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The genomic transcriptional response of female fathead minnows (*Pimephales promelas*) to an acute exposure to the androgen, 17 β -trenbolone

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Abstract

We investigated the genomic transcriptional response of female fathead minnows (*Pimephales promelas*) to an acute (4 day) exposure to 0.1 or 1.0 $\mu\text{g/L}$ of 17 β -trenbolone (TB), the active metabolite of an anabolic androgenic steroid used as a growth promoter in cattle and a contaminant of concern in aquatic systems. Our objectives were to investigate the gene expression profile induced by TB, define biomarkers of exposure to TB, and increase our understanding of the mechanisms of adverse effects of TB on fish reproduction. In female gonad tissue, microarray analysis using a 22K oligonucleotide microarray (EcoArray Inc., Gainesville, FL) showed 99 significantly upregulated genes and 741 significantly downregulated genes in response to 1 $\mu\text{g TB/L}$. In particular, hydroxysteroid (17 β) dehydrogenase 12a (*hsd17b12a*), zona pellucida glycoprotein 2.2 (*zp2.2*), and protein inhibitor of activated STAT, 2 (*pias2*) were all downregulated in gonad. Q-PCR measurements in a larger sample set were consistent with the microarray results in the direction and magnitude of these changes in gene expression. However, several novel potential biomarkers were verified by Q-PCR in the same samples, but could not be validated in independent samples. In liver, Q-PCR measurements showed a significant decrease in *vitellogenin 1* (*vtg1*) mRNA expression. In brain, cytochrome P450, family 19, subfamily A, polypeptide 1b (*cyp19a1b*, previously known as aromatase B) transcript levels were significantly reduced following TB exposure. Our study provides a candidate gene involved in mediating the action of TB, *hsd17b12a*, and two potential biomarkers sensitive to acute TB exposure, hepatic *vtg1* and brain *cyp19a1b*.

Keywords

17 β -Trenbolone; Fathead minnow; Q-PCR; Microarray

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Introduction

Environmental contaminants that interact with the endocrine system have the potential to cause adverse effects on reproduction and development (World Health Organization (WHO), 2002). Among these endocrine-disrupting compounds are those that mimic natural hormones. In particular, environmental contaminants that activate the androgen receptor may be causing adverse effects in wildlife (Gray et al., 2002). For example, investigations of pulp and paper mill effluents from different locations in North America as well as examinations of beef feedlot discharges have associated morphological alterations in fish collected from the field with androgenic activity (Parks et al., 2001; Orlando et al., 2004; Soto et al., 2004; Durhan et al., 2006).

In the United States, approximately 90 % of beef cattle are treated with a growth-promoting mix of estrogens, androgens, and progesterones, including the synthetic androgen trenbolone acetate (17 β -hydroxy-estra-4,9,11-trien-3-one-17-acetate) (Balter, 1999). Administration of trenbolone acetate has multiple effects on the endocrine system, including androgenic, progestin, and anti-glucocorticoid activities measured in mammalian systems (Danhaive and Rousseau, 1986; Bauer et al., 2000). The active metabolite of trenbolone acetate is 17 β -trenbolone (17 β -hydroxy-estra-4,9,11-trien-3-one) (Schiffer et al., 2001), a potent androgen receptor agonist in vertebrates (Wilson et al., 2002; Ankley et al., 2003). 17 β -trenbolone is metabolized by oxidation to trendione, followed by reduction to 17 α -trenbolone (Schiffer et al., 2001), which has about 20-fold less affinity than 17 β -trenbolone for the androgen receptor (Bauer et al., 2000). However, both forms are potent reproductive toxicants in fish, due to reconversion of 17 α -trenbolone to 17 β -trenbolone by fish following uptake of 17 α -trenbolone (Ankley et al., 2003; Jensen et al., 2006).

Runoff from feedlots and fields fertilized with manure has the potential to contaminate aquatic environments with trenbolone. Both the alpha and beta isomers are eliminated from cattle dosed with trenbolone acetate, with the 17 α -trenbolone comprising about 95 % of the excreted product (Schiffer et al., 2001). Schiffer et al. (2001) also demonstrated that both metabolites are quite stable in the environment with half-lives of about 270 days in liquid manure. Durhan et al. (2006) documented the occurrence of both 17 β -trenbolone and 17 α -trenbolone in water with *in vitro* androgenic activity that had been collected in discharge from a beef feedlot where trenbolone acetate implants had been used. Measured concentrations of 17 α - and 17 β -trenbolone in samples from the site varied temporally but both were present, at least occasionally, at concentrations greater than 0.01 $\mu\text{g/L}$ (Durhan et al., 2006) concentrations of 17 α -trenbolone ranged up to 0.125 $\mu\text{g/L}$ in discharge and 0.05 $\mu\text{g/L}$ in receiving water; while concentrations of 17 β -trenbolone ranged up to 0.02 $\mu\text{g/L}$ in discharge and 0.007 $\mu\text{g/L}$ in receiving water (Durhan et al., 2006).

Recent studies have assessed the effects of 17 β -trenbolone on fecundity and reproductive endocrinology of various fish species. Peterson et al. (2001) exposed mature Japanese medaka (*Oryzias latipes*) to 17 β -trenbolone for 14 days at nominal water concentrations ranging from 0.002 to 2 $\mu\text{g/L}$. They found that concentrations ≥ 0.2 $\mu\text{g/L}$ inhibited fecundity of the fish. They also reported an inhibition of vitellogenin production in female medaka exposed to 2 $\mu\text{g/L}$ of 17 β -trenbolone. Ankley et al. (2003) conducted a 21-day reproduction test with fathead minnows (*Pimephales promelas*). They found that a concentration of about 0.027 $\mu\text{g/L}$ of the androgen caused female fathead minnows to develop male secondary sexual characteristics (dorsal nuptial tubercles) and significantly reduced fecundity, plasma vitellogenin, and sex steroid concentrations. An *in vitro* competitive binding study with fathead minnow androgen receptor (Ar) also demonstrated that 17 β -trenbolone had a higher affinity for the receptor than that of the endogenous ligand, testosterone (Ankley et al., 2003). In another study, a 28-day exposure to 17 β -trenbolone (1-10 $\mu\text{g/L}$) induced development of a gonopodium from the

female anal fin accompanied by a transient up-regulation of *ara* and *arβ* mRNA in adult female mosquitofish (*Gambusia affinis affinis*) (Sone et al., 2005). Further studies have also demonstrated the potential effects of 17β-trenbolone on various fish species (Ankley et al., 2004; Miller and Ankley, 2004; Holbech et al., 2006; Miracle et al., 2006; Örn et al., 2006; Seki et al., 2006; Hemmer et al., 2008).

Gene expression changes associated with signal pathway activation can provide chemical-specific information on the mechanistic and biological effects of a chemical (Nuwaysir et al., 1999; Bartosiewicz et al., 2001; Hamadeh et al., 2001). We chose to investigate gene expression changes associated with 17β-trenbolone exposure in the fathead minnow (*Pimephales promelas*), a member of the ecologically important Cyprinidae family, and a standard test species for aquatic toxicology since the 1960s (reviewed by Ankley and Villeneuve, 2006). We chose an acute exposure duration of 96 hours to allow for the assessment of early responses in gene expression following 17β-trenbolone exposure and for consistency with studies of acute effects of other contaminants. Gene expression was analyzed by quantitative PCR (Q-PCR) and by microarray analysis.

The aim of this study was to investigate the gene expression profile induced by 17β-trenbolone and subsequently to understand the molecular mechanism(s) by which 17β-trenbolone causes adverse effects in fish and develop biomarkers of 17β-trenbolone exposure. Since 17β-trenbolone is an androgen and effects on egg production have been previously observed, our study focused on adult female fathead minnows. Exposure levels of 0.1 μg/L and 1 μg/L were chosen based on concentrations of 17β-trenbolone that produced effects in the study by Ankley et al. (2003).

Materials and methods

Animal culture conditions and exposures

Adult fathead minnows (*Pimephales promelas*) were cultured at the Columbia Environmental Research Center (CERC), USGS and both sexes were housed separately before the experiment. Fish were held in the test system for one day prior to initiation of the chemical exposure at 20.3 ± 0.5 °C under an 14:10 light:dark photo-period. Fish were exposed to 17β-trenbolone for 4 days using a flow-through exposure system. Nominal water concentrations of 0.1 and 1 μg/L 17β-trenbolone were used. For each exposure, a stock solution was prepared in 100 % ethanol and slowly added to the exposure tanks in a proportional flow-through diluter system (6.5 L tank capacity; filled with 4 L of well water) at a flow rate of 0.5 ml/12.5 min. Control tanks had only ethanol added (solvent control). The final concentration of ethanol was 0.03 %. There were four replicate tanks for each treatment, with six fish per replicate at a ratio of three males to three females, for a total of 12 females per treatment. After 4 days of chemical exposure, fish were removed from the test tanks and anesthetized with MS-222 (tricaine methanesulfonate). The weight of each fish was recorded and liver, brain (without pituitary) and gonad tissues were collected, placed in RNALater (Ambion, Austin, TX), and stored at -20 °C until used for RNA extraction. The female fathead minnows were adults with a mean weight of 1.1 ± 0.25 g. Animals were not fed during exposure. No mortality was observed during the experiment. For Q-PCR analysis, brain RNA was sampled from 12 females per treatment, liver RNA from 6 females per treatment, and gonad RNA from 10 females per treatment. For microarray analysis, gonad RNA was sampled from 4 females each from the control and 1 μg/L treatment groups only.

Chemical analysis

Water concentrations of 17β-trenbolone were determined in samples from each exposure tank at 0-hours and 96-hours using high performance liquid chromatography with photodiode array

detection (HPLC-PDA). Water samples were extracted by liquid:liquid partitioning with dichloromethane (DCM). Water concentrations of 17 β -trenbolone were stable over the course of the experiment; the concentration means and standard deviations in the 0.1 and in the 1 $\mu\text{g/L}$ treatments were $0.114 \pm 0.025 \mu\text{g/L}$ and $1.47 \pm 0.05 \mu\text{g/L}$, respectively. The limit of detection was $0.014 \mu\text{g/L}$; no analyte was detected in control water.

Microarray analysis of gene expression

Microarray analysis was performed with a commercially available microarray containing 22,000 oligonucleotide probes developed for fathead minnow (EcoArray Inc., Gainesville, FL). Female gonad samples from control and 1 $\mu\text{g/L}$ treatment groups were used for microarray hybridization according to the One-Color Microarray-Based Gene Expression Analysis procedure from Agilent Technologies, Inc. (Santa Clara, CA). Scanned images were processed using Agilent Feature Extraction 9.5.1 software for spot identification and for quantification of the fluorescent signal intensities. The fluorescent signal intensity for each oligo spot was calculated using local background subtraction. Quality control algorithms in the software detected poor quality spots, which were excluded from subsequent analyses. The microarray dataset and MIAME-compliant information have been submitted to the NCBI GEO database, accession number GSE12904.

A statistical analysis was performed in order to identify differentially expressed genes between the control and 1 $\mu\text{g/L}$ treatment groups using SAS 9.1 (SAS Institute, Cary, NC). Data were analyzed using two-stage mixed model ANOVA following the procedure described by Wolfinger et al. (2001). This approach centers around two interconnected ANOVA models, the “normalization” model and the “gene” model. The first stage is to normalize the data by outputting residuals from an ANOVA containing the effects of array and treatment. The treatment effect is fixed and the array effect is random. A second ANOVA uses the residuals from the first ANOVA to test differences among treatments with a separate ANOVA for each gene. The treatment difference for each gene was determined using a one degree of freedom F-test ($P < 0.05$). Because one aim of this project was to discover novel genes and pathways involved in the response to 17 β -trenbolone, we did not use a multiple testing correction. Thus, some of the genes identified as altered are expected to be false positives.

In order to detect changes in groups of genes with related functions, we tested overrepresentation of gene ontology (GO) terms (Ashburner et al., 2000) with the FatiGO (fast assignment and transference of information) web tool (Al-Shahrour et al., 2005; Al-Shahrour et al., 2006). FatiGO uses Fisher’s exact test to compare the GO terms associated with significantly altered probes and the GO terms associated with unaltered probes. It returns a list of GO terms that are significantly over-represented among altered probes, relative to their abundance in the list of unaltered probes. For this analysis, multiple probes assigned to the same gene name are analyzed separately. Since most of the altered genes were downregulated, we tested for significantly enriched GO terms among upregulated and downregulated genes separately. The significance criteria for GO terms were $P < 0.05$ and number of altered genes ≥ 2 .

Quantitative PCR (Q-PCR) analysis of gene expression

The expression of selected genes was evaluated by real-time quantitative PCR (Q-PCR), a technique which allows a rapid and reliable quantification of specific mRNAs (Nolan et al., 2006). Total RNA was extracted from female fathead tissues using the AutoGenprep 245, following manufacturer’s instructions (AutoGen, Holliston, MA). Total RNA concentration and $A_{260 \text{ nm}}/A_{280 \text{ nm}}$ ratios were measured on a SpectraMax 190 plate-reading spectrophotometer (Molecular Devices, Sunnyvale, CA), and RNA concentrations were adjusted to $0.4 \mu\text{g}/7 \mu\text{l}$ in order to produce a constant RNA concentration in all reverse

transcription reactions. All RNA samples had $A_{260\text{ nm}}/A_{280\text{ nm}}$ ratios greater than 1.8. Total RNA quality was verified in two randomly selected samples from each tissue by 1 % agarose gel electrophoresis under denaturing conditions with ethidium bromide detection, to confirm the presence of two sharp rRNA bands indicating that the RNA samples were not degraded (Sambrook and Russell, 2001). Procedural blanks run in parallel with tissue samples confirmed that there was no detectable cross-contamination between samples. The quality of mRNA was not independently assessed (Nolan et al., 2006).

Reverse transcription (RT) reactions for Q-PCR assays were carried out in a volume of 20 μl with 0.04 μg of total RNA, primed with random hexamers using the TaqMan® Reverse Transcription Reagents kit following the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA), with the addition of denaturation of the RNA at 75 °C for 10 min prior to the RT reaction. Real-time quantitative PCR was performed with an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using SYBR-Green PCR Master Mix (Applied Biosystems). One-tenth of the cDNA was used for each Q-PCR reaction in a total volume of 25 μl . Duplicates were run for each sample. The expression levels of target genes were normalized using the expression of a housekeeping gene, *ribosomal protein L8 (rpl8)* (Filby and Tyler, 2005). The mRNA abundance of *rpl8* relative to total RNA did not exhibit any significant differences between experimental groups (data not shown). Normalization to *rpl8* reduced variation among samples and increased the correlation coefficient for Q-PCR data compared to microarray data in the same samples (data not shown).

Target genes were selected based on array results and by screening the literature. Fathead minnow target gene sequences were found in GenBank (NCBI) by search of annotations or by BLAST (Basic Local Alignment Search Tool) (Altschul et al., 1990) search against an annotated homolog in a related species. Primer sets for Q-PCR were designed using Primer Express 2.0 software according to manufacturer's guidelines and synthesized by Integrated DNA Technologies, Inc. (Coralville, IA) (Table 1). Q-PCR assays were optimized and validated to confirm the specific amplification of the target sequence (Nolan et al., 2006). Primer concentrations were optimized by assaying the same sample with primer concentrations from 100 nM to 800 nM, with four replicates per primer concentration. The optimum primer concentration gives the lowest threshold cycle (C_T), lowest variation among replicates, and highest change in fluorescence (ΔR_n). No-template controls confirmed the absence of interfering non-specific amplicons such as primer-dimers. Primer sets that resulted in non-specific amplification were redesigned. The absence of non-specific amplification was also confirmed by agarose gel electrophoresis of an amplification from each primer set to confirm the presence of a single amplicon of the expected size, and by examination of dissociation curve profiles for each sample to confirm a single peak at the expected melting temperature.

The linearity of the Q-PCR assays and the absence of inhibitors in the RNA samples were confirmed using dilution series. Four two-fold dilutions of a randomly selected total RNA sample were prepared using bacterial total RNA to maintain a constant RNA concentration and assayed in parallel with the rest of the RNA samples and controls. This procedure reveals any deviation from linearity in either the reverse transcription or the real-time PCR reactions. The dilution series also reveals the presence of inhibitors in the RNA sample, because inhibitory components will result in anomalously low C_T values and increasing PCR efficiency as the dilution increases (Nolan et al., 2006). Because the endpoints of interest are changes in expression relative to the control group, standard curves with artificial templates to define the copy number of expressed genes were not performed.

Statistical differences in relative mRNA expression between experimental groups were assessed by one-way analysis of variance (ANOVA) followed by Fisher's test. Some data were log-transformed in order to satisfy the assumptions of normality and homogeneity. All

statistical analyses were performed using SAS 9.1 (SAS Institute, Cary, NC). All data are shown as the mean \pm SEM. Differences were considered statistically significant at $P < 0.05$.

Results

Results obtained from the microarray analysis reflect transcriptional changes in gonad tissues of female fathead minnows exposed for 4 days to 1 $\mu\text{g/L}$ of 17β -trenbolone. There were 14,714 genes out of 21,495 probes on the microarray which were expressed in gonad tissue at levels greater than the limit of detection of the microarray. 17β -trenbolone exposure caused 840 genes in female gonad tissue to be significantly altered ($P < 0.05$) in expression relative to control fish. Among those, 99 genes (0.67 % of expressed genes) were up-regulated following 17β -trenbolone exposure while 741 (5.04 % of expressed genes) exhibited a down-regulation relative to control fish. Gene ontology (GO) terms for biological process were examined to determine the function of genes with altered patterns of expression (Fig. 1). GO information for fathead minnow was limited, in that among altered genes, 33.1% had no biological process GO annotation and 16.2% were annotated as biological process unknown (GO:0000004). This is in part a consequence of the fathead minnow genome being less characterized in comparison to other fishes such as the zebrafish (*Danio rerio*) and Japanese medaka (*Oryzias latipes*), although a fully sequenced genome is not necessarily a prerequisite for understanding biological function. Genes annotated as biological process unknown (GO:0000004) were significantly over-represented among down-regulated genes; 6.3 % of the expressed genes annotated as biological process unknown were downregulated (Fig. 1). Other GO terms significantly enriched among down-regulated genes included terms associated with protein synthesis, lipid metabolism, and synthesis of DNA and RNA (Fig. 1).

Each gene may be associated with many biological process GO terms. Several of the GO terms significantly over-represented among down-regulated genes are associated with overlapping sets of genes (Fig. 1). For example, lipid metabolism (GO:0006629) overlaps with sphingolipid metabolism (GO:0006665), fatty acid beta-oxidation (GO:0006635) and prostaglandin metabolism (GO:0006693). DNA replication (GO:0006260) has genes in common with DNA repair (GO:0006281), chromatin remodeling (GO:0006338), DNA replication checkpoint (GO:0000076), traversing start control point of mitotic cell cycle (GO:0007089) and transcription (GO:0006350). Sensory perception of sound (GO:0007605) overlaps with transcription (GO:0006350) and DNA repair (GO:0006281).

A subset of the differentially expressed genes identified by microarray analysis was selected for validation by Q-PCR: *hydroxysteroid (17 β) dehydrogenase 12a (hsd17b12a)*, *zona pellucida glycoprotein 2.2 (zp2.2)*, and *protein inhibitor of activated STAT, 2 (pias2)* (Fig. 2). The fold change, the variation within each treatment, the level of statistical significance, the overall expression level, and the gene's function in reproduction were all factors considered in selection of genes for Q-PCR. Three additional candidate genes which were not significantly altered based on microarray analysis, cytochrome P450, family 19, subfamily A, polypeptide 1a (*cyp19a1a*, previously known as aromatase A); *androgen receptor (ar)*; and *estrogen receptor 1 (esr1)*, previously known as estrogen receptor alpha) were selected by screening the literature and their expression levels were also evaluated by Q-PCR in female gonad tissue. Six of the selected genes fall within three groups of interest: receptors and transcription factors (*ar*, *esr1*, and *pias2*); steroid metabolism (*cyp19a1a* and *hsd17b12a*); and egg maturation (*zp2.2*). Four additional genes were also selected for further analysis based on large fold changes and highly significant p-values in the microarray data, without regard to gene annotation: similar to zebrafish *hypothetical protein LOC565514 (loc565514)*, *clusterin (clu)*, *prostaglandin D2 synthase (ptgds)*, and similar to zebrafish *zgc:55572*, which has homology to human serine/threonine kinase 38 (Fig. 2).

For genes involved in reproduction, agreement between Q-PCR data and microarray data was good as to the direction of gene change and the magnitude of difference between control and exposed groups (Table 2, Fig. 2). The expression of *pias2*, *hsd17b12a*, and *zp2.2* genes determined by Q-PCR was decreased following 17 β -trenbolone exposure, in accordance with microarray data. However, in the case of significant expression changes based on microarray data, only the *hsd17b12a* gene was close to significantly altered (Table 2). Significant changes in gene expression between treatment and control groups may not have been observed due to the small number of replicates, a small magnitude in the changes of expression, and to a large degree of variation among individual fish. Additionally, variation is not unexpected in female gonad tissue, due to the heterogeneous nature of ovarian tissues and differential reproductive staging that may have occurred among females. The difference in significance by these two techniques may also reflect the greater power of normalization to residuals for array data. Lastly, in gonads, *ar* gene expression was reduced in the treated groups compared to controls, while *esr1* and *cyp19a1a* transcript levels tended to increase and also showed increased variation in treated groups (Fig. 2). However, the changes in *ar* and *cyp19a1a* were not statistically significant ($P > 0.05$).

The genes selected based on large fold changes in the microarray data were not closely matched by Q-PCR data (Table 2, Fig. 2). In particular, the magnitudes of the changes in gene expression were much less in the Q-PCR data set than in the microarray data set (Table 2, Fig. 2). This unexpected result was not caused by technical differences in Q-PCR and microarray measurements; for individual samples, the Q-PCR and microarray measurements were in close agreement, with the exception of *zgc:55572* (Fig. 3). The differences between the results based on Q-PCR and microarray are likely to be the result of sample size. The microarray analysis used samples from 4 individual fish per treatment, while the Q-PCR analysis used the same samples run on microarray and 6 additional fish exposed in the same experiment, for a total of 10 samples per treatment (Fig. 3). The deviation between Q-PCR and microarray results for *zgc:55572* could be due to positional effects, because the microarray probe and Q-PCR primer set targeted different regions of the mRNA; or could be due to differential reverse transcription efficiency, because the microarray experiment used oligo-dT primers and the Q-PCR experiment used random hexamers.

One gene (*loc565514*) measured by both Q-PCR and microarray exhibited positional effects on its measured expression levels (Fig. 4). A Q-PCR primer set that overlapped the position of the microarray probe in the mRNA sequence closely matched the microarray data, but two primer sets targeted to other locations in the mRNA sequence deviated from the expression pattern shown by microarray (Fig. 4). Positional effects have previously been observed for microarray probes of the fathead minnow *vtg1* gene (Larkin et al., 2007).

We investigated gene expression changes in brain and liver by Q-PCR. In liver, *vtg1* mRNA was strongly down-regulated in a dose-dependent manner (Fig. 5A). In brain, *cyp19a1b* mRNA was also down-regulated in a dose-dependent manner, but *ar* mRNA was unchanged with treatment ($P > 0.05$) (Fig. 5B). Exposure to 1 μ g 17 β -trenbolone/L caused a 6-fold decrease ($P < 0.001$) in mean *cyp19a1b* mRNA expression.

Discussion

The results of the present study are consistent with the previously observed declines in both estradiol and testosterone levels in female fish in response to 17 β -trenbolone exposure (Ankley et al., 2003; Ankley et al., 2004). In the microarray analysis of gonadal gene expression, the preponderance of down-regulated genes relative to up-regulated genes suggests that the many estrogen-dependent functions of ovarian tissue are declining in response to the drop in endogenous estradiol levels caused by exposure to 17 β -trenbolone. The GO terms associated

with the greatest numbers of down-regulated genes, protein biosynthesis and ubiquitination, lipid metabolism, transcription, and DNA repair and replication, are all basic functions required for cell growth and maintenance. Thus, the effects of 17 β -trenbolone exposure on reproduction may be due to an overall loss of ovarian function. The results for several specific down-regulated genes are consistent with this hypothesis.

Protein inhibitor of activated STAT, 2 (pias2) encodes a transcriptional co-regulatory protein that recognizes the zinc finger region (ZFR) of Ar and modulates Ar-dependent transcription (Moilanen et al., 1999). While the expression of *androgen receptor* in brain was unaffected by the 17 β -trenbolone treatment in the present study, it tended to decrease in female gonads, although the difference was not statistically significant. A similar decrease was observed for gonadal *pias2* expression. In contrast, although gonadal *esr1* expression was not detected in either control or treated samples on the microarray, Q-PCR analysis was able to detect significantly increased gonadal *esr1* expression in response to 17 β -trenbolone exposure. The observations of decreased *ar* and *pias2* and increased *esr1* are suggestive of negative feedback controls on expression of these transcription factors (Shupnik et al., 1989; Nastiuk and Clayton, 1994). Results of the GO term analysis of microarray results also showed down-regulation of several categories associated with transcription.

Cyp19a1a and *cyp19a1b* encode members of the cytochrome P450 superfamily that catalyze the critical reaction converting C19 androgens to C18 estrogens in gonad and brain tissues, respectively (Callard et al., 1978; Callard et al., 2001; Kishida and Callard, 2001). In brain tissue, exposure to 17 β -trenbolone resulted in a strong and significant decrease in *cyp19a1b* expression. The results for gonad indicated a small increase in average *cyp19a1a* transcript level and a large increase in variation among individual fish. Thus, loss of aromatase activity is unlikely to be the cause of the previously observed drop in plasma estrogen levels in response to 17 β -trenbolone exposure (Ankley et al., 2003). However, loss of *cyp19a1b* expression in the brain may have behavioral consequences (Forlano et al., 2006).

The contrast between the brain and ovary results is consistent with reports in the literature indicating that aromatase isoforms are regulated differently (Kazeto et al., 2001; Kishida and Callard, 2001; Villeneuve et al., 2006). The promoter region of *cyp19a1a* contains a SF-1 (steroidogenic factor-1) regulatory element but no estrogen response elements (EREs), while the *cyp19a1b* promoter contains EREs but no SF-1 regulatory element (Callard et al., 2001; Kazeto et al., 2001). Based on the difference in promoters, one could hypothesize that expression of *cyp19a1a* transcript levels would be primarily influenced by gonadotropin-mediated regulation of steroidogenesis facilitated through SF-1, while *cyp19a1b* expression would be more closely tied to endogenous 17 β -estradiol concentrations. Thus, the strong down-regulation of *cyp19a1b* observed in female brain tissue may be reflective of the decrease in plasma concentrations of 17 β -estradiol reported by Ankley et al. (2003) in fathead minnows exposed to 17 β -trenbolone.

Activation of transcription of vitellogenin isoforms in the liver is also controlled by EREs (Brock and Shapiro, 1983). The results for liver indicated a strong and significant decrease in *vitellogenin 1 (vtg1)* mRNA expression, with exposure to 1 μ g 17 β -trenbolone/L causing a 64-fold decrease ($P < 0.01$) in expression relative to controls. This observation is in total agreement with existing data on the down-regulation of hepatic vitellogenin gene expression and plasma vitellogenin levels in female fish exposed to steroidal androgens (Ankley et al., 2004; Miracle et al., 2006). Similar to vitellogenins, egg envelope precursor proteins, also known as zona pellucida or choriogenin proteins, are produced in response to circulating estrogen. In teleosts, depending on the species zona pellucida proteins are expressed in either liver (e.g., winter flounder, seabream, and cod) or gonad (e.g., zebrafish, goldfish, and carp), or in both (medaka) (Conner and Hughes, 2003; Litscher and Wassarman, 2007). In fathead minnow, zona pellucida

proteins appear to be mostly expressed in gonad tissues (NCBI Unigene Expression Profile, Ppr.12560). In the present study, down-regulation of *zp2.2* in gonads of female fathead minnow after exposure to 17 β -trenbolone was observed, as measured by microarray. The decreases in *vtg1* and *zp2.2* observed in liver and gonad, respectively, are likely to reflect decreased concentrations of plasma 17 β -estradiol and testosterone, as previously observed (Ankley et al., 2003; Miracle et al., 2006). Decreases in *vtg1* expression have direct implications for population-level effects on exposed fish, because fish require vitellogenin production in order to reproduce (Miller et al., 2007).

The mechanism leading to decreased concentrations of the steroid hormones 17 β -estradiol and testosterone in response to 17 β -trenbolone exposure is apparently not androgen-receptor dependent, because co-treatment with 17 β -trenbolone and androgen receptor antagonists flutamide or vinclozolin does not restore expression of *vitellogenin 1* mRNA . (Ankley et al., 2004; Martinović et al., 2008). In Atlantic croaker ovary tissue culture, non-aromatizable androgens had direct, non-genomic, antiandrogen-insensitive inhibitory effects on estrogen production (Braun and Thomas, 2003). Thus, the effects of 17 β -trenbolone exposure on plasma levels of the steroid hormones 17 β -estradiol and testosterone are likely mediated by mechanisms other than the classical genomic androgen receptor (Heinlein and Chang, 2002).

One potential candidate gene involved in the decrease in sex steroid concentrations of fish exposed to the strong exogenous androgen 17-trenbolone is *hydroxysteroid (17 β) dehydrogenase 12a (hsd17b12a)*. In our study, *hsd17b12a* was significantly down-regulated in gonads as measured by microarray. Q-PCR measurement validated the direction and magnitude of the change in expression shown by microarray, and was very close to statistical significance. Hydroxysteroid (17 β) dehydrogenase 12a catalyzes the conversion of androstenedione to testosterone which, in turn, is converted to 17 β -estradiol by the aromatase enzymes Cyp19a1a and Cyp19a1b. Thus, down-regulation of *hsd17b12a* is expected to lead to declines in both testosterone and estradiol, as observed by Ankley et al. (2003). In summary, it is plausible that down-regulation of *hsd17b12a* caused decreases in testosterone, leading to depressed estrogen levels which, in turn, decreased *vtg1* expression through decreased activation of the estrogen receptor, and caused reproductive impairment.

The Q-PCR data and array data were in close agreement for individual samples, and were in general agreement for genes involved in reproduction, as to the direction of gene change and the magnitude of difference between control and exposed groups. The link between genes with altered expression and mode of toxic action, and the consistency between array and Q-PCR results supports the use of microarrays for investigations of biomarkers and mode of toxic action of environmental contaminants. However, the differences between the subsample selected for microarray analysis and the larger sample set measured by Q-PCR for genes selected based on large fold changes on the microarray emphasize the need for cross-validation of microarray results in independent samples, particularly when working with small sample sizes.

Conclusions

The gene expression profile for 17 β -trenbolone defined here suggests a general down-regulation of the expression of estrogen-responsive genes (hepatic *vtg1*, brain *cyp19a1b*, and gonad *zp2.2* as suggested by the interference of 17 β -trenbolone with the biosynthesis of endogenous estrogens in fish. Our study also provides a potential candidate gene mediating the action of 17 β -trenbolone, the *hydroxysteroid (17 β) dehydrogenase 12a*, involved in the production of testosterone, the precursor of 17 β -estradiol. Finally, the expression of hepatic *vtg1* and brain *cyp19a1b* appear to be highly responsive to 4-days exposure to 17 β -trenbolone,

and are potential biomarkers of exposure to 17 β -trenbolone or other contaminants that act to lower endogenous estrogen levels.

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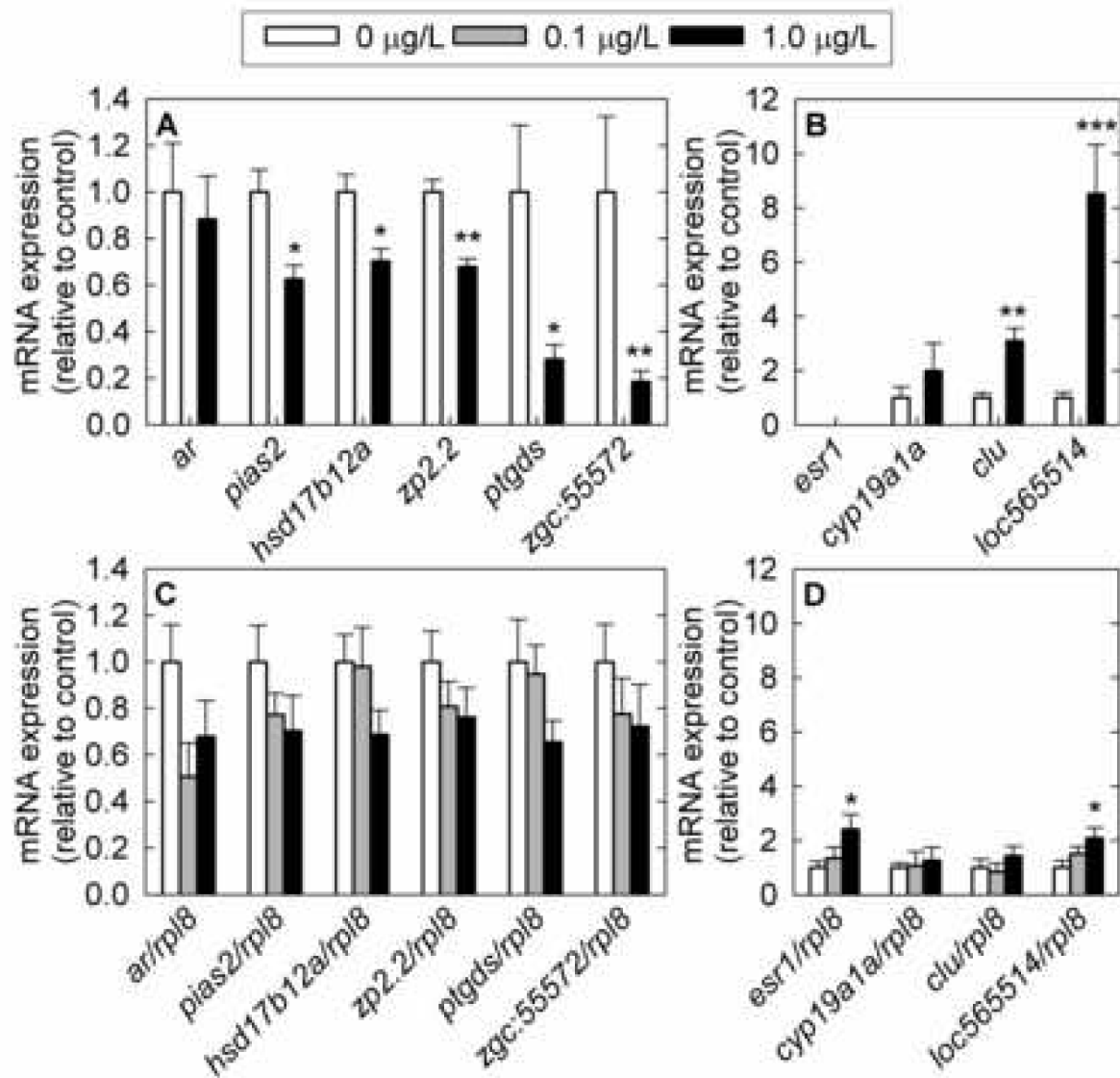


Fig. 2.

Cross-validation of gene expression changes in selected genes measured by microarray (A, B) and validated in a larger set of samples by Q-PCR (C, D) in female fathead minnow gonads following exposure to 17 β -trenbolone for 4 days. A) Microarray data for downregulated genes. B) Microarray data for upregulated genes. C) Q-PCR data for downregulated genes. D) Q-PCR data for upregulated genes. *ar*: androgen receptor; *pias2*: protein inhibitor of activated STAT, 2; *hsd17b12a*: hydroxysteroid (17 β) dehydrogenase 12a; *zp2.2*: zona pellucida glycoprotein 2.2; *ptgds*: prostaglandin D2 synthase; *zgc:55572*: similar to zebrafish *zgc:55572* (homologous to human serine/threonine kinase 38); *esr1*: estrogen receptor 1; *cyp19a1a*: cytochrome P450, family 19, subfamily A, polypeptide 1a; *clu*: clusterin; *loc565514*: similar to zebrafish hypothetical protein LOC565514; *rpl8*: ribosomal protein L8. The Q-PCR data are normalized to *rpl8* expression and the array data by residuals. For microarray analysis, n=4 fish; for Q-PCR analysis, n=10 fish (4 fish measured by microarray and 6 additional fish exposed in the same experiment) at doses 0 μ g/L and 1 μ g/L; n=6 fish at dose 0.1 μ g/L. Error bars denote the standard error of the mean. Statistically significant differences in the expression levels of

fathead minnow genes between control and treated fish are denoted by stars (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

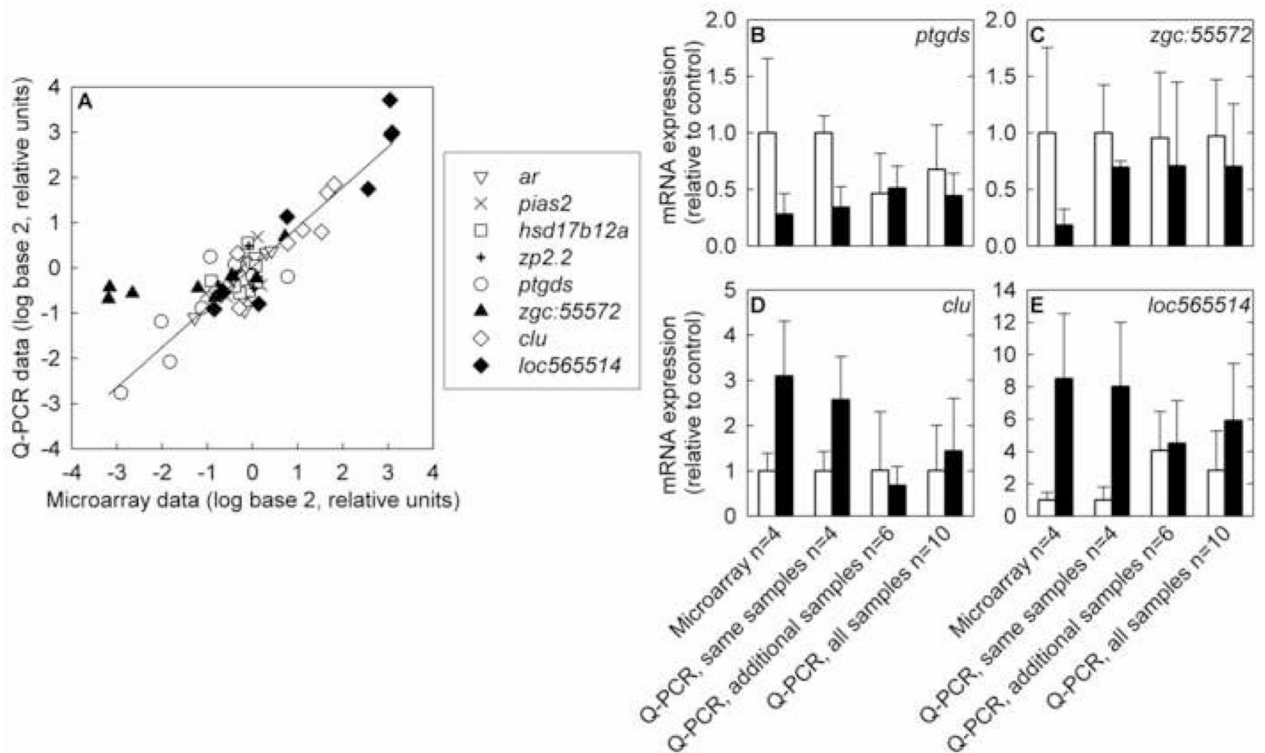


Fig. 3.

Comparison of gene expression measurements by microarray and Q-PCR. A) Correlation of microarray results with Q-PCR results from the same RNA samples. Data for each gene is expressed relative to the average of the untreated samples for that gene. *ar*: androgen receptor; *pias2*: protein inhibitor of activated STAT, 2; *hsd17b12a*: hydroxysteroid (17 β) dehydrogenase 12a; *zp2.2*: zona pellucida glycoprotein 2.2; *ptgds*: prostaglandin D2 synthase; *zgc:55572*: similar to zebrafish *zgc:55572*; *clu*: clusterin; *loc565514*: similar to zebrafish hypothetical protein LOC565514. The regression line shown is for the entire data set, excluding *zgc:55572* ($R^2 = 0.84$, slope = 0.88, intercept = 0.016). If *zgc:55572* is included, the regression parameters are $R^2 = 0.71$, slope = 0.70, intercept = 0.094. B-E) Comparisons of microarray results with Q-PCR results from the same samples, samples of additional fish from the same exposure tanks, and all samples. White bars represent the control group and black bars represent the 1 $\mu\text{g/L}$ 17 β -trenbolone treatment group. Error bars denote one standard deviation. B) prostaglandin D2 synthase, C) similar to zebrafish *zgc:55572*, D) clusterin, E) similar to zebrafish hypothetical protein LOC565514.

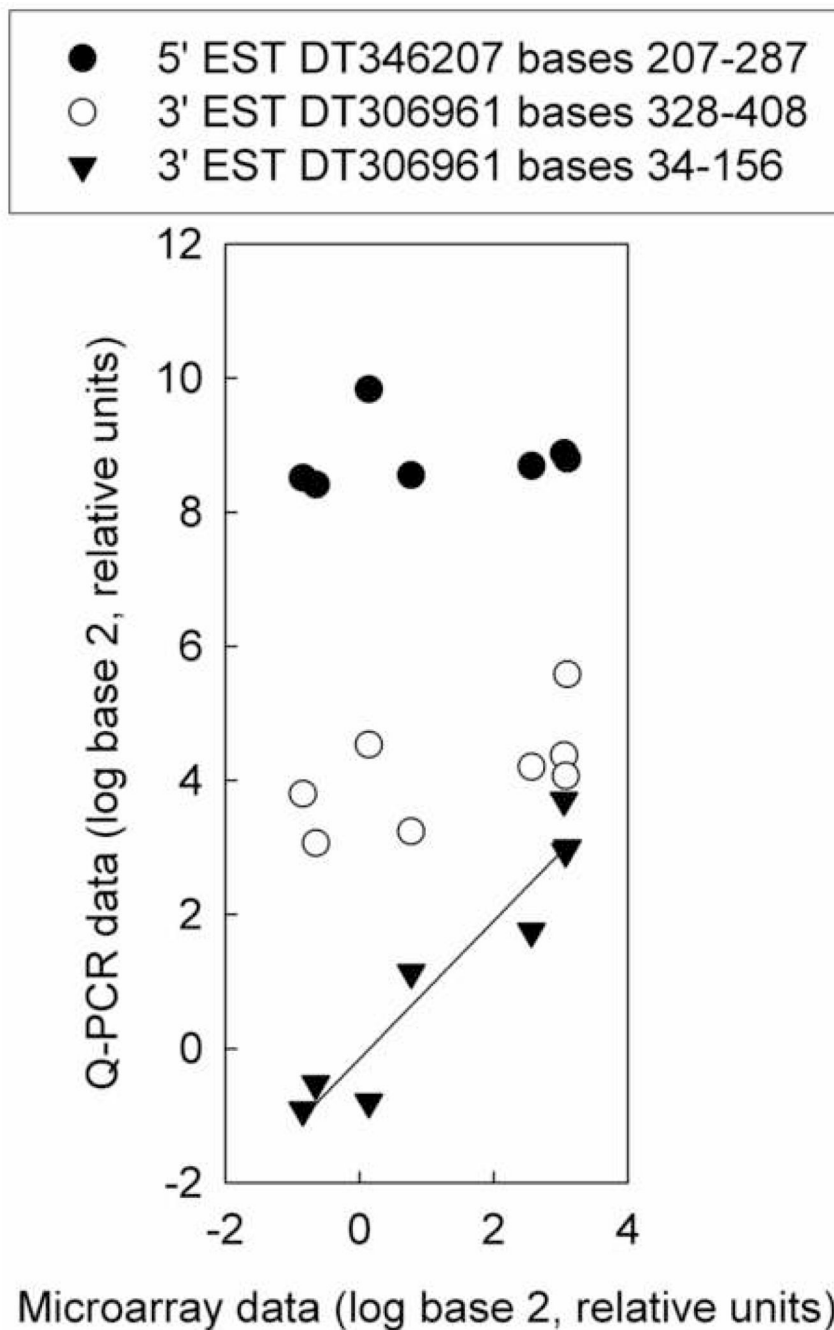


Fig. 4. Position effects shown by three Q-PCR primer sets for *hypothetical protein LOC565514* (*loc565514*). The microarray probe targeted bases 101-160 of 3' EST DT306961. Q-PCR data was normalized to the abundance of *ribosomal protein L8* (*rpl8*). Microarray data are expressed relative to the average of the untreated samples. Q-PCR data for all three primer sets are expressed relative to the average of the 3' EST DT306961 bases 34-156 primer set for the untreated samples. Regression analysis did not show significant relationships for primer sets 5' EST DT346207 bases 207-287 or 3' EST DT306961 bases 328-408. The regression parameters for primer set 3' EST DT306961 bases 34-156 are $R^2 = 0.96$, slope = 1.0, intercept = -0.15.

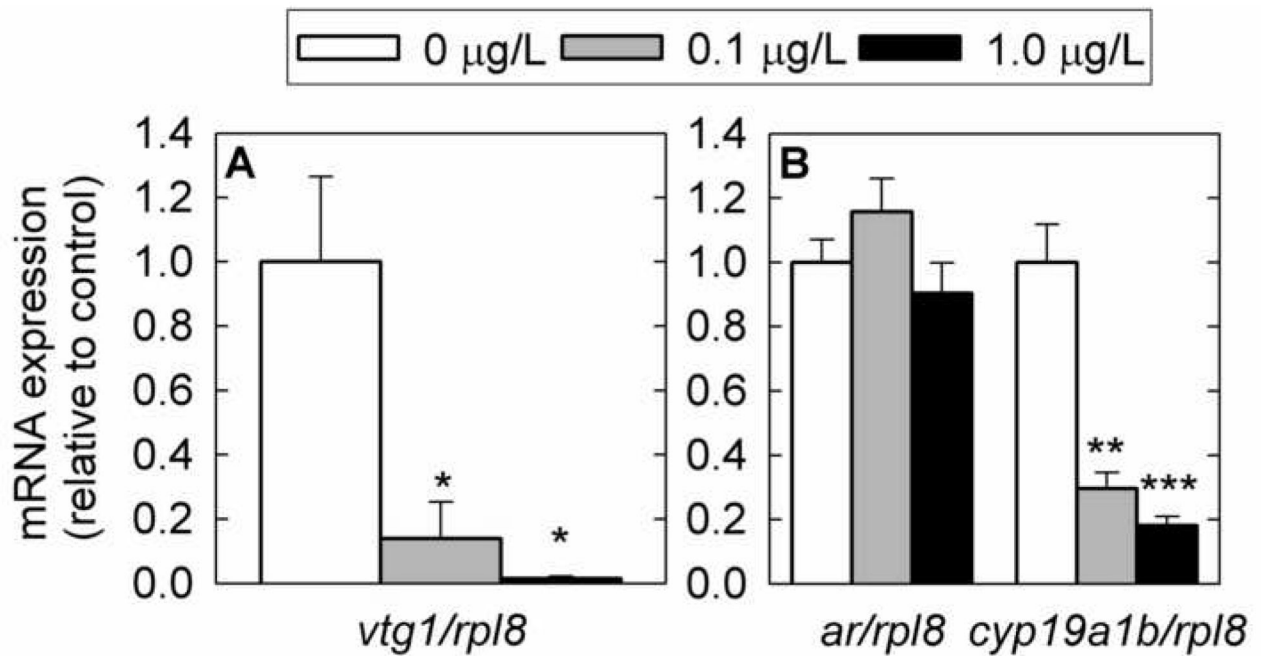


Fig. 5. Gene expression changes in female fathead minnow liver (A) and brain (B) measured by Q-PCR following exposure to 17 β -trenbolone for 4 days. A) *vitellogenin 1* (*vtg1*) in liver tissue. B) cytochrome P450, family 19, subfamily A, polypeptide 1b (*cyp19a1b*) and *androgen receptor* (*ar*) in brain tissue. mRNA expression was normalized to the abundance of *ribosomal protein L8* (*rpl8*). Experimental groups consisted of six fish (liver) or twelve fish (brain) and each fish was analyzed in duplicate. Error bars denote the standard error of the mean. Statistically significant differences in expression levels between control and treated fish are denoted by stars (* P < 0.05; ** P < 0.01; *** P < 0.001).

Table 1

Primer sequences for quantitative PCR. When possible, primer sets were designed over exon boundaries to prevent amplification of genomic DNA. Under Primer sequences, the primers used for quantitation are labeled F=forward and R=reverse. Position refers to numbering within coding sequence (CDS) or expressed sequence tag (EST). Exon boundaries are predicted based on homology with zebrafish (*Danio rerio*).

Gene name (Symbol, accession number)	Primer sequences	Position	Exon boundary
<i>vitellogenin 1</i> (<i>vtg1</i> , AF130354)	F 5'- GCTGCAGAGGCCATTTCTAAGA -3' R 5'- AGCATTGCCAGAACTTTCAG -3'	1393- 1461	none
<i>cytochrome P450, family 19, subfamily A, polypeptide 1b</i> (<i>cyp19a1b</i> , AJ277866)	F 5'- GGACGTTTCCAATAGACTCTTCCTAA -3' R 5'- ATAGCGATGGATCTTTATCAGCAA -3'	585-657	625
<i>cytochrome P450, family 19, subfamily A, polypeptide 1a</i> (<i>cyp19a1a</i> , AF288755)	F 5'- GGAGAGCTGAGCGCTGAGA -3' R 5'- GGAGCCGCGATCAACATCT -3'	181-239	none
<i>estrogen receptor 1</i> (<i>esr1</i> , AY727528)	F 5'- AACTCATCTTTGCTCAGGATCTCA -3' R 5'- AGCCATCCCCTCGACACAT -3'	1109- 1173	1142
<i>androgen receptor</i> (<i>ar</i> , AY727529)	F 5'- GTGCCATGCGCTTCCAA -3' R 5'- CTGACCTTTGTGGGCAAGGA -3'	2568- 2718	2647
<i>protein inhibitor of activated STAT, 2</i> (<i>pias2</i> , DT238876.1)	F 5'- CAGACCTGACAGTGAAATTCG -3' R 5'- GCTCGACAGGGTACCGTAAGG -3'	94-184	149
<i>hydroxysteroid (17β)dehydrogenase 12a</i> (<i>hsd17b12a</i> , DT161033.1)	F 5'- AATTCTTCTCCACATCCCTGAT dehydrogenase -3' R 5'- TCATTGGCACACTGAGGTGAT -3'	260-340	291
<i>zona pellucida glycoprotein 2.2</i> (<i>zp2.2</i> , DT088130.1)	F 5'- ACGAGGAGGTCATTCTGTTTTCA -3' R 5'- TGGGATCCACACCTTTCTTTG -3'	636-746	682
<i>hypothetical protein LOC565514</i> (<i>loc565514</i> , DT346207 (5' EST))	F 5'- GCTGTGTTTGTGGAGCCGTTA -3' R 5'- GTCTGGGACTCTGGTCATCATC -3'	207-287	none
<i>hypothetical protein LOC565514</i> (<i>loc565514</i> , DT306961 (3' EST))	F 5'- ACACAATCTCATTCAGTGAAATCCTT -3' R 5'- TGAGTGCTTGGGTATGAGAAAAC -3'	328-408	none
<i>hypothetical protein LOC565514</i> (<i>loc565514</i> , DT306961 (3' EST))	F 5'- GCAGAGCATCTGCATACATCTTAATT -3' R 5'- TGTGAGCCCCTTGAGAGAGAGTA -3'	34-156	none
<i>clusterin</i> (<i>clu</i> , UniGene Ppr.6588)	F 5'- ACGAGGATGTTGTTATATCGAAACC -3' R 5'- AATTGCGGCGGATTCC -3'	957- 1025	990
<i>prostaglandin D2 synthase</i> (<i>ptgds</i> , UniGene Ppr.12268)	F 5'- TTGGACACCGGCATCCTT -3' R 5'- TTTAAGAGACCCCTCAGGCATCTG -3'	540-620	589
<i>zgc:55572</i> (<i>zgc:55572</i> , UniGene Ppr.6860)	F 5'- TGCGCAAGGCTGATATGCT -3' R 5'- GACTGTCTGCCTCCACAAGGA -3'	385-465	417
<i>ribosomal protein L8</i> (<i>rpl8</i> , AY919670)	F 5'- CATACCACAAGTACAAGGCCAAGA -3' R 5'- ACCGAAGGGATGCTCAACAG -3'	516-598	577

Table 2
Comparison between array data and Q-PCR data.

Gene	Array (n=4)		Q-PCR (n=10)	
	Fold change	p-Value	Fold change	p-Value
Genes selected based on function in reproduction				
<i>androgen receptor (ar)</i>	-1.1	0.6599	-1.5	0.2240
<i>protein inhibitor of activated STAT, 2 (pias2)</i>	-1.6	0.0107	-1.4	0.2071
<i>estrogen receptor 1 (esr1)</i>	ND	--	2.4	0.0157
<i>hydroxysteroid (17β) dehydrogenase 12a (hsd17b12a)</i>	-1.4	0.0137	-1.5	0.0510
<i>cytochrome P450, family 19, subfamily A, polypeptide 1a (cyp19a1a)</i>	2.0	0.2848	1.3	0.6896
<i>zona pellucida glycoprotein 2.2 (zp2.2)</i>	-1.5	0.0017	-1.3	0.2230
Genes selected based on large fold changes in microarray data				
<i>similar to zebrafish zgc:55572 (zgc:55572)</i>	-5.4	0.0054	-1.4	0.1887
<i>prostaglandin D2 synthase (ptgds)</i>	-3.6	0.0119	-1.5	0.2561
<i>clusterin (clu)</i>	3.1	0.0028	1.4	0.2859
<i>similar to zebrafish hypothetical protein LOC565514 (loc565514)</i>	8.5	0.0002	2.1	0.0134

ND = not detected.