

Temporal Gene Induction Patterns in Sheepshead Minnows Exposed to 17 β -Estradiol

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ABSTRACT Gene arrays provide a powerful method to examine changes in gene expression in fish due to chemical exposures in the environment. In this study, we expanded an existing gene array for sheepshead minnows (*Cyprinodon variegatus*) (SHM) and used it to examine temporal changes in gene expression for male SHM exposed to 100 ng 17 β -estradiol (E₂)/L for five time points between 0 and 48 hr. We found that in addition to the induction of genes involved in oocyte development (vitellogenin [VTG], zona radiata [ZRP]), other genes involved in metabolism and the inflammatory response are also affected. We identified five patterns of temporal induction in genes whose expression was modified due to E₂ exposure. We validated the gene array data for the expression of VTG 1, VTG 2, ZRP 2 and ZRP 3 and found that with low levels of exogenous E₂ (100 ng E₂/L) exposure, ZRP expression precedes VTG expression. However, at higher concentrations of E₂ (500 ng E₂/L), the difference in temporal expression appears to be lost. Exposure to high levels of environmental contaminants may affect the normal ordered expression of genes required for reproduction. Gene expression profiling using arrays promises to be a valuable tool in the field of environmental toxicology. As more genes are identified for species used in toxicological testing, researchers will be better able to predict adverse effects to chemical exposures and to understand the relationships between changes in gene expression and changes in phenotype. *J. Exp. Zool.* 305A:707-719, 2006. © 2006 Wiley-Liss, Inc.

How to cite this article: Knoebl I, Blum JL, Hemmer MJ, Denslow ND. 2006. Temporal gene induction patterns in sheepshead minnows exposed to 17 β -estradiol. *J. Exp. Zool.* 305A:[707-719].

The scientific literature is replete with evidence that endocrine disrupting chemicals (EDCs) not only exist in the environment, but also can have adverse effects on wildlife. Of the EDCs in the environment, those acting through estrogenic pathways have received the most attention to date. These xenoestrogens can mimic the activities of the endogenous ligand, 17 β -estradiol (E₂), and enter the aquatic environment via a number of sources including pesticides, byproducts of industry and wastewater treatment plant effluent, among others (Folmar et al., '96; Nimrod and Benson, '96a,b; Sumpter, '98; Solomon and Schettler, 2000).

Among the effects attributed to environmental estrogens is their ability to trigger estrogen receptor activity and female-specific proteins, such

Grant sponsor: US Environmental Protection Agency contract OD-5378-NTGX; Grant sponsor: USEPA cooperative agreement CR826357-10; Grant sponsor: Interdisciplinary Center for Biotechnology Research, University of Florida.

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Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jez.a.314.

as vitellogenin (VTG) and vitelline envelope or zona radiata proteins (ZRP), in male fish. Male fish normally produce only small amounts of VTG in response to normal endogenous levels of E_2 (Copeland et al., '86). However, if exposed to xenoestrogens, plasma VTG levels can rise enough to cause pathology in livers and kidneys (Herman and Kincaid, '88; Folmar et al., 2001). Because of the sensitivity of the estrogenic response in male fish, the synthesis of VTG or ZRP in male fish is a reliable indicator of estrogen exposure (Denslow et al., '96; Arukwe et al., '97a; Folmar et al., 2000).

In addition, E_2 is able to up- or down-regulate other genes involved in natural oogenesis in females. These genes are undoubtedly required to enable the increased level of synthesis and secretion of VTG and ZRPs in the liver and may include other genes of importance to oocyte development.

In addition, exposure to increased levels of E_2 may alter endogenous sex hormone homeostasis by feedback mechanisms. Normal immune function may be altered as well, since there is significant cross-talk between genes involved in the immune response and estrogen receptors (Kalaitzidis and Gilmore, 2005; Mo et al., 2005; Soucy et al., 2005). Reliable tools are needed to measure the genes globally and, by inference, uncover the biochemical pathways that are responsive to E_2 . Changes induced by E_2 exposure are time- and dose-dependent, as has recently been shown for estrogen-induced uterine growth in mice (Moggs et al., 2004).

We have expanded our existing cDNA gene array (Larkin et al., 2002a, 2003) developed for the sheepshead minnow (SHM, *Cyprinodon variegatus*) by including additional genes that may be differentially regulated by EDCs. The expanded array was used to determine temporal responsiveness of hepatic genes to E_2 within the first 48 hr of exposure and reveal various different expression patterns. The genes on the membranes were obtained from suppressive subtractive hybridization (SSH) libraries of liver RNA from male SHM exposed to known EDCs (methoxychlor, nonylphenol) or by differential display (DD) analysis. The arrays were validated and the temporal induction of several estrogen-responsive genes was determined by quantitative real-time PCR.

MATERIALS AND METHODS

cDNA clones were derived from DD RT-PCR as described previously (Denslow et al., 2001a,b) or were obtained from libraries generated by SSH

(Snell et al., 2003; Blum et al., 2004; Sheader et al., 2004). The subtractions were performed as described (Blum et al., 2004) using SHM liver polyA+ mRNA from the following treatments: untreated (control) male vs. female livers, nonylphenol (40 $\mu\text{g/L}$) males vs. control males; methoxychlor (12 $\mu\text{g/L}$) males vs. control males. Nonylphenol and methoxychlor are weak estrogenic compounds (Hemmer et al., 2001) and concentrations known to induce VTG in SHM were chosen for the exposures. A flow-through aqueous exposure procedure described previously (Hemmer et al., 2001) was used to dose the fish. Criteria for the selection of compounds and doses were from previously generated dose-response curves (Hemmer et al., 2001). All subtractions were performed in both directions to isolate genes that were both up- and down-regulated by the treatments. SSH was performed using a kit (Clontech PCR-Select, BD Biosciences, Palo Alto, CA) following the manufacturer's protocol.

The subtracted gene pools were cloned into pGEM T-Easy (Promega, Madison, WI) and sequenced. Approximately 700 cDNA clones were sequenced and of those, over 250 were chosen to be spotted on the array after sequence identification using the Basic Local Alignment Search Tool (BLAST X) on the National Center for Biotechnology Information (NCBI) database. Membrane arrays are limited in the number of spots that can be applied and for this reason only 250 cDNAs were used in duplicate. The following criteria were used to select cDNA clones for spotting onto the arrays. (1) Preference was given to genes that were positively identified by BLAST (with Expect Values (E) below 10^{-5}) and that represented specific biochemical pathways. If more than one clone for a gene was identified, the one with the lower E -value was chosen. (3) Some genes were positively identified as homologs of existing ESTs in the databases. Of these, only those with low E -values were chosen. Clones with E -scores above 1 were not used, as these were usually too short to give high specificity.

Amplification of cDNA for spotting

E. coli containing plasmids of interest were grown overnight in a 96-well plate in 200 μl LB with 20% glycerol and 100 $\mu\text{g/ml}$ ampicillin. The inserts were PCR amplified in a 100 μl final volume of a reaction mix containing 1 \times PCR Buffer A (Promega), 2 mM MgCl_2 , 0.5 mM dNTP mix, 0.3 μM M13 primers (5'-GTT TTC CCA GTC

ACG ACG TTG-3' and 5'-GCG GAT AAC AAT TTC ACA CAG GA-3') and 2.5 U *Taq* polymerase (Promega). PCR conditions were as follows: One cycle at 95°C (10 min), 35 cycles at 95°C (1 min), 57°C (1 min), 72°C (2 min) and one cycle at 72°C (7 min) followed by a 4°C hold.

Aliquots of the PCR reactions were checked on a 1% agarose gel containing ethidium bromide. The PCR products were purified by vacuum filtration through a Millipore Montage plate and were resuspended in 100 µl TE buffer (pH 8.0), and quantified using a SpectraMax Plus 384 plate reader (Molecular Devices, Inc., Piscataway, NJ). Five micrograms of each PCR product were transferred to a 384-well plate and dried using a vacuum centrifuge (Savant).

Spotting of the arrays

The arrays were spotted as previously described (Larkin et al., 2003). Briefly, the dried PCR products were resuspended in 20 µl nuclease-free water, after which 2 µl of 3 M NaOH was added to each sample. The plates were heated for 30 min at 65°C, quenched on ice for 2 min and then 9 µl 20 × SSC (2 M NaCl, 0.3 M sodium citrate, pH 7.0) containing 0.01 mM bromophenol blue was added. The arrays were spotted using a robot (Biomek, 2000; Beckman Coulter, Fullerton, CA, USA). Array controls as previously described (Larkin et al., 2003) were also included as were four blanks randomly distributed on the array. The controls included three *Arabidopsis thaliana* cDNAs (Stratagene, Inc., La Jolla, CA) that are not found in SHM, Cot-1 repetitive sequences, polyA⁺ sequence and an M13 vector sequence lacking an insert. These controls were used to determine similarity of labeling efficiency among arrays. Duplicate spots of each PCR product were deposited onto neutral nylon membranes (Pall Biodyne* B Nylon Membrane, Fisher Scientific), UV cross-linked and stored under vacuum until hybridization.

Sample extraction

Adult male SHM were exposed as previously reported (Hemmer et al., 2001) to E₂ (100 and 500 ng/L) or carrier vehicle for 6, 12, 24 or 48 hr. At each time period, livers from six treated and control fish were sampled. Total RNA was isolated using RNA-STAT 60 Total RNA Isolation Reagent (Tel-Test, Inc., Friendswood, TX) following the manufacturer's protocol. The resulting RNA pellet was resuspended in RNA Secure Resuspension Reagent (Ambion, Inc., Austin, TX) and residual

genomic DNA was removed using DNA-Free (Ambion, Inc.) following the manufacturer's protocol. The purity of the RNA was assessed by measuring optical densities at 260 and 280 nm. An A_{260/280} ratio between 1.8 and 2.0 represented highly pure RNA. RNA integrity was checked by agarose gel electrophoresis.

Labeling, hybridization, imaging and normalization

Total RNA from SHM exposed to 100 ng E₂/L was radiolabeled with α³³P-dATP and hybridized to individual membranes for array analysis as described previously (Larkin et al., 2003) with the exception of the washes, which were increased to 30 min each. The membranes were exposed at room temperature for 48 hr to a phosphor screen (Molecular Dynamics). The screen was scanned on a Typhoon 8600 imaging system (Molecular Dynamics) and quantified using ImageQuant v5.1 (Molecular Dynamics) software. The intensity values for each gene were derived by subtracting the average value of the four blanks on each membrane from the average value of the duplicate cDNA spots. The values were then normalized by generating a normalization factor for each membrane that was calculated by dividing the mean sum of 19 normalization genes for all membranes by the sum of the 19 genes on each individual membrane. The normalization factor for each individual membrane was then used to correct the values for all genes. The normalization genes included the 11 genes previously described (Larkin et al., 2003) plus eight additional ribosomal protein genes. The means were transformed to log base 2 and analyzed by ANOVA. When significant differences ($P \leq 0.05$) were found, further testing was done using Tukey's LSD to determine treatments that were different.

Quantitative PCR (Q-PCR)

cDNA was prepared from 5 µg total RNA in a 50 µl reaction volume using Stratascript (Stratagene) reverse transcriptase following the manufacturer's protocol. Each Q-PCR reaction was performed in a volume of 25 µl, using 1/50th of the product (1 µl) from the cDNA amplification reaction. Four genes of interest (VTG 1 and 2 and ZRP 2 and 3) were measured using cDNA from fish exposed to 100 and 500 ng/L E₂. Q-PCR was performed using the primers and probes as previously described (Knoebel et al., 2004). For each gene, a standard curve of five serial

dilutions was prepared from cloned and sequenced plasmids containing the genes of interest. Each point was analyzed in duplicate on an ABI PRISM 5700 (Applied Biosystems, Inc., Foster City, CA). Each sample was normalized to 18S ribosomal RNA and the amount of mRNA in each sample was calculated as previously described (Knoebl et al., 2004). 18S rRNA was chosen as the normalizer because it is easily quantified as it represents a large portion of the total RNA and is invariable to exposures. Q-PCR data were analyzed by ANOVA and Dunnett's post-test.

RESULTS

Using SSH, we have augmented an SHM macroarray from 30 (Larkin et al., 2003) to over 250 genes. The list of genes and best match from a BLASTX search is found in the Appendix. The membrane array method described here has previously been shown to be reproducible between individual arrays (Larkin et al., 2003). To determine variability in the expanded SHM array, identical liver RNA samples were hybridized to two separate membranes and the log of the pixel intensities of each gene was obtained and compared by linear regression analysis ($r^2 = 0.93$, Fig. 1).

As expected, the genes most highly increased included those for VTGs and ZRPs. Ten genes were identified (by BLASTX) as VTG sequences and 11 as egg membrane sequences (ZRPs or choriogenins) (Table 1). Of the six gene fragments identified as VTG 1, three match most closely with the SHM (*C. variegatus*) sequence in GenBank, however with *E*-values ranging from $4.00E^{-88}$ to $6.00E^{-10}$. Moreover, the lengths of the gene fragments differ from 192 bp for the VTG 1 gene showing a 7.9-fold increase to 462 bp for the gene fragment with a 33.7-fold increase. Only one of the VTG 2 sequences was identified as SHM VTG 2; the other two most closely matched *Fundulus heteroclitus* VTG 2. The SHM VTG 2 gene fragment increased by only 3.1-fold whereas the two fragments matching the *Fundulus* VTG 2, differing only by 100 bp in length increased by 11.2- and 12.2-fold, respectively.

All of the gene fragments identified as ZRP 2 matched the SHM sequence in GenBank, although the *E* values and lengths differed. The level of induction for the ZRP 2 genes was similar, ranging between 8.9 and 13.4-fold. Both of the ZRP 3 gene fragments were identified as SHM ZRP 3; however, both the lengths (652 and 1,166 bp) and

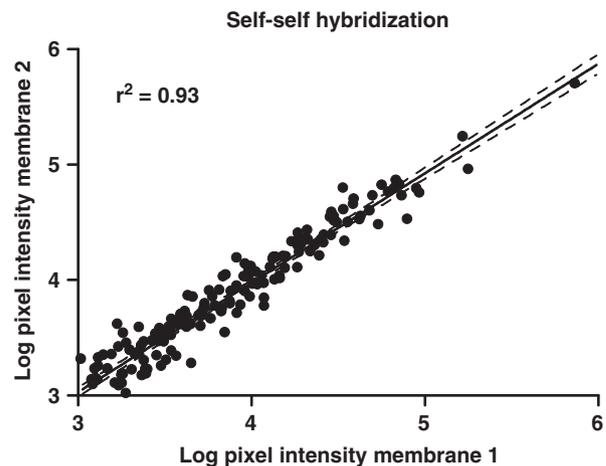


Fig. 1. Scatter plot of a self-self hybridization experiment. Aliquots of identical RNA samples were copied into cDNA and hybridized to two separate membranes. After correction for background and normalization, the pixel intensities for each gene were \log_{10} transformed and plotted for one membrane as a function of the other. A linear regression analysis was then performed.

E values ($3.00E^{-16}$ and 0) differed, as did the induction level (13.0 and 3.1, respectively). The remaining choriogenins and zona pellucida gene fragments were identified as matching Japanese medaka (*Oryzias latipes*) and winter flounder (*Pseudopleuronectes americanus*). Estrogen receptor alpha increased 1.6-fold over time 0. This increase occurred at 6 hr and remained at that level through 48 hr (data not shown).

The array data (Fig. 2) show that the four SHM genes previously known to be E_2 responsive (Knoebl et al., 2004) are up-regulated beginning at 12 hr and are maximally up-regulated at 48 hr. The levels of induction of VTG 1, ZRP 2 and ZRP 3 at 48 hr of exposure are significantly higher ($P \leq 0.01$) compared to all other time points. For VTG 2, the level of induction at 48 hr is significantly higher when compared to the 6 and 12 hr time points. Validation of the array results by Q-PCR for the two VTGs and two ZRPs confirms significant up-regulation ($P \leq 0.001$) after 48 hr of exposure to 100 ng E_2/L (Fig. 3). The temporal response data measured by Q-PCR is similar to the array data response curve and validates the array results. Exposure of SHM to 500 ng E_2/L results in up-regulation of these genes after 12 hr of exposure ($P \leq 0.001$) except for ZRP 3, which is significantly up-regulated ($P \leq 0.01$) after 6 hr of exposure (results not shown). Q-PCR results also reveal that VTG 2 is expressed at a 10-fold lower level than VTG 1, confirming the findings from the arrays.

TABLE 1. Gene fragments on the macroarray identified as either VTG or ZRP and whose expression was induced by treatment of sheepshead minnows with 100 ng/L E_2

Array position	Gene ID	E-value	Species	Size (bp)	Accession number	Fold increase after 48 hr
E18	VTG 1	2.00E-53	<i>F. heteroclitus</i>	422	AAA93123	37.3
C04	VTG 1	4.00E-88	<i>C. variegatus</i>	462	AAG30349	33.7
O01	VTG 1	2.00E-23	<i>C. variegatus</i>	657	AAG30349	15.3
J06	VTG 1	2.00E-86	<i>F. heteroclitus</i>	627	AAA93123	10.7
L05	VTG 1	8.00E-31	<i>F. heteroclitus</i>	469	T43141	10.3
K14	VTG 1	6.00E-10	<i>C. variegatus</i>	192	AF239720	7.9
L09	VTG	4.00E-05	<i>R. marmoratus</i>	852	AAQ16635	14.3
L03	VTG 2 precursor	4.00E-46	<i>F. heteroclitus</i>	569	AAB17152	12.2
K17	VTG 2 precursor	3.00E-52	<i>F. heteroclitus</i>	591	AAB17152	11.2
C09	VTG 2	5.00E-86	<i>C. variegatus</i>	465	AAG30350	3.1
C07	ZRP2	1.00E-102	<i>C. variegatus</i>	632	AAT51698	13.4
C03	ZRP2	3.00E-24	<i>C. variegatus</i>	842	AAT51698	12.2
E22	ZRP2	1.00E-108	<i>C. variegatus</i>	689	AAT51698	10.4
B03	ZRP 2	2.00E-40	<i>C. variegatus</i>	482	AAT51698	10.1
B10	ZRP 2	1.00E-109	<i>C. variegatus</i>	697	AAT51698	8.9
C14	ZRP 3	3.00E-16	<i>C. variegatus</i>	652	AAT51699	13.0
D06	ZRP 3	0	<i>C. variegatus</i>	1166	AAT51699	3.1
H19	Choriogenin H	3.00E-11	<i>O. latipes</i>	521	BAA13994	17.4
O19	Choriogenin H	2.00E-09	<i>O. javanicus</i>	458	AAX09342	12.0
K13	Zona pellucida	9.00E-22	<i>P. americanus</i>	278	AAC59642	4.9
N21	Zona pellucida	8.00E-36	<i>P. americanus</i>	419	AAC59642	1.9

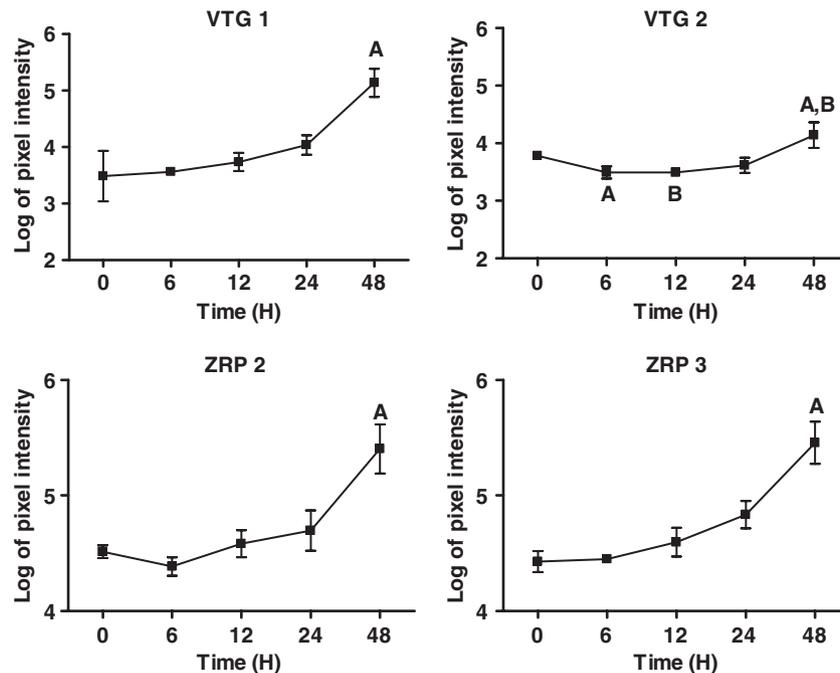


Fig. 2. Quantification of the pixel intensities of four SHM genes ($N = 6$ at each time point) known to be estrogen responsive. Data are plotted as $\log(10)$. Male fish were exposed to 100 ng E_2/L and sampled after 0, 6, 12, 24 or 48 hr. Liver RNA was arrayed on macroarrays. Letters indicate expression levels that are statistically different at $P \leq 0.01$.

In Figure 4, we plot the temporal changes in gene expression as determined by macroarray analysis for genes other than the VTGs and ZRPs.

We find five different patterns of expression. Those genes that were significantly up-regulated only after 48 hr (Fig. 4A1, A2) included alcohol

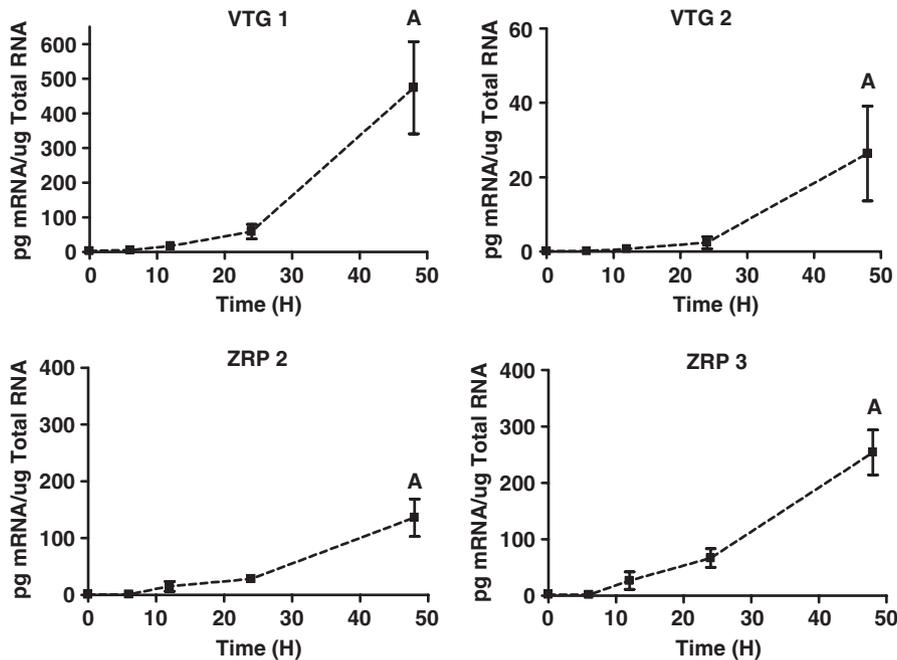


Fig. 3. Quantitative real-time PCR results of hepatic gene induction for the four estrogen-responsive SHM genes shown in Figure 2. Male fish ($N = 6$) were exposed to $100 \text{ ng E}_2/\text{L}$ and sampled after 0, 6, 12, 24 or 48 hr. Note the difference in the Y-axis scales. Results were normalized to 18 S ribosomal RNA. Letters indicate expression levels that are statistically different at $P \leq 0.001$.

dehydrogenase, tryptophan 2,3 dioxygenase, serum amyloid A protein, two genes that match ESTs from green pufferfish (*Tetraodon nigroviridis*), and three novel transcripts. In this same 48 hr time frame, C-type lectin, fibrinogen alpha and carboxypeptidase *N* regulatory subunit were down regulated (Fig. 4B). Some genes including NADH dehydrogenase subunit 1 and ATP synthase 6 are up-regulated at 6 hr and return to control levels by 48 hr of exposure (Fig. 4C). Two genes, liver basic fatty acid binding protein and a gene that matches a zebrafish (*Danio rerio*) EST are down-regulated at 12 hr, but return to control values after 48 hr (Fig. 4D). Three other genes including calreticulin, *N*-acetylneuraminase pyruvate lyase and peroxisomal proliferator-activated receptor beta 1 have a pattern of increasing at 6 hr then dropping to below control levels by 24 hr and returning to control levels at 48 hr (Fig. 4E). Each of these temporal variations would have been missed if only a 48 hr exposure had been performed.

DISCUSSION

Gene array technology is a fairly new technique in the field of environmental toxicology and promises to become a powerful tool to evaluate exposure of aquatic organisms to EDCs and other pollutants. Laboratory studies using macroarrays

to assess gene expression in largemouth bass (*Micropterus salmoides*) (Larkin et al., 2002b; Blum et al., 2004), SHM (Larkin et al., 2002a, 2003) and plaice (*Pleuronectes platessa*) (Brown et al., 2004a,b) exposed to estrogenic chemicals have recently been published. In addition, a rainbow trout (*Oncorhynchus mykiss*) cDNA microarray was used on whole fry to discriminate between effects of several chemical contaminants and determine potential biomarkers (Koskinen et al., 2004). The rainbow trout array was also used to identify gene expression in the brain and kidney in response to stress (Krasnov et al., 2005). The toxic stress response in field-captured European flounder (*Platichthys flesus*) was assessed using a DNA microarray (Williams et al., 2003). The limiting factor in all of these studies is the restricted number of identified gene sequences available for species that are typically used for environmental studies.

The present study expands an earlier macroarray by including additional genes obtained by SSH. The goal was to determine temporal- and dose-response differences in the expression of estrogen-responsive genes after exposure to exogenous E_2 . SSH of hepatic RNA from fish exposed to weak estrogenic chemicals were used to identify additional genes that may react to estrogen exposure. The majority of the identified cDNA

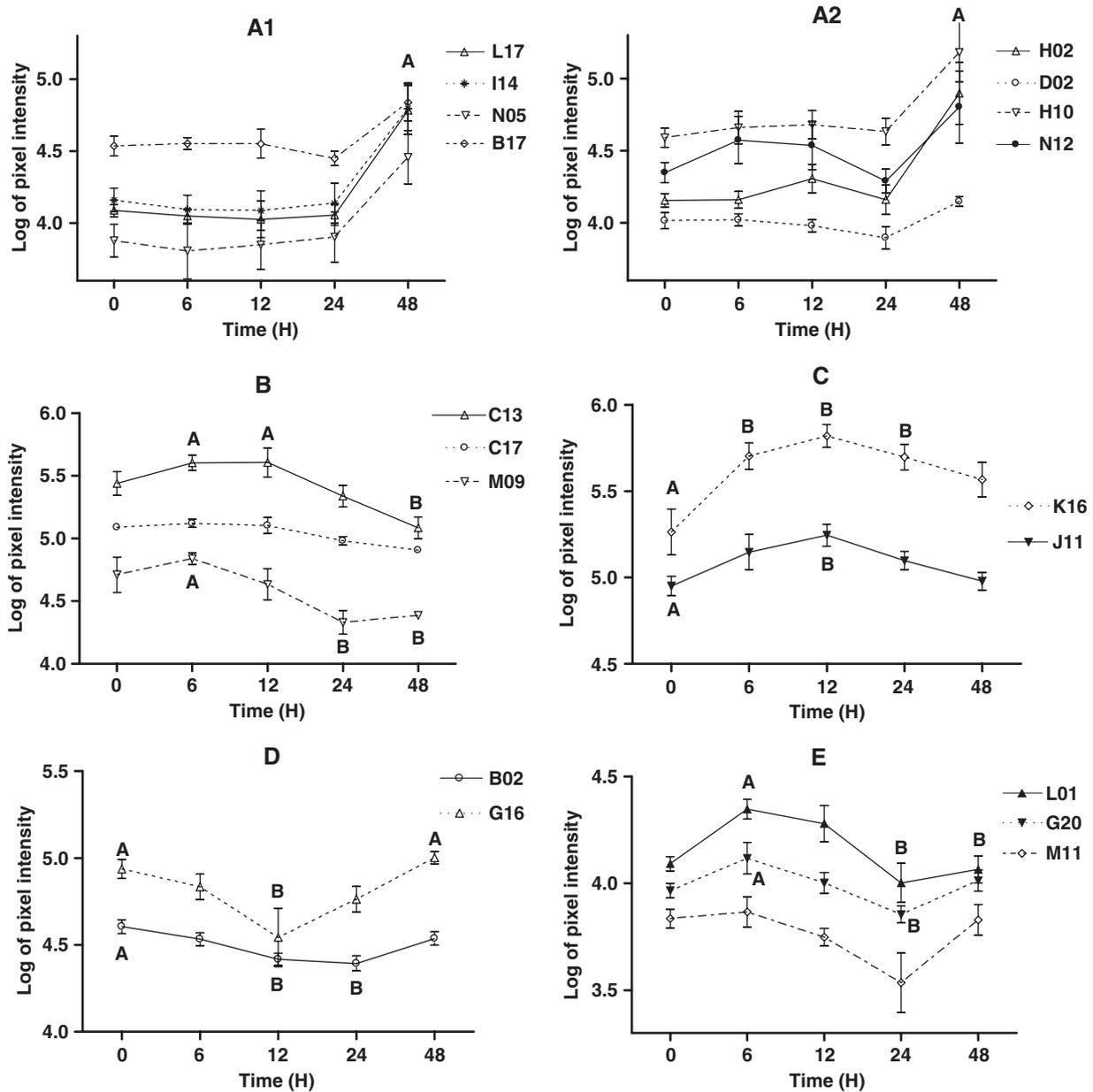


Fig. 4. Quantification of the pixel intensities of genes from liver mRNA of male fish exposed to 100 ng E_2 /L for 0–48 hr ($N = 6$ at each time point). Data are plotted as \log_{10} . Those genes found to have differences in expression during 48 hr of exposure fell into five different patterns of temporal expression. Letters indicate expression levels that are statistically different at $P \leq 0.05$. Pattern A (A1, A2) genes all were significantly different only at 48 hr except D02 with significant differences only between 24 and 48 hr: L17, EST (*T. nigroviridis*); I14, alcohol dehydrogenase; N05, EST (*T. nigroviridis*); B17, tryptophan 2,3 dioxygenase; H02, unknown (MXCc1-D08); D02, unknown; H10, unknown (MXCc1-G02); N12, serum amyloid A. Pattern B: C13, C-type lectin; C17, fibrinogen alpha; M09, carboxypeptidase *N* regulatory subunit. Pattern C: K16, ATP synthase 6; J11, NADH subunit 1. Pattern D: B02, unspecified zebrafish EST; G16, Liver basic fatty acid binding protein. Pattern E: L01, Calreticulin; G20, *N*-acetylneuraminatate pyruvate lyase; M11, peroxisomal proliferator-activated receptor beta 1. EST = expressed sequence tags.

fragments were VTGs and ZRPs (also known as choriogenins). Many of the VTG and ZRP gene fragments may be from different regions of the same genes.

It is not surprising that the array results indicate that VTG and ZRP genes were the most highly up-regulated in fish exposed to E_2 for 48 hr (Table 1). These findings are supported by several

other studies of fish exposed to estrogenic chemicals (Larkin et al., 2002a,b, 2003; Brown et al., 2004b). The variability in the response of genes with similar identities was also observed in plaice (Brown et al., 2004b). This variability suggests that there may be more Vtg and ZRP isotypes that are differentially regulated by E₂. Increasing evidence points to as many as seven distinct VTG genes in zebrafish (Wang et al., 2000) and indications are that other species, such as largemouth bass, may have four or more VTG genes (Larkin et al., 2002b), while trout may have as many as six VTG genes (Buisine et al., 2002). The differences in the length of some gene fragments may also play a role in the variability of expression seen in at least one VTG 1 fragment. To date, only two ZRP genes (ZRP 2 and 3) have been identified in SHM although the existence of a third (ZRP 1) is probable. Thus far, three distinct ZRP genes have been described in rainbow trout (Hyllner et al., 2001) and in arctic char (*Salvelinus alpinus*) (Westerlund et al., 2001). These findings also underscore the importance of validating array results with Q-PCR.

Evidence exists that oogenesis in females requires an ordered expression of genes involved in oocyte development, with ZRP expression preceding VTG expression (Arukwe et al., '97b, 2001). In the present experiment, it appears that with low levels of exogenous E₂ (100 ng E₂/L), this ordered pattern is maintained, since ZRP expression precedes VTG expression (Fig. 3). However, at higher concentrations of E₂, the dissimilar temporal expression appears to be lost (data not shown), implying that exposure to high levels of environmental contaminants may affect the normal ordered expression of genes required for reproduction.

Of those gene fragments that were neither VTGs nor ZRPs, eight were up-regulated only after 48 hr of E₂ exposure (Fig. 4A1, A2). Five of those remain unidentified or match *T. nigroviridis* ESTs of unknown function. The three identified genes are serum amyloid A, alcohol dehydrogenase and tryptophan 2,3 dioxygenase. Both serum amyloid A, an acute-phase inflammatory response protein and alcohol dehydrogenase are induced by E₂ in mammals (Qulali et al., '91; Urieli-Shoval et al., 2000; Abbas et al., 2004). Tryptophan 2,3 dioxygenase is involved in the metabolism of tryptophan.

The three down-regulated genes (Fig. 4B), carboxypeptidase N, C-type lectin and fibrinogen alpha are also involved in inflammatory responses.

Carboxypeptidase is a plasma enzyme that protects against potent vasoactive and inflammatory peptides such as kinins or anaphylatoxins (Matthews et al., 2004). The C-type lectin, a member of the selectin family, is a membrane-bound protein involved in inflammation (Lasky, '92). Fibrinogen is involved in blood clotting and inflammation and has previously been shown to be inhibited by exposure to E₂ in fish (Bowman et al., 2002) and frogs (Wang et al., '83). These results suggest that exposure to E₂ results in modulation of genes that are involved in the inflammatory response, an indication that has received wide support in the mammalian literature (Carlsten, 2005; Karpuzoglu et al., 2005; Opal et al., 2005). The induction of defense and detoxification genes to provide a protective environment for embryo implantation and development has been hypothesized for mice (Moggs et al., 2004) injected with E₂. However, exactly how down regulation of these genes affects the immune response in fish needs additional experimentation.

Two genes were up-regulated between 6 and 24 hr and then returned to control levels at 48 hr (Fig. 4C). NADH dehydrogenase (ubiquinone) catalyses the reduction of ubiquinone to ubiquinol. It is present in mitochondria as part of the respiratory-chain NADH dehydrogenase (also known as complex I or NADH-ubiquinone oxidoreductase), an oligomeric enzymatic complex. The other is ATP synthase 6, which is involved in metabolism and energetics.

Two genes show a pattern of down regulation at either 12 or 24 hr or both (Fig. 4D) and then return to control levels at 48 hr. One is a match for a zebrafish EST. The other, liver basic fatty acid binding protein is a member of a superfamily of lipid binding proteins. It may be involved in the uptake and metabolism of fatty acids, in the maintenance of fatty acid levels in the cell membrane, trafficking of the fatty acids, modulation of specific enzymes involved in lipid metabolism pathways and in the modulation of cell growth and differentiation (Massolini and Calleri, 2003).

Three of the genes exhibited both up and down regulation during the 48 hr exposure (Fig. 4E). Calreticulin, which was up-regulated at 6 and 12 hr during exposure, is a calcium binding protein present in most cells. It may play a role in the storage of calcium in the endoplasmic reticulum. Calreticulin has also been characterized as an inhibitor of steroid hormone-regulated gene expression (Coppolino and Dedhar, '98).

It is clear that many genes other than those directly involved in reproduction are regulated by E_2 in fish. Recently, gene expression profiling in mice during estrogen-induced uterine growth has revealed complex temporal molecular changes (Moggs et al., 2004) that are linked to phenotypic changes. The first changes observed involved genes for transcriptional regulation and signal transduction, followed by genes involving protein biosynthesis and cell proliferation. There likely are other genes affected by E_2 exposure in fish that may be targets for estrogenic EDCs. In future experiments, as more genes are obtained, it may become possible to link specific changes in gene expression to traditional toxicological endpoints of whole systems and changes in phenotype such as the occurrence of intersex fish. This "phenotypic anchoring" (Paules, 2003) may help identify groups of genes whose expression determines the developmental or reproductive patterns of fish.

These experimental results begin to elucidate the many genes that may be regulated by endogenous E_2 . The most highly up-regulated genes identified to date in fish are the VTGs and ZRPs required for developing oocytes. However, other genes appear to be regulated in a temporal and dose-dependent manner upon exposure to E_2 . Gene arrays, along with Q-PCR validation of results, provide powerful tools to examine the relationships between changes in gene expression and phenotype, as well as defining broad biochemical pathways that may be affected by chemical exposures.

ACKNOWLEDGMENTS

This study was funded by US Environmental Protection Agency contract OD-5378-NTGX, USEPA cooperative agreement CR826357-10 and the Interdisciplinary Center for Biotechnology Research, University of Florida. This paper has been reviewed in accordance with official EPA policy. The research described in this article does not necessarily reflect the views of the EPA and no official endorsement should be inferred. N. Denslow is an inventor of the technology discussed in this publication and holds equity in EcoArray Inc., a company commercializing the technology. She may benefit from this technology by receiving royalties and equity growth.

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APPENDIX: A

TABLE A1.

Array position	Gene name	Array position	Gene name
B02	Zebrafish EST sequence	C18	Unknown
B03	zona radiata-2 (<i>Cyprinodon variegatus</i>)	C19	Hepatocyte growth factor activator (<i>Rattus norvegicus</i>)
B04	3-hydroxy-3-methylglutaryl-CoA reductase	C20	glucose-6-phosphatase
B05	SHM49 NORM	C21	Unknown
B06	SHM 48—unknown	C22	Unknown
B07	SHM75-2	D01	SHM-73 NORM
B08	SHM8-3	D02	Unknown
B09	Unknown	D03	SHM34
B10	<i>Cyprinodon variegatus</i> zona radiata-2 mRNA	D04	Zebrafish EST sequence
B11	SHM35-2	D05	SHM-5A
B12	SHM 48-2 unknown	D06	ZRP 3
B13	rat liver regeneration related protein	D07	Glycosylate reductase
B14	Unknown	D08	Unknown
B15	miro2 pending protein	D09	SHM45-2
B16	lysophospholipase (<i>Rattus norvegicus</i>)	D10	Unknown
B17	tryptophan 2,3 dioxygenase	D11	Alpha1-microglobulin/bikunin precursor (AMBP) protein
B18	beta hemoglobin A (<i>Seriola quinqueradiata</i>)	D12	SHM-34-2
B19	Unknown	D13	Cytochrome <i>c</i> oxidase subunit II
B20	C9 protein (<i>Oncorhynchus mykiss</i>)	D14	Similar to chitinase, (<i>D. rerio</i>)
B21	Unknown	D15	Fatty acid binding protein 2, hepatic
B22	Unknown	D16	Beta galactosidase/ubiquitin fusion protein
C01	Estrogen receptor alpha	D17	Perforin 1 (pore forming protein) human
C03	ZRP 2	D18	Orla C3 (<i>O. latipes</i>)
C04	Vitellogenin I	D19	DNAse II homolog F09G8.2 (<i>Caenorhabditis elegans</i>)
C05	IGF I	D20	pentraxin (<i>Cyprinus carpio</i>)
C06	IGF 2	D21	Unknown
C07	ZRP2 (ND92A)	E01	SHM51-3 Unknown
C08	Unknown	E02	40 S ribosomal protein S17 (<i>Ictalurus punctatus</i>)
C09	Vitellogenin II	E03	Unknown SHM 22 NORM
C10	Ubiquitin-conjugating enzyme 9 (Putative)	E04	Beta actin
C12	Hepatic lipase precursor	E05	Transferrin
C13	C-type lectin (<i>Fundulus heteroclitus</i>)	E06	Similar to ribosomal protein L37a, cytosolic
C14	ZRP3 (<i>Cyprinodon variegatus</i>)	E07	Low molecular mass polypeptide subunit (Takifugu)
C15	60 S ribosomal protein L8	E08	Unknown
C16	Complement factor B/C2B (<i>O. mykiss</i>)	E09	Unknown SHM 24 NORM
C17	Fibrinogen alpha (<i>Rattus</i>)	E10	SHM 25 Ribosomal protein S9 like NORM
E11	Ribosomal protein L5	G06	Similar to high-mobility group box 1 (<i>Danio rerio</i>)
E12	Apolipoprotein A/I precursor (<i>Sparus aurata</i>)	G07	Prostaglandin D synthase (<i>Xenopus laevis</i>)
E13	Perlecan (heparan sulfate proteoglycan 2	G08	Endoplasmic reticulum luminal L-amino acid oxidase
E14	Mitochondrial inner membrane protease subunit	G09	Probable complement regulatory plasma protein SB1—
E15	Chemotaxis (<i>O. mykiss</i>)	G10	Leucine-rich alpha-2-glycoprotein (<i>Homo sapiens</i>)
E16	40 S ribosomal protein S3	G11	Complement component C3 (<i>Paralichthys olivaceus</i>)
E17	Chicken fatty acid binding protein	G12	Solute carrier family 27 (fatty acid transporter), member
E18	Vitellogenin I precursor (<i>Fundulus heteroclitus</i>)	G13	Retinol binding protein 4 (<i>D. rerio</i>)
E19	Unknown	G14	Unknown
E20	Orla C4 (<i>Oryzias latipes</i>)	G15	Serotransferrin precursor
E21	Unknown	G16	Liver basic fatty acid bp
E22	Zona radiata-2 (<i>Cyprinodon variegatus</i>)	G17	Ribosomal protein L35 (<i>G. galus</i>)
F01	Recombination repair protein	G18	Similar to 60S ribosomal protein L18A (<i>D. rerio</i>)
F02	No hit	G19	Complement component C9 (<i>Paralichthys olivaceus</i>)
F03	Gene product is related to a protein kinase.	G20	<i>N</i> -acetylneuraminate pyruvate lyase
F04	Dodecenoyl-coenzyme A delta isomerase	G21	Unnamed protein product (<i>Homo sapiens</i>)
F05	Cytochrome P450 3A56 (<i>Fundulus heteroclitus</i>)	H01	Cytochrome <i>c</i> oxidase subunit I (<i>Engraulis japonicus</i>)

TABLE A1. Continued

Array position	Gene name	Array position	Gene name
F06	Kallistatin (<i>Rattus norvegicus</i>)	H02	Unknown
F07	Fibrinogen beta chain precursor	H03	Immunoglobulin domain-containing protein family
F08	Similar to retinol dehydrogenase type III (<i>Danio rerio</i>)	H04	Hypothetical protein (<i>Plasmodium falciparum</i> 3D7)
F09	Beta-2-glycoprotein I precursor (Apolipoprotein H)	H05	Hypothetical protein (<i>Magnetospirillum magnetotacticum</i>)
F10	Tyrosine kinase (<i>Gallus gallus</i>)	H06	Sorting nexin 11 (<i>Homo sapiens</i>)
F11	Ceruloplasmin (<i>Danio rerio</i>)	H07	Warm-temperature-acclimation-related-protein
F12	Hypothetical protein (<i>Ferroplasma acidarmanus</i>)	H08	UDP-glucose pyrophosphorylase (<i>Gallus gallus</i>)
F14	C-type lectin (<i>Fundulus heteroclitus</i>)	H09	Interferon-related developmental regulator 1
F15	Unknown protein for MGC:63946 (<i>D. rerio</i>)	H10	Unknown protein
F16	Sertotransferrin precursor (<i>O. Latipes</i>)	H11	Thyroid hormone receptor interactor 12;
F17	Cytochrome P450 (<i>Ictalurus punctatus</i>)	H12	Ribosomal protein L21 (<i>Mus musculus</i>)
F18	Unnamed protein product (<i>Tetraodon nigroviridis</i>)	H13	Ribosomal protein P2 (<i>I. punctatus</i>)
F19	Peroxisomal long-chain acyl-coA thioesterase	H14	Embryonic epidermal lectin (<i>X. laevis</i>)
F20	Very-long-chain acyl-CoA synthetase	H15	C type lectin s (<i>Fundulus heteroclitus</i>)
F21	Ligand-gated ionic channel family member	H16	Similar to 60S ribosomal protein L21
G01	Putative transmembrane 4 superfamily member protein	H17	Similar to chitinase, acidid (<i>D. rerio</i>)
G02	Phospholipid hydroperoxide glutathione peroxidase	H18	Unknown protein for MGC:64127 (<i>D. rerio</i>)
G04	Aldehyde dehydrogenase 7 family, member A1	H19	Choriogenin Hminor (<i>Oryzias latipes</i>)
G05	Cytochrome b (<i>Orestias silustani</i>)	H20	Dihydroorotate dehydrogenase electron transfer subunit
H21	Unknown	J16	Mesau serum amyloid A/3 protein precursor
I01	Cytochrome c biogenesis factor (<i>Leptospira interrogans</i>)	J17	Natural killer cell enhancement factor (<i>O. mykiss</i>)
I02	Endoplasmic reticulum luminal L-amino acid oxidase	J18	Alpha s HS glycoprotein (<i>Platichthys flesus</i>)
I03	35 kDa serum lectin (<i>Xenopus laevis</i>)	J19	Unknown
I04	SPI-2 serine protease inhibitor (AA 1-407)	J20	Hypothetical protein (<i>Plasmodium yoelii yoelii</i>)
I06	Polyadenylate-binding protein 1	J21	Unknown
I07	Alpha-1-antitrypsin (<i>Sphenodon punctatus</i>)	K01	4-hydroxyphenylpyruvate-dioxygenase
I08	Cytochrome c oxidase, subunit Va	K02	Serine proteinase inhibitor CP9—common carp
I09	KIAA0018 protein (<i>Homo sapiens</i>)	K03	Prothrombin precursor (<i>Takifugu rubripes</i>)
I10	Complement component C5-1 (<i>Cyprinus carpio</i>)	K04	ATPase, H ⁺ transporting, lysosomal,
I11	Fibrinogen, B beta polypeptide	K05	Proteasome Regulatory Particle, ATPase-like
I12	Similar to fibrinogen, gamma polypeptide (<i>Danio rerio</i>)	K06	Expressed sequence AL022852 (<i>Mus musculus</i>)
I13	G protein B subunit (<i>Ambystoma tigrinum</i>)	K07	Similar to monocarboxylate transporter 6
I14	Alcohol dehydrogenase	K08	Elastase 4 precursor (<i>Paralichthys</i>)
I15	Precerebellin like protein (<i>O. mykiss</i>)	K09	Charged amino acid rich leucine zipper factor-1
I16	AMBP protein precursor microglobulin	K10	Dendritic cell protein (<i>Homo sapiens</i>)
I17	Chain A, alcohol dehydrogenase	K11	Chain A, Alcohol Dehydrogenase
I18	Warm temperature acclimation protein (<i>O. latipes</i>)	K12	17-beta-hydroxysteroid dehydrogenase type IV
I19	Alanine-glyoxylate aminotransferase 2	K13	Zona pellucida protein (<i>Pseudopleuronectes americanus</i>)
I20	Apolipoprotein B—Atlantic salmon	K14	Vitellogenin (<i>Sillago japonica</i>)
I21	Similar to transducin (beta)-like 2 (<i>Xenopus laevis</i>)	K15	Similar to ribosomal protein L10 (<i>D. rerio</i>)
J01	Putative delata 6-desaturase (<i>Oncorhynchus masou</i>)	K16	ATPase subunit 6 (<i>Scomberomorus tritor</i>)
J02	Complement control protein factor I-A (<i>Cyprinus carpio</i>)	K17	VTG 2 (<i>Fundulus heteroclitus</i>)
J03	BH2041~unknown conserved protein (<i>Bacillus halodurans</i>)	K18	ATPase subunit 6 (<i>Scomberomorus tritor</i>)
J04	CG4198-PA (<i>Drosophila melanogaster</i>)	K19	KIAA1657 protein (<i>Homo sapiens</i>)
J05	Iron oxidase precursor	K20	aryl-CoA ligase EncN (<i>Streptomyces maritimus</i>)
J06	Vitellogenin I precursor (VTG I)	K21	Sertotransferrin precursor (<i>O. Latipes</i>)
J07	Unknown	L01	Calreticulin (<i>Danio rerio</i>)

TABLE A1. Continued

Array position	Gene name	Array position	Gene name
J08	Unknown	L02	Hypothetical protein APE0566
J09	Group XIII secretory phospholipase A2 precursor	L03	Vitellogenin II precursor (VTG II) (<i>Fundulus heteroclitus</i>)
J11	NADH subunit 1 (<i>Cyprinodon variegatus</i>)	L04	Translation elongation factor 1-alpha (<i>Stylonychia mytilus</i>)
J12	Chain A, Complex Of The Catalytic Portion Of Human	L05	Vitellogenin I (<i>Cyprinodon variegatus</i>)
J13	Transducin beta/like 2 protein	L06	Unknown
J14	Unknown	L08	Ubiquitin A-52 residue ribosomal protein (<i>Homo sapiens</i>)
J15	Unknown	L09	Vitellogenin A (<i>Melanogrammus aeglefinus</i>)
L11	LFA-3(delta TM) (<i>Ovis</i> sp.)	L10	Unknown
L12	CG32659-PA (<i>Drosophila melanogaster</i>)	N06	Hemoglobin beta chain >gi 7439519 pir S70614
L13	Similar to transducin (beta)-like 2 (<i>Xenopus laevis</i>)	N07	Unknown
L14	Predicted CDS, seven TM Receptor S	N08	Cytochrome <i>c</i>
L15	Unknown	N09	RIKIN (<i>Plasmodium falciparum</i> 3D7) >gi 23498329 e
L16	Similar to transducin (beta)-like 2 (<i>Xenopus laevis</i>)	N10	P0699H05.18 (<i>Oryza sativa</i> (japonica cultivar-group))
L17	EST (<i>Tetraodon nigroviridis</i>) CAG02904	N11	Aminotransferase (<i>Bradyrhizobium japonicum</i>)
L18	Unknown	N12	Serum amyloid A protein (<i>Holothuria glaberrima</i>)
L19	Immunoglobulin light chain (<i>Seriola quinqueradiata</i>)	N14	Unknown
L20	Unknown	N15	Unknown
L21	Pre alpha inhibitor heavy chain 3 rat	N16	FUGU complement component C9 precursor
M01	Interferon induced protein 2 (<i>Ictalurus punctatus</i>)	N17	Unknown
M02	Alcohol dehydrogenase	N18	Unknown
M03	14 kDa apolipoprotein (<i>Anguilla japonica</i>)	N19	Cytochrome <i>c</i> oxidase subunit I
M04	Serine (or cysteine) proteinase inhibitor, clade F	N20	Unknown
M05	Ribosomal protein XL1a— <i>Xenopus laevis</i>	N21	Egg envelope protein winter flounder
M06	Microfibrillar-associated protein 4	O01	Vitellogenin I (<i>Cyprinodon variegatus</i>)
M07	Apolipoprotein E (<i>Scophthalmus maximus</i>)	O02	WS beta-transducin repeats protein (<i>Homo sapiens</i>)
M08	Aldehyde reductase AFAR2 subunit	O03	60S ribosomal protein L10a
M09	Carboxypeptidase N regulatory subunit (<i>Gallus gallus</i>)	O04	Similar to transducin (beta)-like 2 (<i>Xenopus laevis</i>)
M10	Similar to ribosomal protein S25, cytosolic	O05	Deoxyribonuclease II precursor (DNase II)
M11	Peroxisomal proliferator-activated receptor beta1	O06	Similar to olfactory receptor MOR149-1 (<i>Mus musculus</i>)
M12	Similar to sperm associated antigen 7 (<i>Homo sapiens</i>)	O07	CG31752-PA (<i>Drosophila melanogaster</i>)
M13	SHM-C01	O08	Heparin cofactor II (<i>Danio rerio</i>)
M14	Unknown	O09	F1F0-type ATP synthase subunit g (<i>Homo sapiens</i>)
M15	Unknown	O10	Unknown
M16	Unknown	O11	Hypothetical protein XP_215519 (<i>Rattus norvegicus</i>)
M17	Unknown	O12	Mitochondrial import receptor subunit TOM7 homolog
M18	Unknown	O14	Unknown
M19	Unknown	O15	ndSHM-NPc1-E11
M21	Beta hemoglobin A	O16	Unknown
N01	Ribophorin I (<i>Danio rerio</i>)	O17	alpha-2-macroglobulin 2 (<i>C. carpio</i>)
N02	KIAA1560 protein (<i>Homo sapiens</i>)	O18	Unknown
N03	ATP synthase alpha chain, mitochondrial precursor	O19	Choriogenin H (<i>Oryzias latipes</i>)
N04	Similar to Tho2 (<i>Homo sapiens</i>) (<i>Rattus norvegicus</i>)	O20	Unknown
N05	Unnamed protein product (<i>Tetraodon nigroviridis</i>)	O21	ndSHM-FT1-H10