Toxicogenomic analysis of the hepatic effects of perfluorooctanoic acid on rare minnows (*Gobiocypris rarus*)

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Abstract

Perfluorooctanoic acid (PFOA) is a ubiquitous environmental contaminant that has been detected in a variety of terrestrial and aquatic organisms. To assess the effects of PFOA in fish and predict its potential mode of action, a toxicogenomic approach was applied to hepatic gene expression profile analysis in male and female rare minnows (*Gobiocypris rarus*) using a custom cDNA microarray containing 1773 unique genes. Rare minnows were treated with continuous flow-through exposure to PFOA at concentrations of 3, 10, and 30 mg/L for 28 days. Based on the observed histopathological changes, the livers from fish exposed to 10 mg/L PFOA were selected for further hepatic gene expression analysis. While 124 and 171 genes were significantly altered by PFOA in males and females, respectively, of which 43 genes were commonly regulated in both sexes. The affected genes are involved in multiple biological processes, including lipid metabolism and transport, hormone action, immune responses, and mitochondrial functions. PFOA exposure significantly suppressed genes involved in fatty acid biosynthesis and transport but induced genes associated with intracellular trafficking of cholesterol. Alterations in expression of genes associated with mitochondrial fatty acid β-oxidation were only observed in female rare minnows. In addition, PFOA inhibited genes responsible for thyroid hormone biosynthesis and significantly induced estrogen-responsive genes. These findings implicate PFOA in endocrine disruption. This work contributes not only to the elucidation of the potential mode of toxicity of PFOA to aquatic organisms but also to the use of toxicogenomic approaches to address issues in environmental toxicology.
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Introduction

Perfluorooctanoic acid (PFOA), the predominant perfluorinated compound (PFC) present in the environment, is a ubiquitous environmental contaminant detected in a wide variety of animals from both terrestrial and aquatic environments (Dai et al., 2006; Kannan et al., 2006; Smithwick et al., 2005; Tao et al., 2006) as well as in the sera of humans from different parts of the world (Ericson et al., 2007; Karrman et al., 2007; Yeung et al., 2006). PFOA is a remarkably stable compound that is not metabolized (Vanden Heuvel et al., 1992).

As a result of both the prevalence and persistence of PFOA in organisms, the potential adverse effects of this compound on diverse species, especially mammals, have been extensively examined. The liver is a primary target organ for both short-term and chronic effects of PFOA (Butenhoff et al., 2002). PFOA has been shown to interfere with fatty acid metabolism, cholesterol synthesis, and lipid transport in the liver (Haughom and Spydevold, 1992). In addition, exposure to this compound increases the incidence of liver tumors (Biegel et al., 2001).

Marked species and gender differences have been observed in the pharmacokinetics and toxicity of PFOA. For example, PFOA caused potent peroxisome proliferation in male rats but exhibited a less potent effect on female rats in repeated exposure studies. This gender difference, however, was not seen in mice (Sohlenius et al., 1992). These pronounced species and gender differences in the pharmacokinetics and toxicity of PFOA preclude the generalization of conclusions regarding its me-

chanism of action based on studies done on one species or one gender.

Although a good deal of work has focused on the adverse effects of PFOA on mammals, few studies have been performed on the effects of PFOA on freshwater fish, which are commonly tested for toxicological risk assessment. Also, gender differences in gene expression elicited by PFOA are not well understood. Thus, we sought to characterize the effects of PFOA on the liver and predict its potential mechanism of toxicity in freshwater fish species. To this end, we employed a customized cDNA microarray to examine the hepatic gene expression profiles in male and female rare minnows (*Gobiocypris rarus*) following PFOA exposure. Rare minnows have many attractive features that make them a suitable organism in aquatic toxicity tests: sensitivity to chemicals, small size, wide temperature range, ease of laboratory culture, and short embryonic development period (Zhong et al., 2005; Zhou and Jiang, 2002). Gene expression patterns obtained in males and females were analyzed separately. The results from this analysis will provide useful data for the assessment of the potential risks involved in the exposure of aquatic species to PFOA and will further help to extend our understanding of its mechanism of action.

Methods

Exposure experiments.   Adult male and female rare minnows (∼9 months old) with an average body weight of 1.4±0.4 g and an average total length of 47.7±3.6 mm were obtained from a laboratory hatchery and held in 20-L glass tanks (∼2 g body weight/L). Fish were acclimated and treated as previously described (Wei et al., 2007). Briefly, fish were supplied with dechlorinated tap water under continuous flow-through conditions at 25±2 °C with a photoperiod of 16 h:8 h (light/dark). Commercial granular food (Tetra, Germany) was supplied at a daily rate of 0.1% body weight. Waste and uneaten food were removed daily. Gender determination was based on the shape of the abdomen and the distance between the abdominal fin and the tail fin. After a 1-week acclimation period, equal numbers of randomly selected male and female rare minnows were assigned to one of four treatment groups: PFOA exposure at 0, 3, 10, or 30 mg/L. These concentrations were selected based on previous studies (Oakes et al., 2004); however, the actual PFOA concentrations in the tanks were not verified by chemical analysis. PFOA (98%) was purchased from Acros Organics (Geel, Belgium). Each treatment group contained ten male and ten female minnows in duplicate tanks. The flow rate of the test solution was 8 L/h. At the end of the 28-day exposure period, all fish were anesthetized on ice. Gonadal tissues from all fish and hepatic tissues from four male and four female fish per treatment group were quickly dissected and fixed in 10% formalin for histological analysis. The livers from the remaining six males and six females per treatment group were removed and immediately frozen in liquid nitrogen and stored at −80 °C until analysis.

Histopathological analysis.   Formalin-fixed tissues were processed sequentially in ethanol, xylene, and paraffin. Tissues were then embedded in paraffin wax, sectioned transversely at 4–5 μm, mounted on slides, and stained with hematoxylin and eosin. Fish sexes and their level of maturation were determined based on the degree of egg and sperm cell development in the gonadal tissues (Grier, 1981).

RNA extraction.   Total RNA extraction was performed according to the manufacturer's instructions. Briefly, for each individual fish analyzed, total RNA was extracted from 20 mg of liver tissue using the RNeasy Mini Kit (Qiagen, Germany) and treated with RNase-free DNase I (Qiagen, Germany) to remove any remaining genomic DNA. Isolated RNA was quantified based on the A_{260} value. The purity of the RNA was assessed by determining the A_{260}/A_{280} ratio as well as visual inspection of 1.0 μg RNA on a denaturing gel (data not shown).

cDNA library construction, EST processing, and annotation.   A pool of RNA was extracted from 30 unexposed adult male and female minnows. The cDNA was synthesized and cloned into the *Eco*RI–*Xho*I site of the pBluescript II SK (+) vector (Promega, USA) to construct a non-normalized cDNA library. A total of 7386 random clones were partially sequenced from the 5′end of the cDNA to generate ESTs. After screening, processing, and eliminating the low-quality ESTs using the PHRED and CROSS_MATCH programs, 6919 high-quality ESTs, ranging in size from 100 to 808 bp with an average read length of 555 bp, were obtained and deposited in the GenBank dbEST database (accession numbers: EE392478–EE399396). The sequence assembly software PHRAP was used to build consensus sequences. A total of 1773 unique genes, which comprised 771 contigs (consensus sequences) and 1002 singlets, was acquired. BLAST homology searches in the non-redundant protein and nucleic acid databases in GenBank were performed for each of the unique genes under *E*-values of 1e−5 and 1e−10. The unique genes were named after the homologous sequences in GenBank. The annotated unique genes were then matched to specific processes or functions using gene ontology (GO).

Rare minnow cDNA microarray construction.   The 1773 clones that corresponded to unique genes were selected for PCR amplification of the inserts. PCR was performed using 2.5 units of ExTaq (Takara, China) in a volume of 50 μL containing 1× PCR buffer, 250 μM of each dNTP, and 0.5 μM of each primer (T3 and T7). The PCR amplification program consisted of 5 min at 94 °C followed by 18 cycles of 40 s at 94 °C, 40 s at 58 °C, 1 min at 72 °C, and a final extension at 72 °C for 5 min. The PCR products were precipitated with the addition of ethanol, dissolved in EasyArray™ spotting solution (CapitalBio, China), and printed in triplicate on a PolymerSlide (CapitalBio, China) using a SmartArray™ microarrayer (CapitalBio, China). Eight sequences derived from the intergenic yeast genomic regions, which exhibited no significant homologies with any existing teleost sequences in GenBank were used as external controls. These sequences were amplified by PCR and cloned into plasmid pSP64Poly(A) (Promega, USA) to produce poly(A)-RNA following *in vitro* transcription. Varying amounts of this poly(A)-RNA were spiked into the RNA samples.

Microarray experiment design.   RNA samples from the 10 mg/L PFOA exposure group were chosen for microarray analysis due to the moderate hepatic histological changes in this group compared with the other two PFOA exposure groups. A total of twelve arrays were used in the experiment. Six pooled RNA samples (three from males and three from females) were obtained by pooling every two individual RNA sample and were analyzed separately. The common reference design was that samples from PFOA-treated fish were co-hybridized with a common control (six individual RNA samples from male or female control group were pooled). A dye-swap replicate was used for each sample to measure reproducibility and reduce systematic bias.

RNA amplification, labeling, and hybridization.   Eberwine's linear RNA amplification method was used to yield the cDNA labeled with fluorescent dye (Cy5 and Cy3–dCTP), and subsequent enzymatic reactions were performed as previously described (Guo et al., 2005; Patterson et al., 2006; Shi et al., 2006). Briefly, double-stranded cDNA containing a T7 RNA polymerase promoter sequence was synthesized with 5 μg total RNA using the Reverse Transcription System, RNase H, DNA polymerase I, and T4 DNA polymerase according to the manufacturer's recommended protocol (Promega, USA). The double-stranded cDNA was then subjected to *in vitro* transcription reactions in a volume of 20 mL at 37 °C for 3 h using the T7 RiboMAX Express large-scale RNA production system (Promega, USA). The Klenow enzyme labeling strategy was utilized following reverse transcription. Labeled controls and test samples were quantitatively adjusted based on the efficiency of Cy5–dCTP or Cy3–dCTP (Amersham Pharmacia Biotech, USA) incorporation. Samples were then dissolved in 80 μL of hybridization solution containing 50% formamide. Arrays were hybridized in a CapitalBio BioMixer™ II Hybridization Station overnight at a temperature of 42 °C and washed with two consecutive solutions (0.2% SDS plus 2× SSC at 42 °C for 5 min and 0.2× SSC for 5 min) at room temperature.

Microarray imaging and data analysis.   Arrays were scanned with a confocal LuxScan™ scanner (CapitalBio, China), and the images were then analyzed using LuxScan™ 3.0 software (CapitalBio, China). For individual channel data, faint spots were removed when the intensity was less than 400 units after

background subtraction from both channels (Cy3 and Cy5). Space- and intensity-dependent normalization based on a LOWESS program was performed to normalize the data (Yang et al., 2002). Fold changes were calculated relative to controls. The fold differences in gene expression levels after PFOA exposure were represented as the average fold changes in both the technical and biological replicates. The two-tailed Student's *t*-test method was used to statistically analyze the data from the 12 independently tested arrays. Data from male and female fish were analyzed separately. A *p*-value less than 0.05 combined with an average fold change greater than or equal to 1.5 was used to identify significantly differentially expressed genes.

Quantitative real-time PCR.   Quantitative real-time PCR was performed to validate the results from the microarray analysis as well as to determine the expression levels of selected genes from the other two exposed groups (3 and 30 mg/L PFOA). 1 µg of total RNA from individual fish was reverse-transcribed by M-MuLV reverse transcriptase using the oligo-dT primer as described by the manufacturer (NEB, USA). The cDNA (1.0 µL) was used as a template in 25-µL reactions containing 12.5 µL of 2× QuantiTect SYBR Green PCR master mix (Takara, China) and 0.1 µM each of forward and reverse gene-specific primers. Gene names, accession numbers in GenBank, forward and reverse primer sequences, and amplicon sizes are listed in Table 1. PCR amplification was conducted on a Stratagene Mx3000P qPCR system (Stratagene, USA). Each sample was processed in triplicate. GAPDH was chosen as an internal control for normalization since the results of the microarray data indicated that expression levels of this gene were not significantly different between fish from control and treatment groups. After verifying that the amplification efficiencies of the selected genes and GAPDH were approximately equal, fold differences in expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). A one-way analysis of variance (ANOVA) was used for statistical analysis between the PFOA-exposed groups and control by SPSS v13.0 software. Differences were considered significant when *p*-values were less than 0.05.

Results

Histopathology

Male and female rare minnows exhibited hepatic histopathological changes after exposure to PFOA as compared to the controls. Exposure to 3 mg/L PFOA elicited moderate hepatocellular hypertrophy in both male and female livers (Figs. 1B and F). Male fish exposed to 10 mg/L PFOA exhibited eosinophilic hyaline droplets in the cytoplasm of the hepatocytes (Fig. 1C). Female fish exposed to 10 mg/L PFOA displayed more eosinophilic hyaline droplets in the cytoplasm of hepatocytes, hepatocellular hypertrophy, and vacuolar degen-

eration (Fig. 1G). Fish exposed to 30 mg/L PFOA showed severe hepatic histopathological changes as compared to the controls. The cellular structure was nearly collapsed, and a wide area of hepatocytes underwent necrosis and cytolysis. The enlargement and pycnosis of nuclei were observed in the necrotic hepatocytes (Figs. 1D and H). In addition, infiltration of inflammatory cells was observed in male livers (Fig. 1D), and apparent vacuolar degeneration was observed in female livers (Fig. 1H).

Global views of gene expression profiles

A 1.5-fold change relative to the control was used as the criterion for differential expression in the microarray experiments. Thus, a total of 124 genes in males and 171 genes in females were found to be significantly altered ($p < 0.05$) in fish exposed to 10 mg/L PFOA (Fig. 2). The degree of overlap of the differential expression in fish after PFOA exposure was examined. The expression of 29 genes was significantly upregulated in both male and female livers, while 36 genes in males and 52 genes in females were specifically induced. The expression of 13 genes were significantly downregulated in both male and female livers, while 45 genes in male livers and 76 genes in female livers were specifically suppressed following PFOA exposure (Fig. 2). Among these significantly altered genes, only one unique gene was altered inversely in males and females; the gene was induced in males and suppressed in females (Fig. 2). The gene was found to be similar to phospholipid hydroperoxide glutathione peroxidase A. Based on the annotation from the nr protein and nt databases in GenBank, 121 significantly altered genes were assigned to known annotation groups. These groups of genes consisted of 54 upregulated genes (Table 2) and 68 downregulated genes (Table 3). The genes for which annotation was unclear were excluded from Tables 2 and 3.

Functional categorization of microarray data

Functional annotation of the PFOA-mediated changes in gene expression revealed differences between the affected genes in both males and females regarding their function in various

Table 1
Gene-specific primers used in quantitative real-time PCR

Gene	Accession no.	Forward primer (from 5′ to 3′)	Reverse primer (from 5′ to 3′)	Amplicon size (bp)
GAPDH	EE397198	CGTGCTGCTGTCCAGTCCAA	GCCGCCTTCTGCCTTAACCT	138
PP	EE398509	TGGGTTTGTGCTTCTGCTGT	CTGTTCCCCTCAAGGTCGTA	159
BMG	EE394886	ACAAGGGAAAGTCTCCAGTC	GGAAGCCACTCACATAACAG	98
WTARP	EE395026	AAGAAAGGGAATATGGGACT	TGTTTGGCTGTGACCACTAG	136
ApoC-I	EE392760	GGAGGTCACATATCAGGAA	TTGCCACTAAAACCAAGAGC	147
ApoB	EE398714	GACATCACCAGCCCAACATT	TGGGAGCAGCATAGCGAAAGA	51
L-FABP	EE398232	TTCCTTCTCCAAGCGGTCTA	TGAAGTCGTTGCCGTTTTGT	200
FAD	EE396030	TTTGCGGATGTCGTTAGGTC	ATTCTCGCTCGTGATTGGTG	120
FAS	EE397675	TATGCTCCATTTTGCTGCTT	TGACATTGCCGTGATACTTG	89
Dio2	EE397646	CCGTATTGACCCAAATGAGC	GAAAATGTTCCCAGCAGCCT	161

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; *PP*, 26–29 kDa-proteinase protein; *BMG*, β-2 microglobulin; *WTARP*, warm-temperature-acclimation-related-65 kDa-protein; *ApoC-I*, apolipoprotein C-I precursor; *ApoB*, apolipoprotein B; *L-FABP*, fatty acid-binding protein 10, liver basic; *FAD*, delta-6 fatty acyl desaturase; *FAS*, fatty acid synthase; *Dio2*, type II iodothyronine deiodinase.
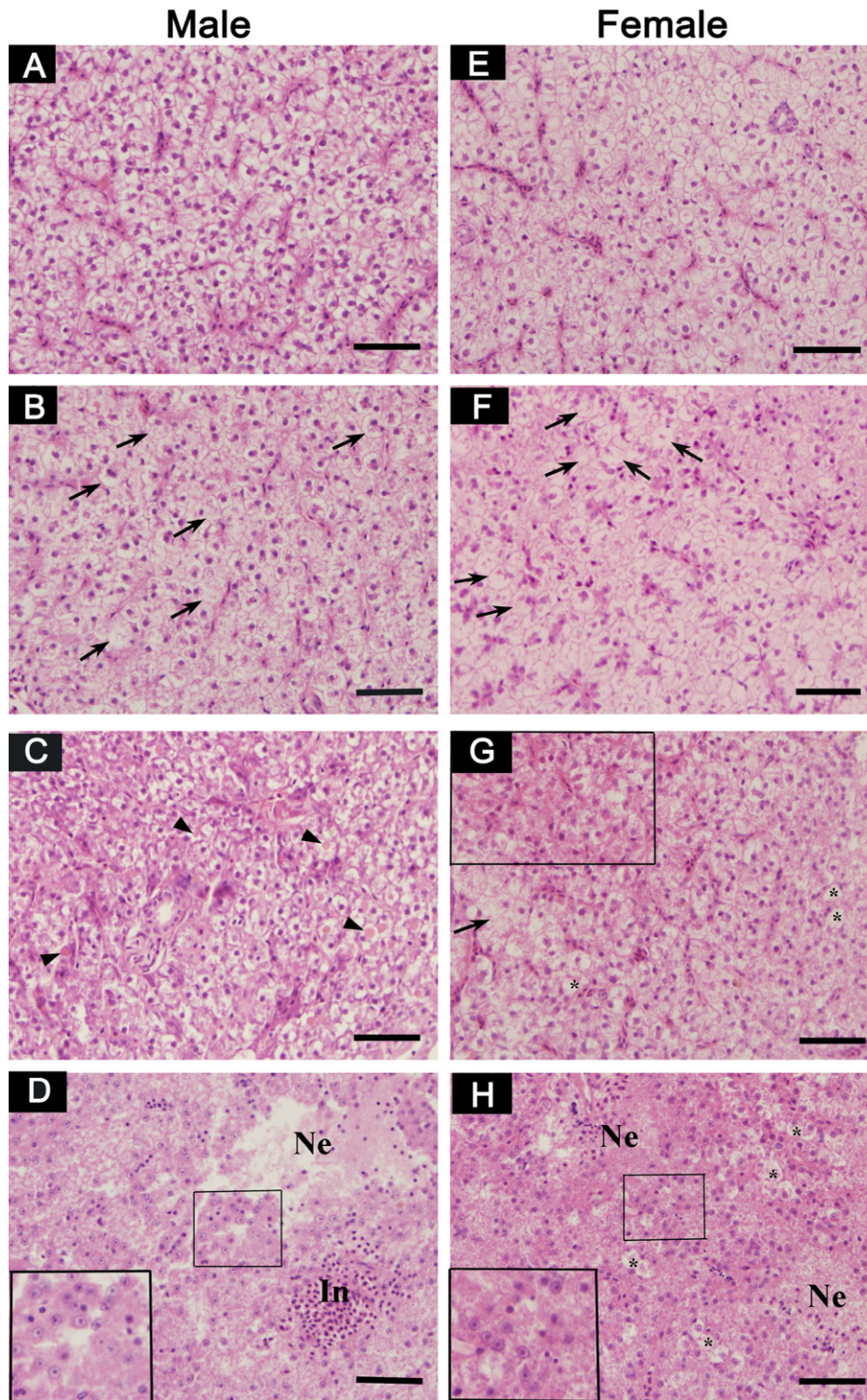
Fig. 1. Liver histopathology in male and female rare minnows following PFOA exposure. Photomicrographs of liver sections (4–5 µm) stained with hematoxylin and eosin. (A and E) Unexposed male and female fish livers. (B and F) 3 mg/L PFOA-exposed male and female fish livers. A number of hepatocytes were swollen (arrow). (C and G) 10 mg/L PFOA-exposed male and female fish livers. Eosinophilic hyaline droplets (arrowhead) were observed in the cytoplasm of male hepatocytes. Female hepatocytes exhibited more eosinophilic droplets in the cytoplasm (see the rectangle area in top left corner of panel G) accompanied by swollen hepatocytes (arrow) and vacuolar degeneration (asterisk). (D and H) 30 mg/L PFOA-exposed male and female fish livers. Hepatocellular necrosis and destruction of cellular structures (marked with Ne) as well as the enlargement and pycnosis of nuclei were observed in this group (see the rectangle area in the middle of the panels D and H, and the corresponding magnified pictures were in the left bottom). Infiltration of inflammatory cells was observed in male livers (marked with In), and apparent vacuolar degeneration was exhibited in female livers (asterisk). Scale bar is equal to 50 µm.

biological processes. For example, phase I enzymes such as CYP3A were specifically altered in males, whereas UDP-glucuronosyltransferase 1, a phase II enzyme, was only induced in females. Several genes involved in immune responses and cell proliferation were specifically induced in females (Table 2). Although many genes exhibited distinct responses in males and
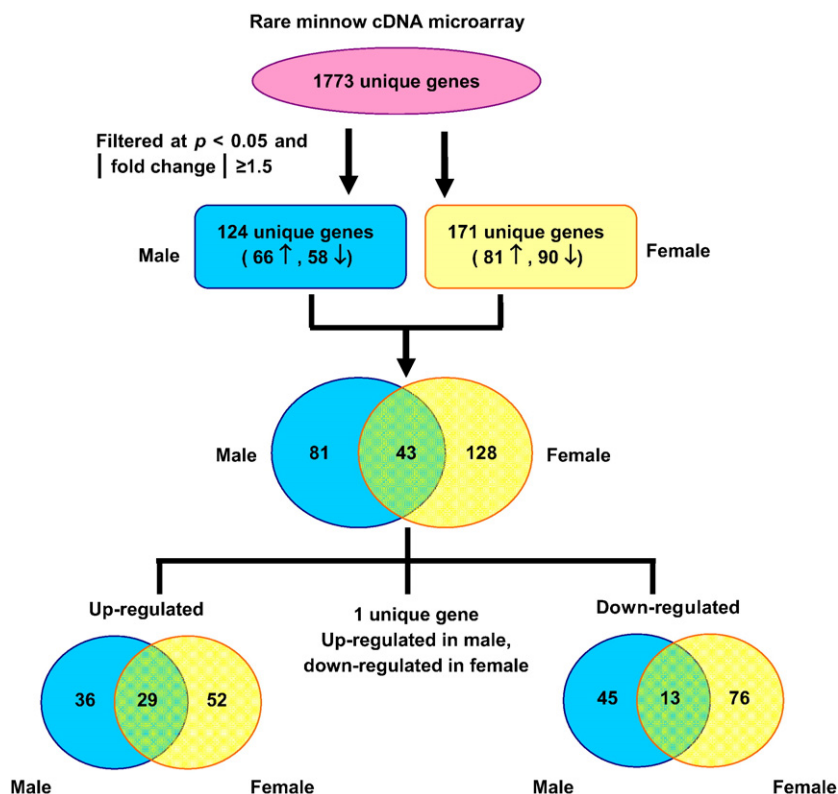
Fig. 2. Overview of comparisons of PFOA-mediated hepatic gene expression profiles in male and female minnows analyzed using a custom cDNA microarray. According to the filtering criteria of the Student's t-test p value less than 0.05 and |fold change| greater than or equal to 1.5, 201 and 238 unique genes were found to be significantly differentially expressed in response to 10 mg/L PFOA in males and females, respectively. The numbers of up- and downregulated genes are given in parentheses; "↑" indicates upregulation and "↓" indicates downregulation. Venn diagrams show the comparisons of differentially expressed genes in males and females. Each complete circle depicts the total number of genes in males or females, and the overlapping region depicts the number of shared altered genes between males and females.

females, eleven genes that matched known annotations displayed similar induced responses in males and females (Table 2).

Among the genes downregulated following PFOA exposure, those involved in metabolism were the most prevalent in both males and females (Table 3). Several genes associated with carbohydrate metabolism, oxidative phosphorylation, and immune responses were significantly suppressed in males, while genes responsible for proteolysis and signal transduction were specifically downregulated in females. In addition, PFOA exposure significantly suppressed a number of genes involved in lipid metabolism and transport in both males and females.

Comparison of gene expression responses in rats exposed to PFOA

In order to identify the similarities and differences in PFOA-mediated gene expression responses between fish and rats, we conducted a comparison of gene expression profiles in male minnows with the results of hepatic gene expression profiles in rats following PFOA exposure (Guruge et al., 2006). Rats were exposed to PFOA for 28 days, and gene expression patterns were analyzed using the Rat Genome 230 2.0 array (Affymetrix, USA). We compared the fold changes in gene expression from our microarray experiments to the significantly affected rat genes that are identical or homologous to that from rare

minnows. Although the microarray analysis methods and the filtering criteria were different, directional responses were identified. Using a p-value of less than 0.0025 rather than a 2-fold change, glycerol-3-phosphate dehydrogenase and cytochrome $P450$ family 4 were induced in both male rare minnows and male rats. With the exception of these genes, the homologous genes of rare minnows and rats were not affected similarly by PFOA exposure. Considering that the number of sequences in our microarray was smaller than the Rat Genome 230 2.0 array and the two microarrays contained different sequences, we could not ensure differential expression of other genes in fish and rats.

Quantitative real-time PCR analysis

Validation and supplementation of the microarray results for nine genes were accomplished by quantitative PCR analysis across all treatment groups. In general, the results obtained from the microarray experiments correlated well with PCR (Table 4). The 26–29 kDa proteinase protein (PP) and β-2 microglobulin (BMG) genes exhibited a notable difference between males and females (Fig. 3). The expression of PP was induced in males but nearly unaffected in females. Conversely, BMG was unchanged in males whereas this gene was significantly upregulated in females except in the group exposed to 30 mg/L PFOA.

Table 2
Significantly upregulated genes in the livers of rare minnows under PFOA exposure (Student's t-test; $p < 0.05$, and fold change ≥ 1.5)

Gene name [a]	Accession no. [b]	Fold change [c]	Functional category [d]
Male (16)			
Cytochrome P450 3A	EE398218	1.86	Xenobiotic metabolism
Cytochrome P450 2Y3	EE395766	2.01	
Putative cytochrome P450 2N3	EE395133	1.79	
Lactate dehydrogenase B4	EE395734	1.72	Lactate metabolism/NAD metabolism
ATP-binding cassette, sub-family A (ABC1), member 2	EE398847	2.04	Regulation of intracellular cholesterol transport/response to steroid hormone stimulus
Histidyl-tRNA synthetase	EE396984	2.11	RNA metabolism/cellular protein metabolism
Uridine phosphorylase-like	EE396551	1.81	Nucleic acid metabolism
Polymerase (RNA) III (DNA directed) polypeptide H	EE397310	1.82	Transcription/citrate metabolism
Activating transcription factor 4	EE393243	1.50	Regulation of transcription
M-phase phosphoprotein 10 (U3 small nucleolar ribonucleoprotein) isoform 1	EE393189	1.81	RNA splicing
Similar to cytochrome c oxidase subunit VIIb precursor	EE396842	1.93	Oxidative phosphorylation
Cytochrome c oxidase subunit IV isoform 2 precursor	EE395679	1.76	Oxidative phosphorylation
Uncoupling protein 1 (UCP1)	EE398516	2.79	Mitochondrial transport
Similar to fibrinogen/angiopoietin-related protein	EE397612	2.16	Angiogenesis
26–29 kDa proteinase protein	EE396440	3.59	
Selenoprotein Pa precursor (zSelPa)	EE393171	1.50	
Female (26)			
UDP-glucuronosyltransferase 1	EE394376	1.70	Xenobiotic metabolic process/estrogen metabolism
Synaptonemal complex protein 3	EE397165	1.56	Lipid biosynthesis
Vitellogenin 3 precursor	EE395982	2.44	Lipid transport
Heat-shock 70 kDa protein 8	EE396844	1.84	Protein metabolism/response to unfolded protein
Similar to haptoglobin precursor	EE393202	1.68	Protein metabolism
Similar to 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase isoform 7	EE395506	1.51	Nucleic acid metabolism/organ regeneration
Similar to pyridoxal kinase (pyridoxine kinase) isoform 3	EE398671	1.54	Pyridoxine metabolism
Multiple coagulation factor deficiency 2	EE395889	1.51	Carboxylic acid metabolism
Insulin-induced gene 1	EE397316	1.74	Regulation of cellular metabolism
Beta-2 microglobulin	EE394886	1.91	Antigen processing
Complement C3	EE397132	2.56	Humoral immune response
Complement C3-H2	EE394762	1.92	Humoral immune response
CCAAT/enhancer binding protein beta	EE397383	1.59	Acute-phase response/inflammatory response/apoptosis/embryonic placenta development/fat cell differentiation/neuron differentiation/regulation of transcription
rRNA promoter binding protein	EE397799	1.53	Cell proliferation/rRNA transcription
X-box binding protein 1B	EE395804	1.57	Regulation of transcription
Ribosomal protein L31	EE393469	1.73	Translation
Angiotensin I-converting enzyme (peptidyl-dipeptidase A) 2	EE395554	2.06	Entry of virus into host cell/muscle contraction/proteolysis/regulation of vasodilation
Transmembrane 4 superfamily member 4	EE398868	1.82	Negative regulation of cell proliferation/protein amino acid N-linked glycosylation
Transcription factor 7-like 1a (T-cell specific, HMG-box)	EE397557	1.84	Maternal determination of anterior/posterior axis, embryo
Similar to quiescin/sulfhydryl oxidase	EE393467	1.63	
Similar to putative secreted protein 4	EE395903	1.61	
Reticulon 4 interacting protein 1	EE396230	1.61	
Partner of Nob1	EE397320	1.51	
Similar to phytanoyl-CoA dioxygenase domain containing 1	EE397387	1.80	
High-mobility group box 2	EE398662	1.69	
Similar to RNA-binding region containing protein 2 (hepatocellular carcinoma protein 1) (splicing factor HCC1) isoform 6	EE394697	1.58	
Male and female (12)			
Dehydrogenase/reductase (SDR family) member 1	EE395412	2.00/1.61	Fatty acid biosynthesis
Vitellogenin	EE395969	6.13/2.15	Lipid transport/response to estrogen stimulus
Vitellogenin precursor	EE396220	3.37/2.22	Lipid transport
Similar to ectonucleoside triphosphate diphosphohydrolase 8 isoform 2	EE397039	1.50/1.51	Nucleoside diphosphate biosynthesis
Warm-temperature-acclimation-related-65 kDa-protein	EE396951	2.74/1.61	Acute phase response
Guanine nucleotide binding protein beta polypeptide 2-like 1	EE396427	1.70/2.10	Signaling pathway
Similar to tripartite motif protein 16	EE396021	1.83/1.60	Potassium channel inhibitor
ATP synthase, H$^+$ transporting, F1 complex, O subunit	EE397484	2.14/1.91	Oxidative phosphorylation
Cytochrome c oxidase subunit Va precursor	EE397037	1.54/1.77	Oxidative phosphorylation

Table 2 (*continued*)

Gene name[a]	Accession no.[b]	Fold change[c]	Functional category[d]
Male and female (12)			
Connexin 32.3	EE394266	1.61/1.54	Intercellular junction
Similar to inositol oxygenase	EE398949	2.12/1.93	
Phospholipid hydroperoxide glutathione peroxidase A	EE396999	1.81/−1.56	Response to oxidative stress

[a] From the annotation by searching for homologies in non-redundant (nr) protein database and nucleic acid (nt) database in GenBank by BLAST and named after the homologous sequences. Number of genes of each subgroup is given in parentheses.

[b] Accession numbers of rare minnow ESTs in GenBank.

[c] Average fold changes determined by microarray analysis. In subgroup of "male and female", the fold changes are referred to as male/female.

[d] Biological processes in GO terms.

Apolipoprotein B (ApoB) was unaffected in both males and females with the exception of fish exposed to 30 mg/L PFOA (Fig. 3). Gender differences in gene expression were also identified in apolipoprotein C-I precursor (ApoC-I), fatty acid-binding protein 10, liver basic (L-FABP), and delta-6 fatty acyl desaturase (FAD). Each of these genes was downregulated in males and unaffected in females (Fig. 3). Apolipoprotein B (ApoB) was unaffected in both males and females with the exception of fish exposed to 30 mg/L PFOA (Fig. 3). Warm-temperature-acclimation-65 kDa-protein (WTARP), fatty acid synthase (FAS), and type II iodothyronine deiodinase (Dio2) exhibited consistent alterations between males and females as compared to the controls in all three treated groups (Fig. 3) WTARP was significantly induced in both genders, whereas FAS and Dio2 were suppressed in both males and females following PFOA exposure. The genes for qRT–PCR validation are involved in metabolism, immune response, environmental adaptation, and hormone action, with special emphasis on fatty acid metabolism and transport. These genes may play important roles in the response to PFOA exposure in accord with previous studies in mammals (Guruge et al., 2006; Martin et al., 2007).

Discussion

The present study used a toxicogenomic approach to assess hepatic gene expression profiles following PFOA exposure in male and female rare minnows. PFOA exposure elicited obvious hepatic lesions in rare minnows as determined by histopathological observations, and no marked differences in histopathology were noted between males and females. The analysis of gene expression profiles using a custom cDNA microarray, however, revealed a number of conserved and gender-specific gene expression responses in rare minnows following PFOA exposure. These altered genes were involved in multiple biological processes, and these associations allow us to predict the potential mechanism of action of PFOA on rare minnows.

Gene expression responses related to lipid metabolism and transport

PFOA acts as a peroxisome proliferator that exerts a wide range of biological effects by activating PPAR in rodents (Klaunig et al., 2003; Permadi et al., 1992). These effects include increased β-oxidation of fatty acids, increases in several CYP450-mediated reactions, and inhibition of the secretion of both very low-density lipoproteins (VLDL) and cholesterol from the liver. In the present study, genes associated with β-oxidation of fatty acids in the mitochondria, including acyl-CoA dehydrogenase, glutaryl-CoA dehydrogenase, and enoyl-CoA hydratase, were unchanged in males but downregulated in females (Fig. 4). This finding suggests that PFOA exposure has suppressive effects on the mitochondrial catabolism of fatty acids in female rather than male minnows. In contrast, a previous study (Guruge et al., 2006) showed that male rats exposed to PFOA exhibited upregulation of genes involved in mitochondrial fatty acid β-oxidation. These results illustrate species and gender differences in the effects of PFOA on mitochondrial fatty acid β-oxidation between fish and rats although the mechanism of these actions is currently unclear.

The possible reason for discrepancy of influences on mitochondrial fatty acid β-oxidation by PFOA between rats and rare minnows may lie in an adaptive downregulation mechanism *in vivo* which could explain the low level of mitochondrial fatty acid β-oxidation observed in the present study. Seacat et al. (2003) has reported that the modest or no increase in palmitoyl CoA oxidase activity were observed in livers of rats fed 20 ppm perfluorooctane sulfonate (PFOS) (a chemical with a similar structure to PFOA) in the diet after 4 or 14 weeks, this result suggested that a mechanism may exist *in vivo* for an adaptive downregulation of hepatic peroxisome proliferation response to PFOS treatment (Shipley et al., 2004). Alternatively, a distinct mechanism may exist in fish that does not relate to mitochondrial fatty acid β-oxidation. It has been established that the biological effects on different species by PFOA are diverse. For example, although peroxisome proliferation had been well demonstrated involved in effects of PFOA on mouse, rats and humans, a study on Cynomolgus monkeys administered up to 0.75 mg/kg/day PFOS for 6 months did not exhibit peroxisome proliferation and no increase in palmitoyl-CoA oxidase activity in livers (Seacat et al., 2002).

A study on the impact of PFOA on the fathead minnow (*Pimephales promelas*) (Oakes et al., 2004) showed that peroxisome proliferation, as quantified by fatty acyl-CoA oxidase (FAO) activity, was elevated in the presence of low PFOA exposure (0.3 mg/L) but attenuated upon exposure to higher PFOA doses. In our study, fatty acid-binding protein 10, liver basic (L-FABP), an intracellular lipid carrier protein that is correlated with peroxisomal fatty acid oxidation, was down-regulated in males and unchanged in females (Fig. 4). An *in vitro* study (Luebker et al., 2002) revealed that PFOA exposure

Table 3
Significantly downregulated genes in the livers of *G. rarus* under PFOA exposure (Student's *t*-test; $p < 0.05$, and |fold change| ≥ 1.5) (68)

Gene name [a]	Accession no. [b]	Fold change [c]	Functional category [d]
Male (25)			
Transketolase	EE394673	−1.79	Carbohydrate metabolism
Phosphogluconate hydrogenase	EE396878	−1.72	Carbohydrate metabolism/water-soluble vitamin metabolism
Malate dehydrogenase 1, NAD (soluble)	EE397605	−1.54	Monosaccharide metabolism
Similar to apolipoprotein C-I precursor (Apo-CI)	EE392756	−1.75	Lipid metabolism
Dolichyl-diphosphooligosaccharide-protein glycosyltransferase	EE397717	−1.75	Protein biosynthesis
Similar to guanine deaminase	EE395320	−1.89	Guanine metabolic process
Similar to angiogenin ribonuclease 2	EE397488	−1.82	Regulation of cellular metabolism/blood vessel morphogenesis/protein biosynthesis
Similar to F-box protein 38 isoform b	EE395924	−2.63	Regulation of cellular metabolism/blood vessel morphogenesis/protein biosynthesis
Deiodinase, iodothyronine, type II (dio2)	EE397646	−2.50	Thyroid hormone metabolism
MHC class II antigen	EE394257	−2.63	Antigen processing
Intelectin	EE397659	−1.89	Innate immune response
C1 inhibitor	EE398721	−1.52	Regulation of immune response
Nucleolar protein 5A (*NOL5A*)	EE396025	−1.67	rRNA processing
Transposase	EE398709	−1.61	Transposition
ATP synthase F0 subunit 6	EE394997	−1.56	Oxidative phosphorylation
NADH dehydrogenase subunit 5	EE394965	−1.59	Oxidative phosphorylation
NADH ubiquinone oxidoreductase subunit 4L	EE399093	−1.72	Oxidative phosphorylation
Annexin A4	EE397590	−1.52	Exocytosis and regulation of epithelial Cl⁻ secretion
Beta-actin2	EE394149	−1.67	Cytoskeleton
Similar to microfibrillar-associated protein 4	EE395194	−1.72	Cell division
Beta-globin	EE399251	−1.69	Gas transport
Similar to organic solute transporter beta	EE398540	−1.72	Transport
Similar to putative transmembrane 4 superfamily member	EE394798	−1.67	
Similar to retinol binding protein 7, cellular	EE396535	−2.04	
Similar to C1q-like adipose specific protein	EE395888	−1.52	
Female (33)			
Cytochrome *P*450 1A	EE396294	−2.44	Xenobiotic metabolism
Similar to Mid1 interacting protein	EE396292	−2.27	Lipid metabolism and transport/steroid and sterol metabolism cellular protein metabolism
Carboxyl ester lipase	EE396450	−1.59	Lipid catabolism
Similar to apolipoprotein B	EE393465	−1.92	Cholesterol transport/lipid metabolism and transport/signal transduction/triacylglycerol mobilization
Phospholipase A2, group XIIB	EE394870	−1.59	Phosphatide metabolism
Similar to anionic trypsin II precursor (pretrypsinogen II)	EE397634	−1.64	Collagen catabolism/regulation of cell adhesion and cell growth/proteolysis
Carboxypeptidase B1 (tissue) isoform 1	EE393601	−1.85	Proteolysis
Carboxypeptidase A	EE393516	−2.00	Proteolysis
Chymotrypsinogen B1	EE394422	−2.17	Proteolysis
Elastase 2 like	EE393532	−1.54	Proteolysis
Elastase A	EE395966	−1.56	Proteolysis
Novel elastase protein (zgc:637440)	EE395967	−1.92	Proteolysis
Trypsin IA	EE397404	−1.64	Proteolysis
tRNA splicing endonuclease 54 homolog	EE396083	−1.61	RNA metabolism
Urate oxidase	EE394791	−1.72	Purine-based catabolism
Eukaryotic translation initiation factor 3, subunit 10 theta	EE392906	−1.61	Translation
Similar to RAB GTPase activating protein 1-like	EE395279	−1.64	Signal transduction
Similar to HIV-1 Rev binding protein	EE395930	−2.86	Regulation of signal transduction, transport and inflammation/ossification/acute-phase response
Fetuin-B	EE398809	−3.33	Regulation of signal transduction, immune response and bone remodeling/vesicle-mediated transport
Similar to cell growth regulator with EF hand domain 1	EE399079	−1.52	Cell growth regulation
Prothymosin, alpha (ptma), mRNA	EE397703	−1.67	Anti-apoptosis/cell differentiation and proliferation
Keratin	EE398842	−1.82	Epidermis development
Angiopoietin-like-3	EE398471	−1.56	Blood vessel morphogenesis
Similar to NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 5, 16 kDa precursor	EE395055	−1.56	Oxidative phosphorylation
Transferrin variant A	EE393592	−1.56	Ion transport
Transferrin variant D	EE394475	−1.72	Ion transport
Similar to Heme-binding protein 2	EE397620	−1.61	

Table 3 (continued)

Gene name [a]	Accession no. [b]	Fold change [c]	Functional category [d]
Female (33)			
High choriolytic enzyme 1 precursor	EE397092	−1.96	
Vtn protein	EE397297	−1.72	
Secreted phosphoprotein 24	EE398244	−2.27	
Similar to pancreatic protein with two somatomedin B domains	EE394127	−2.33	
Serine protease-like protein precursor	EE394226	−1.52	
Brain protein 44	EE398918	−1.56	
Male and female (10)			
Liver glycogen phosphorylase	EE394645	−1.69/−2.38	Glycogen metabolism
Glucokinase (GK)	EE398520	−1.89/−4.76	Glucose homeostasis
Fatty acid synthase	EE397675	−1.79/−1.67	Fatty acid biosynthesis
Putative delta-6 fatty acyl desaturase	EE396030	−1.50/−1.72	Fatty acid biosynthesis
Liver-expressed antimicrobial peptide 2 isoform B precursor	EE393319	−1.54/−1.56	Defense response to bacteria
CCAAT/enhancer binding protein delta	EE396713	−2.13/−1.67	Transcription
Mitochondrial carrier protein	EE395772	−2.04/−2.13	Mitochondrial transport
Novel protein containing a ChaC-like protein domain	EE395339	−5.56/−5.88	
Short-chain dehydrogenase/reductase	EE395263	−1.50/−2.04	
Phospholipid hydroperoxide glutathione peroxidase A	EE396999	1.81/−1.56	Response to oxidative stress

[a] From the annotation by searching for homologies in non-redundant (nr) protein database and nucleic acid (nt) database in GenBank by BLAST and named after the homologous sequences. The number of genes of each subgroup is given in parentheses.
[b] Accession numbers of rare minnows ESTs in GenBank.
[c] Average fold changes determined by microarray analysis. In subgroup of "male and female", the fold changes are referred to as male/female.
[d] Biological processes in GO terms.

interfered with the binding of fatty acids and other endogenous ligands to rat L-FABP. However, due to the few sequences of enzymes responsible for peroxisomal fatty acid β-oxidation that are currently available from the rare minnow library, no conclusions can be drawn about the effects of PFOA exposure on peroxisomal fatty acid β-oxidation that has been well established in rodents.

A number of genes involved in lipid transport and fatty acid biosynthesis were suppressed by PFOA treatment in this study. The downregulated genes related to lipid transport were ApoC-I and ApoC-II, which are major components of the VLDL that are responsible for the transport of endogenous triglycerides. In addition, FAS and FAD were significantly downregulated in both male and female rare minnows by PFOA exposure. These results suggest that PFOA exposure may inhibit biosynthesis of fatty acids and concomitantly reduce the export of endogenous triglycerides (Fig. 4). Apolipoprotein A-I (ApoA-I), which is responsible for the reverse transport of cholesterol to the liver, and ApoB, which is an essential component of low-density lipoproteins (LDL) implicated in the transport of endogenous cholesterol from the liver, were not affected by PFOA exposure at 10 mg/L (data not shown for ApoA-I, level of ApoB was shown in Fig. 3). Another cholesterol transporter, ABCA2, which plays a role in the intracellular trafficking of LDL-derived free cholesterol (Mack et al., 2006), was significantly induced in male rare minnows. These results suggest that PFOA exposure may not affect the transport of cholesterol to and from the liver but may, in fact, interfere with the intracellular trafficking of cholesterol in rare minnows.

Gene expression response related to hormonal effects

In the present study, PFOA exposure significantly suppressed the expression of Dio2 in all treated groups of rare minnows

according to both microarray and PCR results. Dio2 is responsible for the deiodination of T4 (3, 5, 3′, 5′-tetraiodothyronine) into T3 (3,5,3′-triiodothyronine) (Maia et al., 2007). Similarly, a recent study (Martin et al., 2007) showed that PFOA exposure significantly perturbed the expression of iodothyronine deiodinase type I (Dio1) and type III (Dio3) and lowered thyroid hormone levels in rats. These results suggest that PFOA may affect thyroid hormone biosynthesis in rare minnows by inhibiting Dio2. This effect did not exhibit a gender difference. Whether there is a species difference between fish and rats in terms of affected enzyme levels involved in thyroid hormone metabolism after PFOA exposure requires further investigation.

In addition to thyroid hormone disruption, changes in the expression of genes related to estrogen function following

Table 4

Comparison of fold change values of selected genes analyzed using microarray and PCR

Gene name	Male		Female	
	Microarray	PCR	Microarray	PCR
PP	3.59	47.32	−1.19	−1.27
BMG	−1.09	1.03	1.91	2.60
WTARP	2.74	3.00	1.61	1.91
ApoC-I	−1.75	−1.61	−1.36	−1.08
ApoB	−1.26	1.07	−1.92	−1.15
L-FABP	−1.31	−1.46	−1.42	−1.16
FAD	−1.50	−1.62	−1.72	−1.28
FAS	−1.79	−4.17	−1.67	−3.09
Dio2	−2.50	−4.59	−1.30	−2.84

Abbreviations: PP, 26–29 kDa-proteinase protein; *BMG*, β-2 microglobulin; *WTARP*, warm-temperature-acclimation-related-65 kDa-protein; *ApoC-I*, apolipoprotein C-I precursor; *ApoB*, apolipoprotein B; *L-FABP*, fatty acid-binding protein 10, liver basic; *FAD*, delta-6 fatty acyl desaturase; *FAS*, fatty acid synthase; *Dio2*, type II iodothyronine deiodinase.
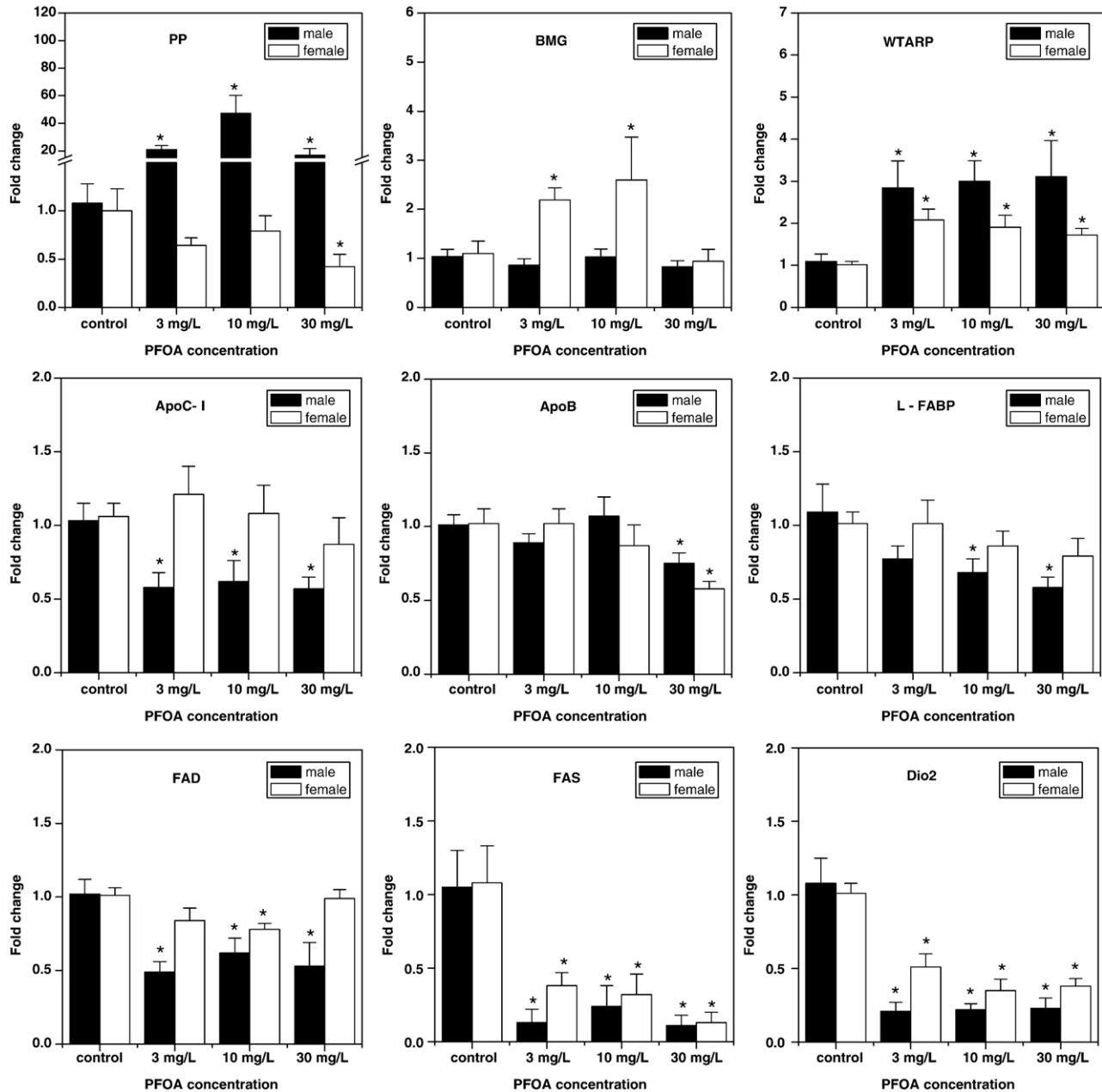
Fig. 3. Real-time PCR analysis of selected genes. Abbreviations: PP, 26–29 kDa-proteinase protein; BMG, β-2 microglobulin; WTARP, warm-temperature-acclimation-related-65 kDa-protein; ApoC-I, apolipoprotein C-I precursor; ApoB, apolipoprotein B; L-FABP, fatty acid-binding protein 10, liver basic; FAD, delta-6 fatty acyl desaturase; FAS, fatty acid synthase; Dio2, type II deiodinase iodothyronine. Bars represent the relative fold changes compared with controls. Error bars represent the SEM for the average fold changes. Statistical significance ($p < 0.05$) between expression following PFOA exposure and the controls is denoted by an asterisk (*).

PFOA exposure were also found in the present study. Vitellogenin (Vtg), a sensitive biomarker for endocrine disruptions, was significantly induced not only in males but also in females in all treatment group as well as at following exposure of only 14 days (data not shown). Moreover, the estrogen receptor β (ERβ) was upregulated and testes–ova were observed in male gonads (Wei et al., 2007). The increase in the estrogen-responsive genes, Vtg and ERβ, together with the testes–ova observed in male gonads strongly confirms the estrogen disruption following PFOA exposure in rare minnows.

Gene expression response related to other functions

PFOA exposure also perturbed a number of genes related to xenobiotic metabolism, immune responses, mitochondrial function, regulation of gene expression, and signal transduction. Many of these genes exhibited gender differences. Similar to expression in male rats exposed to PFOA (Guruge et al., 2006), CYP1A expression was suppressed in female minnows. Similar to male rats exposed to PFOS (Hu et al., 2005), CYP3A was induced in male minnows. Genes involved in immune responses
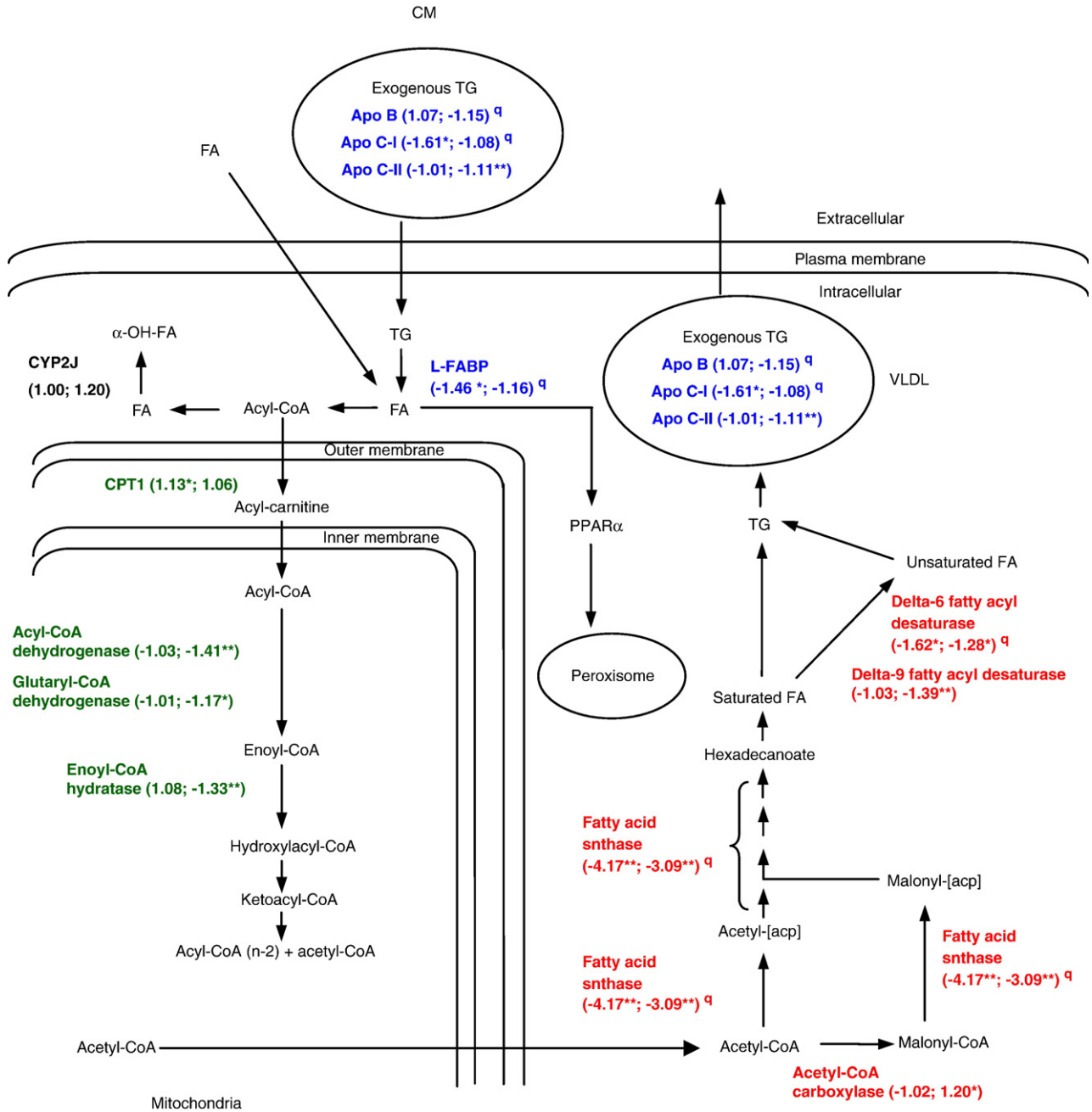
Fig. 4. Diagram of pathways for fatty acid metabolism and transport and the relative fold change of the genes after PFOA exposure in rare minnows. Values in parentheses represent the fold change of male and female gene expression responses after 10 mg/L PFOA exposure (male; female). The average fold changes were derived from microarray analysis with the exception of genes labeled "q" which were derived from the PCR data. Asterisks indicate statistical significance: $*p < 0.05$ and $**p < 0.001$ (microarray analysis), $p < 0.01$ (PCR). The colored names of the genes denote distinct functions: blue for transport, green for catabolic metabolism, and red for fatty acid biosynthesis.

were more likely to be induced in females and suppressed in males. A number of genes involved in mitochondrial transport and oxidative phosphorylation were affected by PFOA in male and/or female rare minnows. This finding supports the mitochondrial dysfunction observed in rats exposed to PFOA (Starkov and Wallace, 2002). With the exception of phospholipid hydroperoxide glutathione peroxidase A, few genes were associated with oxidative stress, suggesting that anti-oxidation is not likely involved in the adaptive response to PFOA in rare minnows.

In summary, a number of genes that were either upregulated or downregulated in the livers of male and female rare minnows following a continuous flow-through exposure to PFOA were identified using a custom cDNA microarray. The altered genes were found to be involved in multiple biological functions and processes, including lipid metabolism and transport, hormone function, immune responses, and mitochondrial function. In addition, the inhibition of thyroid hormone biosynthesis genes and induction of estrogen-responsive genes strongly suggests a role in endocrine disruption for PFOA. These results not only

contribute to the production of a database useful for the elucidation of the mechanism of PFOA toxicity in fish but also demonstrate gender differences in hepatic gene expression elicited by PFOA exposure. As only a limited number of sequences are currently available for rare minnows, the results in the present study represent only a mere fraction of the hepatic gene expression profiles in these fish after PFOA exposure. Further investigation is required to analyze the PFOA-mediated gene expression responses in fish on a larger scale and to identify sensitive biomarkers of exposure to PFOA in environmental toxicology.

Acknowledgments

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