Aquatic Toxicology 101 (2011) 447–458

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

Gene expression profiling of the androgen receptor antagonists flutamide and vinclozolin in zebrafish (*Danio rerio*) gonads

Dalma Martinović-Weigelt[a,b,*], Rong-Lin Wang[c], Daniel L. Villeneuve[a], David C. Bencic[c], Jim Lazorchak[c], Gerald T. Ankley[a]

[a] US Environmental Protection Agency, Office of Research and Development, National Health and Environmental Effects Research Laboratory, Mid-Continent Ecology Division, 6201 Congdon Blvd., Duluth, MN 55804, USA
[b] University of St. Thomas, 2115 Summit Ave, Saint Paul, MN 55105, USA
[c] US Environmental Protection Agency, Office of Research and Development, National Exposure Research Laboratory, Ecological Exposure Research Division, 26W. Martin Luther King Dr., Cincinnati, OH 45268, USA

ARTICLE INFO

Article history:
Received 2 June 2010
Received in revised form
25 September 2010
Accepted 9 October 2010

Keywords:
Transcriptomics
Endocrine disruption
Anti-androgens
Fish
Flutamide
Vinclozolin

ABSTRACT

The studies presented in this manuscript focus on characterization of transcriptomic responses to anti-androgens in zebrafish (*Danio rerio*). Research on the effects of anti-androgens in fish has been characterized by a heavy reliance on apical endpoints, and molecular mechanisms of action (MOA) of anti-androgens remain poorly elucidated. In the present study, we examined effects of a short term exposure (24–96 h) to the androgen receptor antagonists flutamide (FLU) and vinclozolin (VZ) on gene expression in gonads of sexually mature zebrafish, using commercially available zebrafish oligonucleotide microarrays (4 × 44 K platform). We found that VZ and FLU potentially impact reproductive processes via multiple pathways related to steroidogenesis, spermatogenesis, and fertilization. Observed changes in gene expression often were shared by VZ and FLU, as demonstrated by overlap in differentially-expressed genes and enrichment of several common key pathways including: (1) integrin and actin signaling, (2) nuclear receptor 5A1 signaling, (3) fibroblast growth factor receptor signaling, (4) polyamine synthesis, and (5) androgen synthesis. This information should prove useful to elucidating specific mechanisms of reproductive effects of anti-androgens in fish, as well as developing biomarkers for this important class of endocrine-active chemicals.

1. Introduction

Classes of chemicals currently known to interfere with the androgen signaling pathway include dicarboximide fungicides (e.g., vinclozolin), organochlorine-based insecticides (e.g., p,p′-DDT and -DDE), conazole fungicides (e.g., prochloraz), plasticizers (phthalates), urea-based herbicides (e.g., linuron) and pharmaceuticals (e.g., flutamide) (reviewed in Hotchkiss et al., 2008). Even though anti-androgens are widespread environmental contaminants, the assessment of their effects on the fish has received little attention as compared to the other endocrine-active chemicals (EACs) such as estrogens and androgens. Existing research on the effects of anti-androgens in fish has been characterized by a reliance on apical endpoints, such as hormone titers, secondary sexual characteristics, histopathology, and reproduction (Martinovic et al., 2008; Panter et al., 2004; Bayley et al., 2002), but molecular mechanisms of action (MOA) of anti-androgens remain poorly elucidated in fish. Better characterization of MOA for anti-androgens is needed to identify biomarkers of exposure and to link those with endpoints relevant to risk assessment, such as fecundity.

Microarray-based analyses of gene expression potentially can provide mechanistic understanding and generate insights as to biomarker identification and effects assessment, e.g., in field studies. In addition, microarray-based identification of anti-androgen-specific gene profiles could be utilized to screen chemicals for anti-androgenic activity as a basis for prioritizing testing in programs concerned with EACs (Ankley et al., 2009). Gene expression profiling has been used successfully to demonstrate that anti-androgens have a molecular signature distinct from that of estrogens (Larkin et al., 2002; Moens et al., 2006; Filby et al., 2007), and androgens (Leon et al., 2008). However, there are only a limited number of studies that have explored commonalities/disparities in gene expression profiles of different anti-androgens, especially in fish (Moens et al., 2006; Leon et al., 2008). Although anti-androgens acting through the same pathway should have commonalities in their gene expression profiles (Rosen et al., 2005), empirical evi-

* Corresponding author at: University of St. Thomas, Mail OWS 390, 2115 Summit Avenue, St. Paul, MN 55105-1080, USA. Tel.: +1 651 962 5233; fax: +1 651 962 5201.
E-mail addresses: dalma@stthomas.edu, mart6831@stthomas.edu (D. Martinović-Weigelt).

0166-445X/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.aquatox.2010.10.003

dence for this is minimal and results are not consistent among the studies. Leon et al. (2008) compared the expression profiles of the putative androgen receptor (AR) antagonists, flutamide (FLU) and vinclozolin (VZ), in larval medaka and observed that the gene expression profiles for the two were similar. This is consistent with evidence that metabolites of FLU and VZ (VZ-M1, M2; FLU-hydroxyflutamide) bind to the AR both of mammals and fish in a competitive manner (Wilson et al., 2007), and thus should prevent transcription of common androgen-dependent genes (Kelce et al., 1997). However, Moens et al. (2006) also examined responses to VZ and FLU in carp, but found that hepatic gene expression profiles were not similar for the two chemicals. Flutamide was more homologous to another presumed anti-androgen, dibutylphthalate, which is somewhat surprising given that dibutylphtalate has a supposedly distinct mode of anti-androgenic action in that it does not act solely as an AR antagonist (Foster et al., 2001). Similarly, a study that focused on gene expression profiling in rat fetal testis failed to find a common set of genes altered by FLU and VZ treatment (Mu et al., 2006). A number of factors could contribute to a lack of congruence between FLU and VZ gene expression profiles, including differences in tissue-, sex-, developmental stage-specific responsiveness, species-specific differences in toxicant metabolism, and/or experimental analyses and array platforms used (e.g., the source of disparate responses could have been a result of underrepresentation of the anti-androgen responsive target genes).

As part of the U. S. Environmental Protection Agency's (USEPA) Computational Toxicology Program, a research effort was developed to examine the system-wide responses of the fish hypothalamic-pituitary-gonadal (HPG) axis to EACs, with an objective of identifying molecular biomarkers of exposure to, and effects of various classes of EACs and to establish functional links between biomarkers and whole organism outcomes (Ankley et al., 2009). The studies presented in this manuscript represent one portion of this overall program of research, and focus on characterization of genomic responses to the anti-androgens FLU and VZ in zebrafish (*Danio rerio*). The gonads were the focus of this study because reproductive endpoints in fish are consistently altered by exposure to FLU and VZ (Bayley et al., 2002; Jensen et al., 2004; Katsiadaki et al., 2006; Martinovic et al., 2008). The objectives of the studies presented herein were to: (1) propose MOA by which FLU and VZ affect reproduction in fish, (2) determine whether there are commonalities in the gene expression profiles in the gonad exposed to anti-androgens that act via AR receptor antagonism, and (3) identify a list of potential anti-androgen biomarkers in fish.

2. Materials and methods

2.1. Exposure and experimental design

Exposures to FLU or VZ were conducted separately, but using the same protocols and experimental designs (Wang et al., 2008a; Villeneuve et al., 2009). To select exposure concentrations, we first conducted 96 h FLU range-finding experiments with zebrafish. None of the tested concentrations (ranging from 625 to 5000 μg FLU/l) were lethal. We did not conduct VZ range-finding experiments with zebrafish, because vinclozolin was already reported as relatively nontoxic (e.g., no significant effects on mortality rates in larval fish or adults exposed for 34 and 21 days, respectively, to pesticide concentrations on the order of 1 mg/l [Makynen et al., 2000]). We also considered concentration–response data from fathead minnow reproduction tests with the two chemicals (Jensen et al., 2004; Makynen et al., 2000), because comparable data for the zebrafish were not available at the time. The target concentrations for the present study (1000 μg/l for VZ and 1750 μg/l for FLU) were chosen because they did not significantly alter mortality rates and because they were sufficiently high to exert effects on apical reproductive endpoints (e.g., fecundity and ovarian maturation) in fish (Jensen et al., 2004; Makynen et al., 2000).

Reproductively-mature adult zebrafish (ab wild-type strain; 5 months old) were obtained from an on-site culture. Three days prior to exposure initiation, fish were randomly placed into 15 tanks containing 10 l of Lake Superior water (1 μm filtered; 45 ml/min flow rate), at a density of 10 males and 10 females per tank, and allowed to acclimate to exposure-like holding conditions. Fish were held at 25 °C, under a 16 h:8 h light:dark photoperiod and fed to satiation with live brine shrimp (*Artemia* sp.).

A continuous 45 ml/min flow of FLU or VZ dissolved (without solvent) in Lake Superior water or Lake Superior water-only (control) was delivered to 15 other tanks (containing no fish) and chemical concentrations were allowed to equilibrate and stabilize over a 48 h period. To initiate exposures, the zebrafish were transferred from acclimation tanks to the exposure tanks. There were five exposure tanks per treatment. Transfer of fish was staggered by replicate such that the one replicate tank from each treatment group received fish at 6:30 AM, with subsequent replicates from each treatment group receiving fish at 8:00, 10:00, 12:00, 14:00, and 16:00, respectively. After 24 h (±30 min) of exposure, two males and two females were sampled from each replicate tank in the same staggered order that fish had been added to the tanks. After 48 h (±30 min) and 96 h (±60 min) four males and four females were sampled from each replicate tank. Fish were euthanized in a buffered solution of tricaine methanesulfonate (MS 222; Finquel, Argent, Redmond, WA, USA). Gonads were removed using dissection tools washed with RNAseZap® (Ambion, Austin, TX, USA) between each sample, and immediately transferred to pre-weighed microcentrifuge tubes, flash-frozen in liquid nitrogen and stored at −80 °C until extracted. All laboratory procedures involving animals were reviewed and approved by the USEPA Animal Care and Use Committee in accordance with Animal Welfare Act regulations and Interagency Research Animal Committee guidelines.

2.2. Chemicals and analytical measurements

Flutamide (99% purity) used for this study was provided by Sigma (St. Louis, MO, USA). Vinclozolin (99% purity) was obtained from Chem Service (West Chester, PA, USA). Chemical concentrations in exposure tanks were monitored over the course of the experiment. Water samples (1 ml) were collected from each tank 1 h prior to the addition of fish and approximately 1 h prior to tissue collection on each sampling date. Routine quality assurance analyses (i.e., procedural blanks, spiked matrix, and duplicate samples) were conducted with each sample set and constituted approximately 10% of all samples.

Flutamide samples in water were immediately analyzed using reversed phase high pressure liquid chromatography (HPLC). The Agilent (Wilmington, DE, USA) model 1100 HPLC consisted of a capillary pump, chilled auto sampler, heated column compartment, and a diode-array detector. An aliquot of sample was injected onto a Synergi Hydro (Phenomenex, Torrance, CA, USA) RP-C18 column (3 mm × 50 mm) and eluted isocratically with 70% acetonitrile/water at a flow rate of 0.25 ml/min. Flutamide concentrations were determined using the response at a wavelength of 310 nm and an external standard method of quantification. No FLU was detected in the control tanks ($n = 20$) or procedural blanks ($n = 4$). The mean (±SD) recovery of FLU in the spiked matrix samples was 98 ± 0.5% ($n = 4$) and the mean (±SD) percentage agreement among duplicate samples was 99 ± 0.7% ($n = 8$). The analytical quantification limit was 200 μg FLU/l.

Vinclozolin concentrations of the exposure tanks were measured daily by adding 900 μl of tank water samples to vials containing 100 μl of methanol. A 50 μl injection was analyzed on

an Agilent 1100 HPLC equipped with a diode-array detector set at 212 nm. An isocratic method with a mobile phase of 70% acetonitrile and 30% sodium phosphate buffer (0.05 M) at pH 3.3 and 0.4 ml per min was used with a Phenomenex Synergi Hydro RP 4u 50 mm × 3.0 mm column. Retention time of VZ was 4.2 min at a column temp of 25 °C. The external standard method of quantification was used with six standards from 100 to 1200 μg/l in 50% methanol and water. No VZ was detected in the control tanks or procedural blanks ($n = 4$). The mean (±SD) recovery of VZ in the spiked matrix samples was 103 ± 2.5% ($n = 5$) and the mean (±SD) percentage agreement among duplicate samples was 95 ± 6.5% ($n = 5$). The analytical quantification limit was 50 μg VZ/l.

2.3. Microarray analysis

High density zebrafish oligonucleotide microarrays (4 × 44 K, G2519F, 13 Sep 2006, Agilent Technologies, Santa Clara, CA, USA) were used for this study. It was not financially feasible to conduct microarray analyses for all sex, tissue, time-point, and exposure concentration combinations. Use of a "unbalanced incomplete block" design (Wang et al., 2008a,b) enabled us to compare control and FLU (1750 μg/l nominal) treatments for the following conditions: (1) 24 h testes, (2) 48 h testes, (3) 96 h testes, (4) 48 h ovaries, and (5) 96 h ovaries. RNA extracted from the appropriate tissue of one control fish and one FLU-treated fish (1750 μg/l) was labeled with Cy3 (control) or Cy5 (FLU) and hybridized to each microarray. Five microarrays representing ten biological samples (fish) (five for 1750 μg/l FLU and five for a control treatment) were analyzed for each time/sex condition noted above. For the VZ experiment we compared control and fish exposed to 1000 μg/l VZ using the same time/sex treatment combinations as FLU.

Wang et al. (2008a,b) conducted an exhaustive evaluation of the microarray platform, experimental design and bioinformatics analyses used in the present study. To determine the level of congruence between microarray platform and quantitative real time polymerase chain reaction (qPCR) based expression data Wang et al. (2008b) validated the microarray results for 13 genes from gonadal tissue from zebrafish exposed to three endocrine-active chemicals (representing 20 sample pairs). On the basis of individual genes, the correlation coefficients between qPCR and microarray data ranged from 0.34 to 0.96, with a median of 0.8. The qPCR expression profiles of these genes are therefore highly consistent with those observed in microarrays. This level of congruence between microarray data and q PCR data is comparable to previous reports and well within the range of what is considered acceptable (e.g., Morey et al., 2006).

For a detailed description of RNA extraction and purification, labeling and hybridization procedures, microarray scanning, and a comprehensive evaluation of data quality see Wang et al. (2008b). Briefly, microarray data was assessed from a number of perspectives including sample to sample variation, gene-specific dye bias, treatment parameters, and overall distributions of gene expression relative intensities. Comparisons were also made between single and multiple image scans, qPCR and microarray, as well as fluorescent spectra from different sources. Overall, microarray data in this study was found to be highly reproducible, with little variation among arrays and negligible gene specific dye bias. All microarray data described in this study were submitted to the Chemical Effects in Biological Systems (CEBS) database (http://www.niehs.nih.gov/research/resources/databases/cebs/index.cfm). The CEBS accession numbers for these two studies are: 010-00002-0005-000-8 for FLU and 010-00002-0011-000-5 for VZ.

2.4. Differentially-expressed genes

To identify lists of differentially-expressed genes (DEGs) in this study, the text outputs from Agilent Feature Extraction software (Agilent Technologies) were imported into GeneSpring GX 10 (Agilent Technologies). Locally weighted linear regression (LOWESS) analysis was used as a normalization method. This method is suitable for normalizing of expression ratios and can minimize intensity dependent effects on the ratio values (Quackenbush, 2002). Because LOWESS normalization is carried out for individual microarray data set by Feature Extraction software, data preprocessing and normalization in GeneSpring GX10 were limited to value adjustments to a threshold, CY5/Cy3 ratio computing, and logarithmic transformation of those ratios. Lists of differentially-expressed genes were generated using one-class *t*-tests coupled with multiple test corrections by the non-permutation based Benjamini and Hochberg method using GeneSpring GX 10.

Multiple exposure durations were incorporated in the original experimental design to provide temporal data for broader research project that focused on network building and exploration of biomarker discovery methods (Wang et al., 2008b, 2010). We observed significant effects due to the treatment after 24, 48, 96 h for both FLU and VZ treatments (Supplementary Table S.1), but we pooled gene expression data for 24, 48, and 96 h for each sex for each chemical to increase the power and minimize false discovery rate (FDR) (Pawitan et al., 2005). An additional rationale for pooling across time was that genes that emerge as significantly-impacted will likely be those that are regulated in the same fashion at multiple time points; these are the genes that have most potential to serve as reliable biomarkers of effect when establishing time-independent gene expression profiles. The FDR was set to 5% for the purposes of defining lists of DEGs, and the corrected *p*-values.

To examine how FLU and VZ might affect reproduction in fish, we used Venn diagram analyses. We identified the subsets of above defined DEGs that: (1) have known reproductive roles in the teleost HPG axis (83 genes reported by Villeneuve et al., 2007a), and (2) cause reproductive defects—based on the list of 202 mouse single gene mutations that cause reproductive defects (Matzuk and Lamb, 2002).

2.5. Ingenuity pathway analyses

The zebrafish DEGs with at least a 1.3 fold change (FDR 1–5%) between treatment and control were used for input gene lists for Ingenuity Pathway Analyses (IPA) (Ingenuity Systems Inc., Redwood City, CA, USA). The zebrafish probe IDs were first mapped to their human orthologs and the corresponding Entrez human gene IDs were used as IPA IDs. The purpose of this was to increase the mapping success of zebrafish probes to human-mouse-rat pathways in IPA; preliminary tests using zebrafish vs. human IDs show that the Entrez human gene ID had the highest IPA ID mapping success. The statistically enriched canonical pathways and lists of genes responsible for the pathway enrichment were identified ($p < 0.05$). We also examined which toxicity pathways were enriched using the IPA-Tox™ Tool ($p < 0.05$). The IPA-Tox™ toxicity lists are comprised of molecular toxicity pathways and gene lists that, among others, include genes and pathways related to oxidative stress response; xenobiotic metabolism; hepatic, renal, and cardiac toxicity; and cell death.

2.6. Gene ontologies

To determine the enrichment of a GO category in a target gene set, the GO terms are compared for their occurrences in this selected gene group against those in the overall background (e.g., all probes on the microarray). Determination of statistically significant enrichment was based on a hypergeometric test without multiple test correction as calculated using GeneSpring GX 10 software ($p < 0.05$; Agilent Technologies). Enrichment was examined in all three major GO categories (e.g., biological process, cellular

component, molecular function), but only biological process results are reported here, as they were the most relevant category for the purposes of this study.

2.7. Other functional analyses

To develop candidates for EAC toxicity pathways, Wang et al. (2010) used data produced by several zebrafish studies to reverse-engineer transcription factor (TF) regulatory networks and associate them with various chemical treatments by GSEA (gene set enrichment analysis) and Extended-GSEA (E-GSEA). These data encompassed ten EACs, four tissue types, one of the three exposure durations, and both sexes (Ankley et al., 2009). Briefly, with a genome-wide collection of approximately 1000 annotated TFs as regulators (hub genes), their individual networks of target gene were constructed by using the Aracne (Basso et al., 2005), a mutual information-based algorithm, on multiple microarray datasets organized according to tissue types. The TF networks were subsequently considered as gene sets by GSEA/E-GSEA and associated with individual chemical treatments. The basic principle behind GSEA/E-GSEA is to determine, through a non-parametric permutation test, whether members of a gene set defined a priori are statistically enriched with those most differentially expressed among a large list of ranked genes (Subramanian et al., 2005). The ranking statistic could be any measure for comparing two sample means, such as signal-to-noise ratio between a treatment and its control. Such a test not only avoids a subjective statistical cutoff in single gene tests, but also, by considering a group of genes with certain affiliation together, provides a biological context for interpretations.

3. Results

3.1. Exposure verification and survival

Neither VZ nor FLU were detected in the control tanks. Mean concentration of VZ in exposure tanks were 609 ± 38 (SD) μg/l for 24 h, 632 ± 29 μg/l for 48 h, and 567 ± 17 μg/l for 96 h treatments. Mean concentration of FLU in exposure tanks were 1573 ± 20 μg/l for 24 h, 1920 ± 35 μg/l for 48 h, and 1727 ± 28 μg/l for 96 h treatments. There were no significant differences in chemical concentrations among replicate tanks. There were no mortalities during the exposure to either FLU or VZ, and fish exhibited no abnormal behavior possibly indicative of toxic effects.

3.2. Microarray analyses

3.2.1. Differentially-expressed genes

Exposure to VZ (pooled across all exposure durations) altered expression of 523 genes in males and 287 genes in females (one-class *t*-test, FDR < 5%) (Supplementary Table S.1). The DEGs that changed more than 1.3-fold (285 in males, 166 in females) are shown in Supplementary Tables S.2 and S.3. Up-regulated genes accounted for 54% (males) and 72% (females) of the DEGs. Exposure to FLU altered expression of 756 genes in males and 366 genes in females (one-class *t*-test, FDR < 1–5%) (Supplementary Table S.1). The FLU DEGs that changed more than 1.3-fold (530 in males, 239 in females) are shown in Supplementary Tables S.4 and S.5. The FLU up-regulated genes accounted for 36% (males) and 37% (females) of the DEGs.

Venn diagrams indicated that expression of 102 genes was significantly altered (>1.3-fold change, $p < 0.05$) by both VZ and FLU in male zebrafish; the DEGs common to both treatments represented 25% (FLU) to 45% (VZ) of all DEGs (Table 1). In females these percentages were lower; the DEGs common to both treatments in females represent 13% (FLU) to 20% (VZ) of all DEGs (Table 2). Overall, more extensive changes in gene expression were observed in FLU-treated fish of both sexes, with males experiencing more changes in gene expression. The hierarchical clustering analyses indicate that gene expression profiles of VZ and FLU were not readily distinguishable (Fig. 1). Vinclozolin and FLU had 13–45% of the DEGs common to both VZ and FLU. Commonalities in gene expression were more prominent in the males than in the females.

Exposure to VZ and/or FLU affected expression of 12 of 83 genes reported by Villeneuve et al. (2007a) as playing a role in the teleost HPG axis (Table 3). Comparison of DEGs with the list of 202 genes whose mutations cause reproductive defects (Matzuk and Lamb, 2002) resulted in identification of the following nine genes: (1) deleted in azoospermia-like (*dazl*), inhibin a (*inha*), and FSH receptor (*fshr*) in VZ-exposed males; (2) aryl-hydrocarbon receptor (*ahr*), *HIV-1* Rev binding protein (*hrb*), and spermatid perinuclear RNA-binding protein (*spnr*) in FLU-exposed males; and (3) phosphoserine-threonine/tyrosine interaction (*styx*), aromatase (*cyp19*) and *spnr* in FLU-exposed females.

3.2.2. Ingenuity pathway analyses

The statistically enriched canonical human-mouse-rat pathways and the lists of DEGs responsible for their enrichment are reported in Tables 4 and 5. In addition to canonical pathway analyses we also conducted IPA-Tox™ toxicity pathway analyses. These analyses indicate that FLU affected mitochondrial function and hypoxia-inducible signaling in females. In males enriched toxicity pathways include oxidative stress responses, hypoxia-inducible factor signaling, and the cytochrome P450 panel. In addition, there was some indication of retinoid X and/or farnesoid X receptor activation and anti-apoptotic effects ($p = 0.055$). The VZ treatment did not result in statistically significant enrichment on any of the toxicity pathways.

3.2.3. Functional analysis—gene ontologies

In VZ-exposed males there were 97 enriched GO categories, and in females there were 25 ($p < 0.05$). Flutamide exposure resulted in a similar number of GO enriched categories in males (110 categories) and females (100 categories). Twenty most enriched categories are shown in Tables S.6 (VZ) and S.7 (FLU). In the VZ males, the main GO categories included gametogenesis, cation transport, protein biosynthesis, lipoprotein metabolism, and negative regulation of signal transduction. There was little similarity in the enriched categories in VZ females, where the top categories involved alterations in the ubiquitin cycle and embryonic axis specification. In the FLU males, the main GO categories included protein biosynthesis, lipoprotein metabolism, and glutamine biosynthesis. Again, there was little similarity in the enriched categories in FLU females, where the top categories involved alterations in programmed cell death and gametogenesis. Venn diagram analyses suggest that 28 enriched categories were enriched in VZ- and FLU-exposed males (five GO categories were common to both VZ and FLU). The majority of the enriched categories in males indicated effects on the following processes: protein and fatty acid biosynthesis, lipid transport, urea metabolism, apoptosis and ion transport. In females, these included functions involved in embryonic axis specification and synaptic vesicle priming.

3.2.4. Other functional analyses

The GSEA indicated that there were four networks enriched in both VZ- and FLU-treated males. Hub genes for these networks were: estrogen receptor (*esr1*), nuclear receptor subfamily 5, group A member1b (*nr5a1b*), forkhead box O5 (*foxo5*), and endothelial PAS domain protein 1 (*epas1*). These data were visualized with Cytoscape (Shannon et al., 2003), a software tool that displays genes (proteins) and the interactions among them. The Cytoscape visu-

Table 1
The genes whose expression was significantly altered (>1.3 fold change, $p < 0.05$) by both vinclozolin and flutamide in male zebrafish.

Probe name	Description	Fold-change	
		VZ	FLU
Downregulated			
A_15_P101473	Unknown	1.50	2.12
A_15_P104976	Unknown	2.52	2.72
A_15_P110189	Unknown	2.13	1.83
A_15_P113525	Unknown	1.34	1.30
A_15_P117428	Danio rerio actin	1.49	1.78
A_15_P107195	Danio rerio apolipoprotein Eb (apoeb)	4.44	3.19
A_15_P114109	Danio rerio apolipoprotein Eb (apoeb)	4.08	3.06
A_15_P114194	Danio rerio apolipoprotein Eb (apoeb)	3.90	3.37
A_15_P100625	Danio rerio apolipoprotein Eb (apoeb)	3.87	3.33
A_15_P107566	Danio rerio apolipoprotein Eb (apoeb)	3.85	3.10
A_15_P117270	Danio rerio apolipoprotein Eb (apoeb)	3.82	3.13
A_15_P118777	Danio rerio apolipoprotein Eb (apoeb)	3.77	2.95
A_15_P113389	Danio rerio apolipoprotein Eb (apoeb)	3.22	3.10
A_15_P106269	Danio rerio apolipoprotein Eb (apoeb)	3.16	3.12
A_15_P110110	Danio rerio apolipoprotein Eb (apoeb)	3.08	3.05
A_15_P103025	Danio rerio ATPase	1.89	2.90
A_15_P102186	Danio rerio ATPase	1.48	1.86
A_15_P118055	Danio rerio ATPase	1.85	1.53
A_15_P114663	Danio rerio antizyme inhibitor 1 (azin1)	1.58	1.53
A_15_P104015	Unknown	4.42	6.87
A_15_P118750	Danio rerio BCL2/adenovirus E1B interacting protein 3-like (bnip3l)	1.73	1.87
A_15_P114130	Danio rerio cystathionase (cystathionine gamma-lyase) (cth)	2.37	2.30
A_15_P101561	Danio rerio ELOVL family member 5	2.93	3.24
A_15_P111972	Unknown	1.64	1.99
A_15_P115816	PREDICTED: Danio rerio hypothetical protein LOC793616	1.34	1.37
A_15_P100202	Danio rerio hydroxysteroid 11-beta dehydrogenase 2 (hsd11b2)	3.03	3.68
A_15_P104458	Danio rerio isocitrate dehydrogenase 1 (NADP+)	1.97	2.88
A_15_P104022	Danio rerio isocitrate dehydrogenase 1 (NADP+)	1.97	2.86
A_15_P121334	Danio rerio isocitrate dehydrogenase 1 (NADP+)	1.95	2.85
A_15_P121313	Danio rerio isocitrate dehydrogenase 1 (NADP+)	1.89	2.61
A_15_P110723	Danio rerio isocitrate dehydrogenase 1 (NADP+)	1.88	2.60
A_15_P102532	Unknown	2.07	2.32
A_15_P109513	Danio rerio potassium inwardly-rectifying channel	3.39	4.82
A_15_P120126	Danio rerio hypothetical protein LOC553532	2.27	3.56
A_15_P107175	Danio rerio similar to UDP glycosyltransferase 1 family	1.91	2.66
A_15_P117210	Danio rerio similar to cell adhesion molecule NCAM (LOC573953)	1.54	1.91
A_15_P101484	Danio rerio lecithin retinol acyltransferase	3.18	10.15
A_15_P104924	Danio rerio electrogenic Na+ bicarbonate cotransporter (nbce1)	1.77	2.30
A_15_P100569	Danio rerio nuclear receptor subfamily 5	2.64	4.12
A_15_P102087	Danio rerio reticulon 4 receptor (rtn4r)	2.79	3.10
A_15_P100985	Danio rerio si:ch211-192k9.1 (si:ch211-192k9.1)	4.10	3.57
A_15_P109915	Danio rerio si:ch211-192k9.1 (si:ch211-192k9.1)	3.99	3.54
A_15_P101264	Q4S6F5_TETNG (Q4S6F5) Chromosome 10 SCAF14728	2.23	2.20
A_15_P111302	Apolipoprotein Eb precursor (Apo-Eb)	3.80	3.13
A_15_P110124	Rhodanese-like:Thioredoxin-related precursor	2.68	2.47
A_15_P102471	Unknown	1.84	1.59
A_15_P118100	Unknown	1.96	2.37
A_15_P110873	ACT2_ONCVO (P30163) Actin-2	2.32	2.48
A_15_P110622	Danio rerio tropomyosin 4 (tpm4)	1.43	1.70
A_15_P114565	Danio rerio zgc:110003 (zgc:110003)	1.69	1.68
A_15_P104132	Danio rerio zgc:112165 (zgc:112165)	1.93	2.10
A_15_P119129	Danio rerio zgc:112165 (zgc:112165)	1.74	1.64
A_15_P114719	Danio rerio zgc:136650	1.38	1.87
A_15_P102643	Danio rerio zgc:152723 (zgc:152723)	1.31	1.69
A_15_P113645	Danio rerio zgc:153457 (zgc:153457)	1.74	2.19
A_15_P102999	Danio rerio zgc:153815 (zgc:153815)	2.97	3.38
A_15_P120770	Danio rerio zgc:65794 (zgc:65794)	2.33	1.43
A_15_P113388	Danio rerio zgc:85961 (zgc:85961)	1.81	1.94
A_15_P101081	Danio rerio zgc:86709 (zgc:86709)	2.41	4.15
A_15_P108402	Danio rerio zgc:86863 (zgc:86863)	1.38	1.64
A_15_P111771	Danio rerio zgc:92214 (zgc:92214)	4.03	4.35
A_15_P111472	Danio rerio zgc:92215 (zgc:92215)	1.45	1.76
A_15_P118237	Danio rerio zgc:92796 (zgc:92796)	2.69	2.79
A_15_P114616	Danio rerio zgc:92849 (zgc:92849)	4.30	5.89
Upregulated			
A_15_P101052	Unknown	1.47	1.64
A_15_P103599	Unknown	1.47	1.39
A_15_P112686	Unknown	1.44	1.63
A_15_P113751	Unknown	1.46	1.49
A_15_P115824	Unknown	1.48	1.64
A_15_P119998	Unknown	1.46	1.59
A_15_P113543	Unknown	1.44	1.53
A_15_P108774	Danio rerio calcium channel	1.49	1.42

Table 1 (*Continued*)

Probe name	Description	Fold-change	
		VZ	FLU
A_15_P120649	Unknown	1.47	1.73
A_15_P108675	Unknown	1.37	1.61
A_15_P111576	Unknown	1.61	2.47
A_15_P101596	Unknown	1.48	1.61
A_15_P109548	Unknown	1.32	1.40
A_15_P121103	Danio rerio ferrochelatase (fech)	1.55	1.63
A_15_P113403	Danio rerio fibroblast growth factor receptor 1 (fgfr1)	1.42	1.51
A_15_P111886	Danio rerio FK506 binding protein 9 (fkbp9)	1.46	1.61
A_15_P103051	Danio rerio G protein-coupled receptor 19 (gpr19)	1.47	1.39
A_15_P112106	Danio rerio LIM-domain binding factor 4 (ldb4)	1.91	1.75
A_15_P106525	PREDICTED: Danio rerio hypothetical protein LOC797867	1.40	1.48
A_15_P103908	Danio rerio major facilitator superfamily domain containing 2 (mfsd2)	1.48	1.50
A_15_P106506	Q9YHZ8_BRARE (Q9YHZ8) Pax-family transcription factor 6.2	1.48	1.57
A_15_P118764	Q4SKN6_TETNG (Q4SKN6) Chromosome undetermined	1.45	1.70
A_15_P117601	Q3GSY0_9ACTO (Q3GSY0) ATPase (Fragment)	1.46	1.69
A_15_P117718	Q58EL4_BRARE (Q58EL4) Dbx2 protein	1.52	1.71
A_15_P114219	Unknown	2.00	2.21
A_15_P119458	Unknown	1.95	2.66
A_15_P108582	Danio rerio zgc:101682 (zgc:101682)	1.46	1.59
A_15_P113472	Danio rerio zgc:103457 (zgc:103457)	1.39	1.60
A_15_P103497	Danio rerio zgc:110355 (zgc:110355)	1.49	1.62
A_15_P106078	Danio rerio zgc:153041 (zgc:153041)	1.34	1.36
A_15_P106171	Danio rerio zgc:66260 (zgc:66260)	1.38	1.77
A_15_P116228	Danio rerio zgc:77221 (zgc:77221)	1.36	1.40
A_15_P105299	Danio rerio zgc:77784 (zgc:77784)	1.37	1.41
A_15_P100556	Danio rerio zgc:86773 (zgc:86773)	1.49	1.65
A_15_P117498	Danio rerio zgc:91997 (zgc:91997)	1.47	1.69
A_15_P110351	Danio rerio zgc:92022 (zgc:92022)	1.32	1.44
A_15_P116746	Danio rerio zgc:92687 (zgc:92687)	1.43	1.41

alizations of transcription factor networks enriched in the testis of males exposed to VZ or FLU for 24, 48, or 96 h are shown in Fig. 2 and Fig S.1.

In females, 22 networks were enriched by both VZ and FLU treatments. The hubs (excluding 11 hub genes for which reliable annotations were not available) were associated with the following genes: androgen receptor (*ar*), GLI-Kruppel family member 1 (*gli1*), homeodomain leucine zipper gene (*homez*), mothers against decapentaplegic homolog 7 (*smad7*), muscle segment homeobox D (*msxd*), retinoic acid receptor gamma (*rarg*), transcription factor binding to IGHM enhancer 3a (*tfe3a*), D4, zinc and double PHD fingers family 2(*dpf2*), family with sequence similarity 60, member A,

Table 2
The genes whose expression was significantly altered (>1.3 fold change, $p < 0.05$) by both vinclozolin and flutamide in female zebrafish.

Probe name	Description	Fold-change	
		VZ	FLU
Downregulated			
A_15_P109690	Danio rerio CDC42 binding protein kinase beta (DMPK-like) (cdc42bpb)	1.41	1.92
A_15_P105142	AGENCOURT_16623549 NIH_ZGC_10	1.54	1.7
A_15_P107038	Danio rerio X-box binding protein 1 (xbp1)	1.49	1.47
Upregulated			
A_15_P108960	Unknown	1.34	1.35
A_15_P114676	Unknown	1.52	1.35
A_15_P115824	Unknown	1.64	1.63
A_15_P119765	Unknown	1.42	1.49
A_15_P117740	Danio rerio ADP-ribosylation factor-like 6 interacting protein 1 (arl6ip1)	1.32	1.34
A_15_P113543	Similar to contains element LTR5 repetitive element;	1.64	1.47
A_15_P108645	fr80a08.y1 zebrafish adult brain Danio rerio cDNA clone IMAGE:4966935 5'	1.85	1.6
A_15_P116746	calpain 3, (p94)	1.67	1.49
A_15_P118283	Danio rerio CC chemokine 1 (ccl1)	1.71	1.56
A_15_P108675	AGENCOURT_15223819 NIH_ZGC_10 IMAGE:7002585 5′	1.9	1.72
A_15_P101596	FDR202-P00037-DEPE-R_L22 FDR202 Danio rerio	1.83	1.65
A_15_P113403	Danio rerio fibroblast growth factor receptor 1 (fgfr1)	1.61	1.44
A_15_P111886	Danio rerio FK506 binding protein 9 (fkbp9)	1.73	1.54
A_15_P114794	PREDICTED: Danio rerio hypothetical protein LOC100000242 (LOC100000242)	1.31	1.43
A_15_P120917	PRP38 pre-mRNA processing factor 38 (yeast) domain containing A	1.34	1.34
A_15_P103497	Synaptophysin-like 2b	1.82	1.66
A_15_P100191	Q6DRI2_BRARE (Q6DRI2) Clatherin heavy chain	1.37	1.42
A_15_P101835	Q4SH43_TETNG (Q4SH43) Chromosome 8 SCAF14587	1.38	1.46
A_15_P108068	Q6R748_CHICK (Q6R748) Syntaxin 1-binding protein	1.34	1.4
A_15_P111517	Danio rerio thymidine kinase 1	1.47	1.53
A_15_P102575	Danio rerio wdr45 like (wdr45l)	1.52	1.71
A_15_P119458	Similar to SW:EG52_XENLA Q91783 KINESIN-LIKE PROTEIN EG5 2.	1.86	3.15
A_15_P106078	Danio rerio zgc:153041 (zgc:153041)	1.38	1.4
A_15_P119317	Danio rerio zgc:76887 (zgc:76887)	1.47	1.75

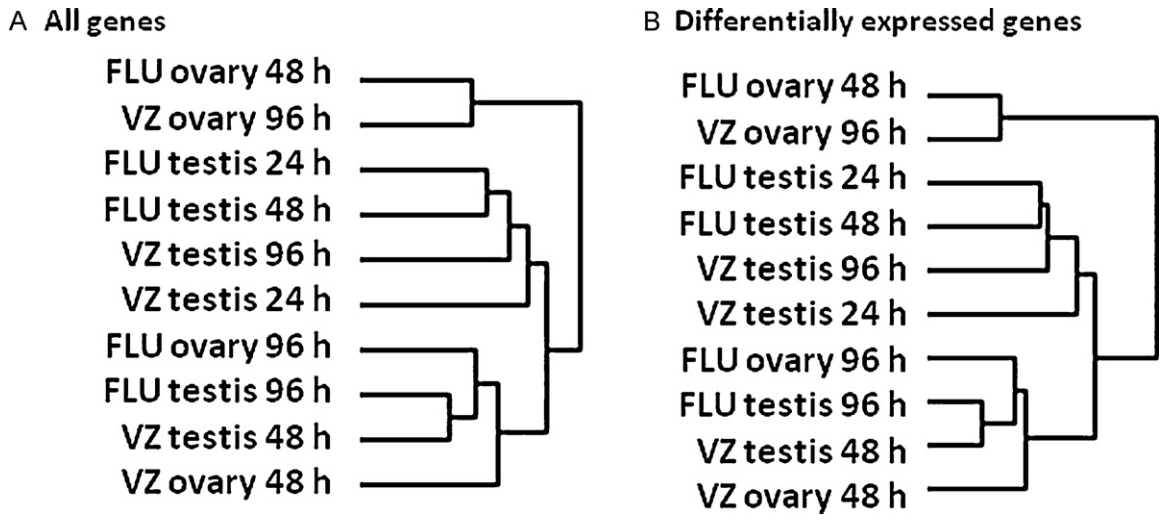

Fig. 1. Hierarchical clustering of (A) all genes that qualified for analyses ($n = 12{,}802$), and (B) differentially-expressed genes ($n = 3947$) for all chemical/sex/time combinations.

Table 3
The list of differentially-expressed genes ($p < 0.05$) from the present study that overlap with the genes with known reproductive roles in the teleost hypothalamo-pituitary gonadal axis.

Treatment		Symbol	Direction of change	Gene description
Vinclozolin				
	Male			
		adx	Downregulated	Adrenodoxin
		fshr	Upregulated	Follicle stimulating hormone receptor
		hsd11b2	Downregulated	Hydroxysteroid 11-beta dehydrogenase 2
		inhba	Downregulated	Inhibin, beta A
	Female			
		smad2	Downregulated	MAD homolog 2
		narg1a	Upregulated	NMDA receptor-regulated gene 1a
Flutamide				
	Male			
		adx	Downregulated	Adrenodoxin
		cbr1l	Downregulated	Carbonyl reductase 1-like
		cyp17a1	Downregulated	Cytochrome P450, family 17, subfamily A, polypeptide 1
		hsd11b2	Downregulated	Danio rerio hydroxysteroid 11-beta dehydrogenase 2
		rnf14	Downregulated	RING finger protein 14 (Androgen receptor-associated protein 54)
		sult2st	Downregulated	Cytosolic sulfotransferase 2
	Female			
		acvr1b	Upregulated	Activin A receptor, type IB
		cyp19a1a	Downregulated	Cytochrome P450, family 19, subfamily A, polypeptide 1a

Table 4
Vinclozolin exposure – statistically enriched canonical human-mouse-rat pathways and the lists of DEGs responsible for their enrichment – Ingenuity Pathway Analyses ($p < 0.1$).

Sex	Enriched canonical pathway	Molecules
Male	C21-Steroid Hormone Metabolism	HSD3B1, HSD11B2
	Synaptic Long Term Potentiation	CALM3, CACNA1C, PPP1CA, CAMK2B
	Methionine Metabolism	CBS, CTH
	Selenoamino Acid Metabolism	CBS, CTH
	Regulation of Actin-based Motility by Rho	MYLK, ACTB, ACTG2 (includes EG:72), GSN, PPP1CA
	Androgen and Estrogen Metabolism	UGT1A5, HSD3B1, HSD11B2
	Glycine, Serine and Threonine Metabolism	CBS, CTH
	Integrin Signaling	MYLK, ACTB, ACTG2, PPP1CA, RAPGEF1, CAPN3
	Insulin Receptor Signaling	JAK1, PPP1CA, RAPGEF1
	Nitric Oxide Signaling in the Cardiovascular System	CALM3, CACNA1C
	FXR/RXR Activation	APOE, G6PC, CYP8B1
	Glutathione Metabolism	GSTP1, IDH1
Female		
	Cardiac Adrenergic Signaling	GNB1, PPP1CA
	Endoplasmic Reticulum Stress Pathway	XBP1
	Folate Biosynthesis	GGH
	Valine, Leucine and Isoleucine Degradation	BCAT2, MUT, GCDH
	Pantothenate and CoA Biosynthesis	BCAT2
	Integrin Signaling	PPP1CA, RAPGEF1, CAPN3

Table 5
Flutamide exposure – statistically enriched canonical human-mouse-rat pathways and the lists of DEGs responsible for their enrichment – Ingenuity Pathway Analyses ($p < 0.1$).

Sex	Enriched canonical pathway	Molecules
Male		
	Integrin Signaling	PAK4,ARF1,TLN2,ARPC1B,MAP2K2,ACTB,CRKL,PIK3R1,ACTA2,ACTG2 (includes EG:72),RAP1A,CAPN3
	Protein Ubiquitination Pathway	PSMB4,HSPA8,TCEB2,PSMB2,HSP90AA1,FBXW7,PSMD4,UBE2E3, PSMA2,SMURF1
	Clathrin-mediated Endocytosis	HSPA8,ARPC1B,ACTB,PPP3R1,PIK3R1,CLTC,ACTA2, VEGFC, ACTG2 (includes EG:72),AP2A2
	NRF2-mediated Oxidative Stress Response	MAP2K6,MAP2K2,PPIB,ACTB,PIK3R1,NQO1,ACTA2, JUNB,ACTG2 (includes EG:72),CBR1,EPHX1
	VEGF Signaling	MAP2K2,ACTB,PIK3R1,ACTA2,VEGFC,ACTG2 (includes EG:72),ELAVL1
	fMLP Signaling in Neutrophils	GNAI2,CALM3,ARPC1B,MAP2K2,PPP3R1,PIK3R1,GNB2L1,GNG5
	Androgen and Estrogen Metabolism	UGT1A5,HSD17B3,HSD3B1,HSD11B2,SULF2,SULT2B1
	Neuregulin Signaling	MAP2K2,HSP90AB1,CRKL,PIK3R1,HSP90AA1,CRK, CDKN1B
	Fc-γ Receptor-mediated Phagocytosis in Macrophages and Monocytes	TLN2,ARPC1B,ACTB,PIK3R1,ACTA2,CRK,ACTG2 (includes EG:72)
	PI3K/AKT Signaling	TP53,MAP2K2,HSP90AB1,PIK3R1,YWHAZ,HSP90AA1, CDKN1B,PPP2R1B
	Ephrin Receptor Signaling	GNAI2,PAK4,ARPC1B,MAP2K2,CRKL,GNB2L1,VEGFC, CRK,GNG5,RAP1A
	Leukocyte Extravasation Signaling	GNAI2,MAP2K2,ACTB,CRKL,PIK3R1,ACTA2,MMP2,CRK,ACTG2 (includes EG:72),RAP1A
	Nitric Oxide Signaling in the Cardiovascular System	CALM3,HSP90AB1,PIK3R1,CACNA1C,HSP90AA1,VEGFC
	C21-Steroid Hormone Metabolism	CYP17A1,HSD3B1,HSD11B2
	FGF Signaling	MAP2K6,FGFR1,CRKL,PIK3R1,CRK,FRS2
	CCR3 Signaling in Eosinophils	GNAI2,PAK4,CALM3,MAP2K2,PIK3R1,GNB2L1,GNG5
	Amyotrophic Lateral Sclerosis Signaling	TP53,PIK3R1,CACNA1C,GLUL,VEGFC,CAPN3
	Axonal Guidance Signaling	PAK4,RND1,ARPC1B,PIK3R1,CRKL,GNB2L1,VEGFC,CRK,RAP1A,GNG5, GNAI2,MAP2K2,PPP3R1,RTN4,RTN4R
	Hypoxia Signaling in the Cardiovascular System	TP53,HSP90AB1,NQO1,HSP90AA1,UBE2E3
	Calcium Signaling	TPM1,CALR,CALM3,TPM3,ATP2B1,PPP3R1,ACTA2, RCAN3,MEF2C,RAP1A
	SAPK/JNK Signaling	TP53,MAP4K3,CRKL,PIK3R1,CRK,GNG5
	Citrate Cycle	IDH3G,SDHC,IDH1
	ERK/MAPK Signaling	PAK4,TLN2,MAP2K2,CRKL,PIK3R1,YWHAZ,CRK, PPP2R1B,RAP1A
	Huntington's Disease Signaling	TP53,HSPA8,PIK3R1,CLTC,GNB2L1,RCOR2,CASP8, AP2A2,GNG5,CAPN3
	Xenobiotic Metabolism Signaling	MAP2K6,HS6ST1,UGT1A5,MAP2K2,HSP90AB1,PIK3R1, NQO1,HSP90AA1,PPP2R1B,AHR,SULT2B1
	Urea Cycle and Metabolism of Amino Groups	CKB,ARG2,ASL
	PDGF Signaling	MAP2K2,CRKL,PIK3R1,PDGFRA,CRK
	Hepatic Fibrosis/Hepatic Stellate Cell Activation	COL1A2,FGFR1,ACTA2,PDGFRA,VEGFC,IGFBP5,MMP2
	Regulation of Actin-based Motility by Rho	PAK4,ARPC1B,ACTB,ACTA2,ACTG2 (includes EG:72)
	Role of NFAT in Regulation of the Immune Response	GNAI2,CALM3,MAP2K2,PPP3R1,PIK3R1,GNB2L1,RCAN3,MEF2C,GNG5
	Actin Cytoskeleton Signaling	PAK4,ARPC1B,MAP2K2,ACTB,CRKL,PIK3R1,ACTA2, CRK,ACTG2 (includes EG:72)
	Aryl Hydrocarbon Receptor Signaling	TP53,CCNA1,HSP90AB1,NQO1,HSP90AA1,CDKN1B,AHR
	CXCR4 Signaling	GNAI2,PAK4,MAP2K2,PIK3R1,GNB2L1,CRK,GNG5
	IGF-1 Signaling	MAP2K2,PIK3R1,YWHAZ,IGFBP5,IGFBP2
	GM-CSF Signaling	MAP2K2,PPP3R1,PIK3R1,GNB2L1
	α-Adrenergic Signaling	GNAI2,CALM3,MAP2K2,GNB2L1,GNG5
	Arginine and Proline Metabolism	CKB,CRYM,ARG2,ASL
	CCR5 Signaling in Macrophages	GNAI2,CALM3,GNB2L1,GNG5
	Endoplasmic Reticulum Stress Pathway	XBP1,MBTPS2
	Neurotrophin/TRK Signaling	MAP2K6,MAP2K2,PIK3R1,FRS2
	Cysteine Metabolism	HS6ST1,CARS,CTH,SULT2B1
	Role of PKR in Interferon Induction and Antiviral Response	TP53,MAP2K6,CASP8
	Caveolar-mediated Endocytosis	FLOT2,ACTB,ACTA2,ACTG2 (includes EG:72)
	CD27 Signaling in Lymphocytes	MAP2K6,MAP2K2,CASP8
	IL-3 Signaling	MAP2K2,PPP3R1,CRKL,PIK3R1
	IL-8 Signaling	GNAI2,MAP2K2,PIK3R1,GNB2L1,VEGFC,MMP2,GNG5
	Synaptic Long Term Potentiation	CALM3,MAP2K2,PPP3R1,CACNA1C,RAP1A
Female		
	Mitochondrial Dysfunction	NDUFB9,PARK7,ND4,PSEN2,SDHC,MAPK12,NDUFA13
	Estrogen Receptor Signaling	TAF9,NCOA2,CTBP2,TAF3,GTF2F1,MAPK12
	Amyloid Processing	PRKCE,PSEN2,MAPK12,CAPN3
	Neuregulin Signaling	HSP90AB1,PTPN11,PRKCE,CDKN1B,MAPK12
	Hypoxia Signaling in the Cardiovascular System	HSP90AB1,UBE2E3,UBE2J1,UBE2E1
	PI3K/AKT Signaling	PPP2CB,HSP90AB1,YWHAZ,CDKN1B,MAPK12
	FGF Signaling	MAP2K6,PTPN11,FGFR1,MAPK12
	TGF-β Signaling	PIAS4,BMP7,ACVR2B,MAPK12
	Parkinson's Signaling	PARK7,MAPK12
	Apoptosis Signaling	PRKCE,LMNA,MAPK12,CAPN3

Table 5 (*Continued*)

Sex	Enriched canonical pathway	Molecules
	Endoplasmic Reticulum Stress P	XBP1,MBTPS2
	IL-6 Signaling	MAP2K6,PTPN11,CYP19A1,MAPK12
	Fc Epsilon RI Signaling	MAP2K6,PTPN11,PRKCE,MAPK12
	Thrombopoietin Signaling	PTPN11,PRKCE,MAPK12
	Ubiquinone Biosynthesis	NDUFB9,ND4,NDUFA13
	JAK/Stat Signaling	PIAS4,PTPN11,MAPK12
	14-3-3-mediated Signaling	YWHAZ,PRKCE,CDKN1B,MAPK12
	Neurotrophin/TRK Signaling	MAP2K6,PTPN11,MAPK12
	Ephrin Receptor Signaling	PTPN11,EFNA3,DOK1,MAPK12,EFNA1
	ERK/MAPK Signaling	PPP2CB,TLN2,YWHAZ,PRKCE,MAPK12
	LPS-stimulated MAPK Signaling	MAP2K6,PRKCE,MAPK12
	Oxidative Phosphorylation	NDUFB9,ND4,SDHC,NDUFA13
	Glucocorticoid Receptor Signaling	TAF9,NCOA2,HSP90AB1,HSPA14,TAF3,GTF2F1,MAPK12
	VDR/RXR Activation	NCOA2,PRKCE,CDKN1B
	Synaptic Long Term Depression	PPP2CB,YWHAZ,PRKCE,MAPK12
	CTLA4 Signaling in Cytotoxic T Lymphocytes	PPP2CB,PTPN11,CLTC
	Fc-γ RIIB Signaling in B Lymphocytes	DOK1,MAPK12
	Nicotinate and Nicotinamide Metab.	MAP2K6,CDK8,PRKCE
	Protein Ubiquitination Pathway	USP7,USP12,UBE2E3,UBE2J1,UBE2E1
	Aryl Hydrocarbon Receptor Signaling	NCOA2,HSP90AB1,CDKN1B,MAPK12
	IGF-1 Signaling	PTPN11,YWHAZ,MAPK12
	Fc-γ Receptor-mediated Phagocytosis in Macrophages and Monocytes	TLN2,PRKCE,MAPK12
	CD27 Signaling in Lymphocytes	MAP2K6,MAPK12
	Wnt/β-catenin Signaling	PPP2CB,CSNK1G2,ACVR2B,SOX11
	Axonal Guidance Signaling	DPYSL2,PTPN11,EFNA3,PRKCE,BMP7,MAPK12,EFNA1
	Glycerophospholipid Metabolism	PPAP2A,PCYT2,YWHAZ
	Toll-like Receptor Signaling	MAP2K6,MAPK12
	Natural Killer Cell Signaling	PTPN11,PRKCE,MAPK12
	IL-2 Signaling	PTPN11,MAPK12

like (*fam60al*), transcription factor 12 (*tcf12*), and zinc finger protein 384 like (*znf384l*).

The results presented here focus on assessing impacts of VZ and FLU on reproduction, but the portions of the presented data were utilized in conjunction with data for 10 other EACs to examine effects on other biological pathways (Wang et al., 2010). The potential biological pathways of significance (for endocrine-active chemicals), have been expanded from initially a single, somewhat subjectively defined HPG-axis compiled to several pathways including TGF-beta, p53 signaling, and their cross-talking network neighbors, and to a still greater number of tentative TF networks peripheral to them, all of which could have a profound impact on cellular functions such as stress response, cell cycle, and apoptosis (Wang et al., 2010).

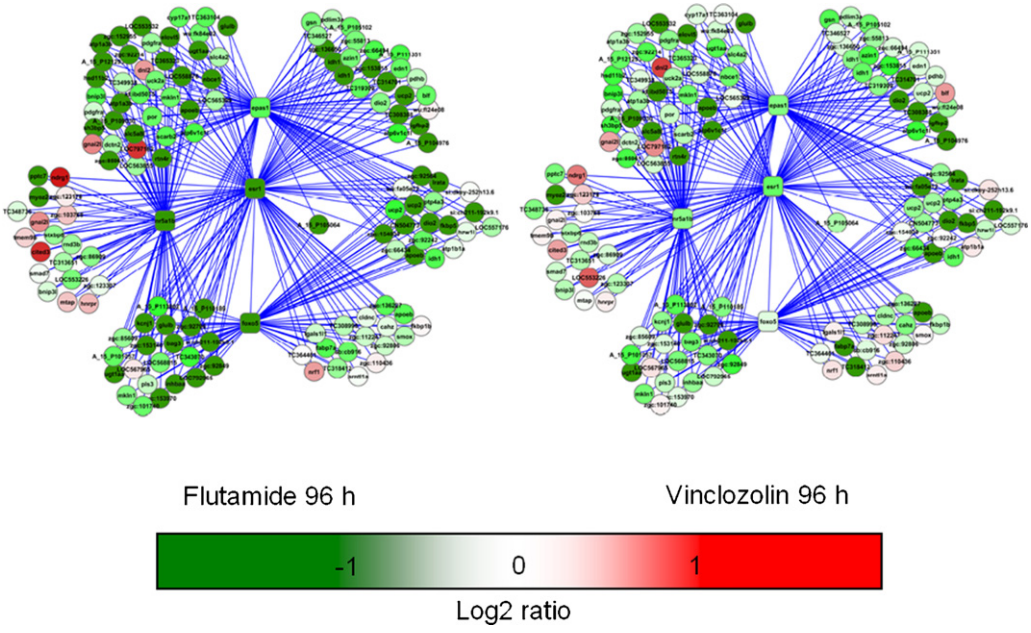

Fig. 2. Cytoscape visualization of transcription factor (TF) networks enriched in the testis of males exposed to vinclozolin or flutamide for 96 h. Rounded rectangles indicate TF network hubs. Circles indicate linked genes (labeled with official gene symbols). Only genes linked to at least two of the four TF hubs are shown. Color key: white indicates log2 ratio = 0. Graded green indicates log2 ratio between 0 and −1 (less than 2-fold down-regulated). Dark green indicates log2 ratio < −1 (greater than 2-fold down-regulated). Graded red indicates log2 ratio between 0 and 1 (less than 2-fold up-regulated). Dark red indicates log2 ratio greater than 1 (greater than 2-fold up-regulated). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

4. Discussion

Both VZ- and FLU-impacted expression of genes involved in multiple aspects of reproductive function, with male reproductive systems more extensively impacted. The following discussion emphasizes effects observed in males as opposed to females, in part because the role androgens play in male fish is well elucidated (Borg, 1994). Nonetheless, it is important to emphasize that FLU- and VZ-induced changes in gene expression in females are indicative of reproductive malfunction. For example, the females exposed to VZ and FLU exhibited alterations in activin signaling (activin receptor [*acvr1*], *smad 2*), which is critical for differential regulation and secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), and thus plays an important role in the oocyte maturation (Ge, 2000; Wu et al., 2000). Likewise, we observed down-regulation of ovarian aromatase (*cyp19a1a*) in FLU-exposed females, which suggests that estradiol production could be depressed by anti-androgens. Similar molecular effects were observed in medaka exposed to FLU (Leon et al., 2008) and are suggestive of adverse effects on vitellogenesis. The molecular effects from the present study concur with the apical level effects (e.g., impaired ovarian maturation and fecundity) observed in female fish exposed to similar concentrations of FLU and VZ (Makynen et al., 2000; Jensen et al., 2004; Martinovic et al., 2008).

In males, exposure to FLU or VZ affected expression of genes involved in androgen biosynthesis (11β-hydroxysteroid dehydrogenase [*11β-hsd*] *and/or* 17α-hydroxylase [*cyp17a1*]). We also observed suppression of adrenodoxin (*adx*), which is important for activation of enzymes that catalyze hydroxylation steps (e.g., CYP11, CYP17, and CYP19) in the biosynthesis of corticosteroids and sex steroids (Muller et al., 2001; Pechurskaya et al., 2007). Paradoxically, *11β-hsd* and/or *cyp17a1* were down-regulated by the anti-androgens. This is contrary to findings of others who observed the over-expression of mRNAs for these enzymes in animals exposed to anti-androgens, and hypothesized that these, combined with an increased LH secretion, represent an adaptive response through which intratesticular testosterone production is increased to compensate for actions of the anti-androgens (Kubota et al., 2003; Ohsako et al., 2003; Filby et al., 2007). Interestingly, the majority of the studies of anti-androgens in fish either reported no change (Filby et al., 2007; Martinovic et al., 2008) or observed decreased circulating androgen concentrations (Sharpe et al., 2004). Furthermore, Hoffmann et al. (2008) observed that exposure to an androgen increased expression of *11β-hsd*, *cyp17a1*, and 3β- hydroxysteroid dehydrogenase (*3β-hsd*) in zebrafish, indicating that anti-androgens could decrease androgen mediated expression of these genes. In the current experiment, VZ males exhibited responses indicative of up-regulation of FSH pathways (e.g., up-regulated FSH receptor mRNA). Down-regulation of inhibin a (*inhba*) could also be contributing to alterations in FSH signaling. Similar to the current study, Villeneuve et al. (2007b) observed up-regulation of *fshb* in pituitaries of males exposed to VZ, and suggested that increased activity of FSH pathway could be a mechanism via which organisms respond to the chemicals that interfere with sperm development. In the present study, several other genes involved in sperm development were altered, including those whose mutations lead to sperm deformities (*hrb*, Kang-Decker et al., 2001), defects in germ cell differentiation (*dazl*, Ruggiu et al., 1997), and spermatogenesis (*spnr*, Pires-daSilva et al., 2001). These molecular findings are in agreement with the results of Jensen et al. (2004) who documented that male fish exposed to flutamide exhibited spermatocyte degeneration and necrosis.

One of our objectives was to determine whether there were commonalities in the gene expression profiles of VZ and FLU. The hierarchical clustering analyses indicate that gene expression profiles of VZ and FLU were not readily distinguishable. This is supported by the observation that a large proportion of the DEGs were common to both chemical treatments. Even though FLU-exposed males exhibited more extensive changes in gene expression (more DEGs, more enriched pathways), many of these reflected a generalized response to toxicity and stress. This is supported by the IPA data which identified enrichment of several toxicity pathways by FLU (oxidative stress responses, hypoxia-inducible factor signaling, and cytochrome P450s) and none by VZ.

Examination of the enriched canonical pathways using IPA also pointed to many commonalities between FLU and VZ; 6 of 12 pathways that were enriched in VZ-treated males were also enriched in FLU-treated males. Most importantly, all of the common pathways were indicative of disruption of sex steroid synthesis and signaling (e.g., C21-steroid hormone metabolism, androgen and estrogen metabolism), or reflected processes that are modulated by androgens (regulation of actin-based motility by *rho*, integrin signaling, synaptic long term potentiation, nitric oxide signaling). Both actin and integrin play an important role in germ cell migration, and spermatid movement, orientation and attachment in the seminiferous epithelium. Specifically, spermatids attach to epithelium via an actin-based adherens junction type known as ectoplasmic specialization (ES) (Lee and Cheng, 2004). Actin reorganization has been shown to be modulated via AR-mediated activation of the Rho/ROCK pathway (Papadopoulou et al., 2008). The AR is also thought to play a role in the regulation of adhesion through influencing the expression of specific integrin subunits, which play a role in disengagement of the sperm and turnover of the basal ES during spermatogenesis (Beardsley and O'Donnell, 2003). Furthermore, the positive correlation between the presence of certain β1-integrin **c**ell adhesion molecules and the fertilizing ability of spermatozoa suggests that integrin**s** may be putative determinants in egg-sperm recognition and interaction (Klentzeris et al., 1995). Several mammalian studies have documented that FLU induces histological deformations of spermatids and ES ultrastructure (Anahara et al., 2004). Our study demonstrates that VZ has similar effect on genes that regulate these processes critical for spermatogenesis in fish (at least in zebrafish). The consistent and extensive disruption of these pathways by both VZ and FLU suggests that they may be of interest when searching for biomarkers of anti-androgenic exposure. Specifically, actins, as well as their binding protein tropomyosin, may be good indicators of anti-androgens and reproductive impairment. The actins in germ cells have a role in the determination of cell shape during spermiogenesis, motility, and sperm capacitation (Yagi and Paranko, 1995). Actin-binding proteins (e.g., tropomyosin) are also active during sperm maturation and control polymerization status of actin, which is important for multiple aspects of sperm function (Howes et al., 2001). In fact, Leon et al. (2008) suggested tropomyosin as a diagnostic gene for chemical effects on reproduction in fish, based on their studies in which they documented its up-regulation by an androgen and down-regulation by anti-androgens in medaka.

Another gene (*nr5a1*) that changed consistently in FLU-and VZ-exposed fish codes for nuclear receptor 5A. It encodes the steroidogenic factor 1 (SF1) in mammals which regulates the cell-specific expression of steroidogenic genes (Luo et al., 1994). In mammals, SF1 is involved in sex determination, which involves synergistic action of SRY and SF1 (Sekido and Lovell-Badge, 2008). While the role of *nr5a1* in zebrafish is not fully elucidated, it has been suggested as one of the main candidate genes involved in sex determination and differentiation (von Hofsten and Olsson, 2005). Antimulerian hormone is a possible target of *nra5a1* clade of genes in zebrafish (von Hofsten and Olsson, 2005). Furthermore, the zebrafish *cyp19a* promoter region contains binding sites for SF-1, which indicates its role in the regulation of *cyp19a* expression (Watanabe et al., 1999; Tong and Chung, 2003). The down-regulation of *nra5a1* by FLU and VZ in the present study

is in agreement with the results of others who exposed fish and mammals to anti-androgens (Borch et al., 2006). Borch et al. (2006) reported reduced mRNA expression of SF-1, and hypothesized that effects of the anti-androgen diethylhexyl phthalate on SF-1 are involved in the down-regulation of steroid synthesis, and thereby underlie disturbed development of the male reproductive system. Several key HPG axis genes, including *srb1*, *star*, *p450scc*, *3βhsd*, and *cyp17* are regulated by SF-1 (Val et al., 2003), and our observation of reduced expression of sf1, which coincided with changes in *3β-hsd* and *p450c17*, provides further support for this hypothesis. The integral role of nr5a1 in anti-androgen induced disruption of reproductive functioning was also confirmed by our GSEA analyses that identified *nr5a1* as one of the main hub genes for networks of genes impacted by both FLU- and VZ-treated males.

Apolipoprotein (*apoeb*) mRNA, also was consistently down-regulated by FLU and VZ. *Apoe* inhibits ovarian androgen synthesis (Dyer and Curtiss, 1988), and some of its forms (*apoe4*) have been shown to decrease cytosolic androgen receptor concentrations (Raber et al., 2002). In spite of its connectivity to the androgen pathway, *apoeb* probably could not be used alone as an indicator of anti-androgens, because past studies in our laboratory have shown that *apoeb* is differentially regulated by variety of non-chemical (Martinovic et al., 2008) and chemical stressors that affect HPG axis (Villeneuve et al., 2009).

In the current study, antizyme inhibitor (*azin1*) mRNA was down-regulated across all time points in VZ and FLU-exposed males. This enzyme is involved in regulation of the polyamine pathway (Dayoub et al., 2006), which is important for spermiogenesis (Coffino, 2000). Levels of polyamines are regulated, in part, by ornithine decarboxylase 1 (ODC), which is a key rate-limiting enzyme involved in their synthesis (Palanimurugan et al., 2004). The synthesis of polyamines in testis depends upon the androgen environment and the regulation of ODC activity appears to be controlled by androgens (de las Heras et al., 1992). Although we did not observe effects on *odc*, the observation of decreased *azin1* is indicative of alterations of polyamine pathways. AZIN1 is an ODC homolog and is responsible for maintaining polyamine levels by binding antizymes, thus protecting ODC from antizyme-induced degradation (Bercovich and Kahana, 2004). Given the extensive documentation of inverse regulation of ODC and *azin* expression by androgens vs. anti-androgens (Kontula et al., 1985; Hoffmann et al., 2008), we suggest that this pathway is another reasonable target in the search for biomarkers of anti-androgenic exposure and effects.

Fibroblast growth factor receptor (FGFR-1) mRNA expression was up-regulated by FLU and VZ in both males and females. This is of interest because FGRF-1 signaling is involved in spermiogenesis and sperm capacitation (Cotton et al., 2006), as well as development of androgen-dependent secondary sex characteristics in at least some fish species (Offen et al., 2008). Furthermore, Petiot et al. (2005) discovered a putative androgen response element within the *fgfr2* promoter, suggesting that *fgfrs* could be direct transcriptional targets of the AR. Ruohola et al. (1995) showed that testosterone increases the expression of *fgfr1*. The up-regulation of *fgfr1* by anti-androgens documented by the present study is somewhat counterintuitive, but nevertheless stood out as a consistent marker of exposure to FLU and VZ across both sexes and multiple time-points.

5. Conclusion

We demonstrated that anti-androgens potentially impact reproductive success via multiple MOAs involved in processes ranging from steroidogenesis to spermatogenesis and fertilization. Many of these mechanisms are common to VZ and FLU as demonstrated by substantial overlap in DEGs and enrichment of common pathways. Finally, we identified several pathways and genes to guide identification of biomarkers of anti-androgenic action that are indicative of both exposure to anti-androgens, and apical effects on reproduction. Given the nature of microarray-based gene expression profiling, as part of final biomarker validation/implementation there is a need to conduct focused follow-up studies to confirm the initial transcriptomic results with additional methods (e.g., QPCR, protein and metabolite analyses), ideally at an expanded range of test times/chemical concentrations in multiple fish species.

Funding information

This work was supported by the USEPA National Center for Computational Toxicology. The manuscript has been reviewed in accordance with USEPA guidelines and approved for publication. Approval does not indicate that the contents reflect the views of either Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use. The views expressed are those of the authors and do not necessarily reflect USEPA policy.

Acknowledgments

We thank our colleagues who have been involved in different aspects of this work, including Lindsey Blake, Elizabeth Durhan, Kathleen Jensen, Michael Kahl, and Elizabeth Makynen. We thank Robin Sternberg for the initial review of the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2010.10.003.

References

Anahara, R., Toyama, Y., Mori, C., 2004. Flutamide induces ultrastructural changes in spermatids and the ectoplasmic specialization between the Sertoli cell and spermatids in mouse testes. Reprod. Toxicol. 18, 589–596.

Ankley, G.T., Bencic, D.C., Breen, M.S., Collette, T.W., Conolly, R.B., Denslow, N.D., Edwards, S.W., Ekman, D.R., Garcia-Reyero, N., Jensen, K.M., Lazorchak, J.M., Martinovic, D., Miller, D.H., Perkins, E.J., Orlando, E.F., Villeneuve, D.L., Wang, R.L., Watanabe, K.H., 2009. Endocrine disrupting chemicals in fish: developing exposure indicators and predictive models of effects based on mechanism of action. Aquat. Toxicol. 92, 168–178.

Basso, K., Margolin, A.A., Stolovitzky, G., Klein, U., Dalla-Favera1, R., Califano, A., 2005. Reverse engineering of regulatory networks in human B cells. Nat. Genet. 37, 382–390.

Bayley, M., Junge, M., Baatrup, E., 2002. Exposure of juvenile guppies to three antiandrogens causes demasculinization and a reduced sperm count in adult males. Aquat. Toxicol. 56, 227–239.

Beardsley, A., O'Donnell, L., 2003. Characterization of normal spermiation and spermiation failure induced by hormone suppression in adult rats. Biol. Reprod. 68, 1299–1307.

Bercovich, Z., Kahana, C., 2004. Degradation of antizyme inhibitor, an ornithine decarboxylase homologous protein, is ubiquitin-dependent and is inhibited by antizyme. J. Biol. Chem. 279, 54097–54102.

Borch, J., Metzdorff, S.B., Vinggaard, A.M., Brokken, L., Dalgaard, M., 2006. Mechanisms underlying the anti-androgenic effects of diethylhexyl phthalate in fetal rat testis. Toxicology 223, 144–155.

Borg, B., 1994. Androgens in teleost fishes. Comp. Biochem. Physiol. 109, 219–245.

Cotton, L., Gibbs, G.M., Sanchez-Partida, L.G., Morrison, J.R., de Kretser, D.M., O'Bryan, M.K., 2006. FGFR-1 [corrected] signaling is involved in spermiogenesis and sperm capacitation. J. Cell. Sci. 119, 75–84.

Coffino, P., 2000. Polyamines in spermiogenesis: not now, darling. PNAS 97, 4421–4423.

de las Heras, M.A., Gonzalez, S.I., Calandra, R.S., 1992. Androgenic control of polyamine concentrations in rat epididymis. J. Reprod. Fertil. 96, 323–330.

Dayoub, W.E., Thasler, A.K., Bosserhoff, T., Singer, K.W., Jauch, H.J., Schlitt, Weiss, T.S., 2006. Regulation of polyamine synthesis in human hepatocytes by hepatotrophic factor augmenter of liver regeneration. Biochem. Biophys. Res. Commun. 345, 181–187.

Dyer, C.A., Curtiss, L.K., 1988. Apoprotein E-rich high density lipoproteins inhibit ovarian androgen synthesis. J. Biol. Chem. 263, 10965–10973.

Filby, A.L., Thorpe, K.L., Maack, G., Tyler, C.R., 2007. Gene expression profiles revealing the mechanisms of anti-androgen- and estrogen-induced feminization in fish. Aquat. Toxicol. 81, 219–231.

Foster, P.M.D., Mylchreest, E., Gaido, K.W., Sar, M., 2001. Effects of phthalate esters on the developing reproductive tract of male rats. Hum. Reprod. Update 7, 231–235.
Ge, W., 2000. Roles of the activin regulatory system in fish reproduction. Can. J. Physiol. Pharmacol. 78, 1077–1085.
Hoffmann, J.L., Thomason, R.G., Lee, D.M., Brill, J.L., Price, B.B., Carr, G.J., Versteeg, D.J., 2008. Hepatic gene expression profiling using GeneChips in zebrafish exposed to 17alpha-methyldihydrotestosterone. Aquat. Toxicol. 87, 69–80.
Hotchkiss, A.K., Ankley, G.T., Wilson, V.S., Hartig, P.C., Durhan, E.J., Jensen, K.M., Martinovic, D., Gray, L.E., 2008. Of mice and men (and mosquitofish): antiandrogens and androgens in the environment. BioScience 58, 1037–1050.
Howes, E.A., Hurst, S.M., Jones, R., 2001. Actin and actin-binding proteins in bovine spermatozoa: potential role in membrane remodeling and intracellular signaling during epididymal maturation and the acrosome reaction. J. Androl. 22, 62–72.
Jensen, K.M., Kahl, M.D., Makynen, E.A., Korte, J.J., Leino, R.L., Butterworth, B.C., Ankley, G.T., 2004. Characterization of responses to the antiandrogen flutamide in a short-term reproduction assay with the fathead minnow. Aquat. Toxicol. 70, 99–110.
Kang-Decker, N., Mantchev, G.T., Juneja, S.C., McNiven, M.A., van Deursen, J.M., 2001. Lack of acrosome formation in Hrb-deficient mice. Science 294, 1531–1533.
Katsiadaki, I., Morris, S., Squires, C., Hurst, M.R., James, J.D., Scott, A.P., 2006. Use of the three-spined stickleback (*Gasterosteus aculeatus*) as a sensitive in vivo test for detection of environmental antiandrogens. Environ. Health Perspect. 114 (Suppl 1), 115–121.
Kelce, W.R., Lambright, C.R., Gray Jr., L.E., Roberts, K.P., 1997. Vinclozolin and p,p′-DDE alter androgen-dependent gene expression: in vivo confirmation of an androgen receptor-mediated mechanism. Toxicol. Appl. Pharmacol. 142, 192–200.
Klentzeris, L.D., Fishel, S., McDermott, H., Dowell, K., Hall, J., Green, S., 1995. A positive correlation between expression of beta 1-integrin cell adhesion molecules and fertilizing ability of human spermatozoa in vitro. Hum. Reprod. 10, 728–733.
Kontula, K.K., Seppanen, P.J., van Duyne, P., Bardin, C.W., Janne, O.A., 1985. Effect of a nonsteroidal antiandrogen, flutamide, on androgen receptor dynamics and ornithine decarboxylase gene expression in mouse kidney. Endocrinology 116, 226–233.
Kubota, K., Ohsako, S., Kurosawa, S., Takeda, K., Qing, W., Sakaue, M., Kawakami, T., Ishimura, R., Tohyama, C., 2003. Effects of vinclozolin administration on sperm production and testosterone biosynthetic pathway in adult male rat. J. Reprod. Dev. 49, 403–412.
Larkin, P., Folmar, L.C., Hemmer, M.J., Poston, A.J., Lee, H.S., Denslow, N.D., 2002. Array technology as a tool to monitor exposure of fish to xenoestrogens. Mar. Environ. Res. 54, 395–399.
Lee, N.P., Cheng, C.Y., 2004. Adaptors, junction dynamics, and spermatogenesis. Biol. Reprod. 71, 392–404.
Leon, A., Wu, P.S., Hall, L.C., Johnson, M.L., Teh, S.J., 2008. Global gene expression profiling of androgen disruption in Qurt strain medaka. Environ. Sci. Technol. 42, 962–969.
Luo, X., Ikeda, Y., Parker, K.L., 1994. A cell-specific nuclear receptor is essential for adrenal and gonadal development and sexual differentiation. Cell 77, 481–490.
Makynen, E.A., Kahl, M.D., Jensen, K.M., Tietge, J.E., Wells, K.L., Van Der Kraak, G., Ankley, G.T., 2000. Effects of the mammalian antiandrogen vinclozolin on development and reproduction of the fathead minnow (*Pimephales promelas*). Aquat. Toxicol. 48, 461–475.
Martinovic, D., Blake, L.S., Durhan, E.J., Greene, K.J., Kahl, M.D., Jensen, K.M., Makynen, E.A., Villeneuve, D.L., Ankley, G.T., 2008. Reproductive toxicity of vinclozolin in the fathead minnow: confirming an anti-androgenic mode of action. Environ. Toxicol. Chem. 27, 478–488.
Matzuk, M.M., Lamb, D.J., 2002. Genetic dissection of mammalian fertility pathways. Nat. Cell Biol. 4 (Suppl.), s41–s49.
Moens, L.N., van der Ven, K., Van Remortel, P., Del-Favero, J., De Coen, W.M., 2006. Expression profiling of endocrine-disrupting compounds using a customized *Cyprinus carpio* cDNA microarray. Toxicol. Sci. 93, 298–310.
Morey, J.S., Ryan, J.C., Dolah, F.M.V., 2006. Microarray validation: factors influencing correlation between oligonucleotide microarrays and real-time PCR. Biol. Proceed. Online 8, 175–193.
Mu, X., Liu, K., Kleymenova, E., Sar, M., Young, S.S., Gaido, K.W., 2006. Gene expression profiling of androgen receptor antagonists in the rat fetal testis reveals few common gene targets. J. Biochem. Mol. Toxicol. 20, 7–17.
Muller, J.J., Lapko, A., Bourenkov, G., Ruckpaul, K., Heinemann, U., 2001. Adrenodoxin reductase-adrenodoxin complex structure suggests electron transfer path in steroid biosynthesis. J. Biol. Chem. 276, 2786–2789.
Offen, N., Blum, N., Meyer, A., Begemann, G., 2008. Fgfr1 signalling in the development of a sexually selected trait in vertebrates, the sword of swordtail fish. BMC Dev. Biol. 8, 98.
Ohsako, S., Kubota, K., Kurosawa, S., Takeda, K., Qing, W., Ishimura, R., Tohyama, C., 2003. Alterations of gene expression in adult male rat testis and pituitary shortly after subacute administration of the antiandrogen flutamide. J. Reprod. Dev. 49, 275–290.
Palanimurugan, R., Scheel, H., Hofmann, K., Dohmen, R.J., 2004. Polyamines regulate their synthesis by inducing expression and blocking degradation of ODC antizyme. EMBO J. 23, 4857–4867.
Panter, G.H., Hutchinson, T.H., Hurd, K.S., Sherren, A., Stanley, R.D., Tyler, C.R., 2004. Successful detection of (anti-)androgenic and aromatase inhibitors in prespawning adult fathead minnows (*Pimephales promelas*) using easily measured endpoints of sexual development. Aquat. Toxicol. 70, 11–21.
Papadopoulou, N., Charalampopoulos, I., Alevizopoulos, K., Gravanis, A., Stournaras, C., 2008. Rho/ROCK/actin signaling regulates membrane androgen receptor induced apoptosis in prostate cancer cells. Exp. Cell Res. 314, 3162–3174.
Pawitan, Y., Michiels, S., Koscielny, S., Gusnanto, A., Ploner, A., 2005. False discovery rate, sensitivity and sample size for microarray studies. Bioinformatics 21, 3017–3024.
Pechurskaya, T.A., Harnastai, I.N., Grabovec, I.P., Gilep, A.A., Usanov, S.A., 2007. Adrenodoxin supports reactions catalyzed by microsomal steroidogenic cytochrome P450s. Biochem. Biophys. Res. Commun. 353, 598–604.
Petiot, A., Perriton, C.L., Dickson, C., Cohn, M.J., 2005. Development of the mammalian urethra is controlled by Fgfr2-IIIb. Development 132, 2441–2450.
Pires-daSilva, A., Nayernia, K., Engel, W., Torres, M., Stoykova, A., Chowdhury, K., Gruss, P., 2001. Mice deficient for spermatid perinuclear RNA-binding protein show neurologic, spermatogenic, and sperm morphological abnormalities. Dev. Biol. 233, 319–328.
Quackenbush, J., 2002. Microarray data normalization and transformation. Nat. Genet. 32 (Suppl.), 496–501.
Raber, J., Bongers, G., LeFevour, A., Buttini, M., Mucke, L., 2002. Androgens protect against apolipoprotein E4-induced cognitive deficits. J. Neurosci. 22, 5204–5209.
Rosen, M.B., Wilson, V.S., Schmid, J.E., Gray, L.E., 2005. Gene expression analysis in the ventral prostate of rats exposed to vinclozolin or procymidone. Reprod. Toxicol. 19, 367–379.
Ruggiu, M., Speed, R., Taggart, M., McKay, S.J., Kilanowski, F., Saunders, P., Dorin, J., Cooke, H.J., 1997. The mouse Dazla gene encodes a cytoplasmic protein essential for gametogenesis. Nature 389, 73–77.
Ruohola, J.K., Valve, E.M., Vainikka, S., Alitalo, K., Harkonen, P.L., 1995. Androgen and fibroblast growth factor (FGF) regulation of FGF receptors in S115 mouse mammary tumor cells. Endocrinology 136, 2179–2188.
Sekido, R., Lovell-Badge, R., 2008. Sex determination involves synergistic action of SRY and SF1 on a specific Sox9 enhancer. Nature 453, 930–934.
Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., Amin, N., Schwikowski, B., Ideker, T., 2003. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res. 13, 2498–2504.
Sharpe, R.L., MacLatchy, D.L., Courtenay, S.C., Van Der Kraak, G.J., 2004. Effects of a model androgen (methyl testosterone) and a model anti-androgen (cyproterone acetate) on reproductive endocrine endpoints in a short-term adult mummichog (*Fundulus heteroclitus*) bioassay. Aquat. Toxicol. 67, 203–215.
Subramanian, A., Tamayoa, P., Moothaa, V.K., Mukherjeed, S., Eberta, B.L., Gillettea, M.A., Paulovich, A., Pomeroy, S.L., Golub, T.R., Lander, E.S., Mesirov, J.P., 2005. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc. Natl. Acad. Sci. U.S.A. 102, 15545–15550.
Tong, S.K., Chung, B.C., 2003. Analysis of zebrafish cyp19 promoters. J. Steroid Biochem. Mol. Biol. 86, 381–386.
Val, P., Lefrancois-Martinez, A.M., Veyssiere, G., Martinez, A., 2003. SF-1 a key player in the development and differentiation of steroidogenic tissues. Nucl. Recept. 1, 8.
Villeneuve, D.L., Larkin, P., Knoebl, I., Miracle, A.L., Kahl, M.D., Jensen, K.M., Makynen, E.A., Durhan, E.J., Carter, B.J., Denslow, N.D., Ankley, G.T., 2007a. A graphical systems model to facilitate hypothesis-driven ecotoxicogenomics research on the teleost brain-pituitary-gonadal axis. Environ. Sci. Technol. 41, 321–330.
Villeneuve, D.L., Blake, L.S., Brodin, J.D., Greene, K.J., Knoebl, I., Miracle, A.L., Martinovic, D., Ankley, G.T., 2007b. Transcription of key genes regulating gonadal steroidogenesis in control and ketoconazole- or vinclozolin-exposed fathead minnows. Toxicol. Sci. 98, 395–407.
Villeneuve, L., Wang, R.L., Bencic, D.C., Biales, A.D., Martinovic, D., Lazorchak, J.M., Toth, G., Ankley, G.T., 2009. Altered gene expression in the brain and ovaries of zebrafish (*Danio rerio*) exposed to the aromatase inhibitor fadrozole: microarray analysis and hypothesis generation. Environ. Toxicol. Chem. 28, 1767–1782.
von Hofsten, J., Olsson, P.E., 2005. Zebrafish sex determination and differentiation: involvement of FTZ-F1 genes. Reprod. Biol. Endocrinol. 3, 63.
Wang, R.L., Bencic, D., Biales, A., Lattier, D., Kostich, M., Villeneuve, D., Ankley, G.T., Lazorchak, J., Toth, G., 2008a. DNA microarray-based ecotoxicological biomarker discovery in a small fish model species. Environ. Toxicol. Chem. 27, 664–675.
Wang, R.L., Biales, A., Bencic, D., Lattier, D., Kostich, M., Villeneuve, D., Ankley, G.T., Lazorchak, J., Toth, G., 2008b. DNA microarray application in ecotoxicology: experimental design, microarray scanning, and factors affecting transcriptional profiles in a small fish species. Environ. Toxicol. Chem. 27, 652–663.
Wang, R.L., Biales, A., Bencic, D., Villeneuve, D.L., Ankley, G.T., Lazorchak, J., Edwards, S., 2010. A transcriptome based biological framework for studying mechanisms of endocrine disruption in small fish species. Aquat. Toxicol. 98, 230–244.
Watanabe, M., Tanaka, M., Kobayashi, D., Yoshiura, Y., Oba, Y., Nagahama, Y., 1999. Medaka (*Oryzias latipes*) FTZ-F1 potentially regulates the transcription of P-450 aromatase in ovarian follicles: cDNA cloning and functional characterization. Mol. Cell. Endocrinol. 149, 221–228.
Wilson, V.S., Cardon, M.C., Gray Jr., L.E., Hartig, P.C., 2007. Competitive binding comparison of endocrine-disrupting compounds to recombinant androgen receptor from fathead minnow, rainbow trout, and human. Environ. Toxicol. Chem. 26, 1793–1802.
Wu, T., Patel, H., Mukai, S., Melino, C., Garg, R., Ni, X., Chang, J., Peng, C., 2000. Activin, inhibin, and follistatin in zebrafish ovary: expression and role in oocyte maturation. Biol. Reprod. 62, 1585–1592.
Yagi, A., Paranko, J., 1995. Actin, α-actinin, and spectrin with specific associations with the postacrosomal and acrosomal domain of bovine spermatozoa. Anatom. Rec. 241, 77–87.