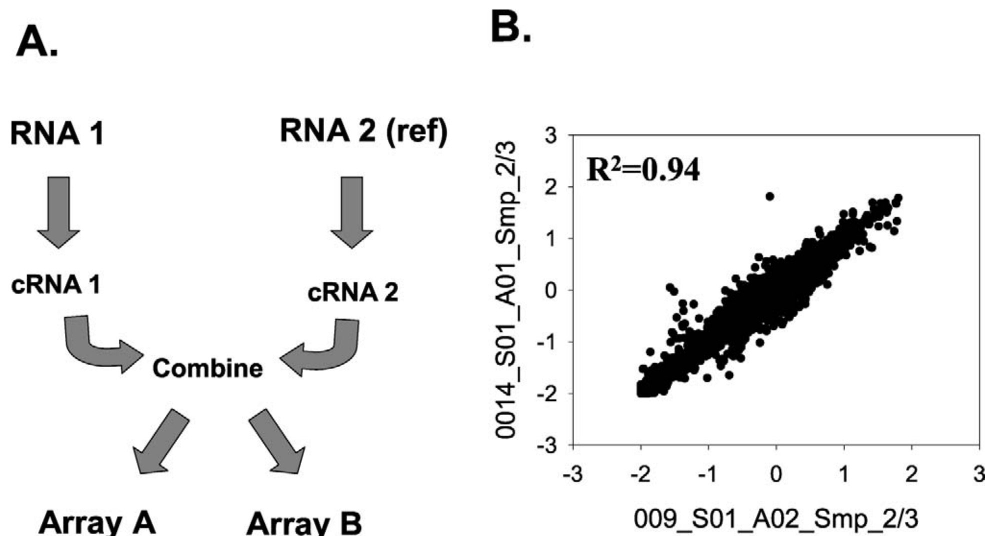


Fig. 2. Evaluation of chip reproducibility. (**A**) Represents the RNA labeling strategy. Ref. = Reference RNA. (**B**) Graphical representation of the gene expression profiles from aliquots of the same RNA that were hybridized onto two separate microarrays.

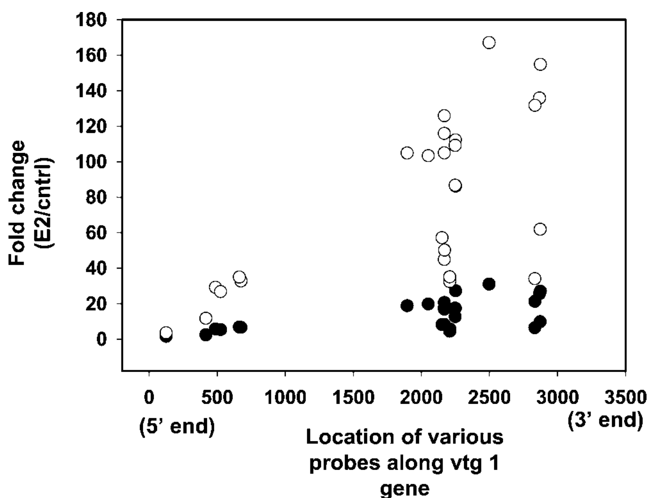


Fig. 3. Relative fold change of vitellogenin 1 probes varies depending on probe location. Open circles = 100 ng/L; closed circles = 40 ng/L. Average (±standard deviation) vitellogenin fold change level for low dose was 13.6 ± 8.6. Average vitellogenin level for high dose was 76.7 ± 47.0.

dependent induction of plasma vitellogenin concentrations in males (Fig. 4).

In addition to vtg 1, we examined the expression patterns for three other well-characterized estrogen-responsive genes, ER α and choriogens 2 and 3. We observed a significant upregulation of estrogen receptor α at both the low (2.4-fold increase relative to controls) and the high treatment groups (2.6-fold increase). No upregulation of choriogenin 2 ($p = 0.53$) or choriogenin 3 ($p = 0.67$) occurred.

In addition to vtg 1 and ER α, we identified 71 additional genes in the male fish that were regulated differentially by estradiol (Table 1). Post hoc analysis (shown in italics in Table 1) revealed that 37 genes were downregulated in one of the treatment groups and 7 genes were downregulated in both of the treatment groups. Nineteen genes were upregulated in one of the treatment groups and three genes were upregulated in both treatment groups. In addition, two genes were regulated differentially (up in one group and down in the other) and, for the last three genes, the post hoc analysis could not differentiate which treatment group was significantly changed.

Where possible, gene ontology information was obtained for each of the genes whose expression was significantly changed in at least one treatment group. This information is

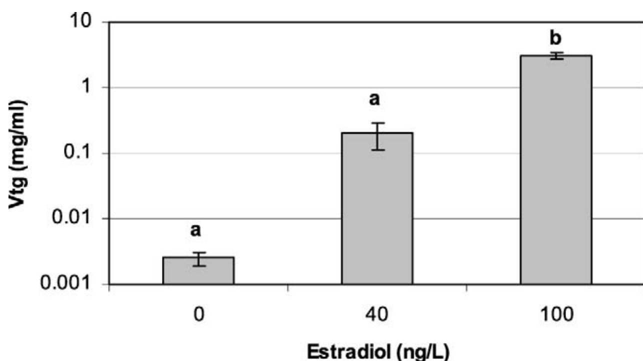


Fig. 4. Plasma vitellogenin (vtg) concentrations for male fathead minnows exposed to 40 or 100 ng 17β-estradiol/L (measured concentrations) for 24 h. Different letter indicates statistically significant difference ($p < 0.05$).

summarized in Figure 5. The differentially regulated genes encompass a variety of biological processes and pathways. For example, some of the dominant groups in the molecular function designation include binding (58.9%) and catalytic activity (27.1%). In terms of cellular components, cell and organelle constituents seem more dominant (33.3% and 35.4%) than extracellular constituents (16.7%). For biological processes, the predominant categories were cellular (66.7%) and physiological (70.6%) processes.

To help validate the microarray data, we measured the expression of four genes (vtg 1, ER α, keratin 18, and reticulocalbin 3) by real-time PCR (Table 2). Vitellogenin 1 was upregulated 43-fold and 531-fold in liver tissue of male fathead minnows exposed to 40 or 100 ng estradiol/L, respectively. Estrogen receptor α was upregulated 1.7-fold and 3.0-fold and reticulocalbin 3 was upregulated 1.7-fold and 2.7-fold at these test concentrations. Keratin 18 was downregulated 1.5-fold and 3.6-fold in the respective treatments.

## DISCUSSION

The purpose of the present study was to develop and validate a large-scale gene microarray in the fathead minnow. For the present study, we sequenced a total of 5,100 clones from several cDNA and subtraction libraries. The strategy that we employed was to sequence clones from the various libraries in batches of 300 to 500, and to stop sequencing when fewer than 10% additional unique genes were obtained from one batch to the next. Using this approach, our overall success rate for obtaining unique genes, compared to the total number of clones sequenced, was 16% for the subtraction and 32% for the cDNA libraries.

In contrast to this strategy, the Joint Genome Institute recently sequenced 250,000 cDNA clones from several tissues from fathead minnows (nucleotide database, National Center for Biotechnology Information). We recently clustered these sequences and obtained approximately 36,000 unique genes based on the pairwise alignments (EcoArray, Alachua, FL, USA). This shotgun approach resulted in a 14% success rate (i.e., unique genes per total number of clones sequenced). Although the success rate in the current study was greater than that, the approach requires more informatics time because each sequencing run must be evaluated before making a decision to continue. However, both methods work well for identifying unique sequences.

From the various sequence data, we identified 2,000 unique genes, which were used to construct the first high-density microarray for the fathead minnow. Of the 2,000 unique genes, 70% (1,400 genes) were identified by Transcript Assembler as being singlets and 30% (600 genes) were identified as contigs. A contig is a single gene that was assembled from two or more clones from the cDNA libraries.

Consistency and reproducibility of the microarrays were documented by examining multiple technical replicates. A similar expression pattern was observed on several microarrays that were hybridized with the same aliquot of cRNA. In addition, the microarray labeling procedure was found to be uniform and robust. Overall, consistency and reproducibility of the fathead minnow microarrays are similar to other cDNA arrays developed for sheepshead minnows and largemouth bass [7,8].

To evaluate the microarray performance, we examined gene expression in liver samples of male fathead minnows exposed to estradiol. A principal role of the native estradiol is as an

Table 1. Genes that were differentially regulated by 17β-estradiol ($p < 0.01$). For each gene, a Tukey post hoc analysis was run. The treatment groups that were changed significantly compared to controls are shown in italics. Two or more probes listed in the same row signify the average fold-change value of the different probes, which were designed to the same gene. ** Denotes genes that also were analyzed by quantitative real-time polymerase chain reaction. Estrogen receptor (ER) α also is listed on this table. Vitellogenin (vtg) 1 is not listed in this table. See Figure 3 for expression of the vtg 1 gene

| EcoArray probe ID | Fold change (low dose/controls) | Fold change (high dose/controls) | Top gene name[a] | Top e-value[b] | Top accession no. |
|---|---|---|---|---|---|
| EA_Pp_12703 | −1.50 | *−2.52* | 3-hydroxyanthranilate 3,4-dioxygenase (*Gallus gallus*) | 1.00E-32 | XP_419453 |
| EA_Pp_10702 | −1.16 | *−1.90* | Alanine-glyoxylate aminotransferase (*Danio rerio*) | 5.00E-101 | AAH56520 |
| EA_Pp_13783 | *−1.39* | *−1.65* | Alanine-glyoxylate aminotransferase 2-like 1 isoform 3 (*D. rerio*) | 1.00E-17 | XP_706982 |
| EA_Pp_11854 | −1.42 | *−3.00* | Aldehyde dehydrogenase 8 family, member A1 (*D. rerio*) | 2.00E-99 | AAH81581 |
| EA_Pp_12380 | *−2.20* | *−3.91* | Alpha-2,8-sialyltransferase ST8Sia VI (*D. rerio*) | 2.00E-97 | CAG29390 |
| EA_Pp_10590 | 1.04 | *−2.03* | Alpha-2-macroglobulin (*Ctenopharyngodon idella*) | 8.00E-175 | AAR00337 |
| EA_Pp_11208 | −1.10 | *−1.51* | Amiloride binding protein 1 (*D. rerio*) | 1.00E-82 | XP_694917 |
| EA_Pp_13092 | −1.21 | *−1.73* | Antithrombin III (*Cyprinus carpio*) | 4.00E-32 | AAC19409 |
| EA_Pp_13105 | 1.01 | *1.88* | ATP synthase, H+ transporting, mitochondrial F0 complex, subunit f, isoform 2 (*Xenopus tropicalis*) | 2.00E-28 | NP_001017095 |
| EA_Pp_13999 | *6.92* | −1.14 | Beta-actin (*Megalobrama amblycephala*) | 3.00E-12 | AY170122 |
| EA_Pp_12347 | −1.10 | *−2.13* | C1R/C1S subunit of Ca2+-dependent complex (*Oncorhynchus mykiss*) | 6.00E-55 | CAD58654 |
| EA_Pp_13741 | *1.50* | *−1.69* | Carnitine deficiency-associated gene expressed in ventricle 3 (*D. rerio*) | 5.00E-21 | AAH53231 |
| EA_Pp_13227 | −1.03 | *−1.50* | Coagulation factor IX (*D. rerio*) | 5.00E-43 | XP_688459 |
| EA_Pp_11407 | −1.13 | *1.60* | Coatomer protein complex, subunit beta 2 (*D. rerio*) | 0.00 | NP_001001940 |
| EA_Pp_12954 & EA_Pp_10402 | −0.02 | *−1.78* | Complement C3-Q1, partial (*D. rerio*) | 2.00E-61 | XP_696303 |
| EA_Pp_14463 | −1.06 | *−3.13* | Complement factor B/C2A (*C. carpio*) | 0.00 | BAA34706 |
| EA_Pp_10667 | −1.21 | *−1.84* | Complement factor B/C2B (*C. carpio*) | 7.00E-82 | BAA34707 |
| EA_Pp_14729 | −1.07 | *−1.67* | Cryopyrin isoform b (*D. rerio*) | 2.00E-13 | XP_696270 |
| EA_Pp_10750 | −1.43 | *−2.77* | Cytochrome P450 (family 2) (*Ictalurus punctatus*) | 3.00E-73 | AAG30296 |
| EA_Pp_10138 | −1.43 | *−2.40* | EST (*Citrobacter freundii*) | 2.00E-09 | AAL59385 |
| EA_Pp_14778 | −1.03 | *1.65* | EST (*D. rerio*) | 1.00E-16 | BC083530 |
| EA_Pp_10144** | 2.40 | 2.80 | Estrogen receptor alpha (*Pimephales promelas*) | 0 | AAV41373 |
| EA_Pp_14674 | *−199.20* | −19.04 | F-box protein 38 isoform b (*D. rerio*) | 2.00E-11 | XP_699063 |
| EA_Pp_11198 & EA_Pp_11199 | 1.08 | *−1.64* | Fibrinogen gamma polypeptide (*D. rerio*) | 0.00 | NP_998219 |
| EA_Pp_14317 | 1.02 | *−1.51* | Fibrinogen, B beta polypeptide (*D. rerio*) | 6.00E-09 | AAH66629 |
| EA_Pp_12490 | 1.45 | *3.68* | Fxr1 protein (*D. rerio*) | 3.00E-85 | AAH68402 |
| EA_Pp_14926 | *−1.29* | *−1.37* | GABA(A) receptor-associated protein (*D. rerio*) | 7.00E-21 | BC056701 |
| EA_Pp_10719 | *1.30* | 1.83 | Heat shock cognate 70 kDa protein (*Carassius auratus gibelio*) | 4.00E-127 | AAO43731 |
| EA_Pp_12735 | −1.65 | *−2.52* | Hyaluronan-binding protein (*D. rerio*) | 6.00E-84 | XP_683106 |
| EA_Pp_10686 | −1.11 | *−2.01* | IGFALS (*D. rerio*) | 6.00E-128 | XP_699366 |
| EA_Pp_10857 | −1.55 | *−2.15* | Igfbp2 protein (*D. rerio*) | 6.00E-132 | AAH65627 |
| EA_Pp_14236 | 1.01 | *−1.48* | Integral membrane protein 2B (*D. rerio*) | 4.00E-27 | BC068357 |
| EA_Pp_11179 & EA_Pp_11006 & EA_Pp_12835 | −1.22 | *−2.25* | Interalpha trypsin inhibitor, heavy chain 3 (*Mus musculus*) | 1.00E-56 | AAH15276 |
| EA_Pp_10193 & EA_Pp_11898** | 1.04 | *−2.26* | Keratin 18 (*D. rerio*) | 0.00 | AAH65848 |
| EA_Pp_13461 | 1.02 | *−1.84* | Kininogen 1 (*D. rerio*) | 3.00E-98 | NP_001005981 |
| EA_Pp_14999 | *−2.64* | −1.51 | Major histocompatibility complex class I gene (*D. rerio*) | 4.00E-11 | BC097061 |
| EA_Pp_13549 | 1.36 | *1.83* | M-phase phosphoprotein 1 (*Rattus norvegicus*) | 0.00 | XP_001080017 |
| EA_Pp_14757 | −1.04 | *1.28* | Orthodenticle homolog 1 (*D. rerio*) | 1.00E-09 | AAH45290 |
| EA_Pp_12233 | −1.40 | *−1.80* | Osmotic stress transcription factor 1 isoform 1 (*D. rerio*) | 2.00E-92 | XP_686637 |
| EA_Pp_12329 | −1.42 | *−1.98* | Phosphoenolpyruvate carboxykinase (*D. rerio*) | 9.00E-132 | NP_998357 |
| EA_Pp_12259 | 1.45 | *6.66* | Phosphoinositol 3-phosphate-binding family protein (*D. rerio*) | 3.00E-80 | CAK05190 |
| EA_Pp_10585 | −1.07 | *−2.04* | Plasminogen (*D. rerio*) | 2.00E-131 | AAH59801 |
| EA_Pp_10237 | −1.17 | *1.83* | Presenilin (*D. rerio*) | 7.00E-71 | NP_571099 |

Table 1. Continued

| EcoArray probe ID | Fold change (low dose/controls) | Fold change (high dose/controls) | Top gene name[a] | Top e-value[b] | Top accession no. |
|---|---|---|---|---|---|
| EA_Pp_14997 | 1.70 | *2.40* | Proliferating-cell nucleolar antigen p120 isoform 5 (*D. rerio*) | 1.00E-29 | XP_707579 |
| EA_Pp_11845 | −1.08 | *1.50* | Prostaglandin E synthase 2-like (*D. rerio*) | 3.00E-61 | AAH49325 |
| EA_Pp_12099 | −1.25 | *−1.53* | Protein C (*G. gallus*) | 2.00E-37 | NP_989772 |
| EA_Pp_12563 | −1.11 | *−1.45* | Rbm5-prov protein (*D. rerio*) | 2.00E-57 | XP_694937 |
| EA_Pp_10245** | *2.38* | *2.61* | Reticulocalbin 3 (*Bos taurus*) | 6.00E-30 | XP_600346 |
| EA_Pp_10965 | 1.10 | *1.58* | Retinoblastoma binding protein 4 isoform 3 (*D. rerio*) | 4.00E-148 | XP_708180 |
| EA_Pp_10480 | *1.30* | *1.38* | Ribosomal protein S4, X-linked (*D. rerio*) | 2.00E-115 | AAH81584 |
| EA_Pp_10258 | *−1.24* | −1.01 | Smad2 (*Carassius auratus*) | 3.00E-68 | AAS57861 |
| EA_Pp_13512 | 1.21 | *1.84* | Sorcin (22 kDa protein) (CP-22) (V19) isoform 3 (*Macaca mulatta*) | 1.00E-22 | XP_001104452 |
| EA_Pp_12356 | −1.06 | *2.02* | Splicing factor, arginine/serine-rich 2 (*D. rerio*) | 4.00E-49 | AAH46045 |
| EA_Pp_12485 | *−1.30* | *−1.77* | Steroid sulfatase (*Canis familiaris*) | 2.00E-68 | CAI85000 |
| EA_Pp_13356 | 1.51 | *3.90* | Stress-associated endoplasmic reticulum protein 1 (*X. tropicalis*) | 4.00E-24 | AAH62484 |
| EA_Pp_14774 | −1.10 | *1.32* | Synaptonemal complex protein 3 (*D. rerio*) | 2.00E-20 | XP_690333 |
| EA_Pp_13654 | *−1.26* | 1.08 | Testis nuclear RNA-binding protein (*D. rerio*) | 5.00E-47 | XP_693240 |
| EA_Pp_10408 | 1.83 | −2.52 | Transferrin variant C (*Carassius cuvieri*) | 7.00E-103 | AAP86289 |
| EA_Pp_10387 | *−1.41* | *1.87* | Tryptophan 2,3-dioxygenase (*G. gallus*) | 1.00E-70 | XP_4203 |
| EA_Pp_11955 | 1.08 | *1.89* | Tubulin, alpha 2 (*D. rerio*) | 5.00E-98 | AAQ91280 |
| EA_Pp_10526 | 1.17 | *2.13* | Tubulin, beta 2c (*D. rerio*) | 8.00E-92 | AAH62827 |
| EA_Pp_12751 | −1.54 | *−3.21* | UDP-glucose pyrophosphorylase 2 (*D. rerio*) | 1.00E-12 | XP_683457 |
| EA_Pp_14612 | −1.27 | *−1.68* | Unknown gene | 9 | — |
| EA_Pp_14417 | *−2.25* | *−1.75* | Unknown gene | 2.30 | — |
| EA_Pp_14697 | −1.18 | *−1.51* | Unknown gene | 1.8 | — |
| EA_Pp_14912 | 1.60 | *2.65* | Unknown gene | 1.40 | — |
| EA_Pp_15119 | *−2.36* | 1.53 | Unknown gene | 1.30 | — |
| EA_Pp_14081 | −1.03 | *−2.39* | Unknown gene | 0.47 | — |
| EA_Pp_14587 | *−1.53* | *−1.49* | Unknown gene | 0.36 | — |
| EA_Pp_15027 | −1.35 | 1.23 | Unknown gene | 0.12 | — |
| EA_Pp_14629 | *−2.64* | *−4.04* | Unknown gene | 0.019 | — |

[a] EST = expressed sequence tag; UDP = uridine diphosphoglucose; GABA = gamma-aminobutyric acid; ATP = adenosine triphosphate; SMAD = [similar to] mothers against decapentaplegic homolog; IGFALS = insulin-like growth factor binding protein, acid labile subunit; and IGFBP2 = insulin-like growth factor binding protein 2.
[b] e-value = expectation value.

ER ligand in the liver of adult female fish that activates synthesis of specific gene transcripts, which encode proteins required for reproduction. Genes known to be activated by this process include those that encode the ER itself, as well as various vtgs and choriogenins [27–29]. Vitellogenins, egg yolk precursor proteins, and the choriogenins, which are required for making the inner covering of the egg, normally increase in the blood of females during oogenesis [30]. However, in males, normal endogenous levels of estradiol are sufficient to induce only very small amounts of these proteins [31]. Hence, when males are exposed to estrogens, the result is an increase in the circulating levels of vtg and choriogenin proteins.

The present study confirmed upregulation of vtg 1, both at the transcript and protein levels. Measurement of plasma vtg in the males helped confirm that the estrogen exposure acted as expected in terms of inducing vtg and also helped anchor the gene response data collected via the microarray analysis. On the microarrays, vtg 1 transcripts increased up to 76.7 (SD = 47.0) in liver tissue of male fish exposed to 100 ng estradiol/L. In the present study, we observed a correlation between fold-change and location of the different vtg 1 probes. It is possible that these results are due to the labeling of the RNA samples. For the experiments, a poly dT-T7 primer was used for the labeling, which may result in a more efficient labeling of the RNA samples closer to the 3′ end of a gene. It would be interesting to see if a similar result could be obtained if a random primer was used for the labeling experiments. In order to obtain accurate fold-change results among treatments and experiments using microarrays, multiple probes for a gene should be examined and extensively characterized because different probe positions may have different bias associated with them.

Expression patterns of ER α and choriogenin 2 and 3 genes also were characterized using the microarrays. Estrogen receptor α was upregulated 2.4- and 2.8-fold in liver tissue of fish exposed to 40 or 100 ng of 17β-estradiol/L. Similar fold changes in the expression of this transcription factor have been observed on macroarrays in other fish species exposed to ER agonists [32,33]. Surprisingly, we did not detect differential gene expression of either choriogenin 2 or 3 using the fathead minnow microarray. Inspection of spot intensity values for the choriogenin genes on the 2,000 gene microarrays revealed that the values for these genes were very close to background for all three treatment groups. Lack of differential regulation of

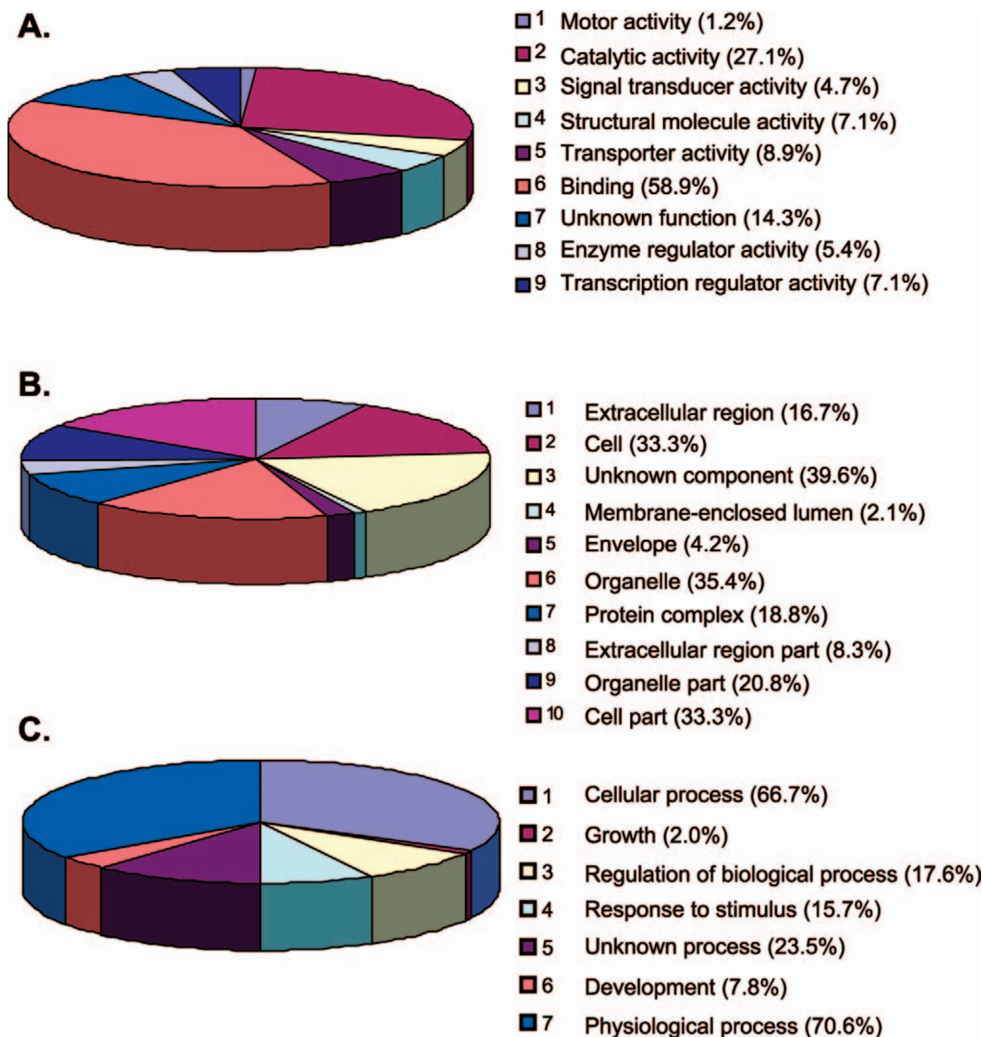


Fig. 5. Gene ontology of the genes that differentially regulated the liver of male fathead minnows exposed to 17β-estradiol. As based on the gene ontology classification system, a gene can have more than one gene ontology designation. (**A**) = Molecular function; (**B**) = Cellular component; (**C**) = Biological process.

the choriogenin genes when male fish were exposed to estradiol also was observed using a 200-gene fathead minnow cDNA-based macroarray that preceded the microarray developed for this project (P. Larkin, unpublished observations). These data suggest that the choriogenin 2 and 3 gene transcript abundance in the fathead minnow may be lower or less inducible than that observed in other fish species. It is possible that, in the fathead minnow, choriogenin genes are expressed in tissues other than the liver. A recent paper by Pinto et al. [34] reported upregulation of choriogenins 2 and 3 in the gonad, as well as the liver tissue, of sea bream exposed to estradiol. Additional experiments to characterize tissue-specific expression of choriogenin genes in the fathead minnow would be helpful in explaining their seeming lack of responsiveness to estradiol in the liver.

The expression patterns of four genes (vtg 1, ER α, keratin 18, and reticulocalbin 3) were compared by both microarray analysis and real-time PCR. Vitellogenin 1, ER α, reticulocalbin 3 all had higher expression levels in the liver tissue of male fish exposed to estradiol compared to the control fish, whereas keratin 18 was downregulated.

These observations help confirm that the fathead minnow microarrays provide reliable data in order to identify genes that are regulated differentially for a particular compound.

In addition to identifying differential expression for known estrogen-responsive genes like vtg and the ER, we found 71 additional genes that were differentially expressed in the male fathead minnows. This is the first characterization of these genes being estrogen responsive in the fathead minnow. The genes encompass a variety of biological processes and pathways, including regulation of transcription, proteolysis, complement activation, metabolism, and other pathways (see Fig. 5). Exposure to estrogens in other species also has been found to affect several of these genes/pathways.

Five of the 71 genes that were expressed differentially are part of the blood coagulation process. These genes, which all were downregulated in estrogen-exposed fish, include antithrombin III, fibrinogen, protein kinase C, and coagulation factor IX. These results are consistent with experiments by Owens et al. [35], which have shown that proteins involved in the blood coagulation process are regulated differentially by diethylstilbestrol, a potent synthetic estrogen ER agonist. Plasma (activity) concentrations of Factor II (prothrombin), Factor VII, antithrombin III, and plasminogen were decreased,

Table 2. Comparison of microarray data with quantitative real-time polymerase chain reaction (PCR) for select genes. For each gene, analysis of variance was run followed by Tukey post hoc analysis. The treatment group that was significantly changed compared to controls is shown in italics. ER = estrogen receptor; Vtg = vitellogenin

| | Microarray | | | Real-time PCR | | |
|---|---|---|---|---|---|---|
| | Fold change | | | Fold change | | |
| Gene | 40 ng/L | 100 ng/L | *p* value | 40 ng/L | 100 ng/L | *p* value |
| Vtg 1 | *13.6 ± 8.6* | *76.7 ± 47.0* | fold change reflects average value of multiple probes | 43.2 | *531.3* | 0.005 |
| ER alpha | 2.4 | *2.8* | 0.02 | 1.7 | 3.0 | 0.07 |
| Keratin 18 | 1 | *−2.3* | 0.01 | −1.5 | −3.6 | 0.06 |
| Reticulocalbin 3 | *2.4* | *2.6* | 0.01 | 1.7 | 2.7 | 0.1 |

although Factor VIII activity increased in male orchiectomized rats after they were administered diethylstilbestrol for 28 d [35].

Another gene that was downregulated in the liver of male fathead minnows exposed to estradiol was phosphoenolpyruvate carboxykinase, whose gene transcripts encode a protein that is a key enzyme in gluconeogenesis. Similar downregulation of this gene has been reported in male rats administered estradiol for 42 d [36].

Heat shock protein 70 was upregulated differentially by estradiol in the fish. Heat shock proteins protect cells against various injuries by binding and refolding damaged proteins [37]. Similar upregulation of heat shock protein 70 has been reported in male goby injected with estradiol or nonylphenol, a known ER agonist in fish [38].

The field of ecotoxicogenomics is young, and because of this, there is not a wealth of microarray data available for fish species. Thus it can be difficult to make extensive among-species comparisons. However, we have shown previously, using a 30-gene macroarray in sheepshead minnows, that gene expression profiles in male fish exposed to estradiol and the synthetic estrogen, 17α-ethinylestradiol, are similar [7]. Hence, we felt insights could be gained through comparisons of the gene expression patterns in our current data set with a recent study by Hoffmann et al. [39], who examined gene expression profiles in liver tissue of female zebrafish exposed to 17α-ethinylestradiol using a 14,900-gene microarray. Keeping in mind gender and exposure differences between these two studies, examination of the gene ontology terms for significant genes nonetheless revealed some common pathways and processes affected by estrogens in the two studies. Some of these include blood coagulation, metabolism, protein biosynthesis, electron transport, and regulation of cell growth. Although similarities between the two studies exist, it is uncertain what the biological significance of some of the gene expression changes may be in terms of responses to estrogens. Linking alterations in molecular responses such as gene expression to adverse apical outcomes is, in fact, a critical need if genomic data are to be used for risk assessments [4]. As more microarray data for fish become available, modeling approaches such as those described by Villeneuve et al. [21] will help understand changes in gene expression relative to adverse outcomes.

## CONCLUSION

In conclusion, our results indicate that the microarrays developed yield reproducible results. The microarrays also were used successfully to characterize responses of the estradiol, in terms of identifying known both estrogen-responsive genes and novel genes. Future studies include examining and characterizing the genetic ''fingerprints'' of compounds present in the environment. The lessons learned from this study also served as an important basis for designing a larger 22,000-gene microarray in fathead minnows, which is now available.

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