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Effects of a short-term exposure to the fungicide prochloraz on endocrine function and gene expression in female fathead minnows (*Pimephales promelas*)

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ABSTRACT

Prochloraz is a fungicide known to cause endocrine disruption through effects on the hypothalamic–pituitary–gonadal (HPG) axis. To determine the short-term impacts of prochloraz on gene expression and steroid production, adult female fathead minnows (*Pimephales promelas*) were exposed to the chemical (0 or 300 μg/L) for a time-course of 6, 12 and 24 h. Consistent with inhibition of cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17) and aromatase (CYP19), known molecular targets of prochloraz, plasma 17β-estradiol (E2) was reduced within 6 h. *Ex vivo* E2 production was significantly reduced at all time-points, while *ex vivo* testosterone (T) production remained unchanged. Consistent with the decrease in E2 levels, plasma concentrations of the estrogen-responsive protein vitellogenin were significantly reduced at 24 h. Genes coding for CYP19, CYP17, and steroidogenic acute regulatory protein were up-regulated in a compensatory manner in ovaries of the prochloraz-treated fish. In addition to targeted quantitative real-time polymerase chain reaction analyses, a 15k feature fathead minnow microarray was used to determine gene expression profiles in ovaries. From time-point to time-point, the microarray results showed a relatively rapid change in the differentially expressed gene (DEG) profiles associated with the chemical exposure. Functional analysis of the DEGs indicated changes in expression of genes associated with cofactor and coenzyme binding (GO:0048037 and 0050662), fatty acid binding (GO:0005504) and organelle organization and biogenesis (GO:0006996). Overall, the results from this study are consistent with compensation of the fish HPG axis to inhibition of steroidogenesis by prochloraz, and provide further insights into relatively rapid, system-wide, effects of a model chemical stressor on fish.

1. Introduction

Prochloraz is an imidazole fungicide known to affect endocrine function through interactions with the hypothalamic–pituitary–gonadal (HPG) axis (Ankley et al., 2005, 2009; Vinggaard et al., 2000). Imidazole fungicides are designed to inhibit the enzyme 14α-demethylase, a cytochrome P450 (CYP) isoform important for ergosterol biosynthesis in fungal cells (Henry and Sissler, 1984; van den Bossche et al., 1978). In animals, CYP 14α-demethylase (CYP51) demethylates lanosterol. This demethylation is an important reaction in the metabolic pathway leading to the biosynthesis of cholesterol (KEGG PATHWAY database: Kanehisa and Goto, 2000). In addition to affecting CYP51, prochloraz can cause endocrine disruption in mammals (e.g., human placental cell-lines and rats) through other mechanisms, including antagonism of the androgen and estrogen receptors (Andersen et al., 2002; Laier et al., 2006; Noriega et al., 2005), and inhibition of CYP enzymes important to steroidogenesis such as CYP 17α-hydroxylase/17,20-lyase (CYP17) (Blystone et al., 2007a,b; Vinggaard et al., 2005, 2006), and aromatase (CYP19) (Andersen et al., 2002; Mason et al., 1987; Vinggaard et al., 2000).

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Studies from our lab indicate that prochloraz also affects the HPG axis function in fish through inhibition of CYP17 and CYP19 both *in vivo* and *in vitro* (Ankley et al., 2005, 2009; Villeneuve et al., 2007a). Due to the ability of the fungicide to inhibit steroidogenic CYPs, prochloraz has been used as a model chemical for validating assays designed to detect HPG-active chemicals (Organisation for Economic Co-operation and Development, 2006; US Environmental Protection Agency, 2007).

Reproduction in fish is a complex process modulated by numerous internal and external cues via the HPG axis (Thomas, 2008; Villeneuve et al., 2007b). Production of the sex steroids testosterone (T) and 17β-estradiol (E2) is a critical component of the system. Gonadal sex steroids are produced from cholesterol, which is converted to T through a series of reactions, some of which are catalyzed by CYPs, including CYP cholesterol side-chain cleavage (CYP11A) and CYP17. Conversion of T to the estrogen E2 is catalyzed by CYP19. Thus, the expression and activity of CYP19 represent important points of control in maintaining appropriate T:E2 ratios (Cheshenko et al., 2008; Tanaka et al., 1992). The ovarian secretion of E2, in particular, is a key signal relative to female fish reproduction. Synthesis of vitellogenin (VTG), a lipoprotein precursor of egg yolk proteins, occurs in the liver through stimulation of the estrogen receptor by E2. After VTG is produced in the hepatocytes, it is secreted into the bloodstream and transported to the ovaries for incorporation into maturing oocytes (Wallace, 1985). As a result, chemicals that affect steroid production can impact vitellogenesis and have a profound effect on reproduction. For example, female fathead minnows (*Pimephales promelas*) exposed to prochloraz exhibit decreased gonadal E2 production, depressed VTG levels in the plasma, and reduced fecundity (Ankley et al., 2005, 2009).

To maintain homeostatic conditions supporting reproduction, fish can respond to chemical disruption of HPG function via a variety of compensatory mechanisms. For example, Ankley et al. (2007) demonstrated that ketoconazole, a fungicide known to inhibit enzymes involved in steroid biosynthesis, significantly reduced steroid production in both ovary and testis tissue in fathead minnows exposed to the compound for 21 d. The fish responded to the chemical stressor through increased gonadosomatic index (GSI), interstitial (steroid producing) cell proliferation in the testis, and increased expression of genes coding for enzymes thought to be inhibited by ketoconazole. Other studies with the model aromatase inhibitor fadrozole have shown that female fathead minnows may compensate for exposure to the chemical through up-regulation of a number of ovarian HPG-axis genes, including steroidogenic acute regulatory protein (*star*), *cyp11a* and *cyp19a1a* (Villeneuve et al., 2006, 2009). Thus, both changes in gonadal mass and transcription of genes coding for key regulatory proteins are two potential compensatory responses to chemicals that inhibit steroid biosynthesis.

Similar compensatory responses have been seen in studies with prochloraz in our lab. For example, depression of E2 production in female fathead minnows, accompanied by up-regulation of *cyp17* and *cyp19a1a* were seen after as little as 1 d of exposure to 300 μg prochloraz/L (Ankley et al., 2009). Past studies concerning the dynamics of changes in the HPG-axis of fish exposed to chemicals have usually not considered time-points of less than 24 h, so it was unclear how rapid these responses may be. The objective of the present study was to investigate the effects of an exposure to 300 μg prochloraz/L for 6, 12 or 24 h on basic endocrine function in female fathead minnows. In addition to determining steroid and VTG concentrations, gene expression was analyzed by quantitative real-time polymerase chain reaction (QPCR) and by microarray. The QPCR analysis enabled us to focus on specific genes related to HPG function, while the aim of the microarray analysis was to investigate broader gene expression profiles in ovarian tissue that may be indicative of other pathways of toxicological concern.

2. Materials and methods

2.1. Prochloraz exposure

Adult female fathead minnows (5–6 months old) from an on-site culture facility at the US Environmental Protection Agency (USEPA) lab in Duluth, MN, USA were used for the experiment. Prochloraz (99.5% purity) was obtained from Sigma–Aldrich (St. Louis, MO, USA). Solvent-free stock solutions of the fungicide were prepared in Lake Superior water as described elsewhere (Ankley et al., 2005). A target test concentration of 300 μg prochloraz/L was chosen based on effect–concentrations from a previous 21 d fathead minnow reproduction assay with prochloraz (Ankley et al., 2005), as well as a 16 d time-course study with the fungicide (Ankley et al., 2009). Glass 20-L test tanks were divided into three sections with nylon mesh screen, and two female fathead minnows were placed in each section. There were four replicate tanks per treatment (control and prochloraz). The fish were held for an acclimation period of 4 d in clean water prior to initiation of the exposure and fed thawed adult brine shrimp twice per day. Each tank contained 10 L of Lake Superior water (control) or prochloraz solution, delivered at a continuous-flow of about 45 mL/min. The fish were kept at 25 ± 1 °C under a 16:8 h light:dark photoperiod. Animal research protocols were approved by the on-site Animal Care and Use Committee in accordance with Animal Welfare Act regulations and Interagency Research Animal Committee guidelines.

At 6, 12 and 24 h after starting the prochloraz exposure, two fish were removed from one of the screened sections in each exposure tank, yielding a total of eight fish per treatment at each sampling time. The fathead minnows were anesthetized in buffered tricaine methanesulfonate (MS-222; 100 mg/L buffered with 200 mg $NaHCO_3$/L, Argent, Redmond, WA, USA). The animals were weighed and blood was collected from the caudal vein/artery with a heparinized microhematocrit tube. Plasma was separated by centrifugation and stored at −80 °C until analyzed for E2 and VTG. The ovaries were removed from the fish and weights recorded for calculation of the GSI. The ovaries then were sectioned into four pieces. Two portions were snap-frozen in liquid nitrogen and stored until used for gene expression analyses. A third portion was preserved in Davidson's fixative for possible histological analysis. The final piece of the gonad was used immediately for an *ex vivo* steroid production assay. The brain was removed from the fish and snap-frozen in liquid nitrogen, and the pituitary was removed, placed in RNAlater (Sigma–Aldrich) and stored until used for gene expression analyses.

To confirm exposure conditions, water samples (1 mL) were collected from all the test tanks at the start of the test and at each sampling period. Prochloraz was measured using reverse-phase high pressure liquid chromatography with diode array detection, as described elsewhere (Ankley et al., 2005, 2009). No prochloraz was detected in the control tanks ($n = 16$) or in the procedural blanks ($n = 4$). Measured concentrations of prochloraz were about 30% lower than the target concentration, but remained relatively constant with mean values (SD, n) of 218.3 (1.7, 4), 202 (1.5, 4), 201 (0.82, 4) and 195 (1.6, 4) μg/L at 0, 6, 12 and 24 h, respectively. The mean (SD, n) recovery of prochloraz in spiked Lake Superior water samples was 93.8% (2.1, 4), and the agreement among duplicate samples was 99.5% (1, 4).

2.2. Biochemical analyses

Plasma VTG concentrations in the females were quantified using an enzyme-linked immunosorbent assay with a polyclonal antibody to the fathead minnow VTG and purified fathead minnow VTG as a standard (Korte et al., 2000). Plasma E2 was quantified using radioimmunoassay (RIA) when the available sample volume

was ≥5 μL (Jensen et al., 2001). *Ex vivo* production of T and E2 in the ovary tissue was determined with an adaptation of the method of McMaster et al. (1995), as described by Villeneuve et al. (2009), using RIA to measure steroids in the culture medium.

2.3. Real-time polymerase chain reaction

Gonad and brain samples were extracted with TRI Reagent® (Sigma–Aldrich) following the manufacturer's protocol. Total RNA was extracted from individual pituitary samples using RNeasy micro kits (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. The resulting total RNA samples were resuspended in RNase-free water (Ambion, Austin, TX, USA), and the RNA concentration was measured using a Nanodrop® ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). The RNA samples had optical density 260/280 ratios between 1.8–2.2 (gonad and brain) and 1.4–2.5 (pituitary). Total RNA samples were diluted to 10 ng/μL (gonad and brain) or to 0.5 ng/μL (pituitary) and stored at −80 °C until analyzed using QPCR.

Expression of several important genes in the HPG-axis was determined. Genes measured in the ovaries included those for three CYP isozymes involved in gonadal steroidogenesis: *cyp11a*, *cyp17* and *cyp19a1a* (the isoform of aromatase expressed predominantly in the ovary). Two additional genes measured in the ovary were *star* and follicle-stimulating hormone receptor (*fshr*). Pituitary transcripts measured included luteinizing hormone β subunit (*lhb*), follicle-stimulating hormone β subunit (*fshb*) and gonadotropin-releasing hormone receptor 1 (*gnrhr1*). Transcripts measured in the brain of female fish were aromatase B (*cyp19a1b*; isoform of aromatase expressed predominantly in the brain), *cyp51* and chicken-II-type gonadotropin-releasing hormone (*cgnrh*). Primer information for the genes listed above can be found in Supplemental Table S1.

For the most of the genes (*star*, *cyp11a*, *cyp17*, *cyp19a1a*, *fshr*, *fshb*, *lhb* and *cgnrh*) QPCR assays were performed directly from total RNA using a one-step Taqman® EZ RT-PCR kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's recommended protocol. Each 25-μL reaction contained 150 nM of a gene-specific Taqman probe, 200 nM of forward and reverse primers and 20 ng (ovary and brain) or 1 ng (pituitary) total RNA. Samples were amplified over 40 cycles and the relative transcript abundance was estimated as described by Ankley et al. (2009). Relative transcript abundance was estimated based on a standard curve generated by analyzing multiple dilutions of a gene-specific DNA amplicon, without correction for amplification efficiency. Amplicons used as standards were amplified from fathead cDNA with the same gene-specific primers used for QPCR. Dilution of the amplicon used as a standard was optimized to yield a standard curve over threshold cycle numbers ranging from about 15 to 35.

The QPCR assays for the rest of the genes were conducted with a one-step procedure using Power SYBR Green PCR Master Mix (Applied Biosystems). Template RNA (2.0 μL) was combined with 200 nM forward and reverse primers and 2× Master Mix in a 12-μL reaction. Samples were amplified over 40 cycles, and relative transcript abundance was estimated based on a standard curve generated by analyzing multiple dilutions of a gene-specific amplicon as described by Ankley et al. (2009). Specificity of the amplified signal was determined from melting curve analysis. Relative transcript abundance was estimated based on a standard curve generated as mentioned above.

The one-step QPCR procedure also was used to evaluate relative gene expression changes in the female gonads of a subset of differentially expressed genes (DEGs) determined by the microarray analysis, to help confirm the microarray results by QPCR (Supplemental Fig. S1). These genes were selected based on fold-change over time, confidence in annotation and knowledge of the gene's function. Genes, primer pairs and expressed sequence tag information for target genes for this analysis are provided in Supplemental Table S1.

2.4. Microarray analysis of gene expression

Half of the RNA samples ($n = 4$) for each treatment/time point combination were used for microarray analysis (total of 24 arrays). For the microarray work, total RNA was isolated from ovary samples using RNeasy kits (Qiagen, Valencia, CA, USA). The RNA quality was assessed with an Agilent 2100 Bioanalyzer (Agilent, Wilmington, DE, USA) and quantity was determined using a Nanodrop® ND-1000 spectrophotometer. Total RNA was stored at −80 °C until analyzed with oligonucleotide microarrays. Microarray analysis was conducted at the US Army Engineer Research and Development Center (Vicksburg, MS, USA) using a 15k feature fathead minnow microarray (GEO: http://www.ncbi.nlm.nih.gov/geo/; Accession platform number GPL9248) designed by Dr. Nancy Denslow (University of Florida, Gainesville, FL, USA) and manufactured by Agilent Technologies (Palo Alto, CA, USA). The Agilent one-color microarray hybridization protocol (One-Color Microarray-Based Gene Expression Analysis, version 5.7, Agilent Technologies) was used for microarray hybridizations. 1 μg of total RNA was used for all hybridizations. The cDNA synthesis, cRNA labeling, amplification and hybridization were performed following the manufacturer's kits and protocols (Quick Amp Labeling kit; Agilent Technologies). An Axon GenePix® 4000B Microarray Scanner (Molecular Devices Inc., Sunnyvale, CA, USA) was used to scan microarray images at 5 μm resolution. Data were resolved from microarray images using Agilent Feature Extraction software (Agilent Technologies). Consistent with the Minimum Information About a Microarray Experiment standards (Brazma et al., 2001), text versions of the Agilent raw data from this study have been deposited at the Gene Expression Omnibus website (GEO: http://www.ncbi.nlm.nih.gov/geo/; accession series record number GSE26958).

2.5. Statistical analysis

Statistical analyses were conducted using Statistica 8 (StatSoft Inc., Tulsa, OK, USA) and GraphPad Instat v. 3.01 (GraphPad Software, San Diego, CA, USA). Data were tested for normality and homogeneity of variance. When data conformed to parametric assumptions, data were analyzed using a one-way ANOVA with chemical treatment as the independent variable. When data did not conform to parametric assumptions, they were either transformed (log 10) or analyzed using a non-parametric Kruskall–Wallis test ($p \leq 0.05$). The data are presented as means with standard errors of the mean (SEM). Differences were considered significant at $p \leq 0.05$.

Microarray data were imported into the Rosetta Resolver 7.2 system for gene expression analysis (Rosetta Biosoftware, Kirkland, WA, USA). Data were normalized using the default, text loader, intensity profile builder settings in the Rosetta Resolver system. Significant DEGs were identified using one-way ANOVA ($p < 0.01$) with no multiple testing correction. Two approaches were applied. The first used one-way ANOVA to identify genes significantly differentially expressed, between control and prochloraz-exposed fish, at each time point (Supplemental Table S2). The second approach assessed DEGs associated with prochloraz treatment, across all time-points (Supplemental Table S3). Analysis of significantly enriched functional Gene Ontology (GO) categories was carried out using the 413 DEGs identified using the second approach (Supplemental Table S3). Enrichment analysis was conducted using eGOn v.2.0 (explore GeneOntology; http://www.genetools.microarray.ntnu.no/egon/; Beisvag et al., 2006). GenBank accession numbers corresponding to the 413 DEGs

Table 1
Gene expression changes determined by the 15k feature fathead minnow microarray in the ovaries of female fathead minnows exposed to 300 μg prochloraz/L for 6, 12 or 24 h. The number of up- and down-regulated differentially expressed genes identified by ANOVA ($p \leq 0.01$) for each time-point.

Time-point	Up-regulated	Down-regulated	Total
6 h	130	15	145
12 h	92	2	94
24 h	64	7	71

sequences (Supplemental Table S3) were loaded as reporter lists in eGOn. GenBank accession numbers corresponding to every feature on the array were loaded as a master reporter list. Of the 15,208 genes uploaded as the master list, 4264 genes (28%) had associated GO-terms. For the 413 DEGs uploaded in to eGOn, 147 genes (35.5%) had associated GO-terms. The statistical test applied was eGOn's Master-Target comparison, employing Fisher's exact test, with *p*-value of 0.05. This was used to determine GO categories over- or under-represented on the DEG list, compared with their overall representation on the entire microarray. The analyses examined molecular function, biological process and cellular component GO-terms. In general, results are shown with auto grouping, with the limit set to 20. Categories with only one DEG represented were excluded.

3. Results

There was no prochloraz-induced mortality during the test, nor were there any observations of abnormal behavior. There were no significant effects of prochloraz on the GSI of the female fathead minnows with mean (SEM) values of 13.5% (0.91) and 14.3% (0.79) for control and treated fish, respectively.

Prochloraz reduced plasma E2 concentrations in the fish, with significant effects at 12 and 24 h, and a large, but not significant, decrease at 6 h (Fig. 1a). The *ex vivo* production of E2 by ovarian tissue reflected the observed depression of plasma concentrations of the estrogen (Fig. 1b). *Ex vivo* E2 production was significantly depressed within 6 h and remained decreased throughout the 24 h exposure to the pesticide. *Ex vivo* ovarian production of T did not vary significantly between the control and treatment groups (Fig. 1c). A significant decrease in plasma VTG concentration was observed at 24 h of exposure (Fig. 1d).

Several gene products measured by QPCR in the ovaries, *star*, *cyp11a*, *cyp17* and *cyp19a1a*, displayed a relatively similar expression pattern in response to the prochloraz exposure (Fig. 2a–d). All four transcripts were significantly elevated by the 12 h time-point, and three of the four genes remained statistically higher than controls at 24 h (*star*, *cyp17* and *cyp19a1a*). Expression of *cyp19a1a* and *star* were significantly increased within 6 h (Fig. 2a and d). Ovarian *fshr* was significantly decreased at the 6 h time-point and returned to control levels at the 12 and 24 h sampling times.

There was no significant difference between the control versus treated groups in expression of three genes examined in the brain, *cyp19a1b*, *cyp51* and *cgnrh* (Fig. 3a–c). However, expression of *cyp51* (the CYP isozyme targeted by prochloraz in fungi) appeared elevated at 6 h ($p = 0.056$) before returning to control levels at 12 and 24 h (Fig. 3b). Expression of *lhb*, *fshb* and *gnrhr1* in pituitary tissue did not show any significant treatment-related effects (Supplemental Fig. S2).

The number and identity of significantly up- and down-regulated DEGs from the microarray analysis between control and prochloraz-exposed fish varied markedly over time (Supplemental Table S2). For example, at 6, 12 and 24 h there were, respectively, 130/15, 92/2 and 64/7 up/down regulated genes (Table 1). The number of significantly up-regulated genes detected outnumbered the down-regulated genes, which accounted for just 2–10% of those

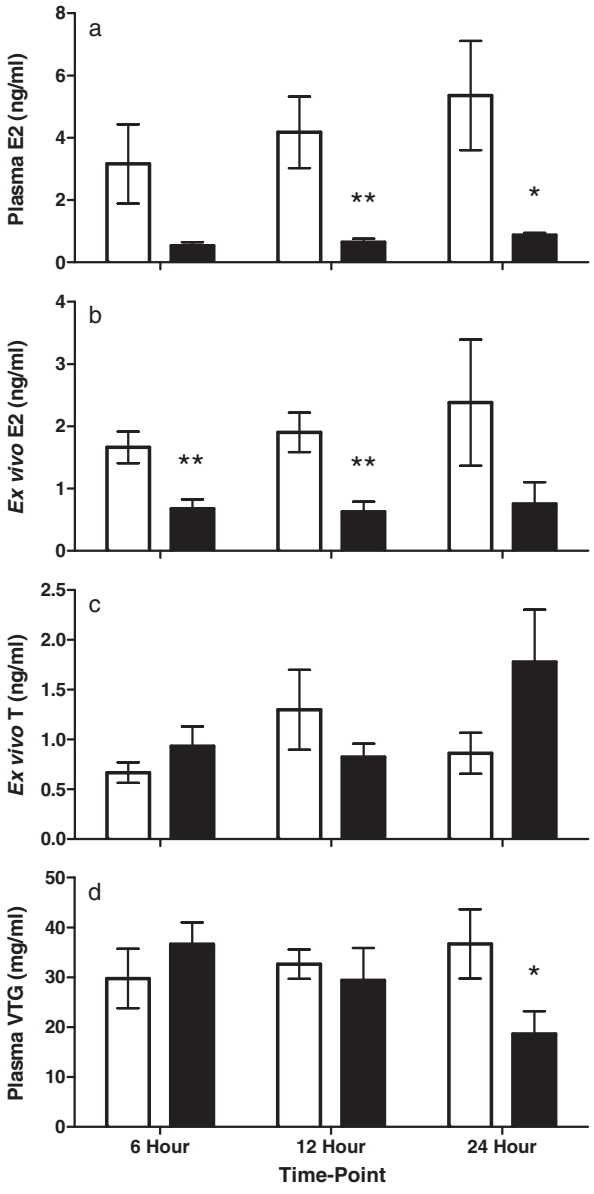

Fig. 1. Effects of prochloraz on (a) plasma estradiol (E2), and (b and c) *ex vivo* ovary synthesis of E2 and testosterone (T), and (d) plasma vitellogenin (VTG) concentrations, in female fathead minnows exposed to 0 (control) or 300 μg prochloraz/L for 6, 12 or 24 h. Open bars show results for Lake Superior water controls; solid bars show results for prochloraz treatment. Bars indicate mean ± standard error of the mean ($n = 7$–8). Asterisks ($^{*}p \leq 0.05$; $^{**}p \leq 0.01$) indicate a significant treatment-related effect compared to Lake Superior water (controls) for any given time-point.

differentially expressed. The QPCR genes selected for validation were quite consistent with the microarray data, both in terms of direction (up or down) and fold-changes at each time-point, indicating no evidence of bias for up-regulated genes in the microarray data. Among the 310 DEGs detected as statistically differentially expressed for a specific time-point, only four genes overlapped among the different times. The four genes that did overlap, similar to neogenin, solute carrier family 7 member 9, hypothetical loc559801 and similar to transport-associated protein, were significantly expressed at 6 h and 24 h, but not at 12 h.

To further illustrate the time-dependent differences in the effects of prochloraz treatment on gene expression, the group of 306 DEGs associated with prochloraz treatment at each time-point (Supplemental Table S2), were ordered relative to fold-change in expression at the 6 h time-point and subsequently compared with

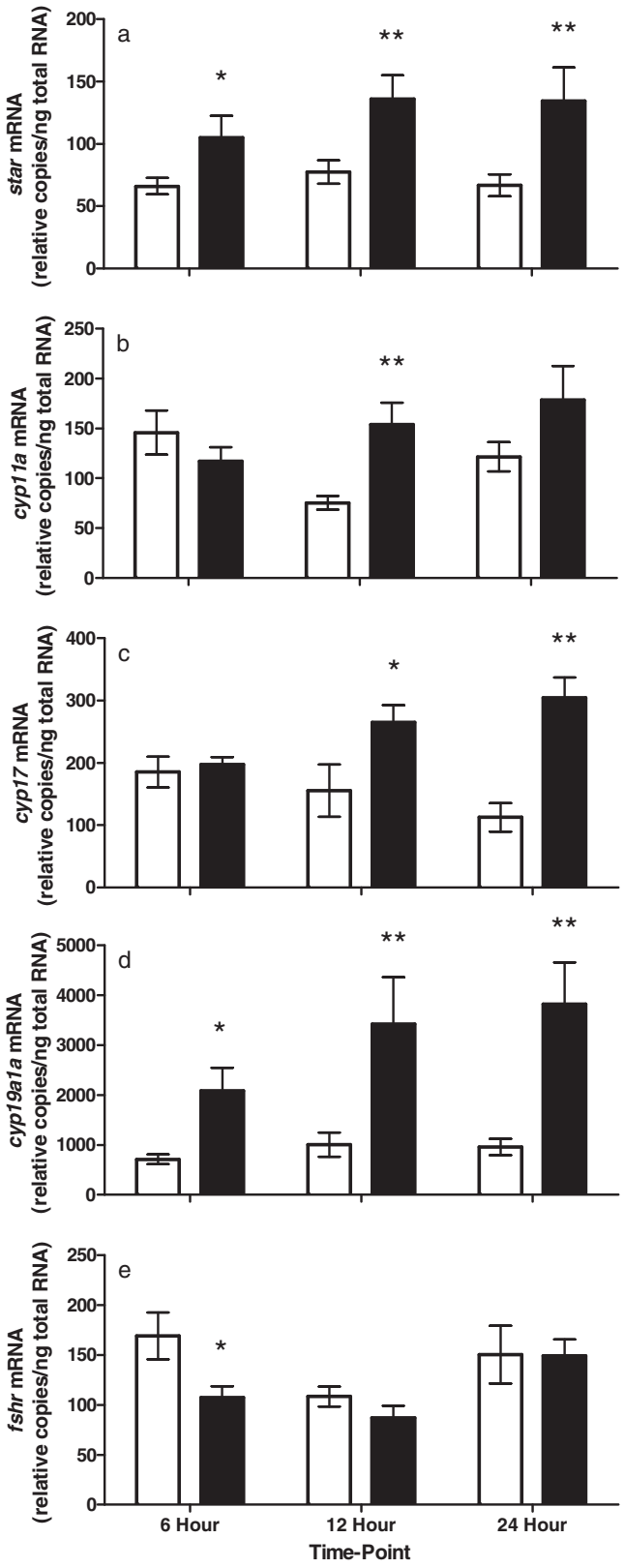

Fig. 2. Relative abundance of five gene products in ovary tissue from female fathead minnows exposed to 0 (control) or 300 μg prochloraz/L for 6, 12 or 24 h. Genes monitored were: (a) *star*: steroidogenic acute regulatory protein, (b) *cyp11a*: cytochrome P450 cholesterol side-chain cleavage, (c) *cyp17*: cytochrome P450 17α-hydroxylase/17,20 lyase, (d) *cyp19a1a*: ovarian aromatase, and (e) *fshr*: follicle-stimulating hormone receptor. Open bars show results for Lake Superior water controls; solid bars show results for prochloraz treatment. Bars indicate mean ± standard error of the mean (n = 7–8). Asterisks ($^*p \leq 0.05$; $^{**}p \leq 0.01$) indicate a significant treatment-related effect compared to Lake Superior water (controls) for any given test time-point.

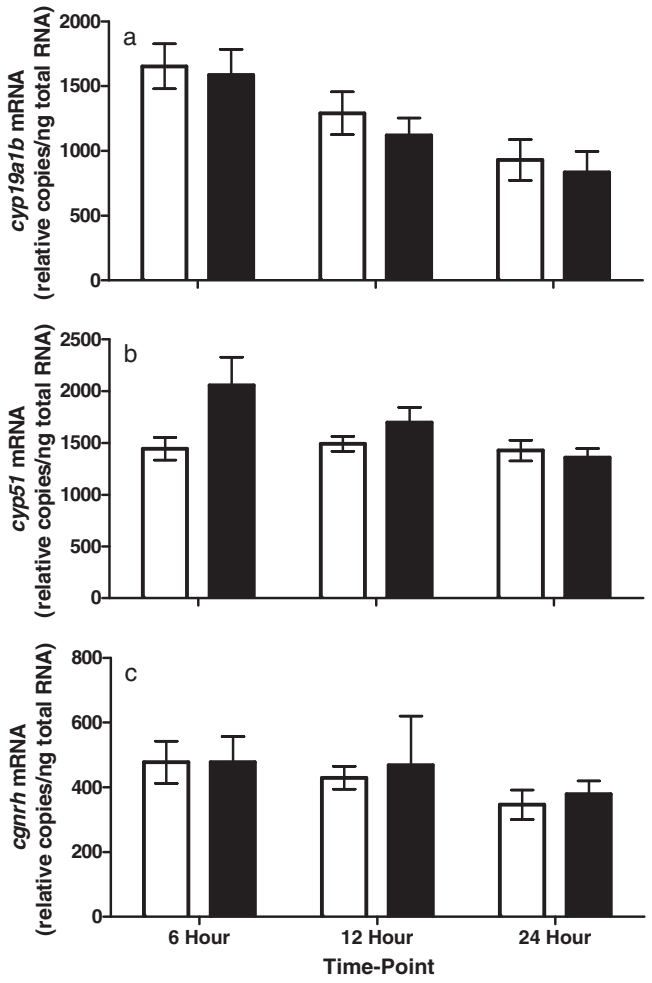

Fig. 3. Relative abundance of three gene products in brain tissue from female fathead minnows exposed to 0 (control) or 300 μg prochloraz/L for 6, 12 or 24 h. Genes monitored were: (a) *cyp19a1b*: brain aromatase, (b) *cyp51*: cytochrome P450 14α-demethylase, and (c) *cgnrh*: chicken-II-type gonadotropin-releasing hormone. Open bars show results for Lake Superior water controls; solid bars show results for prochloraz treatment. Bars indicate mean ± standard error of the mean (n = 7–8).

expression profiles at both 12 and 24 h (Fig. 4a–c). Relative expression of this initial set of 306 genes changed substantially over time. For example, of the 65 genes up-regulated by 2-fold or greater at 6 h, 43 were down-regulated at 12 h and 37 at 24 h. Of the 16 genes down-regulated by 2-fold or greater at 6 h only three were down-regulated at 12 h and seven at 24 h.

In order to assess the enriched GO categories associated with prochloraz exposure, the set of 413 DEGs associated with prochloraz treatment across all time-points (Supplemental Table S3), were categorized with eGOn. These GO categories were subsequently separated on the basis of their classification as molecular function, biological process or cellular component (Table 2; Supplemental Table S4). Analysis of the microarray data for these categories using eGOn showed differential regulation associated with cofactor, coenzyme and fatty acid binding (GO:0048037, 0050662 and 0005504). Some of the differentially expressed features associated with cofactor, coenzyme and fatty acid binding were annotated as similar to glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), acyl-coenzyme A binding domain containing 6 (*acbd6*) and Zgc:112043 (also known as acyl-coenzyme A binding domain containing 5a, *acbd5a*). Under the biological process category, differentially expressed features annotated with organelle and chromosome organization and biogenesis (GO:0006996 and

Table 2
Enriched gene ontology (GO) categories[a] associated with 413[b] differentially expressed genes (identified by ANOVA ($p \leq 0.01$)) determined by the 15k feature fathead minnow microarray in the ovaries of female fathead minnows exposed to 300 μg prochloraz/L for 6, 12 or 24 h. Categories were determined by eGOn ($p \leq 0.05$).

	Name	Genes in category	Genes in list category[c]	*p*-Value
GO term moleuclar function				
GO:0003674	Molecular_function	3905	122	
GO:0048037	Cofactor binding	67	7	0.004
GO:0050662	Coenzyme binding	49	6	0.004
GO:0005504	Fatty acid binding	4	2	0.006
GO:0017076	Purine nucleotide binding	451	23	0.014
GO:0030554	Adenyl nucleotide binding	354	19	0.016
GO:0032559	Adenyl ribonucleotide binding	338	19	0.013
GO:0005524	ATP binding	338	19	0.013
GO:0032555	Purine ribonucleotide binding	435	23	0.012
GO:0032553	Ribonucleotide binding	435	23	0.012
GO:0019842	Vitamin binding	24	3	0.037
GO:0016614	Oxidoreductase activity, acting on CH–OH group of donors	22	3	0.03
GO:0016616	Oxidoreductase activity, acting on the CH–OH group of donors, NAD or NADP as acceptor	21	3	0.026
GO:0019208	Phosphatase regulator activity	5	2	0.009
GO term biological process				
GO:0008150	Biological_process	3555	116	
GO:0006996	Organelle organization and biogenesis	134	9	0.04
GO:0051276	Chromosome organization and biogenesis	45	5	0.015
GO:0006325	Establishment and/or maintenance of chromatin architecture	38	0.034	
GO:0045596	Negative regulation of cell differentiation	4	2	0.006
GO:0044249	Cellular biosynthetic process	217	13	0.028
GO:0009123	Nucleoside monophosphate metabolic process	11	3	0.005
GO:0009165	Nucleotide biosynthetic process	34	4	0.023
GO:0006163	Purine nucleotide metabolic process	29	4	0.014
GO:0009259	Ribonucleotide metabolic process	28	4	0.012
GO:0055086	Nucleobase, nucleoside and nucleotide metabolic process	55	5	0.032
GO:0007423	Sensory organ development	60	5	0.045
GO:0043010	Camera-type eye development	38	4	0.034
GO term cellular component				
GO:0005575	Cellular_component	3564	123	
GO:0015629	Actin cytoskeleton	11	3	0.005

[a] Categories with only one differentially expressed genes represented were excluded.
[b] List of 413 differentially expresses genes in Supplemental Table S3.
[c] Genes in list category in Supplemental Table S4.

0051276) were ribosomal L24 domain containing 1 (*rsl24d1*) and structural maintenance of chromosomes 4 (*smc4*).

Although our QPCR measurements clearly showed that prochloraz increased the transcription of several ovarian genes, the microarray did not highlight these as differentially expressed. This could be because overall transcript abundance was relatively low for the genes of interest (e.g., *star*, *cyp11a*, *cyp17* and *cyp19a1a*) as determined by the microarray. Additionally, QPCR primers (and associated amplicon) were not specifically designed to overlap the 60-mer oligonucleotide probes on the microarrays, suggesting that binding affinities may have varied somewhat between the two methods. Most notably, the microarray analysis included only half the sample size analyzed by QPCR. As a result, there was less power to detect the differences using microarray. However, a comparison of the microarray results to the genes selected for QPCR confirmation (Supplemental Fig. S1) showed good overall good agreement.

4. Discussion

The results of the present study are consistent with, and build on, previous work from our lab concerning effects of prochloraz on the fathead minnow (Ankley et al., 2005, 2009). Ankley et al. (2005) reported that a 21 d exposure to 300 μg prochloraz/L caused a decrease in plasma E2 and VTG concentrations in females, which coincided with a significant decrease in fecundity of the fish. Ankley et al. (2009) exposed fathead minnows to 300 μg prochloraz/L for 8 d, followed by an 8 d depuration phase, with periodic sampling during both phases to examine direct impacts, adaptation/compensation and/or recovery. They found a significant depression of plasma E2 and *ex vivo* E2 production within 1 d of exposure to prochloraz. Ankley et al. (2009) also reported that key genes relative to steroidogenesis (e.g., *cyp19a1a* and *cyp17*), were elevated during the exposure phase of the test and returned to control levels after cessation of the prochloraz treatment. In the present work, we have expanded on those previous studies by examining effects of prochloraz treatment within the first 24 h of an exposure. Prochloraz caused a rapid (within 6 h) depression in E2 production and circulating E2 levels in female fish. Also observed in the present study was a relatively quick up-regulation of several genes directly involved in steroid synthesis: *star*, *cyp11a*, *cyp17* and *cyp19a1a*. This indicates that the animals were capable of a compensatory gene expression response to inhibition of steroid synthesis by prochloraz within a matter of hours. Among the genes examined by QPCR, the most significantly affected gene, based on fold change, in the prochloraz-treated fish was *cyp19a1a*, the key control point in the conversion of androgens to estrogens in the gonad.

Prochloraz had a profound effect on E2 status in the female fathead minnows in terms of both *ex vivo* production and *in vivo* plasma concentrations. As previously mentioned, decreases in E2 and T production due to prochloraz have been reported in adult fathead minnows, as well as in another small fish species, zebrafish (Liu et al., 2010). In addition, prochloraz has been shown to reduce T and E2 production in mammalian systems such as the human adrenocortical carcinoma H295R cell line (Sanderson et al., 2002;

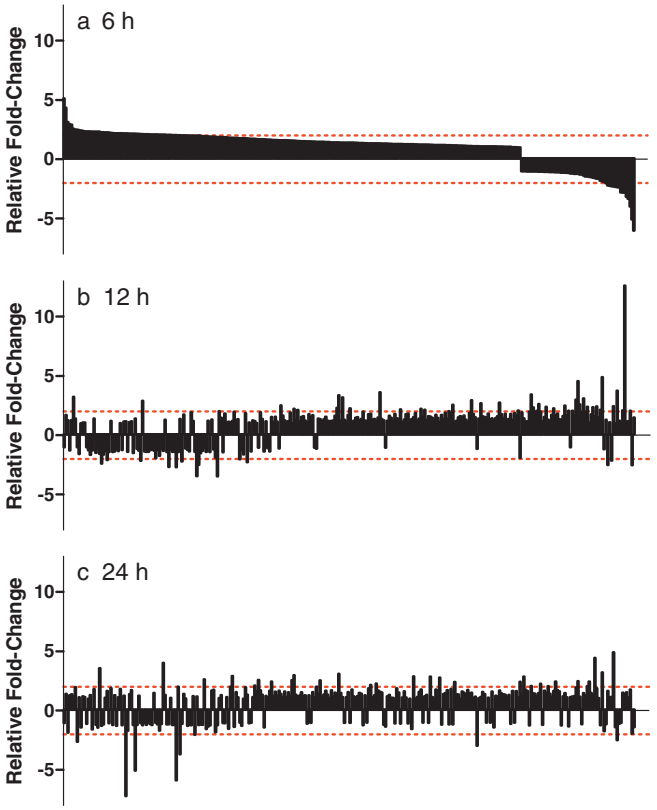

Fig. 4. Comparison of genes (identified by ANOVA ($p \leq 0.01$)) on the 15k feature fathead minnow microarray in the ovaries of female fathead minnows exposed to 300 μg prochloraz/L for (a) 6 h, (b) 12 h, and (c) 24 h. Genes are expressed as relative fold-change compared to controls. Genes were arranged from most up-regulated to most down-regulated according to expression profile at 6 h and kept in the same order for all time-points. Dotted line indicates 2-fold change.

Hecker et al., 2006; Laier et al., 2006; Villeneuve et al., 2007a). Available data suggest that prochloraz can disturb multiple steps in the synthesis of steroid hormones, primarily the enzymes CYP17 and CYP19, through binding to the heme-group within the active site of the enzyme (Andersen et al., 2002; Ankley et al., 2005; Blystone et al., 2007b; Vinggaard et al., 2002). While CYP19 is key to estrogen production, CYP17 is important in the formation of androgens. In the present study, the inhibition of E2 production in the female fathead minnow likely resulted from a decrease in CYP19 activity versus CYP17 activity, as there was no indication of decreased *ex vivo* synthesis of T in the fish. The differential effect of prochloraz on CYP19 versus CYP17 in the present study could be related to the test concentrations of the chemical, i.e., aromatase activity may be affected by a lower concentration of the pesticide than CYP17 activity. This would be consistent with the results of Villeneuve et al. (2007a) with a fathead minnow ovary explant assay where E2 production was reduced 50% at 1.6 μM (approximately 350 μg prochloraz/L), while a prochloraz concentration over two times greater (3.5 μM) was needed to reduce T production by 50%. In the same study, prochloraz treatment also was found to be a more potent suppressor of E2 production in H295R human adrenocarcinoma cells compared to T production.

Exposure to 300 μg prochloraz/L significantly decreased plasma VTG within 24 h, coinciding with the depression in circulating E2 concentrations. This has been seen in previous studies with prochloraz (Ankley et al., 2005, 2009) and is consistent with reduced activation of the hepatic estrogen receptor, the initiating step in VTG production (Sumpter and Jobling, 1995). Normal VTG production is important to reproductive physiology of oviparous species. Ankley et al. (2005) found that female fathead minnows exposed for 21 d to 300 μg prochloraz/L experienced reduced VTG concentrations and egg production. In the present study, it was shown that the effects of prochloraz on vitellogenesis are rapid (within 24 h), as is compensation (up-regulation of genes) by the fish in response to the exposure. However, based on the 21 d reproduction test in which 300 μg prochloraz/L inhibited VTG and egg production in fathead minnows (Ankley et al., 2005), it appears that the early compensatory response of up-regulation of key steroidogenic genes observed in the present study may not adequate to overcome toxic effects of this level of prochloraz exposure in terms of regaining/maintaining homeostasis.

The genes that were up-regulated in our study serve a number of functions relative to steroid production. In addition to the two CYP enzymes (CYP19 and CYP17) mentioned above, transcription was elevated for the genes coding for StAR, which mediates the uptake of cholesterol into the mitochondria, and CYP11A which catalyzes the first step in the conversion of cholesterol to steroids in the mitochondria. Two of the four significantly up-regulated ovarian genes in the present study (*cyp19a1a* and *cyp17*) also were also up-regulated by prochloraz exposure in longer-term exposures with both fathead minnows (Ankley et al., 2009) and medaka (Zhang et al., 2008). In addition, Liu et al. (2010) also showed that ovarian *fshr*, *star*, *cyp19a* and *cyp17* expression were up-regulated in female zebrafish after exposure to 300 μg prochloraz/L for 48 h. Other aromatase inhibitors, such as the pharmaceutical fadrozole, also have been shown to cause up-regulation of *star*, *cyp11a* and *cyp19a1a* expression in the fathead minnow (Villeneuve et al., 2006, 2009). This suggests that generalized inhibition of aromatase in fish may cause the compensatory transcription of a common suite of genes important to steroidogenesis. This information could be useful to the development of biomarkers for detection of chemical inhibitors of aromatase.

Follicle-stimulating hormone receptor is important to the feedback regulation of steroidogenesis, and has been shown to be up-regulated in the ovary following inhibition of aromatase in fathead minnows by fadrozole (Villeneuve et al., 2009). We hypothesized that *fshr* would be similarly up-regulated following inhibition of aromatase by prochloraz. In the present study however, *fshr* gene expression was down-regulated at 6 h of exposure and returned to control levels thereafter. In a longer term study, Ankley et al. (2009) did not find a significant decrease in expression of *fshr* in fathead minnows exposed to prochloraz. Differences in expression of *fshr* between the two prochloraz studies could be related to when fish were sampled during the exposures. However, in the present study, lack of significant effects in *fshr* signaling at 12 or 24 h is consistent with the lack of significant effects seen in the transcript abundance of *lhb* and *fshb* in the pituitary.

No significant effects of prochloraz on expression of genes investigated in the brain (*cyp19a1b*, *cyp51* and *cgnrh*) were seen within the 24 h of treatment. One possible exception to this was the up-regulation ($p = 0.056$) of *cyp51* at 6 h, which we hypothesized might be altered by prochloraz treatment in terms of being the CYP isozyme targeted by prochloraz in fungi. With regard to the brain isoform of aromatase, in medaka, Zhang et al. (2008) found *cyp19b* expression, in brain tissue, to be decreased after a 7 d exposure to 300 μg prochloraz/L. Liu et al. (2010) found *cyp19b* expression in zebrafish to be decreased after 12 and 48 h of exposure to 300 μg prochloraz/L. Lack of concordance in the present study could be due to species differences and/or differences in exposure times.

Relative to genes investigated in the pituitary (*lhb*, *gnrhr1* and *fshb*), we had hypothesized that expression of those coding for the gonadotropins (*lhb*, *fshb*) which are regulated through negative feedback by the gonadal steroids, might be altered by prochloraz exposure; however, this was not the case. This is consistent with the results of Ankley et al. (2009) where no significant effects were seen in the expression of *lhb* and *fshb* in the pituitary of female fat-

head minnows exposed to prochloraz for a longer period of time. In addition, Liu et al. (2010) found no significant effects in the expression of *lhb* and *fshb* of female zebrafish exposed to prochloraz for up to a 48 h period.

Our microarray analysis provided several potentially relevant observations relative to the short-term effects of prochloraz on global gene expression in the fish. The number and identity of up- and down-regulated genes varied substantially from time-point to time-point, indicating significant temporal variability in gene expression associated with exposure. We hypothesize that attempts to compensate for the chemical stressor by transcriptional changes may be reflected in this temporal variability. Also highlighted by our microarray results were rapid changes in gene expression profiles associated with chemical exposure, and the fact that comparatively few DEGs remained up- or down-regulated over the entire time-course. Specifically, genes that were up-regulated early on may subsequently have become down-regulated due, perhaps, to feedback mechanisms. The numbers of genes up- or down-regulated over 2-fold decreased over time, suggesting gradual return to control gene expression profiles after some initial perturbation.

Functional analysis of the DEGs from prochloraz-treated animals could provide insights into pathways other than the HPG axis that could be affected by chemical treatment. Based on the available annotations that could be derived from public databases, functional analysis with eGOn showed differential regulation of several biological process categories associated with organelle and chromosome organization and biogenesis. Two of the genes in the category, *smc4*, a structural component of chromosomes, and *rsl24d1*, a structural component of ribosomes, are important to the structural assembly and stability of the respective constituents (Egebjerg et al., 1987; Hirano et al., 1997). The categories associated with organelle and chromosome organization may be indicative of a generalized cellular remodeling in the ovary due to the chemical exposure. Within the molecular function categories, one gene differentially regulated associated with cofactor, coenzyme and fatty acid binding was *gapdh*, which codes for a multifunction protein within glycolysis and other cellular functions such as DNA replication and repair (Sirover, 1999). Two additional genes, *acbd5a* and *acbd6*, for acyl-coenzyme A binding domain are important in fatty acid metabolism and gene regulation (Kragelund et al., 1999; Mikkelsen and Knudsen, 1987). These results suggest a potential change in energy and lipid metabolism requirements.

Past microarray studies with rats and mice have demonstrated that some triazole fungicides similar to prochloraz affect hepatic lipid, sterol and steroid pathways (Goetz et al., 2006; Goetz and Dix, 2009a,b; Tully et al., 2006). The effects of prochloraz on steroid pathways are evident from the plasma and QPCR data from the present study. Because cholesterol is the precursor for steroid production in the gonads, lipid metabolism pathways also are important to endocrine function. Effects on lipid metabolism were suggested by our microarray data from the fathead minnow ovaries. For example, up-regulated DEGs involved in lipid metabolism included farnesyl diphosphate synthase and lanosterol synthase (both involved in *de novo* cholesterol synthesis), along with several other genes important to lipid metabolism, such as peroxisome proliferator-activated receptor gamma coactivator 1 alpha, low density lipoprotein receptor-related protein associated protein 1, elongation of very long chain fatty acids-like 1, acyl-coenzyme A binding domain containing 6 and mitochondrial trans-2-enoyl-CoA reductase. The responsiveness of these genes to prochloraz suggests that a potential indirect pathway of endocrine disruption by the fungicide in fish could be related to changes in lipid metabolism.

In conclusion, this study indicates that the effects of prochloraz on E2 production and subsequent compensatory up-regulation of genes related to steroidogenesis occur rapidly in the fathead minnow – within just a few hours of exposure. This demonstrates the dynamic nature of the HPG-axis relative to maintenance of homeostasis in response to chemical stress. Although a number of genes comprised the observed compensatory response, that coding for CYP19 was affected the most, consistent with its established role in maintaining estrogen and androgen balance. Our study also showed some interesting changes in global gene expression indicated by microarray analysis; e.g., transcript abundance of DEGs changed substantially over the 24 h test with regard to up/down-regulation. The microarray data also contributed to the identification of potential pathways of importance to the toxicity of prochloraz in fish. For example, the analysis revealed a molecular functional category of chemically responsive genes that were related to lipid metabolism. This observation provides direction for future work aimed at understanding the connections between lipid metabolism, maintenance of homeostasis, steroidogenesis and potential effects on reproduction in fish.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2011.02.016.

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