2008

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Journal of Fish Biology (2008) **72,** 2281–2340
doi:10.1111/j.1095-8649.2008.01897.x, available online at http://www.blackwell-synergy.com

Altered gene expression in the brain and liver of female fathead minnows *Pimephales promelas* Rafinesque exposed to fadrozole

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The fathead minnow *Pimephales promelas* is a small fish species widely used for ecotoxicology research and regulatory testing in North America. This study used a 2000 gene oligonucleotide microarray to evaluate the effects of the aromatase inhibitor, fadrozole, on gene expression in the liver and brain tissue of exposed females. Reproductive measures, plasma vitellogenin and gene expression data for the brain isoform of aromatase (cytP19B), vitellogenin precursors and transferrin provided evidence supporting the efficacy of the fadrozole exposure. Unsupervised analysis of the microarray results identified 20 genes in brain and 41 in liver as significantly up-regulated and seven genes in brain and around 45 in liver as significantly down-regulated. Differentially expressed genes were associated with a broad spectrum of biological functions, many with no obvious relationship to aromatase inhibition. However, in brain, fadrozole exposure elicited significant up-regulation of several genes involved in the cholesterol synthesis, suggesting it as a potentially affected pathway. Gene ontology-based analysis of expression changes in liver suggested overall down-regulation of protein biosynthesis. While real-time polymerase chain reaction analyses supported some of the microarray responses, others could not be verified. Overall, results of this study provide a foundation for developing novel hypotheses regarding the system-wide effects of fadrozole, and other chemical stressors with similar modes of action, on fish biology.

Key words: aromatase inhibitor; cholesterol; endocrine disruption; fish; transcriptomics; vitellogenin.

INTRODUCTION

The fathead minnow *Pimephales promelas* Rafinesque, 1820, is an animal model widely used in aquatic ecotoxicology research and regulatory programmes in North America (Ankley & Villeneuve, 2006). Over the years, the field of aquatic toxicology and associated regulatory needs have gradually evolved from a focus on

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overt toxicity to concerns over more subtle impacts on reproduction, development, immune function, behaviour and, or other critical functions of fish and wildlife within their ecosystems. This shift in focus has evoked changes in the tools being used to conduct ecotoxicology research. There is increasing reliance on molecular and biochemical analyses as a foundation for determining or predicting potential mode(s) of action of unknown or poorly characterized chemicals. Additionally, there is increasing need to understand the ways biological systems respond to stressors to improve the ability to predict ecologically relevant outcomes and risks based on chemical mode of action. Transcriptomic analyses with high-density oligonucleotide microarrays represent a research tool that can be applied to help address some of the critical research needs and challenges facing the fields of ecotoxicology and ecological risk assessment.

To facilitate the widespread use of transcriptomic data to support research and regulation, there is a need to develop commercially available, high-density, general purpose, oligonucleotide microarrays for common ecotoxicity test organisms with rich historical databases of regulatory toxicity data, such as the fathead minnow (Ankley & Villeneuve, 2006; Ankley *et al.*, 2006). In 2004, the US Environmental Protection Agency (EPA) initiated a cooperative research and development agreement with EcoArray (Alachua, FL, U.S.A.) aimed at addressing this need. Larkin *et al.* (2007) reported on the initial validation of a 2000 gene fathead minnow microarray. In that study, fathead minnows were exposed to 17β-oestradiol, and gene expression profiles in the livers of males were examined using the microarray (Larkin *et al.*, 2007). Gene expression responses observed in the study were generally consistent with those observed in previous cDNA macroarray studies with largemouth bass *Micropterus salmoides* (Lacepède, 1802) (Larkin *et al.*, 2002) and sheepshead minnow *Cyprinodon variegatus* Lacepède, 1803 (Larkin *et al.*, 2003; Knoebl *et al.*, 2006) and in oligonucleotide microarray studies with Japanese medaka *Oryzias latipes* (Temminck & Schlegel, 1846) (Kishi *et al.*, 2006).

The present study was designed to complement the initial validation of the 2000 gene fathead minnow microarray by using it to examine transcript changes in fathead minnows exposed to the aromatase inhibitor, fadrozole. Aromatase is a cytochrome P450 enzyme that catalyses the critical reaction that converts C19 androgens (*e.g.* testosterone and androstenedione) to C18 oestrogens (*e.g.* 17β-oestradiol and oestrone). The reaction catalysed by aromatase is thought to be the rate-limiting step in oestrogen biosynthesis (Simpson *et al.*, 1994). As far as is known, this is the first microarray study with a fish species that has examined the effects of an aromatase inhibitor on gene expression. Villeneuve *et al.* (2007) previously reported a limited analysis of the microarray results as a demonstration of the utility of graphical systems models for conducting hypothesis-driven ecotoxicogenomics research. However, in addition to their utility for systems-oriented hypothesis-based investigation, microarrays are also ideally suited for conducting unsupervised analyses (Ankley *et al.*, 2006). Because they can be used effectively to screen expression of thousands of different genes without *a priori* knowledge of which may be affected, microarrays are well suited for discovery and hypothesis generation. Unsupervised microarray analyses have considerable potential for use in field monitoring and, or exposure assessment as a means to identify the types of stressors to

which organisms are being exposed (Ankley *et al.*, 2006). Furthermore, in screening unknown or poorly characterized chemicals, unsupervised analyses may be useful for identifying unsuspected modes of action (Ankley *et al.*, 2006).

This study focused on the use of an unsupervised analysis to identify novel genes that may be differentially expressed as a result of the direct or indirect effects of fadrozole on the brain and livers of exposed female fathead minnows. Effects of fadrozole on plasma vitellogenin concentrations and reproductive output were examined to help establish efficacy of fadrozole in the experiment and provide some phenotypic anchoring of the transcript-level responses. Additionally, quantitative real-time polymerase chain reaction (QPCR) assays were used to evaluate selected microarray responses and follow up on several hypotheses derived from the microarray analysis. Overall, results of this study provided both novel information regarding the molecular response of fathead minnows to an aromatase inhibitor and further evaluation of the 2000 gene microarray, which helped inform the subsequent development of a 22 000 gene microarray for the fathead minnow (http://www.ecoarray.com).

METHODS

FADROZOLE EXPOSURE

Fadrozole was provided by Novartis, Inc. (purity $\geq$99%; Dr H. Cooper Eckhardt, Summit, NJ, U.S.A.). Adult (*c.* 6 month old) male (4·65 $\pm$ 0·93 g) and female (1·90 $\pm$ 0·44 g) fathead minnows obtained from an on-site aquatic culture unit at the US EPA Mid-Continent Ecology Division were paired and placed in tanks with a breeding substrate and allowed to acclimate to exposure-like conditions (25° C, 16:8h light:dark photoperiod, fed adult brine shrimp twice daily) over a period of at least 1 week. Exposures were initiated by transferring randomly selected pairs (one male and one female) to exposure tanks supplied with a continuous flow-through of Lake Superior water containing measured concentrations of 0 (<2·0), 6 and 60 $\mu g\ l^{-1}$ fadrozole (Villeneuve *et al.*, 2007; supporting information SI-5) delivered without the use of a carrier solvent. There were four replicate tanks per treatment with two pairs of fish (separated by a mesh divider) per tank. Spawning activity and fecundity (number of eggs) were monitored daily. After separate exposures, either 24 h ($\pm$90 min) or 7 days ($\pm$4 h) in duration, fish were humanely euthanized in buffered tricaine methanesulphonate (Finquel; Argent, Redmond, WA, U.S.A.). Plasma, liver, gonad and brain samples were then collected and tissue samples transferred directly to pre-weighed vials containing RNAlater® (Sigma, St Louis, MO, U.S.A.). Plasma samples were stored frozen at −80° C. Vitellogenin concentrations in the plasma of individual females from all treatments were quantified by enzyme-linked immunosorbent assay with a fathead minnow polyclonal antibody (Korte *et al.*, 2000; US EPA, 2002). Average water quality characteristics ($\pm$S.D.) monitored over the course of the 24 h and 7 days experiments, respectively, were temperature 25·1 $\pm$ 0·3 and 25·5 $\pm$ 0·1° C, pH 7·39 $\pm$ 0·17 and 7·21 $\pm$ 0·04 and dissolved oxygen 5·15 $\pm$ 0·60 and 5·74 $\pm$ 0·52 $mg\ l^{-1}$. All laboratory procedures involving the animals were reviewed and approved by the local Animal Care and Use Committee in compliance with Animal Welfare Act regulations and Interagency Research Animal Committee guidelines.

MICROARRAY ANALYSIS

Expression of *c.* 2000 genes in the whole liver and brain of female fathead minnows exposed to 60 $\mu g\ l^{-1}$ fadrozole or Lake Superior water (control) for 7 days was analysed with the fathead minnow microarray validated and described by Larkin *et al.*

(2007; gene expression omnibus (GEO) accession no. GPL6516). Briefly, a variety of fathead minnow cDNA and suppression subtractive hybridization libraries from multiple tissues including gonad, liver, brain, heart and spleen from both male and female fathead minnows were constructed and clones sequenced (Larkin *et al.*, 2007). In total, the sequencing efforts yielded *c.* 2000 unique genes that were positively matched (e value <1E-5) with clones found in the National Center for Biotechnology Information (NCBI) non-redundant or nucleotide database (Larkin *et al.*, 2007). Probes for each gene were designed using Agilent software (Agilent Technologies, Palo Alto, CA, U.S.A.), and 60-mer oligonucleotides were synthesized *in situ* on treated glass slides using inkjet deposition technology (Larkin *et al.*, 2007). Each unique probe spotted on the array was assigned an identification number (EA_Pp_###) and annotated with the following information: target sequence name; query sequence name; a gene name derived from the highest confidence match in the non-redundant (NR) or nucleotide (NT) database (NCBI); biological process, cellular component and molecular function gene ontology (GO) terms (where available; Gene Ontology Consortium, 2006); the top basic local alignment search tool (BLAST) hit from the NR database along with accession number and e value; and the top BLAST hit from the NT database along with its accession number and e value. Approximately 90% of the sequences were annotated with a name corresponding to a BLAST hit in the NT or NR database and biological process, cellular component and molecular function GO annotations were available for 51, 37 and 62% of the sequences, respectively. Duplicates of all probes were spotted at random locations on the slide surface, and in cases where the direction of a gene could not be determined, probes were synthesized from both the strands (forward and reverse complement; Larkin *et al.*, 2007). For selected genes (*e.g.* vitellogenin), multiple probes spanning different portions of the gene sequence or designed based on different target sequences for the gene were synthesized on the microarray. This multiple spotting approach took advantage of the extra capacity of the microarray format (*i.e.* 11 000 features, including Agilent platform control spots, two arrays per glass slide) to examine whether there were significant differences in probe performance (Larkin *et al.*, 2007).

Total RNA was isolated from tissue samples using TRI reagent lysis solution (Molecular Research Center, Cincinnati, OH, U.S.A.) following the manufacturer's protocol. Samples were DNAse treated and quality of the total RNA was evaluated by denaturing agarose gel electrophoresis and optical density (260/280 ratio 1·8–2·0). Aliquots of total RNA extracted from the liver or brain of two females from the same replicate tank were pooled, and poly-A RNA was reverse transcribed from 500 ng of pooled total RNA using a poly-dT-T7 primer and then cRNA was labelled with cyanine 5 (experimental samples) or cyanine 3 (reference) using Agilent's low-input fluorescent linear amplification kit (for additional details see Larkin *et al.*, 2007). Amplification yields and dye incorporation were quantified using a Nanodrop spectrophotometer (Agilent). Three replicate-pooled samples (cyanine 5 labelled), per treatment, per tissue, were then hybridized to the 2000 gene microarrays along with reference samples labelled with cyanine 3 (Larkin *et al.*, 2007), following the manufacturer's recommended protocol (Agilent; 60-mer oligo microarray processing protocol). The same reference sample, consisting of pooled liver, brain and gonad samples from adult male and female fathead minnows, was used for all analyses (aliquots from a single labelling reaction stored at −80° C until used).

The slides were scanned with an Agilent DNA microarray scanner and raw images processed using Agilent's Feature Extraction software. Resulting data were analysed using the AnalyzeIt statistical package (University of Florida, Gainesville, FL, U.S.A.). Data were first filtered to remove flagged features (*e.g.* saturated, non-uniform spots). Following filtering, data from each spot were local background subtracted, Lowess normalized and $\log_{10}$ transformed. The mean response for each probe (spot) across chips ($n = 3$ for each tissue–treatment) was determined, and a *t*-test performed to estimate the statistical significance of differences between the control and 60 $\mu g\ l^{-1}$ fadrozole treatment group. Probes were ranked in ascending order based on *P*-value (*i.e.* those responses with the lowest probability of being a false positive to those with the greatest probability) and then two quantitative decision criteria were applied to

identify putative differentially expressed genes while minimizing the reporting of false positives. First, in all cases where a duplicate (same oligonucleotide sequence) probe was spotted on the microarray (and data were not removed by filters), fold change and *t*-test *P*-values of the duplicate probes were compared as means to assess the reliability of the response. Any duplicate probes with contradictory fold-change data (*i.e.* one duplicate probe up-regulated and one down-regulated) or with average *P*-values $>0{\cdot}05$ were considered unreliable and not included in the list of differentially expressed genes. In the case of brain, this criterion narrowed the list of differentially expressed genes to a relative handful; consequently, no additional criterion was applied, except in cases where a duplicate probe response could not be identified. In the case of liver, the list was narrowed further by applying a Benjamini and Hochberg false discovery rate (FDR) multiple test correction (Benjamini & Hochberg, 1995) to ranked *P*-values for individual probes. FDR $= 0{\cdot}1$ was assigned as a cut off value and applied such that only genes with an average uncorrected *P*-value $<0{\cdot}05$ among duplicate probes and a Benjamini and Hochberg corrected *P*-value $<0{\cdot}1$, for at least one of the two duplicate probes, were included on the list of differentially expressed genes. In cases where comparison to a duplicate probe response was not feasible (generally because of data being flagged and removed by filters), only probes meeting the FDR $<0{\cdot}1$ criterion were included.

GENE ONTOLOGY-BASED ANALYSIS

Annotation of the 2000 gene microarray provided by EcoArray was loaded into GeneSpring GX 7.3.1 software (Agilent Technologies) as a custom genome, and annotations were updated based on NT accession numbers using the Genespider function. 'Enriched' biological process GO categories associated with genes differentially expressed in this study were identified using a hypergeometric test without multiple testing correction (GeneSpring GX 7.3.1, Gene Ontology Browser). For the purposes of this study, statistical enrichment was defined as hypergeometric $P < 0{\cdot}01$ with at least three differentially expressed genes in the category. GO-based analysis was performed using both the moderately conservative lists of differentially expressed genes identified using the criteria described above and the expanded lists based on relaxed statistical criteria (*i.e.* $P < 0{\cdot}05$ for a single probe and no conflicting fold-change data for duplicate or multiple probes; non-redundant by gene name; see Appendices 4, 5 and 6). While the relaxed criteria allows for more false positives to be identified as differentially expressed, it was considered suitable for GO analysis that considers profiles of multiple genes with similar function, rather than any one specific gene. Furthermore, the additional gene representation on the relaxed list provided a means to assess the robustness of conclusions based on more restricted lists, which place greater weight on individual responses.

QUANTITATIVE REAL-TIME PCR

QPCR was used to assess relative expression of fathead minnow genes coding for transferrin, cytochrome P450 51 (cytP51; EC 1.14.13.70), pre-proguanylin (PPG), fibroin-like substance (FLS), vitellogenin, steroidogenic acute regulatory protein (StAR) and sterol response element binding protein 2 (SREBP-2). The intent of the QPCR analyses was to evaluate–validate selected microarray responses (in the case of transferrin, cytP51, PPG, FLS and vitellogenin) or investigate specific hypotheses (in the case of StAR and SREBP-2). In addition, QPCR was used to assess gene expression responses in the liver of female fathead minnows at the additional concentration (6 $\mu g\ l^{-1}$) and time-point (24 h) tested. Total RNA was isolated from whole liver or brain tissue of individual females as described for the microarray analysis (Larkin *et al.*, 2007). In the case of females from the 7 days control and 60 $\mu g\ l^{-1}$ fadrozole treatments, the total RNA samples used for QPCR were aliquots of the same total RNA samples analysed by microarray but neither pooled nor cyanine 5 labelled. Gene-specific primers were

designed based on partial cDNA sequences (StAR and transferrin) or expressed sequence tags (ESTs) available in GenBank (Table I) using PrimerExpress software (Applied Biosystems, Foster City, CA, U.S.A.). ESTs corresponding to fathead minnow genes of interest were identified by nucleotide–nucleotide BLAST (BLASTn) search of the *P. promelas* EST database using the top NT database hits from the microarray annotation as a query sequence (Table I). In the case of SREBP-2, which was not represented on the microarray, the amino acid sequence for pufferfish *Tetraodon nigroviridis* Marion de Procé, 1822; CAG01649), which had significant homology to mouse SREBP-2 (AAK54763), was used as a query sequence in a translated BLASTn search of the *P. promelas* EST database (Table I). Amplification of 18S ribosomal RNA (18S) sequences for normalization was performed using QuantumRNA Universal 18S Primer Mix (Ambion, Austin, TX, U.S.A.). Total RNA from the samples was reverse transcribed with 50 μM random hexamers and 50 μM oligo $d(T)_{23}VN$, using reverse transcriptase reagents and the protocol recommended by Applied Biosystems. QPCR reactions were carried out in duplicate in a 20 μl reaction volume consisting of 10 μl 2× HS DyNAmo SYBR green mix, 0·5 μl each forward and reverse primer (10 μM) (Table I), 7 μl nuclease-free water and 2 μl cDNA. Amplification was carried out with 40 cycles of 95° C for 10 s, 60° C for 20 s and 72° C for 20 s. Target fluorescence was measured at the end of the 72° C step for each cycle. The cDNA expression levels for all samples were normalized to 18S expression levels. Relative expression values for each sample were calculated using the 2Δct method (Ramakers *et al.*, 2003).

RESULTS

GONADAL-SOMATIC INDEX, VITELLOGENIN AND FECUNDITY

Fadrozole exposure did not elicit treatment-related differences in body or tissue masses or gonadal-somatic index. Exposure to fadrozole for 24 h or 7 days caused a significant decrease in plasma vitellogenin in female fathead minnows [Fig. 1(a)]. The degree of depression in the 60 μg l^{-1} treatment group was greater after 7 days than that observed after 24 h, suggesting that the effect was dependent on both concentration and duration of exposure. Spawning and fecundity data were not sufficient to support robust statistical analysis. Nonetheless, analysis of total spawns and total fecundity by treatment group suggests that 7days of fadrozole treatment lowered reproductive output [Fig. 1(b)].

MICROARRAY ANALYSIS – BRAIN

The 2000 gene fathead minnow microarray was used to examine gene expression changes in whole brains from female fathead minnows exposed to 60 μg l^{-1} fadrozole for 7 days (GEO series accession no. GSE10722). Of the *c.* 2000 genes surveyed, 27 unique genes were identified as differentially expressed using the statistical criteria defined for this study (Table II and Appendix 1). Two different probe sequences for cytochrome *b* and putative Δ-6 fatty acyl desaturase were among the list of genes identified as differentially expressed in brain. Among the unique genes, 20 were up-regulated, while just seven were down-regulated. Estimated fold change, either up or down, relative to controls tended to be small. Among those genes identified as significantly differentially expressed, the gene coding for the isoform of aromatase predominantly expressed in brain showed the greatest fold down-regulation (−2·96), while the

TABLE I. Nucleotide sequences of primers used for quantitative real-time PCR (QPCR)

Gene coding	Query sequence[a]	Fathead minnow sequence accession numbers		Primer sequence5′ → 3′	Amplificon length
cytP51	NM_001001730	DT309699	F	GCCATCCTCATCATGGTCATTAT	66
			R	TGCTGGACCGCTGTCTGA	
Fibroin-like substance	AF309416	DT355656	F	AGAGACTGTGCTGGAGGACATAAAT	71
			R	CATGAAAACAGGTGGCACACAT	
Pre-proguanylin	BC067691	DT353877	F	CGCCCTGTGTGTCGCTTT	72
			R	ATCGCCTTCCTGGACTTGTACA	
SREBP-2	CAG01649[b]	DT285479	F	CGGTAGCGCTTCTCGATGAT	63
			R	CGGGAGGAGTCGTGAAAGAG	
StAR	N/A	DQ60497[c]	F	CTTGAACAGCAAACAGATGACCTT	62
			R	CTCCCCCATTTGTTCCATGT	
Transferrin	N/A	DQ676851[d]	F	AAGCTGGTGACGTTGCTTT	79
			R	TTTCTTTGCCCATTCTGGTC	
Vitellogenin	N/A	AF130354[e]	F	CACAATCCCAGCTCTGCGTGA	106
			R	TGGCCTCTGCAGCAATATCAT	

cytP51, cytochrome P450 51; EST, expressed sequence tag; SREBP-2, sterol response element binding protein 2; StAR, steroidogenic acute regulatory protein.
[a]Query sequence refers to a known cDNA sequence for the gene of interest that was used for a BLASTn search of the *Pimephales promelas* EST database in order to identify putative partial cDNA sequences specific for fathead minnow.
[b]An amino acid sequence used for a tBLASTn search of the *P. promelas* EST database.
[c]Villeneuve *et al.* (2007).
[d]Wintz *et al.* (2006).
[e]Korte *et al.* (2000).

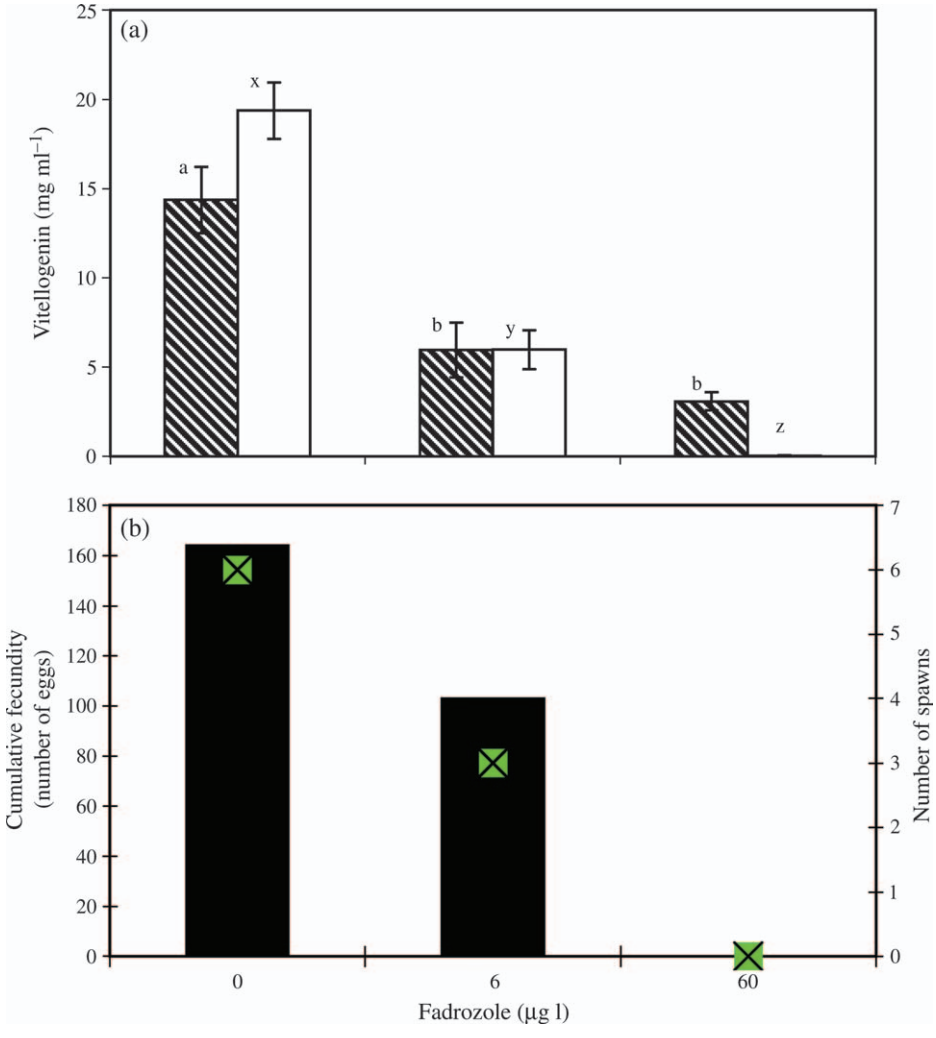

FIG. 1. (a) Plasma vitellogenin concentrations for female fathead minnows exposed to Lake Superior water (control, 0), 6 or 60 μg l^{-1} fadrozole for 24 h (▨) or 7 days (□) and (b) cumulative fecundity and numbers of spawns for corresponding pairs of male and female fathead minnows exposed for 7 days. Error bars = standard error ($n = 8$). Different letters indicate statistically significant differences among treatments for a given experiment (24 h or 7 days; $P < 0{\cdot}05$). Cumulative fecundity and total numbers of spawns are totals across all replicate pairs for each treatment group. ■, number of eggs; ⊠, number of spawns.

gene coding for 7-dehydrocholesterol reductase showed the greatest fold up-regulation (2·06).

The majority of genes identified as differentially expressed had some degree of functional annotation. Using the GO enrichment criteria defined for this study, no GO categories were identified as significantly down-regulated in the brain of fish exposed to fadrozole, even using an expanded gene list based

TABLE II. Genes identified as differentially expressed* in the brain of female fathead minnows exposed to 60 μg l^{-1} fadrozole for 7 days

	Probe ID	Gene name	Duplicate probe	
			Average fold†	Average *P*†
1	EA_Pp_10085	Cytochrome P450 aromatase (brain isoform)	−2·72	0·0081
2	EA_Pp_10078	Cytochrome *b*	−1·65	0·0256
3	EA_Pp_10343	14 kDa apolipoprotein	−1·58	0·0277
4	EA_Pp_11183	Angio tensinogen precursor	−1·80	0·0474
5	EA_Pp_13140	NADH dehydrogenase subunit 1	−1·47	0·0347
6	EA_Pp_10454	Cytochrome *b*	−1·53	0·0219
7	EA_Pp_10492	Unknown (protein for MGC:103693) (*Danio rerio*)	−1·32	0·0361
8	EA_Pp_12674	Complement C3-H1 (*Cyprinus carpio*)	−1·23	0·0338
9	EA_Pp_11744	Similar to heterogeneous nuclear ribonucleoprotein C (C1/C2)	1·34	0·0473
10	EA_Pp_11389	Potassium channel tetramerization domain containing 10 (*Danio rerio*)	1·31	0·0361
11	EA_Pp_11679	Zgc:55461 (*Danio rerio*), beta tubulin	1·54	0·0104
12	EA_Pp_11363	Similar to heterogeneous nuclear ribonucleoprotein H (hnRNP H)	1·24	0·0498
13	EA_Pp_12277	Hypothetical protein MGC63587 (*Danio rerio*)	1·35	0·0484
14	EA_Pp_12559	Zgc:56546, ribosomal protein L18a	1·52	0·0053
15	EA_Pp_13257	Unknown (protein for IMAGE:7432353) (*Danio rerio*)	1·37	0·0262
16	EA_Pp_11687	Cct4 protein	1·47	0·0487
17	EA_Pp_10659	Alpha-tubulin	1·48	0·0306
18	EA_Pp_11955	Tubulin, alpha 8 like (*Danio rerio*)	1·50	0·0360
19	EA_Pp_12239	Unknown (protein for MGC:114731) (*Xenopus laevis*)	1·50	0·0372
20	**EA_Pp_13324**	**14α-demethylase (*Danio rerio*) cytochrome P450, family 51**	**1·61**	**0·0298**
21	EA_Pp_12217	Transmembrane protein 49 (*Danio rerio*)	1·53	0·0475
22	EA_Pp_11608	Cyclase-associated protein-1	1·52	0·0397
23	EA_Pp_10854	S-adenosylhomocysteine hydrolase (*Danio rerio*)	1·54	0·0485
24	EA_Pp_10952	Glucose phosphate isomerase a	1·56	0·0240
25	EA_Pp_10090	Putative Δ-6 fatty acyl desaturase (*Cyprinus carpio*)	1·78	0·0297
26	EA_Pp_10720	Heat shock cognate 70 kDa protein (*Carassius auratus gibelio*)	1·67	0·0321

TABLE II. Continued

	Probe ID	Gene name	Duplicate probe	
			Average fold†	Average *P*†
27	EA_Pp_11292	Putative Δ-6 fatty acyl desaturase (*Cyprinus carpio*)	1·70	0·0472
28	EA_Pp_12035	Farnesyl diphosphate synthase	2·11	0·0067
29	EA_Pp_12430	7-dehydrocholesterol reductase	1·72	0·0457
a	EA_Pp_10371	Lanosterol synthase (oxidosqualene–lanosterol cyclase)	1·52	0·1300
b	EA_Pp_14181	Diphosphomevalonate decarboxylase zgc:100824 (*Danio rerio*)	2·13‡	0·0430‡
c	EA_Pp_11177	HMG-CoA reductase isoform 1 (*Danio rerio*)	1·56	0·2700

HMG-CoA, hydroxymethylglutaryl-CoA. Additional data and annotations for all genes identified can be found in Appendix 1.

a, b, c: Genes shaded in grey did not meet the criteria for identification as differentially expressed but are shown here to support discussion of a putative effect on the cholesterol synthesis pathway.

Bold indicates expression was also examined by quantitative real-time PCR.

*Average *P*-value for duplicate probes <0·05; if data were only available for one probe, a Benjamini and Hochberg false discovery rate <0·1 was used as a cut-off.

†The average fold change and *P*-values for the two duplicate probes (same oligonucleotide sequence) spotted on the microarray are provided.

‡Data were only available for one probe, individual probe fold-change and *P*-value reported.

on the relaxed differential expression criteria ($n = 47$ genes). However, a number of up-regulated GO categories were identified. Prominent biological processes represented by the genes up-regulated in brain of fadrozole-exposed fish included lipid biosynthesis–metabolism as well as protein folding and polymerization (Table III). Specific up-regulated genes associated with lipid biosynthesis included putative Δ-6 fatty acyl desaturase (multiple probes), farnesyl diphosphate synthase and 7-dehydrocholesterol reductase. Genes coding for heat-shock cognate 70 kDa, Cct4 protein, beta tubulin and unknown protein MGC:114731 were associated with protein polymerization and, or folding. The same GO categories, as well as other related categories, were identified as enriched when an expanded gene list based on the relaxed differential expression criteria was analysed ($n = 61$ genes), adding confidence to the functional analysis of genes up-regulated in brain.

MICROARRAY ANALYSIS – LIVER

Of the *c.* 2000 genes surveyed in the livers of females exposed to 60 μg l^{-1} fadrozole for 7 days (GEO series accession no. GSE10722), 41 unique genes were identified as significantly up-regulated using the statistical criteria defined for this study (Appendix 2). Two different probe sequences for a 14 kDa apoplipoprotein were identified as up-regulated (Table IV). Transcripts that hybridized to 69 different oligonucleotide probe sequences were identified as significantly down-regulated (Appendix 3). However, 19 of those probe sequences represented multiple probes for the same gene, vitellogenin. Additionally, three different probe sequences for CG5020-PA isoform A and two different probe sequences for fast muscle troponin I and runt-related transcription factor b, were among the list of genes down-regulated in liver. Overall, 46 unique genes were identified as down-regulated.

The range of fold change observed for genes identified as differentially expressed in liver was greater than that observed for brain. While some differentially expressed genes had fold changes as little as 1·4, transducer of ERBB2 was down-regulated nearly 20-fold relative to controls, while type 1 cytokeratin (similar to zgc:109868) was up-regulated 8·23-fold (Table IV). Averaging across multiple probes, vitellogenin was the most down-regulated gene (*c.* 43 ± 17-fold), but the fold change estimate varied significantly with probe sequence (Appendix 3).

Around 70% of the genes differentially expressed in liver had some degree biological process GO annotation. Based on that annotation, there was significant enrichment of genes associated with immune–inflammatory response and endocytosis among those identified as up-regulated in the liver of fadrozole-exposed fish (Table V). Enrichment in these categories was driven largely by the up-regulation of three isoforms of complement C3 (Table IV). GO analysis based on an expanded list of putative up-regulated genes ($n = 161$ using relaxed differential expression criteria) supported the results of the more conservative analysis, identifying immune response and complement activation as enriched categories. Additionally, the expanded analysis suggested that genes associated with carbohydrate metabolism, specifically gluconeogenesis were among those potentially up-regulated in response to fadrozole treatment.

TABLE III. Statistically 'enriched' gene ontology categories[a] (biological process only) associated with genes identified as up-regulated in the brains of female fathead minnows exposed to 60 μg l^{-1} fadrozole for 7 days, based on conservative[b] (no shading) or relaxed[c] (shaded) criteria

	Category	Genes in category on microarray	Percentage on microarray in category	Genes in list in category	Percentage of genes in list in category	*P*-value
Brain (up-regulated)	GO:8610: lipid biosynthesis	61	1·7	4	22·2	0·000204
	GO:51258: protein polymerization	34	1·0	3	16·7	0·0006
	GO:6457: protein folding	67	1·9	3	16·7	0·00432
	GO:6629: lipid metabolism	142	4·0	4	22·2	0·0049
	GO:8610: lipid biosynthesis	61	1·7	7	12·5	3·82E-05
	GO:6457: protein folding	67	1·9	7	12·5	7·07E-05
	GO:51258: protein polymerization	34	1·0	5	8·9	0·000163
	GO:19538: protein metabolism	820	23·2	24	42·9	0·000788
	GO:6694: steroid biosynthesis	16	0·5	3	5·4	0·00182
	GO:46394: carboxylic acid biosynthesis	38	1·1	4	7·1	0·00279
	GO:6996: organelle organization and biogenesis	168	4·7	8	14·3	0·00441
	GO:8152: metabolism	1898	53·7	40	71·4	0·00473
	GO:7010: cytoskeleton organization and biogenesis	105	3·0	6	10·7	0·00576
	GO:6629: lipid metabolism	142	4·0	7	12·5	0·00645
	GO:9058: biosynthesis	399	11·3	13	23·2	0·00788
	GO:902: cellular morphogenesis	52	1·5	4	7·1	0·00868

GO, gene ontology.

[a]Statistical enrichment defined as hypergeometric $P < 0{\cdot}01$ with at least three genes in list in category; to accommodate display in the table, highly redundant gene ontology categories with identical or strongly overlapping gene representation are not shown.

[b]Average *P*-value for duplicate probes <0·05 ($n = 21$; Table II; Appendix 1).

[c]*P*-value for a single probe <0·05, no conflicting fold-change data for duplicate or multiple probes ($n = 61$; Appendix 4).

TABLE IV. Selected genes identified as differentially expressed[a] in the liver of female fathead minnows exposed to 60 μg l^{-1} fadrozole for 7 days

Numbers[b]	Probe ID	Gene name	FDR[a]	Average fold[c]	Average P[c]
2	EA_Pp_14382	Receptor (calcitonin) activity modifying protein 2 isoform 2 (*Danio rerio*)	<0·05	1·47[d]	7·07E-05[d]
7	**EA_Pp_14267[e]**	***Cyprinus carpio* transferrin variant A mRNA, complete cds ($n = 2$)**	**<0·05**	**2·79[d]**	**0·000412[d]**
10	EA_Pp_12706	Complement C3-S (*Cyprinus carpio*)	<0·05	2·10	0·034241
12	EA_Pp_13543	Pentraxin (*Salmo salar*)	<0·05	2·24	0·000353
16	EA_Pp_13640[e]	14 kDa apolipoprotein (*Carassius auratus gibelio*) ($n = 2$)	<0·05	1·53	0·004594
22	EA_Pp_12750	Complement C3 (*Ctenopharyngodon idella*)	<0·10	2·98	0·002138
29	EA_Pp_12148	Complement C3-Q2	<0·10	2·45	0·028423
30	**EA_Pp_13491**	**Preprorenoguanylin (*Anguilla japonica*)**	**<0·10**	**2·12**	**0·007282**
38	EA_Pp_11124	zgc:109868 similar to type I cytokeratin, enveloping layer	<0·10	5·47	0·009219
8	EA_Pp_14138	Transducer of ERBB2, 1a, mRNA, *Danio rerio* (tob1a)	<0·05	−19·25[d]	0·00021[d]
9	EA_Pp_11059	Fast muscle troponin I	<0·05	−3·74[d]	0·00022[d]
12	EA_Pp_14804	Zgc:92215 (*Danio rerio*) >gi|52219026|ref|NP_001004586.1|	<0·05	−2·50[d]	0·00033[d]
21	EA_Pp_14257[e]	Runt-related transcription factor b (*Danio rerio*) ($n = 2$)	<0·05	−1·57	0·00194
29	EA_Pp_14778	ADAM metallopeptidase domain 12 (meltrin alpha)	<0·10	−4·23[d]	0·00198[d]
32	EA_Pp_14157[e]	CG5020-PA, isoform A (*Danio rerio*) ($n = 3$)	<0·10	−2·21	0·00125
34	EA_Pp_11809	Filamin A	<0·10	−2·69	0·00454
40	EA_Pp_13990	Cholinergic receptor, nicotinic, alpha polypeptide 1 (*Danio rerio*)	<0·10	−1·42[d]	0·00329[d]
44	**EA_Pp_14703**	***Cyprinus carpio* ovarian fibroin-like substance-3 mRNA**	**<0·10**	**−4·08[d]**	**0·00397**
51	EA_Pp_11051[e]	Vitellogenin 3 precursor (*Danio rerio*) ($n = 5$)	<0·05	−15·57	0·01022
60	EA_Pp_11042[e]	Vitellogenin precursor (*Pimephales promelas*) ($n = 14$)	<0·05	−50·87	0·00008

Additional data and annotations for all genes identified can be found in Appendices 2 and 3.

Bold indicates expression was also examined by quantitative real-time PCR.

[a]Benjamini and Hochberg false discovery rate (FDR).

[b]Provides cross reference to corresponding entry in Appendices 2 (upregulated) or 3 (downregulated).

[c]The average fold change and P-values for the two duplicate probes (same oligonucleotide sequence) spotted on the microarray are provided.

[d]Data were only available for one probe, individual probe fold-change and P-value reported.

[e]Multiple probes for the same gene ($n = x$ in gene name column indicates how many) were identified as differentially expressed using FDR <0·1 as a cut-off, only one representative probe is listed here.

TABLE V. Statistically 'enriched' gene ontology categories[a] (biological process only) associated with genes identified as differentially expressed in the livers of female fathead minnows exposed to 60 μg l^{-1} fadrozole for 7 days, based on conservative[b] (no shading) or relaxed[c] (shaded) criteria

	Category	Genes in category on microarray	Percentage on microarray in category	Genes in list in category	Percentage in list in category	*P*-value
Liver (up-regulated)	GO:6897: endocytosis	47	1·3	4	13·8	0·000509
	GO:50776: regulation of immune response	32	0·9	3	10·3	0·00209
	GO:50727: regulation of inflammatory response	32	0·9	3	10·3	0·00209
	GO:6956: complement activation	84	2·4	11	8·5	0·000203
	GO:6955: immune response	131	3·7	14	10·8	0·000253
	GO:5975: carbohydrate metabolism	154	4·4	14	10·8	0·00132
	GO:9968: negative regulation of signal transduction	47	1·3	7	5·4	0·00142
	GO:6094: gluconeogenesis	12	0·3	3	2·3	0·00836
Liver (down-regulated)	GO:6869: lipid transport	129	3·6	19	38·8	9·37E-16
	GO:51179: localization	1140	32·2	34	69·4	8·81E-08
	GO:6810: transport	1100	31·1	32	65·3	7·24E-07
	GO:7519: striated muscle development	25	0·7	3	6·1	0·00464
	GO:6412: protein biosynthesis	172	4·9	17	12·3	0·000296
	GO:9058: biosynthesis	399	11·3	26	18·8	0·00516

GO, gene ontology.

[a]Statistical enrichment defined as hypergeometric $P < 0{\cdot}01$ with at least three genes in list in category; to accommodate display in the table, highly redundant gene ontology categories with identical or strongly overlapping gene representation are not shown.

[b]Average *P*-value for duplicate probes <0·05, Benjamini and Hochberg false discovery rate <0·1 ($n = 42$ up; $n = 69$ down; Appendices 2 and 3, respectively).

[c]*P*-value for a single probe <0·05, no conflicting fold-change data for duplicate or multiple probes ($n = 161$ up, Appendix 5; $n = 187$ down, Appendix 6).

GO analysis based on the more conservative differential expression criteria identified lipid transport, localization, transport and striated muscle development as categories enriched in the list of genes down-regulated in the liver of fadrozole-exposed fish (Table V). Down-regulated genes ostensibly associated with striated muscle development included those coding for filamin B, cholinergic receptor and ADAM metallopeptidase domain 12. Enrichment of lipid transport was solely because of the 19 probes for vitellogenin included on that list. The more general transport and localization categories were associated with the 19 probes for vitellogenin plus 10 other unique down-regulated genes. However, GO analyses based on an expanded, although non-redundant, list did not identify similar terms as enriched. Based on the expanded list, genes associated with biosynthesis, particularly protein biosynthesis, were disproportionately down-regulated in the livers of fadrozole-exposed fish.

QPCR ANALYSES

QPCR was used to examine the relative expression of six genes in the brains of fadrozole-exposed *v.* control female fathead minnows (Fig. 2). Among the genes examined, cytP51 (14α-demethylase) was the only one that was identified as differentially expressed in brain (Table II). When individual (non-pooled) RNA samples ($n = 6$) were analysed by QPCR, the fold-change in relative abundance of cytP51 transcripts was similar (1·44 *v.* 1·5–1·6 by microarray), but the difference in means was not statistically significant. Overall, no significant alterations in transcript abundance were detected when individual brain RNA samples were analysed using QPCR.

QPCR analysis of the relative abundance of selected transcripts in liver was more comprehensive. Expression of cytP51, FLS, PPG, transferrin, StAR and SREBP-2 at both time points (24 h and 7days), and all exposure concentrations (0, 6 and 60 $\mu g\, l^{-1}$ fadrozole) was examined (Fig. 3). Vitellogenin expression was evaluated for all three concentrations tested, but only at the 7 days time point. Fadrozole exposure caused significant up-regulation of transferrin expression after both 24 h and 7 days of exposure. Conversely, the relative abundance of vitellogenin transcripts was significantly reduced by fadrozole exposure. Although cytP51 expression in liver was not significantly affected after 7 days of exposure, cytP51 transcripts were significantly less abundant in the livers of females exposed for 24 h. For the remainder of the genes examined, there were no statistically significant differences in mean transcript abundance.

DISCUSSION

FATHEAD MINNOW OLIGONUCLEOTIDE MICROARRAYS

The microarray used for this study was the first generation of high-density oligonucleotide microarrays developed for the fathead minnow, a species with a long history of use in aquatic toxicology research and regulation (Ankley & Villeneuve, 2006). At the time of its initial development, very few fathead minnow cDNA sequences or ESTs were publicly available and only a 200 gene macroarray had been developed (Klaper *et al.*, 2006; Larkin *et al.*, 2007). Thus, the

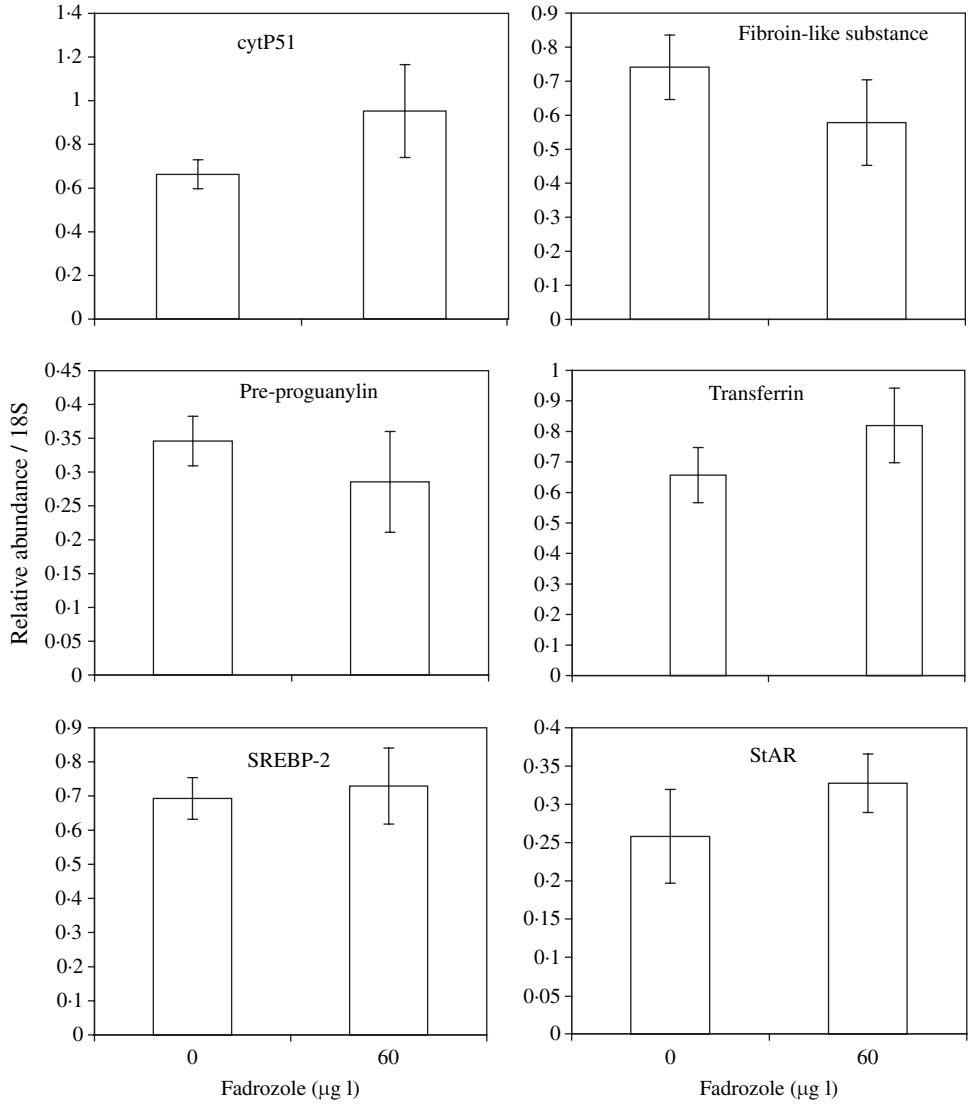

FIG. 2. Transcript abundance of six genes, relative to 18S ribosomal RNA abundance, in whole brains from female fathead minnows exposed to Lake Superior water (control, 0) or 60 μg l^{-1} fadrozole for 7 days as determined by quantitative real-time PCR. Error bars = standard error ($n = 6$). cytP51, cytochrome P450 51 (14α-demethylase); SREBP-2, sterol response element binding protein 2; StAR, steroidogenic acute regulatory protein.

2000 gene microarray, which was commercially available to the scientific community starting in June 2005, was a significant step forward in facilitating both ecotoxicogenomic and more general transcriptomic research with this species.

However, the technology has progressed rapidly. Subsequent to the initial development and application of the 2000 gene microarray (Larkin *et al.*, 2007; Villeneuve *et al.*, 2007; this study), the Joint Genome Institute sequenced

250 000 cDNA clones from fathead minnows and made the ESTs publicly available in August 2005 (NCBI nucleotide database). Clustering of these EST sequences and addition of more recent cDNA sequences submitted to GenBank yielded over 22 000 unique fathead minnow gene sequences that have been incorporated into a new generation of high-density fathead minnow oligonucleotide microarrays available in a variety of formats (http://www.ecoarray.com). These include 22 000 gene microarrays with *c.* 80% of the genes matched to named genes found in publicly available databases, as well as 15 000 gene microarrays that exclude all 'no hit' sequences and duplicates (by accession number). As far as is known, no attempts have been made to validate the use of the fathead minnow microarrays for other species. However, a number of ecotoxicogenomic studies with these higher density commercial fathead minnow microarrays have been conducted (D. Tillitt, pers. comm.; N. Denslow, pers. comm.; B. Carter, pers. comm.), and we expect that the 22 000 gene and 15 000 gene fathead minnow microarrays will soon be fully described in the peer-reviewed literature. Additionally, public availability of the extensive library of fathead minnow ESTs has made it feasible for other investigators and institutions to design and print high-density fathead minnow oligonucleotide microarrays suited to specific research objectives and needs.

EFFICACY OF FADROZOLE EXPOSURE

Based on its mode of action, inhibition of aromatase activity, fadrozole would be expected to reduce circulating concentrations of oestradiol in exposed fish. Data collected in this study suggest that fadrozole was acting in a manner consistent with its expected mode of action, causing effects similar to those observed in previous experiments with the chemical. For example, fadrozole exposure caused significant, concentration-dependent reductions in plasma vitellogenin concentrations and reduced both the number of spawning events and number of eggs produced by exposed fish. This was consistent with a previous 21 days reproduction test in which fadrozole inhibited brain aromatase activity and reduced plasma concentrations of oestradiol and vitellogenin, vitellogenin uptake into ooctyes and fecundity of the exposed fish (Ankley *et al.*, 2002). Additionally, microarray results from this study indicated significant down-regulation of the gene coding for the brain isoform of aromatase (cytP19B). This effect was consistent with previous work by Villeneuve *et al.* (2006) as well as reports that cytP19B has an oestrogen response element in its promoter region (Callard *et al.*, 2001; Kazeto *et al.*, 2001). Additional evidence of the efficacy of the fadrozole exposure and its impacts on oestradiol concentrations were provided by gene expression results for liver. Most notably, both microarray and QPCR results showed significant down-regulation of vitellogenin gene expression in the liver. Oestrogen-dependent transcription and translation of vitellogenin has been well established (Arukwe & Goksøyr, 2003). Furthermore, reduced vitellogenin gene expression agrees with both the results of Ankley *et al.* (2002) and the plasma vitellogenin concentrations measured in this study. Transferrin was among the genes identified as up-regulated in the livers of fadrozole-exposed females. Transferrin is a key iron transport protein in plasma (Dunn *et al.*, 2007). In medaka, the 5′-promoter region of the transferrin gene contains

an oestrogen-responsive element (Mikawa *et al.*, 1996), and previous studies with both the fathead minnow and sheepshead minnow reported down-regulation of transferrin expression following oestrogen exposure (Denslow *et al.*, 2001; Larkin *et al.*, 2003, 2007), opposite the effect observed in the fadrozole-exposed fish. As a whole, the consistency of these results with both previous experiments and fadrozole's anticipated mode of action provides strong evidence for the efficacy of the fadrozole exposure.

QPCR VALIDATION

Relative transcript abundance of five of the genes identified as differentially expressed in either brain or liver, based on microarray analysis, was examined by QPCR. Identification of vitellogenin and transferrin as significantly down-regulated and up-regulated in liver, respectively, was supported by consistent, statistically significant effects measured by QPCR. However, the up-regulation of cytP51 (14α-demethylase) in brain and PPG in liver were not supported. The mean transcript abundance of cytP51 in the brain and PPG in the liver of fadrozole-treated fish was somewhat greater than that in control fish; however, because of the variability of the QPCR data the differences were not statistically significant. The QPCR results for FLS in liver indicated non-significant up-regulation of this gene and appeared to contradict the microarray results indicating significant down-regulation, suggesting that the response may be a false positive.

As much as the QPCR results raise questions regarding the validity of specific microarray responses, they raise some question about the validity of the comparison itself. At least in this study, there were numerous factors that could confound meaningful comparison of the statistical significance of the QPCR and microarray results. For example, the microarray analyses were based on three replicate-pooled RNA samples (RNA from two fish per pool) per treatment, while QPCR analyses were conducted on total RNA samples from six to eight individual fish per treatment. Results for cytP51 in brain and PPG in liver suggest that the pooling of RNA samples for the microarray analysis may have masked some of the fish-to-fish variation, resulting in different statistical conclusions for the QPCR and microarray results. Statistical analysis of virtual pools generated by averaging the QPCR data from each pair of samples pooled in the microarray analyses altered the statistical conclusion for PPG expression in the liver (Fig. 3) but not cytP51 expression in brain. This suggested that pooling alone contributed to some of the statistical discrepancies, although not all. While the same RNA samples were used for both analyses, pooled aliquots used for the microarray analyses were reverse transcribed with a poly dT-T7 primer, whereas aliquots used for QPCR analysis were reverse transcribed with random hexamers and oligo $d(T)_{23}$ VN. Furthermore, QPCR responses were normalized to 18S ribosomal RNA, while microarray data were globally normalized to a reference sample on a per-gene basis (Larkin *et al.*, 2007). Finally, hybridization efficiencies for the probes spotted on the microarray may differ from those for the primers in a QPCR reaction well. Given these confounding factors, corroborating results can provide a considerable measure of confidence, while a lack of statistical agreement between the two measures

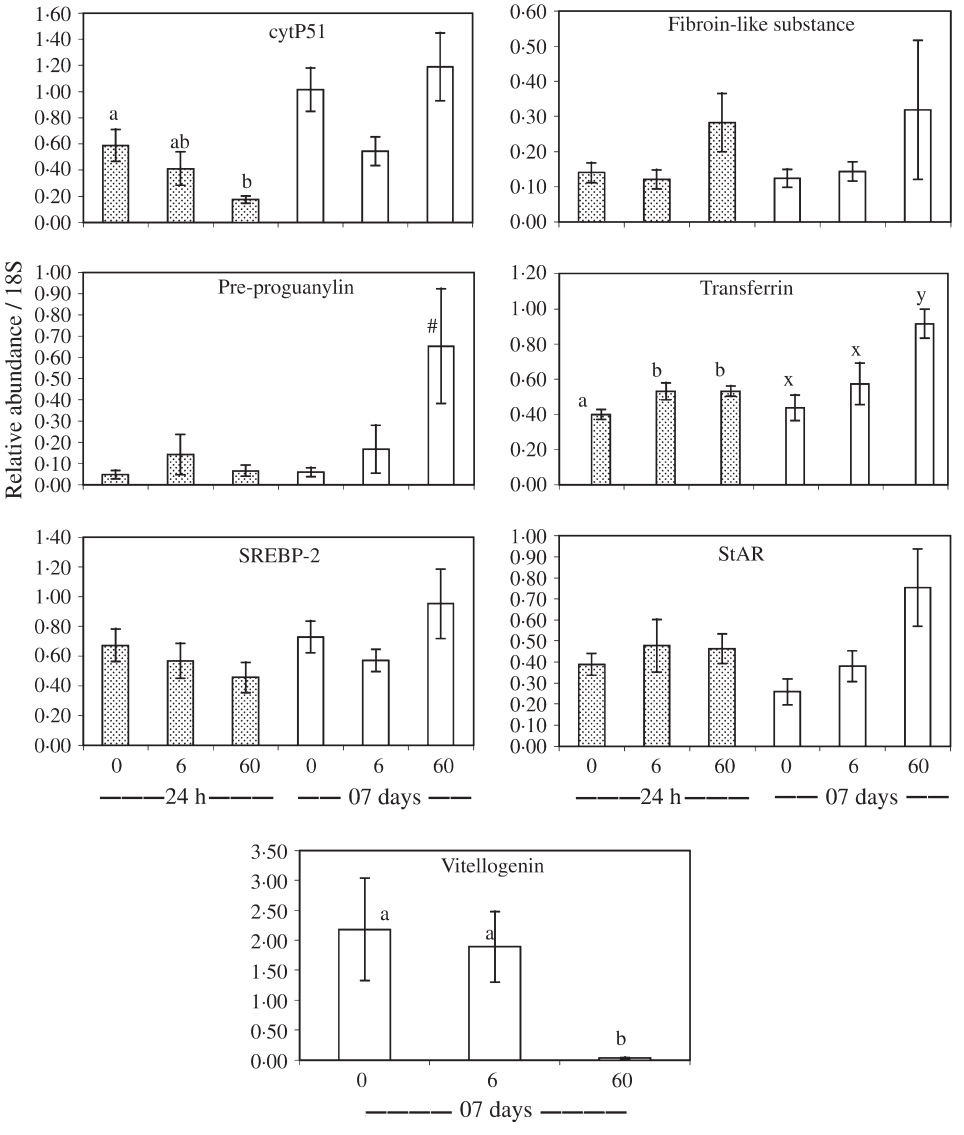

FIG. 3. Transcript abundance of seven genes, relative to 18S ribosomal RNA abundance, in livers from female fathead minnows exposed to Lake Superior water (control, 0), 6 or 60 μg l^{-1} fadrozole for 24 h or 7 days as determined by quantitative real-time PCR. Error bars = standard error (n = 6–8). Different letters indicate statistically significant differences among treatments for a given experiment (24 h or 7 days; $P < 0{\cdot}05$). # indicates a significant difference ($P < 0{\cdot}05$) from control following virtual pooling by averaging data for the same samples pooled for the microarray analysis ($n = 3$). cytP51, cytochrome P450 51 (14α-demethylase); SREBP-2, sterol response element binding protein 2; StAR, steroidogenic acute regulatory protein.

may neither support nor refute a given response. The more these types of confounding factors can be controlled and accounted for, the more informative such comparisons will be. Overall, the authors feel that their results highlight

the need to recognize that a portion of the 'significant' responses identified may be erroneous.

UNSUPERVISED ANALYSIS

The primary aim of this study was to take advantage of the microarray's capabilities for unsupervised analysis to identify some of the novel molecular responses to fadrozole as a foundation for developing hypotheses that could be investigated in future experiments. In an effort to minimize the reporting of false positives, while still allowing for the identification of some putative differentially expressed genes (*i.e.* controlling false negative rates), a moderately stringent criterion was applied for defining differentially expressed genes. While this approach should have identified those genes least likely to be false positives, the variability common in microarray experiments, particularly those based on relatively small sample sizes, dictates that one should not ascribe too much significance or confidence to any individual response, as was reinforced by the QPCR results for FLS (above). Consequently, analyses that consider multiple microarray responses (*e.g.* gene ontology and pathway-based analyses) and weight of evidence (*e.g.* information in the literature; significance of multiple probes for the same gene) should be more reliable than examination of the genes on a list in isolation, and ultimately microarray responses should be replicated in additional hypothesis-driven experiments.

With these caveats in mind, the microarray analyses did yield a few hypotheses that were based on the weight of evidence of multiple microarray responses. For example, one of the most compelling and internally consistent responses was the up-regulation of multiple enzymes associated with the cholesterol biosynthesis pathway (Fig. 4). Genes coding for three enzymes in the cholesterol synthesis pathway, lanosterol synthase (EC 5.4.99.7), 14α-demethylase (cytP51; EC 1.14.13.70) and 7-dehydrocholesterol reductase (EC 1.3.1.21), were identified as significantly up-regulated in brain after 7 days of exposure to 60 $\mu g\ l^{-1}$ fadrozole. Further evaluation of the microarray annotation and data revealed that genes coding for diphosphomevalonate decarboxylase (EC 4.1.1.33) and farnesyl diphosphate synthase (EC 2.5.1.1) may also have been up-regulated (at least one probe $P < 0{\cdot}05$). Overall, based on the available annotation and data, the gene coding for hydroxymethylglutaryl-CoA reductase (EC 1.1.1.34) was the only gene from the cholesterol synthesis pathway that was represented on the microarray but did not appear up-regulated in the brain of fadrozole-exposed fish ($P > 0{\cdot}25$). Genes involved in the cholesterol synthesis pathway along with that coding for putative Δ-6 fatty acyl desaturase were responsible for the statistical enrichment of lipid biosynthesis–metabolism identified by GO analyses. As a whole, the pattern of responses observed was suggestive of an overall up-regulation of cholesterol synthesis in the brain.

Cholesterol is both an important structural component of plasma membranes and a precursor for steroid hormones, sterols and bile acids (Chang *et al.*, 2006). Because most plasma lipoproteins cannot cross the blood–brain barrier, with the possible exception of receptor-mediated transcytosis, nearly all cholesterol in the brain is synthesized in the brain (Dehouck *et al.*, 1997; Dietschy & Turley, 2001; Chang *et al.*, 2006). At the same time, teleost fish

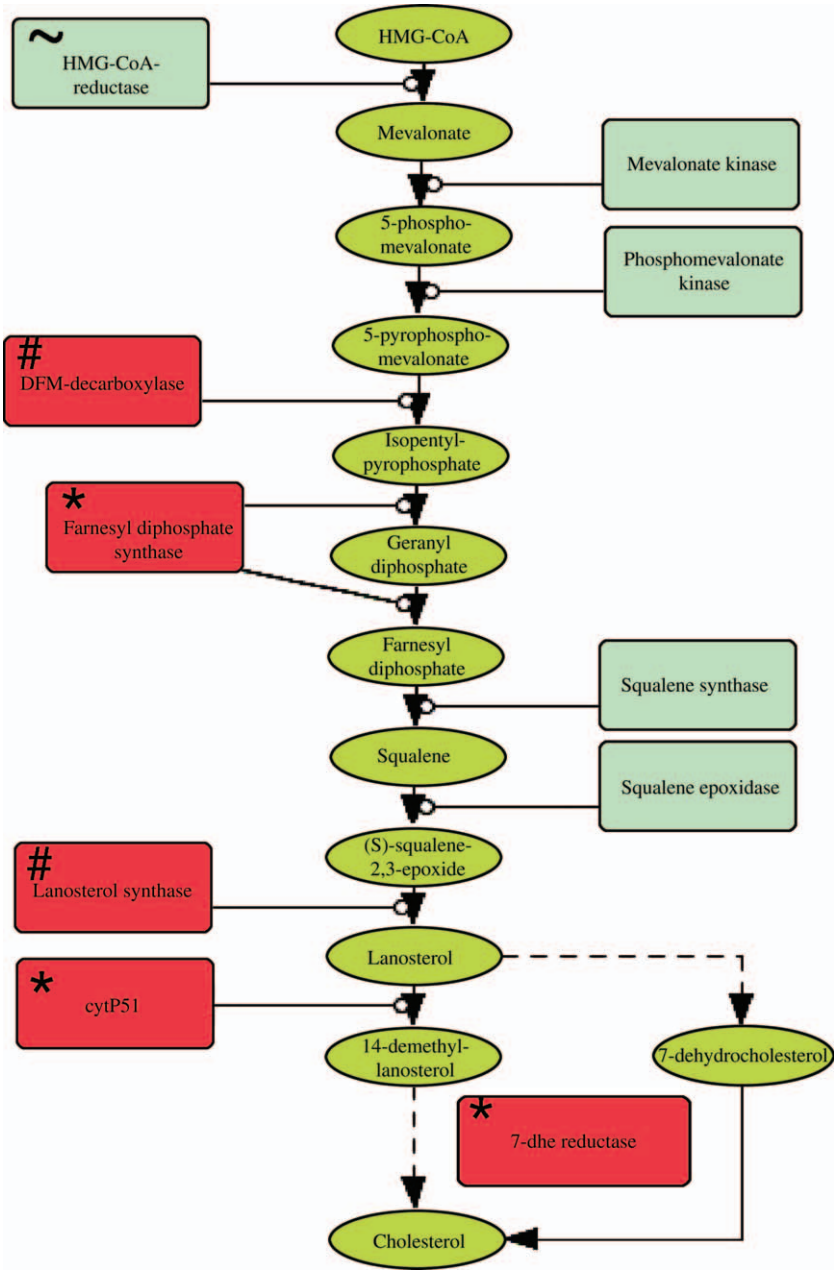

FIG. 4. Graphical model of the cholesterol synthesis pathway showing the role of five enzymes whose corresponding gene expression was up-regulated in the brains of female fathead minnows exposed to 60 $\mu g\, l^{-1}$ fadrozole for 7d (red boxes). * denotes genes that conformed to the criteria used to identify differentially expressed genes as part of this study. # denotes genes present on the microarray with at least one probe response identified as significant using a one-factor *t*-test ($P < 0{\cdot}05$). ~ denotes a gene that was present on the microarray but did not appear affected by fadrozole. Green boxes indicate other enzymes involved in the pathway whose genes were not represented on the microarray. Green ovals indicate cholesterol precursors.

are known to have very high brain aromatase activity relative to other vertebrates and it has been hypothesized that locally produced oestrogens modulate neurogenesis in adult fish (see reviews by Pellegrini *et al.*, 2005; Forlano *et al.*, 2006). Consequently, up-regulation of cholesterol synthesis in the brain could be viewed as part of a compensatory response to fadrozole's direct inhibition of brain aromatase activity.

Based on that view, the authors hypothesized that expression of StAR or SREBP-2, neither of which were represented on the 2000 gene microarray, might also be up-regulated as part of such a compensatory response. StAR plays a critical role in transporting cholesterol from the outer to inner mitochondrial membrane to support steroidogenesis (Stocco & Clark, 1996; Stocco, 2001). SREBPs are a class of transcription factors known to activate multiple genes associated with the cholesterol synthesis pathway, including those identified as differentially expressed based on the microarray results. However, based on QPCR analysis neither of these genes was significantly up-regulated in either the brain or liver of fadrozole-exposed female fathead minnows. Over time, additional focused investigation should help test the hypothesis that fadrozole or potentially other steroidogenesis inhibitors will elicit up-regulation of the cholesterol synthesis in brain and reveal the significance and relevance of that response.

A second hypothesis that emerged from examination of the differentially expressed gene lists relates to the down-regulation of cytochrome *b* and NADH dehydrogenase subunit 1 in brain. These genes are two of just 13 protein-coding genes found within the mitochondrial genome of teleost fish (Miya & Nishida, 1999; Kartavtsev *et al.*, 2007). Assuming all 13 of the protein-coding mitochondrial genes were present on the 2000 gene microarray, these genes would constitute just 0·65% of the genes present on the microarray. Given that they represent two of the seven (28·6%) unique genes identified as down-regulated in brain, the results suggest an enrichment of down-regulated genes in this class. While two genes in a class might still not seem particularly substantial, additional weight of evidence was provided by the fact that two different probe sequences provided corroborating evidence of an effect on cytochrome *b* expression. While by no means definitive, the microarray data provides a foundation for hypothesizing that fadrozole treatment affected oxidative phosphorylation and, or mitochondrial abundance in the brain of female fathead minnows.

In liver, the GO-based analyses indicated significant up-regulation of immune–inflammatory response, primarily associated with genes coding for complement factors, and down-regulation of rather general functional categories including localization, transport and possibly protein biosynthesis. Given the large magnitude of hepatic mRNA and protein production needed to support vitellogenesis (Arukwe & Goksøyr, 2003), one could hypothesize that inhibition of vitellogenin synthesis in female fathead minnows exposed to fadrozole could result in a detectable decrease in the overall amount of protein biosynthesis occurring in the liver, as the GO analyses suggest. If this were true, one would expect to see similar GO-based results in ecotoxicogenomic experiments with other endocrine active chemicals that reduce vitellogenin production in females. The up-regulation of inflammatory–immune response suggests possible tissue damage or trauma in the liver of fadrozole-exposed

fish. While overt liver toxicity was not an anticipated effect of fadrozole, the authors were not aware of any studies that have explicitly examined this possibility through histological analysis or other methods. Such examination may help reveal whether the complement response observed in this study was an indication of hepatotoxicity or part of a more general stress response to the chemical perturbation.

The hypotheses described above provide examples of utility of unsupervised microarray analysis as a tool for hypothesis generation. Pending further validation of the transcript-level responses and evaluation of their specificity, sensitivity and transience–persistence, some of the genes identified in this study may have utility as either markers of exposure to aromatase inhibitors or predictive indicators of adverse effects similar to those caused by fadrozole. Additionally, examination of the potential linkages between aromatase inhibition and processes like riboflavin metabolism (*e.g.* down-regulation of flavin adenine dinucleotide synthetase), blood pressure regulation (*e.g.* down-regulation of angiotensinogen precursor) and ethanol metabolism (*e.g.* up-regulation of alcohol dehydrogenase 5), among others, through focused testing should lead to an improved and integrated understanding of fish biology. Together, all these lines of investigation should advance the ability to detect and predict responses to chemical stressors.

The work was supported in part through a cooperative research and development agreement (CRADA) between the US EPA and EcoArray (CRADA 301-03). Additional support was provided by the US EPA, Office of Research and Development, National Center for Computational Toxicology. Initial support for D.L.V. was provided by a National Research Council Postdoctoral Research Associateship. We thank D. Bencic for review comments on an earlier version of the manuscript. Thanks also to D. Martinović for helpful discussion. N. Denslow and P. Larkin developed the fathead minnow microarrays discussed in this publication and hold equity in EcoArray Inc., a for-profit company commercializing the technology. They may benefit from this technology by receiving royalties and equity growth. The information in this document has been subjected to review by the US EPA National Health and Environmental Effects Research Laboratory and approved for publication. Approval does not signify that the contents reflect the views of the Agency nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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Appendix 1. Genes identified as differentially expressed in the brain of female fathead minnows exposed to 60 μg^{-1} fadrozole for 7 days

Numbers	Probe ID	Gene name	Individual probe		Duplicate probe		NR accession numbers	NT accession numbers	GO biological process	GO cellular component	GO molecular function
			Fold	*P*-value	Average fold	Average *P*					
1	EA_Pp_10085	Cytochrome P450 aromatase (brain isoform)	−2·94	0·0062	−2·72	0·0081	CAC38767	AJ277866	GO:0006118; electron transport	GO:0016020; membrane	GO:0004497; monooxygenase activity
2	EA_Pp_10078	Cytochrome *b*	−1·81	0·0021	−1·65	0·0256	AAS90392	U66602	0	0	0
3	EA_Pp_10343	14 kDa apolipoprotein	−1·61	0·0203	−1·58	0·0277	AAW82445	AY445924	0	0	0
4	EA_Pp_11183	Angiotensinogen precursor	−1·56	0·0051	−1·80	0·0474	AAL12168	AY049731	0	0	GO:0004867; serine-type endopeptidase inhibitor
5	EA_Pp_13140	NADH dehydrogenase subunit 1	−1·52	0·0258	−1·47	0·0347	NP_818813	AF391466	0	0	0
6	EA_Pp_10454	Cytochrome *b*	−1·51	0·0150	−1·53	0·0219	AAQ05178	AF352263	0	0	0
7	EA_Pp_10492	Unknown (protein for MGC:103693) (*Danio rerio*)	−1·44	0·0217	−1·32	0·0361	AAH86845	AF533646	GO:0006096; glycolysis	0	GO:0016829; lyase activity
8	EA_Pp_12674	Complement C3-H1 (*Cyprinus carpio*)	−1·19	0·0336	−1·23	0·0338	BAA36619	AB016211	0	0	GO:0004866; endopeptidase inhibitor activity
9	EA_Pp_11744	Similar to heterogeneous nuclear ribonucleoprotein C (C1/C2) (*Danio rerio*) >gi|41055873|ref| NP_957287.1|	1·15	0·0077	1·34	0·0473	AAH46891	BC046891	0	GO:0030529; ribonucleoprotein complex	GO:0000166; nucleotide binding
10	EA_Pp_11389	Potassium channel tetramerization domain containing 10 (*Danio rerio*) >gi|68359990|ref| XP_702665.1|	1·19	0·0344	1·31	0·0361	AAH61457	BC061457	GO:0006813; potassium ion transport	GO:0016020; membrane	GO:0005216; ion channel activity

Appendix 1. Continued

Numbers	Probe ID	Gene name	Individual probe		Duplicate probe		NR accession numbers	NT accession numbers	GO biological process	GO cellular component	GO molecular function
			Fold	*P*-value	Average fold	Average *P*					
11	EA_Pp_11679	Zgc:55461 (*Danio rerio*), beta tubulin	1·33	0·0037	1·54	0·0104	AAI07977	AF102890	0	0	0
12	EA_Pp_11363	Similar to heterogeneous nuclear ribonucleoprotein H (hnRNP H)	1·35	0·0209	1·24	0·0498	AAH44161	NM_212589	0	0	GO:0000166; nucleotide binding
13	EA_Pp_12277	Hypothetical protein MGC63587 (*Danio rerio*)	1·35	0·0335	1·35	0·0484	AAH55151	BC055151	GO:0007049; cell cycle	0	GO:0000166; nucleotide binding
14	EA_Pp_12559	Zgc:56546, ribosomal protein L18a	1·35	0·0039	1·52	0·0053	CAI12014	AL928834	0	0	GO:0003824; catalytic activity
15	EA_Pp_13257	Unknown (protein for IMAGE:7432353) (*Danio rerio*)	1·38	0·0250	1·37	0·0262	AAH97240	XM_309089	GO:0006457; protein folding	0	GO:0051082; unfolded protein binding
16	EA_Pp_11687	Cct4 protein	1·40	0·0437	1·47	0·0487	AAH65324	NM_200583	GO:0006457; protein folding	0	GO:0051082; unfolded protein binding
17	EA_Pp_10659	Alpha-tubulin	1·41	0·0057	1·48	0·0306	AAP89018	AY394971	GO:0007018; microtubule-based movement	GO:0005874; microtubule	GO:0005525; GTP binding
18	EA_Pp_11955	Tubulin, alpha 8 like (*Danio rerio*) >gi|52353322|ref|NP_997937.1| tubulin, alpha 8 like (*Danio rerio*)	1·44	0·0290	1·50	0·0360	AAH67582	AY391468	GO:0007018; microtubule-based movement	GO:0005874; microtubule	GO:0005525; GTP binding

Appendix 1. Continued

Numbers	Probe ID	Gene name	Individual probe		Duplicate probe		NR accession numbers	NT accession numbers	GO biological process	GO cellular component	GO molecular function
			Fold	*P*-value	Average fold	Average *P*					
19	EA_Pp_12239	Unknown (protein for MGC:114731) (*Xenopus laevis*)	1·47	0·0277	1·50	0·0372	AAH92101	BC044501	GO:0030041; actin filament polymerization	0	GO:0005525; GTP binding
20	EA_Pp_13324	14-alpha demethylase (*Danio rerio*) >gi|48926643| ref|NP_001001730.1| cytochrome P450, family 51	1·52	0·0190	1·61	0·0298	AAR89625	NM_001001730	GO:0006118; electron transport	GO:0016020; membrane	GO:0004497; monooxygenase activity
21	EA_Pp_12217	Transmembrane protein 49 (*Danio rerio*) >gi|46309489 |ref|NP_996943.1| transmembrane protein 49	1·55	0·0211	1·53	0·0475	AAH66412	BC055534	0	0	0
22	EA_Pp_11608	Cyclase-associated protein-1	1·57	0·0280	1·52	0·0397	AAO24759	BC053124	GO:0007190; adenylate cyclase activation	GO:0016020; membrane	GO:0003785; actin monomer binding
23	EA_Pp_10854	S-adenosylhomocysteine hydrolase (*Danio rerio*)	1·57	0·0438	1·54	0·0485	AAQ97740	BC064895	GO:0006730; one-carbon compound metabolism	0	GO:0016787; hydrolase activity
24	EA_Pp_10952	Glucose phosphate isomerase a	1·60	0·0164	1·56	0·0240	AAH83507	BC044450	GO:0006096; glycolysis	0	GO:0016853; isomerase activity
25	EA_Pp_10090	Putative Δ-6 fatty acyl desaturase (*Cyprinus carpio*)	1·67	0·0034	1·78	0·0297	AAG25711	AF309557	GO:0008610; lipid biosynthesis	GO:0016020; membrane	GO:0016491; oxidoreductase activity
26	EA_Pp_10720	Heat shock cognate 70 kDa protein (*Carassius auratus gibelio*)	1·80	0·0149	1·67	0·0321	AAO43731	AY195744	GO:0006457; protein folding	0	GO:0000166; nucleotide binding

Appendix 1. Continued

Numbers	Probe ID	Gene name	Individual probe		Duplicate probe		NR accession numbers	NT accession numbers	GO biological process	GO cellular component	GO molecular function
			Fold	*P*-value	Average fold	Average *P*					
27	EA_Pp_11292	Putative Δ-6 fatty acyl desaturase (*Cyprinus carpio*)	1·82	0·0222	1·70	0·0472	AAG25711	AF309557	GO:0008610; lipid biosynthesis	GO:0016020; membrane	GO:0016491; oxidoreductase activity
28	EA_Pp_12035	Farnesyl diphosphate synthase (farnesyl pyrophosphate synthetase, dimethylallyltranstransferase)	1·87	0·0064	2·11	0·0067	AAH97112	BX005429	GO:0008299; isoprenoid biosynthesis	0	0
29	EA_Pp_12430	7-dehydrocholesterol reductase	2·06	0·0173	1·72	0·0457	AAH55631	BC055631	GO:0008610; lipid biosynthesis	GO:0016020; membrane	GO:0016491; oxidoreductase activity
a	EA_Pp_10371	Lanosterol synthase (Oxidosqualene–lanosterol cyclase) (2,3-epoxysqualene–lanosterol cyclase)	1·87	0·0073	0·13	1·5195	XP_872930	BX255930	GO:0008610; lipid biosynthesis		GO:0016829; lyase activity
b	EA_Pp_14181	Diphosphomevalonate decarboxylase zgc:100824 (*Danio rerio*)	2·13	0·0430			AAH85325	NM_077978	GO:0008299; isoprenoid biosynthesis		GO:0016301; kinase activity
c	EA_Pp_11177	HMG-CoA reductase isoform 1 (*Danio rerio*) PREDICTED: similar to	1·91	0·2396	0·27	1·5568	XP_684400	AY424801	GO:0008610; lipid biosynthesis	GO:0016020; membrane	GO:0016491; oxidoreductase activity

GO, gene ontology, NR, non-redundant; NT, nucleotide.

Individual probe fold-change and *P*-values are for the probe ID listed.

The average fold change and *P*-values for the two duplicate probes (same oligonucleotide sequence) spotted on the microarray are provided. No value in this column indicates that data for only one probe was available because of filtering.

NR accession numbers, top translated BLAST hit from the non-redundant protein database.

NT accession numbers, top (lowest *e*-value) BLASTn hit from the nucleotide database.

GO terms, to accommodate display in the table, only one representative term is shown per category. 0 indicates no GO annotation was available.

a, b, c: Genes highlighted in grey did not meet the criteria for identification as differentially expressed but are shown here to support discussion of a putative effect on the cholesterol synthesis pathway.

APPENDIX 2. Genes identified as up-regulated in the liver of female fathead minnows exposed to 60 μg^{-1} fadrozole for 7 days

Numbers	Probe ID	Gene name	Individual probe		FDR	Duplicate probe		NR accession numbers	NT accession numbers	GO biological process	GO cellular component	GO molecular function
			Fold	*P*-value		Average fold	Average *P*					
1	EA_Pp_10568	Vtn protein (*Danio rerio*); vitronectin	1·67	2·63E-05	<0·05	1·74	0.000864	AAH55570	BC055570	0	0	0
2	EA_Pp_14382	Receptor (calcitonin) activity modifying protein 2 isoform 2 (*Danio rerio*) PREDICTED: similar to	1·47	7·07E-05	<0·05			XP_709602	AL929523	GO:0006898; receptor mediated endocytosis	GO:0016021; integral to membrane	GO:0001664; G-protein-coupled receptor binding
3	EA_Pp_14245	Unknown	1·41	0·000112	<0·05			No hit	AC000159	0	0	0
4	EA_Pp_14048	Megalobrama amblycephala beta-actin gene, complete cds	3·16	0·000146	<0·05	4·35	0·000364	EAM94181	AY170122	GO:0006839; mitochondrial transport	GO:0016021; integral to membrane	GO:0005215; transporter activity
5	EA_Pp_10746	Calcium-activated chloride channel (*Danio rerio*) PREDICTED: similar to	1·59	0·000197	<0·05			XP_697896	NM_201419	GO:0006821; chloride transport	GO:0005886; plasma membrane	GO:0005254; chloride channel activity
6	EA_Pp_14828	Unknown	6·43	0·000229	<0·05			EAA75323	BX322794	0	0	0
7	EA_Pp_14267	*Cyprinus carpio* transferrin variant A mRNA, complete cds	2·79	0·000412	<0·05			CAG00297	AF457152	0	0	0
8	EA_Pp_11767	Dimethylanaline monooxygenase-like	2·54	0·000431	<0·05	1·91	0·034537	AAQ94599	BC066367	GO:0006118; electron transport	0	GO:0016491; oxidoreductase activity

Appendix 2. Continued

Numbers	Probe ID	Gene name	Individual probe		FDR	Duplicate probe		NR accession numbers	NT accession numbers	GO biological process	GO cellular component	GO molecular function
			Fold	*P*-value		Average fold	Average *P*					
9	EA_Pp_15109	Vertebrate adiponectin receptor 2 (ADIPOR2) (*Danio rerio*) >gi|70887627| ref|NP_001020677.1|	1·61	0·000469	<0·05			CAH68962	BX842699	0	GO:0016021; integral to membrane	GO:0004872; receptor activity
10	EA_Pp_12706	Complement C3-S (*Cyprinus carpio*)	1·98	0·000495	<0·05	2·10	0·034241	BAA36621	AB016214	GO:0006958; complement activation	GO:0005576; extracellular region	GO:0004866; endopeptidase inhibitor activity
11	EA_Pp_14145	Carboxypeptidase PREDICTED: hypothetical protein (*Danio rerio*) unnamed protein product (*Tetraodon nigroviridis*)	4·57	0·000533	<0·05			CAG00997	AL359741	0	0	0
12	EA_Pp_13543	Pentraxin (*Salmo salar*)	2·19	0·000756	<0·05	2·24	0·000353	CAA67765	AL954359	GO:0006457; protein folding	GO:0005615; extracellular space	GO:0051082; unfolded protein binding
13	EA_Pp_12302	Sck isoform 1 (*Danio rerio*) PREDICTED: similar to	1·97	0·000772	<0·05	1·82	0·031863	XP_685785	BX470234	GO:0007242; intracellular signalling cascade	0	GO:0003824; catalytic activity
14	EA_Pp_11243	Alcohol dehydrogenase 5	2·13	0·000928	<0·05	2·01	0·001126	AAH67170	AF399909	0	0	GO:0016491; oxidoreductase activity
15	EA_Pp_10712	Fetuin long form	5.55	0·001035	<0·05	3·74	0·005061	AAO74862	AY225965	GO:0007018; microtubule-based movement	GO:0005874; microtubule	GO:0004869; cysteine protease inhibitor activity

Appendix 2. Continued

Numbers	Probe ID	Gene name	Individual probe			Duplicate probe		NR accession numbers	NT accession numbers	GO biological process	GO cellular component	GO molecular function
			Fold	*P*-value	FDR	Average fold	Average *P*					
16	EA_Pp_13640	14 kDa apolipoprotein (*Carassius auratus gibelio*)	1·64	0·001046	<0·05	1·53	0·004594	AAW82445	AY445924	0	0	0
17	EA_Pp_11466	P2AB_RABIT Serine/threonine protein phosphatase 2A, catalytic subunit, beta isoform (PP2A-beta)	2·05	0·001091	<0·05	1·75	0·026805	CAA68732	BC044495	0	0	GO:0005506; iron ion binding
18	EA_Pp_13077	Zgc:92414 (*Danio rerio*) >gi|50539724|ref|NP_001002332.1| hypothetical protein LOC436604	3·00	0·001108	<0·05	2·63	0·001212	AAH76466	BC076466	GO:0006144; purine base metabolism	0	GO:0004846; urate oxidase activity
19	EA_Pp_14023	Ctenopharyngodon idella transferrin mRNA, complete cds	3·41	0·001239	<0·10			No hit	AY383546	0	0	0
20	EA_Pp_13573	14 kDa apolipoprotein (*Carassius auratus gibelio*)	1·61	0·001451	<0·10	1·58	0·028231	AAW82445	AY445924	0	0	0
21	EA_Pp_12792	Multiple inositol polyphosphate histidine phosphatase 1 (*Danio rerio*) PREDICTED: similar to	1·87	0·001641	<0·10	2·10	0·008256	XP_690245	BX088545	0	0	GO:0003993; acid phosphatase activity
22	EA_Pp_12750	Complement C3 (*Ctenopharyngodon idella*)	2·87	0·002071	<0·10	2·98	0·002138	AAQ74974	AY374472	GO:0006958; complement activation	GO:0005576; extracellular region	GO:0004866; endopeptidase inhibitor activity

Appendix 2. Continued

Numbers	Probe ID	Gene name	Individual probe		FDR	Duplicate probe		NR accession numbers	NT accession numbers	GO biological process	GO cellular component	GO molecular function
			Fold	*P*-value		Average fold	Average *P*					
23	EA_Pp_13589	Toxin-1 (*Oncorhynchus mykiss*)	1·60	0·002139	<0·10	1·80	0·001887	AAM21198	AJ293408	0	0	0
24	EA_Pp_11033	Ferritin, heavy polypeptide 1	2·36	0·002153	<0·10	2·24	0·004365	NP_571660	BC045278	GO:0006826; iron ion transport	0	GO:0008199; iron ion binding
25	EA_Pp_10442	Sex hormone-binding globulin type-I (*Cyprinus carpio*)	2·04	0·002164	<0·10	2·27	0·007129	BAE48780	AL929346	0	0	GO:0005496; steroid binding
26	EA_Pp_11884	Unnamed protein product (*Tetraodon nigroviridis*)	3·39	0·002355	<0·10			CAG07125	XM_316594	GO:0008610; lipid biosynthesis	GO:0005737; cytoplasm	GO:0016874; ligase activity
27	EA_Pp_13754	Urocanase domain containing 1 (*Danio rerio*)	2·46	0·002469	<0·10	2·66	0·002167	XP_684713	AL391737	GO:0006547; histidine metabolism	0	GO:0016829; lyase activity
28	EA_Pp_14448	Zebrafish DNA sequence from clone DKEY-38N6, complete sequence	6·53	0·002604	<0·10			CAG07851	BX323816	0	GO:0005882; intermediate filament	GO:0005198; structural molecule activity
29	EA_Pp_12148	Complement C3-Q2	2·30	0·002707	<0·10	2·45	0·028423	BAA36623	AY374472	GO:0006958; complement activation	GO:0005576; extracellular region	GO:0004866; endopeptidase inhibitor activity
30	EA_Pp_13491	Preprorenoguanylin (*Anguilla japonica*)	2·12	0·002885	<0·10	2·12	0·007282	BAC76010	BC067691	GO:0006118; electron transport	0	GO:0008047; enzyme activator activity
31	EA_Pp_14537	Unknown	2·54	0·003216	<0·10			XP_670433	BX663505	0	0	0

Appendix 2. Continued

Numbers	Probe ID	Gene name	Individual probe			Duplicate probe		NR accession numbers	NT accession numbers	GO biological process	GO cellular component	GO molecular function
			Fold	*P*-value	FDR	Average fold	Average *P*					
32	EA_Pp_11934	Inter-alpha-trypsin inhibitor heavy chain H3 zgc: 110377 hypothetical protein LOC553614 (*Danio rerio*)	1·70	0·00338	<0·10	1·81	0·002464	NP_001018424	CR730748	GO:0030212; hyaluronan metabolism	0	GO:0004867; serine-type endopeptidase inhibitor
33	EA_Pp_13936	Immunoglobulin heavy chain delta constant domain gene segment (*Danio rerio*)	2·03	0·003389	<0·10			CAI11477	BX510335	0	0	0
34	EA_Pp_13341	PREDICTED: similar to glutathione S-transferase, theta 3 (*Danio rerio*)	1·56	0·003436	<0·10	2·36	0·006589	XP_692427	BC058294	0	0	GO:0016740; transferase activity
35	EA_Pp_13241	Keratin 4 (*Danio rerio*) >gi|5880677|gb| AAD54774.1| type II basic cytokeratin (*Danio rerio*)	2·81	0·003619	<0·10	2·42	0·005060	NP_571584	BC066728	0	GO:0005882; intermediate filament	GO:0005198; structural molecule activity
36	EA_Pp_14404	Unknown	1·89	0·004253	<0·10			No hit	AC114761	0	0	0
37	EA_Pp_10274	Transferrin precursor (*Carassius auratus gibelio*)	4·29	0·004646	<0·10	4·23	0·018338	AAK92216	AY323918	GO:0006826; iron ion transport	GO:0005576; extracellular region	GO:0008199; ferric iron binding

Appendix 2. Continued

Numbers	Probe ID	Gene name	Individual probe		FDR	Duplicate probe		NR accession numbers	NT accession numbers	GO biological process	GO cellular component	GO molecular function
			Fold	*P*-value		Average fold	Average *P*					
38	EA_Pp_11124	Zgc:109868 similar to type I cytokeratin, enveloping layer hypothetical protein LOC550522 (*Danio rerio*)	8·23	0·004660	<0·10	5·47	0·009219	NP_001017824	BC065653	0	0	GO:0005198; structural molecule activity
39	EA_Pp_14312	*Danio rerio* zgc:77882, mRNA (cDNA clone MGC:77882 IMAGE:6996576), complete cds	4·31	0·004999	<0·10			No hit	BC063991	0	0	GO:0003676; nucleic acid binding
40	EA_Pp_12170	Glycerol kinase 5 (putative); MGC80591 protein (*Xenopus laevis*)	1·72	0·005158	<0·10	1·483	0·008943	AAH73246	BC073246	GO:0005975; carbohydrate metabolism	0	GO:0004370; glycerol kinase activity
41	EA_Pp_15007	Unknown	2·57	0·005242	<0·10			CAF97312	AL109935	0	0	0
42	EA_Pp_10254	Selenoprotein Pa precursor (zSelPa)	1·64	0·005310	<0·10	1·63	0·008938	Q98SV1	BC059656	0	GO:0005576; extracellular region	GO:0008430; selenium binding

GO, gene ontology; NR, non-redundant; NT, nucleotide.

Individual probe fold-change and *P*-values are for the probe ID listed.

The average fold change and *P*-values for the two duplicate probes (same oligonucleotide sequence) spotted on the microarray are provided. No value in this column indicates that data for only one probe was available because of filtering.

FDR, Benjamini and Hochberg false discovery rate.

NR accession number, top translated BLAST hit from the non-redundant protein database.

NT accession number, top (lowest *e*-value) BLASTn hit from the nucleotide database.

GO terms, to accommodate display in the table, only one representative term is shown per category. 0 indicates no GO annotation was available.

Appendix 3. Genes identified as down-regulated in the liver of female fathead minnows exposed to 60 μg^{-1} fadrozole for 7days

Numbers	Probe ID	Gene name	Individual probe			Duplicate probe		NR accession number	NT accession number	GO biological process	GO cellular component	GO molecular function
			Fold	*P*-value	FDR	Average fold	Average *P*					
1	EA_Pp_12927	Thrombospondin (162·1 kDa) (5I321) isoform 1 (*Danio rerio*) PREDICTED: similar to	−3·27	0·00004	<0·05	−2·67	0·00090	XP_685697	BX005374	GO:0006928; cell motility	GO:0016021; integral to membrane	GO:0004867; serine-type endopeptidase inhibitor activity
2	EA_Pp_14623	FAD1 flavin adenine dinucleotide synthetase homologue (flad1) (zgc:91843 (*Danio rerio*)	−7·88	0·00007	<0·05			NP_001003997	CR711508	GO:0006777; Mo-molybdopterin cofactor biosynthesis	0	GO:0016740; transferase activity
3	EA_Pp_12244	Solute carrier organic anion transporter family, member 1C1 (solute carrier family 21, member 14) (organic anion transporter F) (OATP-F)	−3·12	0·00013	<0·05	−3·23	0·00307	XP_686981	BX323818	GO:0006810; transport	GO:0016020; membrane	GO:0005215; transporter activity
4	EA_Pp_11552	Seven in absentia 1A isoform 2 (*Danio rerio*) PREDICTED: similar to	−4·44	0·00015	<0·05	−3·14	0·01743	XP_707669	BC045870	GO:0016567; protein ubiquitination	GO:0000151; ubiquitin ligase complex	GO:0004842; ubiquitin-protein ligase activity
5	EA_Pp_14349	Zebrafish DNA sequence from clone DKEY-15F17, complete sequence	−5·30	0·00015	<0·05			No Hit	BX072537	0	GO:0005783; endoplasmic reticulum	0

Appendix 3. Continued

Numbers	Probe ID	Gene name	Individual probe			Duplicate probe		NR accession number	NT accession number	GO biological process	GO cellular component	GO molecular function
			Fold	*P*-value	FDR	Average fold	Average *P*					
6	EA_Pp_14335	CG5020-PA, isoform A (*Danio rerio*) PREDICTED: similar to	−2·38	0·00017	<0·05			XP_683949	BX004964	0	0	0
7	EA_Pp_11394	DEAD (Asp-Glu-Ala-Asp) box polypeptide 20 (*Danio rerio*) PREDICTED: similar to	−5·72	0·00017	<0·05	−3·98	0·00937	XP_683348	BC031062	GO:0000398; nuclear mRNA splicing, *via* spliceosome	GO:0005681; spliceosome complex	GO:0005524; ATP binding
8	EA_Pp_14138	Transducer of ERBB2, 1a, mRNA, *Danio rerio* (tob1a; cDNA clone MGC:76997 IMAGE:6802403), complete cds	−19·25	0·00021	<0·05			EAN88606	BC066569	0	0	0
9	EA_Pp_11059	Fast muscle troponin I	−3·74	0·00022	<0·05			AAQ13340	AF425744	0	0	0
10	EA_Pp_12369	PREDICTED: similar to CG6729-PA (*Danio rerio*)	−3·97	0·00028	<0·05	−2·79	0·00172	XP_695811	AL139163	0	GO:0005615; extracellular space	GO:0004872; receptor activity GO:0003824
11	EA_Pp_10246	Reticulocalbin 3, EF-hand calcium-binding domain (rcn3) hypothetical protein LOC415248 (*Danio rerio*) >gi|47937870 |gb|AAH71338.1| zgc:86646	−2·39	0·00033	<0·05	−2·53	0·00044	NP_001002158	BC071338	0	GO:0005783; endoplasmic reticulum	GO:0005509; calcium ion binding

Appendix 3. Continued

Numbers	Probe ID	Gene name	Individual probe			Duplicate probe		NR accession number	NT accession number	GO biological process	GO cellular component	GO molecular function
			Fold	*P*-value	FDR	Average fold	Average *P*					
12	EA_Pp_14804	Zgc:92215 (*Danio rerio*) >gi|52219026| ref|NP_001004586.1| hypothetical protein LOC447847 (*Danio rerio*)	−2·50	0·00033	<0·05			AAH81624	BX890575	GO:0006418; tRNA aminoacylation for protein translation	GO:0005737; cytoplasm	GO:0004812; tRNA ligase activity
13	EA_Pp_12259	PREDICTED: similar to KIAA1686 protein (*Danio rerio*)	−18·85	0·00041	<0·05	−10·09	0·01789	XP_695616	XM_417955	0	0	GO:0005545; phosphatidylinositol binding
14	EA_Pp_14953	*Danio rerio* casein kinase 2 alpha 2, mRNA (cDNA clone MGC:55229 IMAGE:2600897), complete cds	−4·37	0·00046	<0·05			No hit	BC044342	GO:0006468; protein amino acid phosphorylation	0	GO:0004672; protein kinase activity
15	EA_Pp_14775	PREDICTED: similar to CG5020-PA, isoform A (*Danio rerio*)	−2·36	0·00051	<0·05			XP_683949	BX004964	0	0	0
16	EA_Pp_11191	LOC407646 protein (*Danio rerio*)	−1·79	0·00055	<0·05	−1·65	0·03249	AAH57247	BC057247	GO:0006824; cobalt ion transport	0	GO:0050897; cobalt ion binding
17	EA_Pp_11061	Fast muscle troponin I	−2·42	0·00060	<0·05			AAQ13340	AF425744	0	0	0
18	EA_Pp_14290	Unknown	−1·82	0·00065	<0·05			No hit	AL731763	0	0	0
19	EA_Pp_12637	Multiple coagulation factor deficiency 2 mcfd2 zgc:103713 (*Danio rerio*) >gi|54400378| ref|NP_001005939.1| hypothetical	−2·74	0·00073	<0·05	−2·62	0·00295	AAH83471	BX004962	GO:0015031; protein transport	GO:0005783; endoplasmic reticulum	GO:0005509; calcium ion binding

Appendix 3. Continued

Numbers	Probe ID	Gene name	Individual probe			Duplicate probe		NR accession number	NT accession number	GO biological process	GO cellular component	GO molecular function
			Fold	*P*-value	FDR	Average fold	Average *P*					
20	EA_Pp_12462	Hi37 (*Danio rerio*) >gi|50539670|ref| NP_001002300.1| hypothetical protein LOC336578 (*Danio rerio*)	−3·31	0·00079	<0·05	−2·41	0·04646	AAT68026	NM_001002300	0	0	0
21	EA_Pp_14257	Runt-related transcription factor b (*Danio rerio*) PREDICTED: similar to	−1·59	0·00084	<0·05	−1·57	0·00194	XP_688654	AC090311	GO:0007603; phototransduction	GO:0005929; cilium	GO:0004872; receptor activity
22	EA_Pp_14966	DEAD (Asp-Glu-Ala-Asp) box polypeptide 21, partial (*Danio rerio*) PREDICTED: similar to	−1·74	0·00085	<0·05			XP_691013	AC084423	0	0	0
23	EA_Pp_14166	*Cyprinus carpio* mRNA for myosin regulatory light chain, complete cds, clone:pLC2 10-2	−1·32	0·00087	<0·05			BAA95134	AB037014	0	0	GO:0005509; calcium ion binding
24	EA_Pp_12527	Novel protein containing a ChaC-like protein domain (*Danio rerio*) >gi|56207301|emb| CAI20656.1| novel protein	−7·95	0·00168	<0·10	−7·82	0·00145	CAI 11900	AL929049	0	0	0

Appendix 3. Continued

Numbers	Probe ID	Gene name	Individual probe			Duplicate probe		NR accession number	NT accession number	GO biological process	GO cellular component	GO molecular function
			Fold	*P*-value	FDR	Average fold	Average *P*					
25	EA_Pp_13948	Synaptotagmin-like protein 2-a delta 2S-I (*Danio rerio*) PREDICTED: similar to	−1·42	0·00173	<0·10			XP_694069	AE017355	0	0	0
26	EA_Pp_14179	Kelch like 15; *Danio rerio* cDNA clone MGC:101051 IMAGE:7152174, complete cds	−2·32	0·00192	<0·10			AAH77093	BC077093	0	0	GO:0005515; protein binding
27	EA_Pp_13065	Ndufab1, NADH dehydrogenase (ubiquinone) 1, alpha–beta subcomplex, 1 unnamed protein product (*Tetraodon nigroviridis*)	−2·47	0·00195	<0·10	−2·17	0·00294	CAG02699	NM_001003418	GO:0006633; fatty acid biosynthesis	0	GO:0031177; phosphopantetheine binding
28	EA_Pp_12731	PREDICTED: similar to DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 26 (*Danio rerio*)	−3·12	0·00196	<0·10	−2·18	0·04642	XP_697939	BC059197	0	GO:0005615; extracellular space	GO:0005524; ATP binding
29	EA_Pp_14778	Unknown	−4·23	0·00198	<0·10			No Hit	AC022015	0	0	0
30	EA_Pp_13356	Ribosome associated membrane protein similar to hypothetical protein LOC436846 (*Danio rerio*) >gi|60551987|gb|AAH90909.1| Zgc:92744 (*Danio rerio*)	−3·05	0·00207	<0·10	−2·96	0·00267	NP_001002573	BC076222	0	0	0

Appendix 3. Continued

Numbers	Probe ID	Gene name	Individual probe			Duplicate probe		NR accession number	NT accession number	GO biological process	GO cellular component	GO molecular function
			Fold	*P*-value	FDR	Average fold	Average *P*					
31	EA_Pp_14175	PREDICTED: similar to Zinc finger and BTB domain containing protein 17 (Zinc finger protein 151) (Myc-interacting zinc finger protein)	−5·55	0·00216	<0·10			XP_693378	AC009243	0	0	0
32	EA_Pp_14157	CG5020-PA, isoform A (*Danio rerio*) PREDICTED: similar to	−2·04	0·00232	<0·10	−2·21	0·00125	XP_683949	BX004964	0	0	0
33	EA_Pp_15046	Zebrafish DNA sequence from clone DKEY-42I9 in linkage group 22, complete sequence	−1·60	0·00239	<0·10			No Hit	BX465868	0	0	0
34	EA_Pp_11809	Filamin A	−3·61	0·00243	<0·10	−2·69	0·00454	BAD52435	AL137574	GO:0007016; cytoskeletal anchoring	GO:0016021; integral to membrane	GO:0051015; actin filament binding
35	EA_Pp_14170	*Danio rerio* methionine adenosyltransferase II, alpha, mRNA (cDNA clone MGC:56664 IMAGE:5916397), complete cds	−11·44	0·00257	<0·10			AAZ62871	BC052136	GO:0006730; one-carbon compound metabolism	0	GO:0004478; methionine adenosyltransferase activity
36	EA_Pp_13268	Ski-interacting protein (*Danio rerio*) >gi|50838798|ref|NP_001002864.1| SKI-interacting protein (*Danio rerio*)	−1·72	0·00284	<0·10	−1·76	0·02379	AAT68034	NM_001002864	0	GO:0005634; nucleus	0

Appendix 3. Continued

Numbers	Probe ID	Gene name	Individual probe			Duplicate probe		NR accession number	NT accession number	GO biological process	GO cellular component	GO molecular function
			Fold	*P*-value	FDR	Average fold	Average *P*					
37	EA_Pp_14708	Runt-related transcription factor b (*Danio rerio*) PREDICTED: similar to	−1·55	0·00303	<0·10			XP_688654	AC090311	GO:0007603; phototransduction	GO:0005929; cilium	GO:0004872; receptor activity
38	EA_Pp_12370	CG6729-PA (*Danio rerio*) PREDICTED: similar to	−1·61	0·00315	<0·10	−2·79	0·00172	XP_695811	AL139163	0	GO:0005615; extracellular space	GO:0004872; receptor activity
39	EA_Pp_14095	Zebrafish DNA sequence from clone DKEY-253A1 in linkage group 9, complete sequence	−2·52	0·00325	<0·10			ZP_00316640	BX247868	0	0	0
40	EA_Pp_13990	Cholinergic receptor, nicotinic, alpha polypeptide 1 (*Danio rerio*) >gi|51858465|gb |AAH81554.1| cholinergic receptor, nicotinic, alpha polypeptide	−1·42	0·00329	<0·10			NP_571520	U70438	GO:0008045; motor axon guidance	GO:0016021; integral to membrane	GO:0030594; neurotransmitter receptor activity
41	EA_Pp_12673	Complement C3-H1 (*Cyprinus carpio*)	−2·09	0·00358	<0·10	−1·88	0·02122	BAA36619	AB016211	0	0	GO:0004866; endopeptidase inhibitor activity
42	EA_Pp_14854	Unknown	−2·02	0·00386	<0·10			No Hit	AL731763	0	0	0
43	EA_Pp_14069	Unknown	−1·41	0·00388	<0·10			BAE06476	BX950468	0	0	0
44	EA_Pp_14703	*Cyprinus carpio* ovarian fibroin-like substance-3 mRNA, complete cds	−4·08	0·00397	<0·10			P58911	AF309416	0	0	0

Appendix 3. Continued

Numbers	Probe ID	Gene name	Individual probe			Duplicate probe		NR accession number	NT accession number	GO biological process	GO cellular component	GO molecular function
			Fold	*P*-value	FDR	Average fold	Average *P*					
45	EA_Pp_13898	Zgc:92747; aquaporin 12 - similar to protein (*Danio rerio*)	−2·50	0·00403	<0·10	−2·66	0·00541	AAH95564	BC076225	GO:0006810; transport	GO:0016021; integral to membrane	GO:0005215; transporter activity
46	EA_Pp_14098	Fibrinogen-like protein 1 precursor (hepatocyte-derived fibrinogen-related protein 1) (HFREP-1) (Hepassocin) (HP-041) (*Danio rerio*)	−2·32	0·00432	<0·10			XP_693746	AL935291	0	0	GO:0003824; catalytic activity
47	EA_Pp_10475	NADH ubiquinone oxidoreductase subunit 4 (*Distoechodon tumirostris*)	−1·77	0·00470	<0·10	−1·78	0·04365	AAD10065	AF036181	0	0	0
48	EA_Pp_10120	Hypothetical protein LOC550609 (*Danio rerio*) >gi|62185659|gb |AAH92365.1| hypothetical protein LOC550609 (*Danio rerio*)	−1·46	0·00487	<0·10			NP_001017910	CR690715	0	0	0
49	EA_Pp_12638	Multiple coagulation factor deficiency 2 (mcfd2) zgc:103713 (*Danio rerio*) >gi|54400378|ref| NP_001005939.1| hypothetical	−2·50	0·00517	<0·10	−2·62	0·00295	AAH83471	BX004962	GO:0015031; protein transport	GO:0005783; endoplasmic reticulum	GO:0005509; calcium ion binding

Appendix 3. Continued

Numbers	Probe ID	Gene name	Individual probe			Duplicate probe		NR accession number	NT accession number	GO biological process	GO cellular component	GO molecular function
			Fold	*P*-value	FDR	Average fold	Average *P*					
50	EA_Pp_12063	Signal sequence receptor, gamma (translocon-associated protein gamma) (*Danio rerio*) >gi|29126995|gb |AAH47859.1| signal sequence receptor, gamma (translocon-associated protein gamma) (*Danio rerio*)	−1·68	0·00530	<0·10	−1·71	0·01662	NP_956347	BC047859	GO:0006613; cotranslational protein targeting to membrane	GO:0030176; integral to endoplasmic reticulum membrane	GO:0004872; receptor activity
51	EA_Pp_11051	Vitellogenin 3 precursor (*Danio rerio*)	−24·00	0·00112	<0·05	−15·57	0·01022	AAG30407	AF254638	GO:0006869; lipid transport	0	GO:0005319; lipid transporter activity
52	EA_Pp_11054	Vitellogenin 3 precursor (*Danio rerio*)	−13·66	0·00023	<0·05			AAG30407	AF254638	GO:0006869; lipid transport	0	GO:0005319; lipid transporter activity
53	EA_Pp_11131	Vitellogenin 3 precursor (*Danio rerio*)	−96·80	0·00203	<0·10			AAG30407	AF254638	GO:0006869; lipid transport	0	GO:0005319; lipid transporter activity
54	EA_Pp_12555	Vitellogenin 3 precursor (*Danio rerio*)	−81·94	0·00001	<0·05			AAG30407	AF254638	GO:0006869; lipid transport	0	GO:0005319; lipid transporter activity
55	EA_Pp_11130	Vitellogenin 3 precursor (*Danio rerio*) PREDICTED: similar to	−7·79	0·00094	<0·05			XP_693881	AF254638	GO:0006869; lipid transport	0	GO:0005319; lipid transporter activity

Appendix 3. Continued

Numbers	Probe ID	Gene name	Individual probe			Duplicate probe		NR accession number	NT accession number	GO biological process	GO cellular component	GO molecular function
			Fold	*P*-value	FDR	Average fold	Average *P*					
56	EA_Pp_10292	Vitellogenin precursor (*Pimephales promelas*)	−29·83	0·00029	<0·05	−24·10	0·00039	AAD23878	AF130354	GO:0006869; lipid transport	0	GO:0005319; lipid transporter activity
57	EA_Pp_11036	Vitellogenin precursor (*Pimephales promelas*)	−35·15	0·00008	<0·05	−47·00	0·00454	AAD23878	AF130354	GO:0006869; lipid transport	0	GO:0005319; lipid transporter activity
58	EA_Pp_11038	Vitellogenin precursor (*Pimephales promelas*)	−20·76	0·00009	<0·05	−29·42	0·00009	AAD23878	AF130354	GO:0006869; lipid transport	0	GO:0005319; lipid transporter activity
59	EA_Pp_11039	Vitellogenin precursor (*Pimephales promelas*)	−27·04	0·00001	<0·05	−16·77	0·00003	AAF07183	AF130354	GO:0006869; lipid transport	0	GO:0005319; lipid transporter activity
60	EA_Pp_11042	Vitellogenin precursor (*Pimephales promelas*)	−54·05	0·00007	<0·05	−50·87	0·00008	AAD23878	AF130354	GO:0006869; lipid transport	0	GO:0005319; lipid transporter activity
61	EA_Pp_11133	Vitellogenin precursor (*Pimephales promelas*)	−53·97	0·00086	<0·05	−62·30	0·00126	AAD23878	AF130354	GO:0006869; lipid transport	0	GO:0005319; lipid transporter activity
62	EA_Pp_11138	Vitellogenin precursor (*Pimephales promelas*)	−52·86	0·00046	<0·05	−53·17	0·00056	AAD23878	AF130354	GO:0006869; lipid transport	0	GO:0005319; lipid transporter activity
63	EA_Pp_11140	Vitellogenin precursor (*Pimephales promelas*)	−54·37	0·00019	<0·05	−54·44	0·00938	AAD23878	AF130354	GO:0006869; lipid transport	0	GO:0005319; lipid transporter activity
64	EA_Pp_11141	Vitellogenin precursor (*Pimephales promelas*)	−33·66	0·00075	<0·05	−48·02	0·00081	AAD23878	AF130354	GO:0006869; lipid transport	0	GO:0005319; lipid transporter activity

Appendix 3. Continued

Numbers	Probe ID	Gene name	Individual probe			Duplicate probe		NR accession number	NT accession number	GO biological process	GO cellular component	GO molecular function
			Fold	*P*-value	FDR	Average fold	Average *P*					
65	EA_Pp_11145	Vitellogenin precursor (*Pimephales promelas*)	−52·42	0·00069	<0·05	−37·03	0·00393	AAD23878	AF130354	GO:0006869; lipid transport	0	GO:0005319; lipid transporter activity
66	EA_Pp_11153	Vitellogenin precursor (*Pimephales promelas*)	−38·73	0·00118	<0·10	−37·52	0·00191	AAD23878	AF130354	GO:0006869; lipid transport	0	GO:0005319; lipid transporter activity
67	EA_Pp_11155	Vitellogenin precursor (*Pimephales promelas*)	−66·72	0·00541	<0·10			AAD23878	AF130354	GO:0006869; lipid transport	0	GO:0005319; lipid transporter activity
68	EA_Pp_11978	Vitellogenin precursor (*Pimephales promelas*)	−49·74	0·00004	<0·05	−64·61	0·00013	AAD23878	AF130354	GO:0006869; lipid transport	0	GO:0005319; lipid transporter activity
69	EA_Pp_12999	Vitellogenin precursor (*Pimephales promelas*)	−76·64	0·00359	<0·10	−63·40	0·00333	AAD23878	AF130354	GO:0006869; lipid transport	0	GO:0005319; lipid transporter activity

GO, gene ontology; NR, non-redundant; NT, nucleotide.
Individual probe fold-change and *P*-values are for the probe ID listed.
The average fold change and *P*-values for the two duplicate probes (same oligonucleotide sequence) spotted on the microarray are listed. No value in this column indicates that data for only one probe was available due to filtering.
FDR, Benjamini and Hochberg false discovery rate.
NR accession number, top translated BLAST hit from the non-redundant protein database.
NT accession number, top (lowest e value) BLASTn hit from the nucleotide database.
GO terms, to accommodate display in the table, only one representative term is shown per category. 0 indicates no gene ontology annotation was available.

Appendix 4. Expanded list of genes whose expression was potentially up-regulated in the brain of female fathead minnows exposed to 60 μg l^{-1} fadrozole for 7 days based on relaxed differential expression criteria[a]

Probe ID	Gene name
EA_Pp_12430	7-dehydrocholesterol reductase
EA_Pp_10659	Alpha-tubulin
EA_Pp_11679	Beta tubulin zgc:123194
EA_Pp_11687	Cct4 chaperonin-containing T-complex 4 protein
EA_Pp_11616	Chaperonin-containing TCP1, subunit 3 (gamma)
EA_Pp_10565	Ctsd (cathepsin D) protein
EA_Pp_11608	Cyclase-associated protein-1
EA_Pp_12322	Cytochrome P450 39A1 (Oxysterol 7-alpha-hydroxylase) (hCYP39A1) (*Danio rerio*)
EA_Pp_13324	Cytochrome P450, family 51 (sterol 14 demethylase)
EA_Pp_10090	Δ-6 fatty acyl desaturase (*Cyprinus carpio*) putative
EA_Pp_13257	DnaJ homologue subfamily C member 8 unknown (Hsp 40) (protein for IMAGE:7432353)
EA_Pp_12665	Eukaryotic translation initiation factor 3, subunit 8 (*Danio rerio*)
EA_Pp_12035	Farnesyl diphosphate synthase (farnesyl pyrophosphate synthetase)
EA_Pp_11675	Fas-associated factor 1
EA_Pp_10951	Glucose phosphate isomerase a
EA_Pp_10849	Heat shock cognate 70 kDa protein (*Carassius auratus gibelio*)
EA_Pp_11743	Heterogeneous nuclear ribonucleoprotein C (C1/C2) zgc:55733 similar to (*Danio rerio*)
EA_Pp_11204	Hsp90b protein (*Danio rerio*)
EA_Pp_12933	KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 3 (*Danio rerio*)
EA_Pp_11382	Kinesin-like protein (KIF1C) XP_697372 (*Danio rerio*) PREDICTED: hypothetical protein
EA_Pp_12239	Ras-related C3 botulinum toxin substrate (rho family, small GTP binding protien) unknown
EA_Pp_10854	S-adenosylhomocysteine hydrolase (ahcy)
EA_Pp_11058	Titin-like
EA_Pp_12874	Tropomyosin 3
EA_Pp_11984	Tubulin, alpha 1 (*Strongylocentrotus purpuratus*) PREDICTED: similar to
EA_Pp_11955	Tubulin, alpha 8 like (tuba8l) tubulin, alpha 8 like (*Danio rerio*)
EA_Pp_12384	(StARD12) (START domain-containing protein 12) (*Danio rerio*) Rho-GTPase-activating
EA_Pp_12198	ATPase type 13A2 (*Danio rerio*) PREDICTED: similar to
EA_Pp_10525	Beta tubulin
EA_Pp_12516	cAMP-regulated phosphoprotein 19
EA_Pp_10880	Chaperonin containing TCP1, subunit 6A (zeta 1)
EA_Pp_11415	Chaperonin containing TCP1, subunit 7 (eta)
EA_Pp_11399	COBW domain-containing protein zgc:77617
EA_Pp_10385	Cyclin G1
EA_Pp_14181	Diphosphomevalonate decarboxylase zgc:100824 (*Danio rerio*)

Appendix 4. Continued

Probe ID	Gene name
EA_Pp_12111	DNA replication licensing factor MCM6 (Mis5 homologue) (*Danio rerio*) PREDICTED:
EA_Pp_10540	Elongation of very long chain fatty acids protein 2 (*Danio rerio*) PREDICTED: similar to
EA_Pp_12507	Epoxide hydrolase 1, microsomal (xenobiotic)
EA_Pp_11396	Eukaryotic translation initiation factor 3, subunit 6 interacting protein
EA_Pp_10158	Glutamic acid decarboxylase isoform 65
EA_Pp_12005	Guanine nucleotide-binding protein G(i), alpha-2 subunit (adenylate cyclase-inhibiting G)
EA_Pp_11899	H3 histone, family 3A
EA_Pp_14944	HLCS gene for holocarboxylase synthetase, complete cds Homo sapiens
EA_Pp_10858	Insulin-like growth factor binding protein 2
EA_Pp_10371	Lanosterol synthase (oxidosqualene–lanosterol cyclase) (2,3-epoxysqualene–lanosterol)
EA_Pp_11222	Myosin IXB (*Danio rerio*) PREDICTED: similar to
EA_Pp_11555	Nucleosome assembly protein 1, like 1
EA_Pp_12575	Peptidase D
EA_Pp_11389	Potassium channel tetramerization domain containing 10
EA_Pp_10979	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 3
EA_Pp_10934	Protein phosphatase 1 regulatory subunit 7 zgc:123325 hypothetical protein LOC560323
EA_Pp_11255	Solute carrier family 37 (glycerol-3-phosphate transporter), member 3 (*Gallus gallus*)
EA_Pp_14436	Solute carrier family 4 member 11, sodium bicarbonate transporter XP_687848 (*Danio rerio*)
EA_Pp_11443	Thioredoxin domain containing (Txndc) protein (*Danio rerio*)
EA_Pp_12947	Translation elongation factor 1 epsilon-1 (multisynthetase complex auxiliary component)
EA_Pp_11362	Ubiquitin-specific protease 14 (tRNA-guanine transglycosylase) ubiquitin-specific protease
EA_Pp_10377	Ubiquitin-conjugating enzyme E2D 3 (*Danio rerio*) >gi|29124433|gb|AAH48896.1|
EA_Pp_14021	Zebrafish DNA sequence from clone DKEY-31N5, complete sequence
EA_Pp_12157	Zgc:100785
EA_Pp_11412	Zgc:112031 hypothetical protein LOC550364 (*Danio rerio*) >gi|62202639|gb|AAH93166.1|
EA_Pp_11722	Zgc:85809

[a]*t*-test *P*-value for a single probe <0·05, no conflicting fold change data for duplicate or multiple probes.

Appendix 5. Expanded list of genes whose expression was potentially up-regulated in the liver of female fathead minnows exposed to 60 μg l^{-1} fadrozole for 7 days based on relaxed differential expression criteria[a]

Probe ID	Gene name
EA_Pp_14382	(Calcitonin) activity modifying protein 2 isoform 2 (*Danio rerio*)
EA_Pp_10293	14 kDa apolipoprotein
EA_Pp_15112	16S ribosomal RNA gene *Plasmodiophora brassicae*, partial sequence; mitochondrial gene for
EA_Pp_10680	3-oxoacid CoA transferase 1
EA_Pp_13871	AAA ATPase containing von Willebrand factor type A (vWA) domain (*Magnetococcus* sp. MC-1)
EA_Pp_10993	Actin, alpha, cardiac muscle like
EA_Pp_13113	Adenylate kinase hypothetical protein LOC445486 (*Danio rerio*) >gi|51327295|gb|AAH80261.1| Zgc:91930 (*Danio rerio*)
EA_Pp_15109	Adiponectin receptor 2 (ADIPOR2) novel protein similar to vertebrate (*Danio rerio*)
EA_Pp_10702	Alanine-glyoxylate aminotransferase
EA_Pp_12505	Alanine-glyoxylate aminotransferase 2-like 1 isoform 1 hypothetical protein MGC63486 (*Danio rerio*)
EA_Pp_10554	Alcohol dehydrogenase
EA_Pp_10017	Alcohol dehydrogenase 5
EA_Pp_10837	Aldehyde dehydrogenase 1 family, member L2 (*Danio rerio*)
EA_Pp_11323	Aldehyde dehydrogenase 9 family, member A1
EA_Pp_10275	Alpha-1-microglobulin–bikunin hypothetical protein LOC394093 precursor (*Danio rerio*)
EA_Pp_11207	Amiloride binding protein 1 (*Danio rerio*)
EA_Pp_10546	Angiopoietin-like-3
EA_Pp_10735	Apobec-1 complementation factor isoform 1 isoform 2 (*Danio rerio*) PREDICTED: similar to
EA_Pp_12974	Apolipoprotein B (including Ag(x) antigen) (APOB)novel protein similar to vertebrate (*Danio rerio*)
EA_Pp_14362	Apolipoprotein Eb Apoeb protein (*Danio rerio*)
EA_Pp_10045	Apolipoprotein Eb, mRNA (cDNA clone MGC:77232 IMAGE:6963043), complete cds *Danio rerio*
EA_Pp_11953	Bbs2 Bardet-Biedl syndrome 2 protein
EA_Pp_14477	Beta-microseminoprotein precursor (prostate secreted seminal plasma protein)
EA_Pp_14191	Bubblegum, Acyl-CoA synthetase, lipidosin – similar to MGC53673 protein isoform 1 (*Danio rerio*)
EA_Pp_10699	C1 inhibitor, partial (*Danio rerio*)
EA_Pp_10543	Cadherin 1, epithelial
EA_Pp_10746	Calcium-activated chloride channel (*Danio rerio*) PREDICTED: similar to
EA_Pp_13842	Carboxylesterase 2 isoform 1 (*Danio rerio*) PREDICTED: similar to
EA_Pp_10487	Carp desaturase 2 (CDS2)
EA_Pp_14596	Carp DNA sequence from clone carpf-GC2H, complete sequence
EA_Pp_11275	Casein kinase 2 alpha 1
EA_Pp_11297	Cbs centrosomins beautiful sister protein

Appendix 5. Continued

Probe ID	Gene name
EA_Pp_12984	CCCH zinc finger protein C3H-1 (*Danio rerio*) PREDICTED: similar to
EA_Pp_10669	Ceruloplasmin
EA_Pp_12770	Chromobox homologue 1 (*Danio rerio*) >gi|49902655|gb|AAH75782.1| Chromobox homologue 1
EA_Pp_12763	Cold-inducible RNA-binding protein (*Danio rerio*) >gi|28856196|gb|AAH48027.1| Cold inducible
EA_Pp_12010	Collagen, type I, alpha 1
EA_Pp_12841	Complement B/C2-A2 (*Cyprinus carpio*)
EA_Pp_12182	complement C3
EA_Pp_12512	Complement C3 precursor (HSE-MSF) (*Danio rerio*) PREDICTED: similar to
EA_Pp_10401	Complement C3-Q1, partial (*Danio rerio*) PREDICTED: similar to
EA_Pp_12148	Complement C3-Q2
EA_Pp_10359	Complement C3-S
EA_Pp_12819	Complement C4-2 (*Cyprinus carpio*)
EA_Pp_13889	Complement C4-2 (*Danio rerio*) PREDICTED: similar to
EA_Pp_12899	Complement component 8 alpha polypeptide hypothetical protein LOC445102 (*Danio rerio*)
EA_Pp_10423	Complement component 9
EA_Pp_10383	Complement component C9
EA_Pp_11202	Complement control protein factor I-A
EA_Pp_10667	Complement factor B/C2B
EA_Pp_11301	Complement factor B/C2B (*Danio rerio*)
EA_Pp_13327	Complement factor H zgc:123186 unknown (protein for MGC:123186) (*Danio rerio*)
EA_Pp_13673	Cytochrome *c* oxidase subunit 4 hypothetical protein MGC73355 (*Danio rerio*)
EA_Pp_12321	Cytochrome P450 39A1 (Oxysterol 7-alpha-hydroxylase) (hCY-P39A1) (*Danio rerio*)
EA_Pp_10749	Cytochrome P450 family 2, subfamily J zgc:91876 (*Danio rerio*)
EA_Pp_10847	Deiodinase, iodothyronine, type II
EA_Pp_11767	Dimethylanaline monooxygenase-like
EA_Pp_12825	Enolase 1, (alpha) (*Danio rerio*) >gi|47551317|ref|NP_999888.1| enolase 3, (beta, muscle) (*Danio rerio*)
EA_Pp_11405	Eukaryotic translation initiation factor 3, subunit 3 (gamma)
EA_Pp_12231	Eukaryotic translation initiation factor 3, subunit 9 eta, 116kDa
EA_Pp_12015	Eukaryotic translation initiation factor 4E binding protein 3
EA_Pp_12740	Expressed sequence AW548124 (*Danio rerio*)
EA_Pp_12053	F-box only protein 9
EA_Pp_10036	F-box protein 38 isoform b (*Danio rerio*)
EA_Pp_11033	Ferritin, heavy polypeptide 1
EA_Pp_10711	Fetuin long form
EA_Pp_10698	Fetuin-B
EA_Pp_10856	Fibronectin 1b
EA_Pp_11167	Fibronectin variant 3

Appendix 5. Continued

Probe ID	Gene name
EA_Pp_14585	FK506 binding protein 8 hypothetical protein hypothetical protein MGC77672 (*Danio rerio*)
EA_Pp_13531	Fructose-1,6-bisphosphatase (*Esox lucius*)
EA_Pp_10587	Fructose-1,6-bisphosphatase 1
EA_Pp_11851	Fucosidase, alpha-L-1, tissue
EA_Pp_10730	G protein pathway suppressor 1 isoform 2 isoform 1 (*Danio rerio*) PREDICTED: similar to
EA_Pp_12173	Gcat glycine C-acetyltransferase (2-amino-3-ketobutyrate-coenzyme A ligase) LOC402822
EA_Pp_11044	Gliacolin (C1Q) novel protein similar to vertebrate (*Danio rerio*) >gi|68356446|ref|XP_708224.1|
EA_Pp_10951	Glucose phosphate isomerase a
EA_Pp_10154	Glucose-6-phosphate-1-dehydrogenase; G6PD
EA_Pp_10752	Glutamate dehydrogenase 1
EA_Pp_12885	Glutathione S-transferase, theta 3 (*Danio rerio*) PREDICTED: similar to
EA_Pp_10662	Glycogen phosphorylase hypothetical protein LOC493916 (*Danio rerio*)
EA_Pp_11899	H3 histone, family 3A (*Mus musculus*) >gi|56388767|gb|AAH87725.1| H3f3b protein (*Rattus norvegicus*)
EA_Pp_10367	Hemopexin precursor (*Danio rerio*) PREDICTED: similar to
EA_Pp_10839	Hgd homogentisate 1,2-dioxygenase protein (*Danio rerio*)
EA_Pp_13209	HIV-1 Rev binding protein, partial (*Danio rerio*) PREDICTED: similar to
EA_Pp_10485	Hyaluronic acid binding protein 2 isoform 3 (*Danio rerio*) >gi|68370901|ref|XP_686209.1|
EA_Pp_10761	Hypothetical protein LOC406484 (*Danio rerio*) >gi|47085999|ref|NP_998368.1| hypothetical
EA_Pp_12655	Hypothetical protein LOC554105 (*Danio rerio*) >gi|66773128|ref|NP_001019577.1| hypothetical
EA_Pp_12032	Hypoxia-inducible factor-1alpha (*Ctenopharyngodon idella*)
EA_Pp_13936	Immunoglobulin heavy chain delta constant domain gene segment (*Danio rerio*)
EA_Pp_10857	Insulin-like growth factor binding protein 2 Igfbp2 protein (*Danio rerio*)
EA_Pp_11180	Inter-alpha trypsin inhibitor (similar to) hypothetical protein LOC402873 (*Danio rerio*)
EA_Pp_11212	Inter-alpha (globulin) inhibitor H2 novel protein (zgc:56119) (*Danio rerio*)
EA_Pp_11933	Inter-alpha-trypsin inhibitor heavy chain 3 hypothetical protein LOC553614 (*Danio rerio*)
EA_Pp_10678	Intestinal fatty acid binding protein 2 (*Danio rerio*) >gi|6687439|emb|CAB64945.1| intestinal fatty
EA_Pp_11124	Keratin (similar to) hypothetical protein LOC550522 (*Danio rerio*) >gi|62205109|gb|AAH92718.1|
EA_Pp_13241	Keratin 4 (*Danio rerio*) >gi|5880677|gb|AAD54774.1| type II basic cytokeratin (*Danio rerio*)

Appendix 5. Continued

Probe ID	Gene name
EA_Pp_13461	Kininogen 1 zgc:103569 (*Danio rerio*) >gi|54400464|ref|NP_001005981.1| hypothetical protein
EA_Pp_10831	Lecithin cholesterol acyltransferase (*Gallus gallus*) PREDICTED: similar to
EA_Pp_10591	LOC322493 (*Danio rerio*) PREDICTED: hypothetical protein
EA_Pp_10895	Lysophospholipase 3 Lypla3 protein (*Danio rerio*)
EA_Pp_13999	Megalobrama amblycephala beta-actin gene, complete cds
EA_Pp_12169	MGC80591 protein (*Xenopus laevis*)
EA_Pp_11705	Minichromosome maintenance protein 8 isoform 1; DNA replication licensing factor MCM8; chromosome 20 open reading frame 154 (*Gallus gallus*) PREDICTED: similar to
EA_Pp_11333	Mitochondrial ribosomal protein S5 isoform 2 (*Danio rerio*) >gi|68387293|ref|XP_708293.1|
EA_Pp_12791	Multiple inositol polyphosphate histidine phosphatase 1 (*Danio rerio*) PREDICTED: similar to
EA_Pp_11489	MYST histone acetyltransferase 2 (*Danio rerio*) >gi|28279513|gb|AAH45314.1| MYST histone
EA_Pp_12596	Novel protein (*Danio rerio*)
EA_Pp_10983	Nucleolar protein 5A NOP56 (*Danio rerio*)
EA_Pp_11103	Opsin 1 (cone pigments), medium-wave-sensitive, 1 (*Danio rerio*)
EA_Pp_13595	PDZ and LIM domain 2, isoform 2 (*Homo sapiens*) >gi|47940543|gb|AAH71774.1| PDZ and LIM
EA_Pp_13543	Pentraxin (*Salmo salar*)
EA_Pp_11807	Peroxisomal membrane protein 2 Zgc:92599 (*Danio rerio*) >gi|52219060|ref|NP_001004607.1|
EA_Pp_12330	Phosphoenolpyruvate carboxykinase (*Danio rerio*) >gi|39645931|gb|AAH63985.1| Zgc:77867
EA_Pp_12276	Phosphoenolpyruvate carboxylase (*Erythroculter ilishaeformis*)
EA_Pp_10564	Phosphoglucomutase 1 (*Danio rerio*) >gi|41056111|ref|NP_957319.1| phosphoglucomutase 1 (*Danio rerio*)
EA_Pp_10494	Phospholipid hydroperoxide glutathione peroxidase A (*Danio rerio*)
EA_Pp_10593	Plasminogen (*Danio rerio*) >gi|41393105|ref|NP_958880.1| plasminogen (*Danio rerio*)
EA_Pp_10236	Poly(A) polymerase (*Carassius auratus*)
EA_Pp_12297	PREDICTED: similar to cadherin 1, epithelial, partial (*Danio rerio*)
EA_Pp_11662	Receptor accessory protein 3 (reep3) hypothetical protein MGC55529 (*Danio rerio*)
EA_Pp_10251	Retinol binding protein 4, plasma (*Danio rerio*) >gi|6687453|emb|CAB64947.1| retinol binding
EA_Pp_12264	Rev-Erb beta 2 (*Danio rerio*)
EA_Pp_15074	Reverse transcriptase (*Danio rerio*)
EA_Pp_14251	Ring finger protein 128 zgc:77843, mRNA (cDNA clone MGC:77843 IMAGE:7002238), complete
EA_Pp_12526	RNA (guanine-7-) methyltransferase (*Danio rerio*)

Appendix 5. Continued

Probe ID	Gene name
EA_Pp_12302	Sck isoform 1 (*Danio rerio*)
EA_Pp_10253	Selenoprotein Pa precursor (zSelPa)
EA_Pp_11322	Serine hydroxymethyltransferase 1 Shmt1 protein (*Danio rerio*)
EA_Pp_10441	Sex hormone-binding globulin type-I (*Cyprinus carpio*)
EA_Pp_14066	SH3 and multiple ankyrin repeat domains 2 isoform 1, partial (*Danio rerio*) PREDICTED:
EA_Pp_13481	Signal transducer and activator of transcription 1 isoform beta (*Danio rerio*) PREDICTED: similar to
EA_Pp_11658	Solute carrier family 1 (glial high affinity glutamate transporter), member 3 (*Danio rerio*)
EA_Pp_10684	Solute carrier family 35 (UDP-glucuronic acid–UDP-N-acetyl-galactosamine dual transporter)
EA_Pp_13563	Toxin-1 (*Oncorhynchus mykiss*)
EA_Pp_13621	Transcobalamin I precursor (TCI) (TC I) (*Danio rerio*) PREDICTED: similar to
EA_Pp_11015	Transferrin (*Ctenopharyngodon idella*)
EA_Pp_14023	Transferrin mRNA, complete cds *Ctenopharyngodon idella*
EA_Pp_14843	Transferrin precursor (*Carassius auratus gibelio*)
EA_Pp_13381	Transferrin variant A (*Cyprinus carpio*)
EA_Pp_14267	Transferrin variant A mRNA, complete cds *Cyprinus carpio*
EA_Pp_11821	Transferrin variant A1 (*Carassius auratus*)
EA_Pp_11012	Transferrin variant D (*Carassius auratus gibelio*)
EA_Pp_14012	Ubiquitin-like protein 2 mRNA, complete cds *Carassius auratus*
EA_Pp_10741	Uncoupling protein 1 (*Cyprinus carpio*)
EA_Pp_11466	Unnamed protein product (*Oryctolagus cuniculus*) >gi|129336|sp|P11611|P2AB_RABIT
EA_Pp_12143	Urate oxidase (uricase) zgc:92414 (*Danio rerio*) >gi|50539724|ref|NP_001002332.1| hypothetical
EA_Pp_10348	Ureidopropionase, beta
EA_Pp_13753	Urocanase domain containing 1 (*Danio rerio*) PREDICTED: similar to
EA_Pp_13963	Vertebrate KIN, antigenic determinant of recA protein homologue (mouse) (KIN) (*Danio rerio*)
EA_Pp_10567	Vitronectin (Vtn) protein (*Danio rerio*)
EA_Pp_14866	Zebrafish DNA sequence from clone CH211-233E16 in linkage group 16, complete sequence
EA_Pp_14892	Zebrafish DNA sequence from clone CH211-260P9 in linkage group 18, complete sequence
EA_Pp_15125	Zebrafish DNA sequence from clone CH211-63O20 in linkage group 20, complete sequence
EA_Pp_14204	Zebrafish DNA sequence from clone DKEY-117N4, complete sequence
EA_Pp_14448	Zebrafish DNA sequence from clone DKEY-38N6, complete sequence
EA_Pp_10893	Zgc:55468 hypothetical protein MGC55468 (*Danio rerio*) >gi|41055857|ref|NP_956451.1|

Appendix 5. Continued

Probe ID	Gene name
EA_Pp_11927	Zgc:55908 hypothetical protein MGC55908 (*Danio rerio*) >gi|41055722|ref|NP_956477.1|
EA_Pp_10777	Zgc:64076 (*Danio rerio*) >gi|47085915|ref|NP_998315.1| hypothetical protein LOC406424 (*Danio rerio*)
EA_Pp_12034	Zgc:77348 (*Danio rerio*) >gi|47085735|ref|NP_998128.1| hypothetical protein LOC405899 (*Danio rerio*)
EA_Pp_14312	Zgc:77882, mRNA (cDNA clone MGC:77882 IMAGE:6996576), complete cds *Danio rerio*

[a]*t*-test *P*-value for a single probe <0·05, no conflicting fold change data for duplicate or multiple probes.

Appendix 6. Expanded list of genes whose expression was potentially up-regulated in the liver of female fathead minnows exposed to 60 μg l^{-1} fadrozole for 7 days, based on relaxed differential expression criteria[a]

Probe ID	Gene name
EA_Pp_13716	40S ribosomal protein S24 (*Pagrus major*)
EA_Pp_10374	40S ribosomal protein S24-like protein
EA_Pp_11299	Apolipoprotein A-I binding protein zgc:92263 (*Danio rerio*) >gi|50540304|ref|NP_001002618.1|
EA_Pp_13897	Aquaporin similar to zgc:92747 protein (*Danio rerio*)
EA_Pp_12725	Beta-carotene 15, 15-dioxygenase 2 (bcdo2) (*Danio rerio*) >gi|13872742|emb|CAC37567.1|
EA_Pp_11353	BING4-like (WD repeat domain 46)
EA_Pp_10245	Calumenin (similar to -isoform unclear) hypothetical protein LOC415248 (*Danio rerio*)
EA_Pp_13751	Cdc2l1 cell division control 2 like 1 protein (*Mus musculus*)
EA_Pp_12265	Cyclic nucleotide gated channel beta 1 (*Danio rerio*) >gi|68365256|ref|XP_684092.1|
EA_Pp_11996	Cytochrome P450, family 3, subfamily a, polypeptide 13 (*Danio rerio*)
EA_Pp_11393	DEAD (Asp-Glu-Ala-Asp) box polypeptide 20 (*Danio rerio*)
EA_Pp_10539	Elongation of very long chain fatty acids protein 2 (*Danio rerio*) PREDICTED: similar to
EA_Pp_10102	Eukaryotic translation elongation factor 1 gamma
EA_Pp_11059	Fast muscle troponin I
EA_Pp_11809	Filamin A
EA_Pp_14896	Glutamate-ammonia ligase (glutamine synthase) b, mRNA (cDNA clone MGC:56052
EA_Pp_10168	Heat shock protein 90-beta (*Danio rerio*) >gi|2791863|gb|AAB96969.1| heat shock protein
EA_Pp_12579	Huntingtin-interacting protein K (*Danio rerio*) PREDICTED: similar to

Appendix 6. Continued

Probe ID	Gene name
EA_Pp_12422	Hypothetical protein LOC549556 (*Xenopus tropicalis*)
EA_Pp_11391	Lectin mannose-binding 1 (similar to) LOC559775 protein (*Danio rerio*)
EA_Pp_12637	Multiple coagulation factor deficiency 2 zgc:103713 (*Danio rerio*)
EA_Pp_10219	NADH ubiquinone oxidoreductase subunit 4 (*Abbottina rivularis*)
EA_Pp_10475	NADH ubiquinone oxidoreductase subunit 4 (*Distoechodon tumirostris*)
EA_Pp_11620	Natural killer specific antigen Klip (similar to) hypothetical protein LOC334511 (*Danio rerio*)
EA_Pp_12527	Novel protein containing a ChaC-like protein domain (*Danio rerio*)
EA_Pp_12487	Nucleobindin 2b (*Danio rerio*) >gi|41393115|ref|NP_958887.1| nucleobindin 2b (*Danio rerio*)
EA_Pp_13297	Ovulatory protein-2 precursor (*Danio rerio*) PREDICTED: similar to
EA_Pp_11135	Parvalbumin (*Danio rerio*) >gi|62204541|gb|AAH93135.1| Parvalbumin like (*Danio rerio*)
EA_Pp_14257	PREDICTED: similar to runt-related transcription factor b (*Danio rerio*)
EA_Pp_13483	Rho guanine nucleotide exchange factor 5 isoform 1 (*Danio rerio*)
EA_Pp_13281	Ribosomal protein L14 (*Danio rerio*)
EA_Pp_12097	Ribosomal protein L18 (60S) Zgc:92872 (*Danio rerio*) >gi|51010947|ref|NP_001003432.1|
EA_Pp_13526	Ribosomal protein S23 (*Rattus norvegicus*) PREDICTED: similar to
EA_Pp_13903	Ribosomal protein S25 hypothetical protein MGC73391 (*Danio rerio*)
EA_Pp_13353	Ribosome associated membrane protein 4 hypothetical protein LOC436846 (*Danio rerio*)
EA_Pp_12305	Sb:cb283 protein (*Danio rerio*)
EA_Pp_11551	Seven in absentia 1A isoform 2 (*Danio rerio*) PREDICTED: similar to
EA_Pp_12063	Signal sequence receptor, gamma (ssr3) (translocon-associated protein gamma) (*Danio rerio*)
EA_Pp_12189	Solute carrier family 41, member 1 isoform 1 (*Danio rerio*)
EA_Pp_12243	Solute carrier organic anion transporter family, member 1C1 (solute carrier family 21)
EA_Pp_13654	Testis nuclear RNA-binding protein (*Danio rerio*) PREDICTED: similar to
EA_Pp_12927	Thrombospondin (162.1 kDa) (5I321) isoform 1 (*Danio rerio*) PREDICTED: similar to
EA_Pp_10268	Tnfrsf1a-associated via death domain (*Danio rerio*) >gi|18859509|ref|NP_571682.1| tnfrsf1a-
EA_Pp_11191	Transcobalamin II precursor LOC407646 protein (*Danio rerio*)
EA_Pp_12215	Transmembrane protein 49 (*Danio rerio*) >gi|46309489|ref|NP_996943.1| transmembrane

Appendix 6. Continued

Probe ID	Gene name
EA_Pp_10387	Tryptophan 2,3 dioxygenase MGC107895 protein isoform 1 (*Danio rerio*) PREDICTED:
EA_Pp_13035	Unknown (*Homo sapiens*)
EA_Pp_13527	Vitellogenin 3 precursor (*Danio rerio*)
EA_Pp_11130	Vitellogenin 3 precursor (*Danio rerio*) PREDICTED: similar to
EA_Pp_11977	Vitellogenin precursor (*Pimephales promelas*)
EA_Pp_15046	Zebrafish DNA sequence from clone DKEY-42I9 in linkage group 22, complete sequence
EA_Pp_14039	Zinc finger and BTB domain containing protein 17 (Zinc finger protein 151) (Myc-interacting)
EA_Pp_10293	14 kDa apolipoprotein
EA_Pp_13862	Actin filament associated protein hypothetical protein LOC566757 (*Danio rerio*)
EA_Pp_11759	Alas2 protein aminolevulinate synthase
EA_Pp_12876	Alpha-2-macroglobulin-1 isoform 14 (*Danio rerio*) PREDICTED: similar to
EA_Pp_12014	Anaphase promoting complex subunit 5
EA_Pp_12974	Apolipoprotein B (including Ag(x) antigen) (APOB)novel protein similar to vertebrate (*Danio rerio*)
EA_Pp_14672	Arginase, type II (ARG2) mRNA, complete cds *Danio rerio* clone RK126A3H11
EA_Pp_12822	Barrier to autointegration factor (similar to) zgc:77767 hypothetical protein LOC334717
EA_Pp_12482	BCL2–adenovirus E1b 19 kDa interacting protein 3a
EA_Pp_10768	Carnitine O-palmitoyltransferase I, mitochondrial liver isoform (CPT I) (CPTI-L) (Carnitine)
EA_Pp_14953	Casein kinase 2 alpha 2, mRNA (cDNA clone MGC:55229 IMAGE:2600897), complete cds
EA_Pp_12895	Centrosomal protein 70 kDa (*Danio rerio*)
EA_Pp_12369	CG6729-PA (*Danio rerio*) PREDICTED: similar to
EA_Pp_11769	Chloride channel 3
EA_Pp_13990	Cholinergic receptor, nicotinic, alpha polypeptide 1 (*Danio rerio*)
EA_Pp_11515	Chromobox homologue 2 (drosophila Pc class)
EA_Pp_11409	Chromosome segregation 1-like
EA_Pp_12673	Complement C3-H1 [Cyprinus carpio]
EA_Pp_10067	Creatine kinase
EA_Pp_14793	Cyclin-B (*Danio rerio*) >gi|20373137|ref|NP_571588.1| cyclin B1 (*Danio rerio*)
EA_Pp_14453	Cyprinid microsatellite library MRB-2003 microsatellite Cyp-G1 sequence
EA_Pp_11642	Cysteine-rich hydrophobic domain 2
EA_Pp_10085	Cytochrome P450 aromatase B
EA_Pp_11991	Cytosolic alanine aminotransferase; cAAT; cGPT (*Sparus aurata*)
EA_Pp_11703	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 19 (ddx19) (DBP5 homologue, yeast)
EA_Pp_12731	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 26 (*Danio rerio*) PREDICTED: similar to

Appendix 6. Continued

Probe ID	Gene name
EA_Pp_11291	Delta-6 fatty acyl desaturase (*Cyprinus carpio*) putative
EA_Pp_10091	Desmoglein 1 (DSG1), mRNA *Homo sapiens*
EA_Pp_11825	Echinoderm microtubule associated protein like zgc:110445 hypothetical protein elongation factor 1-gamma (EF-1-gamma) (eEF-1B gamma) >gi|15528539|dbj|BAB64568.1|
EA_Pp_13325	Elongation factor-1 gamma (*Carassius auratus*)
EA_Pp_10144	Oestrogen receptor alpha
EA_Pp_10776	Eukaryotic translation elongation factor 2 FHM_JGI.355.C1_-1
EA_Pp_12844	Eukaryotic translation initiation factor 2-alpha kinase 3 precursor (PRKR-like endoplasmic)
EA_Pp_11894	Eukaryotic translation initiation factor 5 novel protein (zgc:77026) (*Danio rerio*)
EA_Pp_14376	F10 protein (*Danio rerio*)
EA_Pp_14703	Fibroin-like substance-3 mRNA, complete cds *Cyprinus carpio* ovarian
EA_Pp_12938	Fibronectin type III and SPRY domain containing 1 isoform 1 (*Danio rerio*) PREDICTED:
EA_Pp_10158	GAD 64 glutamic acid decarboxylase isoform 65
EA_Pp_13168	General transcription factor IIA, 1, 19/37 kDa (*Danio rerio*) >gi|29124431|gb|AAH48894.1|
EA_Pp_12759	Gigaxonin (*Homo sapiens*) >gi|11545731|ref|NP_071324.1| gigaxonin (*Homo sapiens*)
EA_Pp_11257	GPI (glycophosphatidyl inositol)-anchored membrane protein 1
EA_Pp_12462	hi37 (*Danio rerio*) >gi|50539670|ref|NP_001002300.1| hypothetical protein LOC336578
EA_Pp_14804	Histidyl-tRNA synthetase Zgc:92215 (*Danio rerio*) >gi|52219026|ref|NP_001004586.1|
EA_Pp_12602	Homeoboxes protein ZHX1 (*Danio rerio*) >gi|38488710|ref|NP_942109.1| zinc-fingers and
EA_Pp_12211	HSPC171 (similar to) hypothetical protein LOC404632 (*Danio rerio*)
EA_Pp_12624	Hydroxysteroid dehydrogenase like 2 (*Danio rerio*) >gi|41054573|ref|NP_955893.1|
EA_Pp_13556	Hypothetical protein LOC550051 (*Xenopus tropicalis*)
EA_Pp_12878	Hypothetical protein LOC74868 (*Mus musculus*) >gi|66396509|gb|AAH96426.1| hypothetical
EA_Pp_13920	Hypoxia up-regulated 1 Oxygen regulated protein (150 kDa) (*Danio rerio*)
EA_Pp_11503	Im:6901326 protein (*Danio rerio*) FHMinnow-GonadLib-1S4-H09.g_-2
EA_Pp_14647	Im:7150989 protein (*Danio rerio*)
EA_Pp_12970	IMP (inosine monophosphate) dehydrogenase 2 (*Danio rerio*)
EA_Pp_14973	Influenza virus NS1A binding protein a, mRNA (cDNA clone MGC:77050 IMAGE:6959874),
EA_Pp_11427	Integrator complex subunit 7 (similar to) hypothetical protein LOC286776 (*Danio rerio*)

Appendix 6. Continued

Probe ID	Gene name
EA_Pp_12027	Ionotropic N-methyl D-aspartate glutamate receptor 1 (GRIN1, NR1, NMDAR1) (*Danio rerio*)
EA_Pp_12816	Isocitrate dehydrogenase 2 (NADP+), mitochondrial (*Danio rerio*)
EA_Pp_13687	K123 protein (*Danio rerio*) PREDICTED: similar to AIWA434.g1_-2
EA_Pp_14179	Kelch-like 15 (similar to) Zgc:101051 *Danio rerio* cDNA clone MGC:101051
EA_Pp_13908	KIAA0188 (possibly similar to lipin) (*Danio rerio*) PREDICTED: similar to
EA_Pp_14364	Kielin (*Danio rerio*) PREDICTED: similar to
EA_Pp_11381	Kinesin-like protein KIF1C (*Danio rerio*) PREDICTED: hypothetical protein XP_697372 [
EA_Pp_11752	Leucine rich repeat containing 28 (*Danio rerio*) PREDICTED: similar to
EA_Pp_13239	LOC553488 protein (*Danio rerio*)
EA_Pp_14318	Matriptase (*Danio rerio*) PREDICTED: similar to
EA_Pp_10796	Methionine adenosyltransferase 2 hypothetical protein LOC541483 (*Danio rerio*)
EA_Pp_14170	Methionine adenosyltransferase II, alpha, mRNA (cDNA clone MGC:56664
EA_Pp_10313	Mitochondrial uncoupling protein 2 (*Leuciscus cephalus*)
EA_Pp_13675	Myosin heavy chain (*Cyprinus carpio*)
EA_Pp_14166	Myosin regulatory light chain, complete cds, clone:pLC2 10-2 *Cyprinus carpio* mRNA for
EA_Pp_10771	NADH dehydrogenase subunit 5 (*Sarcocheilichthys variegatus microoculus*)
EA_Pp_14615	Nuclear receptor coactivator 4 (*Danio rerio*) Similar to
EA_Pp_12907	Parvalbumin (*Cyprinus carpio*) FHM_JGI.62.C8_-1
EA_Pp_10234	Peroxisome proliferator activated receptor isoform b (*Pimephales promelas*)
EA_Pp_11726	Phosphatase and tensin-like protein A long splice variant (*Danio rerio*)
EA_Pp_12253	Phosphoglucomutase 2 hypothetical protein LOC405822 (*Danio rerio*)
EA_Pp_13858	Pim1 protein (*Danio rerio*)
EA_Pp_12681	Pleckstrin homology domain containing, family M (with RUN domain) member 1 (*Danio rerio*)
EA_Pp_12259	Pleckstrin homology domain containing KIAA1686 protein (*Danio rerio*) PREDICTED: similar
EA_Pp_12050	Potassium channel tetramerization domain containing 6 hypothetical protein LOC436601
EA_Pp_14205	Potassium inwardly-rectifying channel, subfamily J, member 1 (*Danio rerio*) PREDICTED:
EA_Pp_14157	PREDICTED: similar to CG5020-PA, isoform A (*Danio rerio*)
EA_Pp_14824	PREDICTED: similar to CG6406-PB, isoform B, partial (*Danio rerio*)

Appendix 6. Continued

Probe ID	Gene name
EA_Pp_14427	PREDICTED: similar to RIKEN cDNA 1110032A13 isoform 2 (*Danio rerio*)
EA_Pp_13629	Preprohepcidin (*Danio rerio*) >gi|38351815|gb|AAR18592.1| preprohepcidin 1 (*Danio rerio*)
EA_Pp_10237	Presenilin-1 (*Danio rerio*) >gi|37082580|sp|Q9W6T7|PSN1_BRARE Presenilin-1 (PS1) (Zf-
EA_Pp_10725	Prosaposin (*Danio rerio*)
EA_Pp_11294	Protein disulfide isomerase-associated 4 (*Danio rerio*)
EA_Pp_14241	Protein disulfide isomerase-related protein (provisional), mRNA (cDNA clone MGC:55846)
EA_Pp_13213	Protein transport protein Sec24D (SEC24-related protein D) (*Danio rerio*) PREDICTED:
EA_Pp_10241	Rap1ga1 protein (Rap1 GTPase activating protein) (*Danio rerio*) PREDICTED: similar to
EA_Pp_14934	Regulator of G-protein signaling 9 (RGS9) (*Danio rerio*)
EA_Pp_10969	Ribosomal protein L21 (*Danio rerio*) >gi|68404101|ref|XP_689959.1| PREDICTED: similar to
EA_Pp_11861	RNF141 protein (*Danio rerio*)
EA_Pp_15100	Rpl17 protein (*Mus musculus*)
EA_Pp_13265	Sarcoglycan delta (*Danio rerio*) >gi|49227301|ref|NP_001001816.1| sarcoglycan, delta
EA_Pp_13216	SH2 domain containing 4A (*Gallus gallus*) PREDICTED: similar to
EA_Pp_13267	Ski-interacting protein (*Danio rerio*) >gi|50838798|ref|NP_001002864.1| SKI interacting
EA_Pp_12496	Solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 23 (*Danio rerio*)
EA_Pp_10833	Solute carrier family 38, member 4 (SLC38A4) (*Danio rerio*) novel protein similar to vertebrate
EA_Pp_11949	Sperm associated antigen 6 hypothetical protein LOC431757 (*Danio rerio*)
EA_Pp_14735	Structure specific recognition protein 1, mRNA (cDNA clone MGC:66086 IMAGE:6797288),
EA_Pp_11636	Sulphatase FP1c, partial (*Danio rerio*)
EA_Pp_12001	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin a-like 1; HepA-
EA_Pp_12513	Synapse-associated protein 1 (*Danio rerio*) >gi|41055612|ref|NP_957236.1| synapse
EA_Pp_14774	Synaptonemal complex protein 3 PREDICTED: similar to (*Danio rerio*)
EA_Pp_14006	Synaptopodin 2-like (*Danio rerio*)
EA_Pp_13948	Synaptotagmin-like protein 2-a delta 2S-I (*Danio rerio*)
EA_Pp_11559	Tetratricopeptide repeat domain 15 (*Danio rerio*) PREDICTED: similar to
EA_Pp_13391	Transmembrane protein-precursor (*Danio rerio*)

Appendix 6. Continued

Probe ID	Gene name
EA_Pp_13583	Tubulin, alpha 4 like (*Danio rerio*). Unknown (protein for MGC:55727) (*Danio rerio*)
EA_Pp_11476	Ubiquitin C (*Homo sapiens*)
EA_Pp_12468	Unknown (*Danio rerio*)
EA_Pp_13121	Vacuolar protein sorting 4b Vps4b-prov protein (*Xenopus laevis*)
EA_Pp_11182	Vigilin high density lipoprotein-binding protein (vigilin) (*Danio rerio*)
EA_Pp_11623	Vitamin K epoxide reductase complex, subunit 1-like hypothetical protein LOC553717
EA_Pp_15083	WD repeat domain 34 isoform 1 (*Danio rerio*) PREDICTED: similar to
EA_Pp_13881	x-box binding protein 1B Xbp1 protein (*Danio rerio*) >gi|18419453|gb|AAL69333.1| (*Danio rerio*)
EA_Pp_14699	XP_677890 isoform 1 (*Danio rerio*)
EA_Pp_11650	X-prolyl aminopeptidase (aminopeptidase P) 1, soluble (*Danio rerio*)
EA_Pp_14584	Zebrafish DNA sequence from clone CH211-119H23 in linkage group 2, complete sequence
EA_Pp_14140	Zebrafish DNA sequence from clone CH211-138G9 in linkage group 20, complete sequence
EA_Pp_14349	Zebrafish DNA sequence from clone DKEY-15F17, complete sequence
EA_Pp_14016	Zebrafish DNA sequence from clone DKEY-170H8 in linkage group 7, complete sequence
EA_Pp_14095	Zebrafish DNA sequence from clone DKEY-253A1 in linkage group 9, complete sequence
EA_Pp_15101	Zebrafish DNA sequence from clone DKEY-81P7, complete sequence
EA_Pp_14724	Zebrafish DNA sequence from clone DKEY-90M5 in linkage group 20, complete sequence
EA_Pp_14186	Zgc:103404 (*Danio rerio*) >gi|54400596|ref|NP_001006047.1| hypothetical protein
EA_Pp_11569	Zgc:110112 hypothetical protein LOC550237 (*Danio rerio*) >gi|62204278|gb|AAH92736.1|
EA_Pp_10120	Zgc:113320 hypothetical protein LOC550609 (*Danio rerio*) >gi|62185659|gb|AAH92365.1|
EA_Pp_12720	Zgc:123187 major facilitator superfamily domain containing hypothetical protein
EA_Pp_11539	Zgc:55549 hypothetical protein MGC55549 (*Danio rerio*) >gi|41056171|ref|NP_956624.1|
EA_Pp_14261	Zgc:56141 (zgc:56141), mRNA *Danio rerio*
EA_Pp_11749	Zinc finger, RAN-binding domain containing 1 (*Danio rerio*) PREDICTED: similar to
EA_Pp_11935	Zona pellucida-like domain novel protein containing a (wu:fi27h02) (*Danio rerio*)
EA_Pp_11682	ZP2, partial (*Danio rerio*) PREDICTED: similar to

[a] *t*-test *P*-value for a single probe $<0{\cdot}05$, no conflicting fold change data for duplicate or multiple probes.