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APPLICATION OF TOXICOGENOMIC APPROACHES TO STUDY CHEMICAL-INDUCED EFFECTS ON THE HYPOTHALAMIC- PITUITARY- GONADAL (HPG) AXIS OF THE JAPANESE MEDAKA (*ORYZIAS. LATIPES*)

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By

Xiaowei Zhang

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ABSTRACT

APPLICATION OF TOXICOGENOMIC APPROACHES TO STUDY CHEMICAL-INDUCED EFFECTS ON THE HYPOTHALAMIC- PITUITARY- GONADAL (HPG) AXIS OF THE JAPANESE MEDAKA (*ORYZIAS. LATIPES*)

By

Xiaowei Zhang

System models utilizing genomic approaches can be powerful tools for mechanistic toxicological research. This dissertation describes the development and validation of a real time polymerase chain reaction (RT-PCR) array for studying chemical-induced effects on gene expression of selected endocrine pathways along the hypothalamic-pituitary-gonadal (HPG) axis of the small, oviparous fish, the Japanese medaka (*Oryzias latipes*). The Japanese medaka HPG PCR array combines the quantitative performance of SYBR® Green-based real-time PCR with the multiple gene profiling capabilities of a microarray to examine expression profiles of 36 genes associated with endocrine pathways in brain, liver and gonad. The performance of the Japanese medaka HPG PCR array was evaluated by examining effects of five model compounds, the synthetic estrogen, 17α-ethinylestradiol (EE2), the anabolic androgen, 17β-trenbolone (TRB), the aromatase inhibitor, fadrozole (FAD), the imidozole-type fungicides, prochloraz (PCZ) and ketoconazole (KTC) on the HPG axis of the Japanese medaka. A pathway-based approach was implemented to analyze and visualize concentration-dependent mRNA expression in the HPG axis of Japanese medaka. Four-month-old medaka were exposed to different concentration of chemicals for 7 d in a static renewal exposure system and the exposure concentrations were EE2 (5, 50, 500 ng/L), or TRB (50, 500, 5000 ng/L) or PCZ (3, 30, 300 μg/L) or KTC (3, 30, 300 μg/L) or 50μg

FAD/L. TRB, PCZ, KTC or FAD caused time- dependent reductions in fecundity by Japanese medaka, but EE2 did not. The compensatory response to EE2 exposure included the down-regulation of male brain *GnRH R I* and testicular *CYP17*. Despite their different biochemical properties, TRB or FAD caused similar responses in Japanese medaka, such as lesser fecundity and down-regulation of *VTG* and *CHG* genes in the liver of females. Compensatory responses to TRB in the female HPG axis included up-regulation of brain *GnRH R II* and ovary *CYP19A*. Exposure to FAD for 8 h resulted in an 8-fold and 71-fold down-regulation of expression of estrogen receptor alpha (*ER-α*) and *CHG L*, respectively in female liver. TRB caused similar down-regulation but the effects were not observed until 32 h of exposure. These results support the hypothesis that FAD reduces plasma E2 more quickly by inhibiting aromatase enzyme activity than does TRB, which inhibits production of the E2 precursor testosterone. Exposure to KTC or PCZ significantly down-regulated expression of *ER-α* and egg precursors in livers of males and females. However, PCZ was more potent than KTC both in modulating transcription and in causing lesser fecundity. Correlation analysis indicated that *ER-α* plays a primary role in the transcription of *VTG* and *CHG* genes in livers. The mRNA level of the five egg precursors and *ER-α* in livers of females was log-log related to the ecologically relevant endpoint, fecundity. Overall, the organ- gender- and concentration–specific gene expression profiles derived by the Japanese medaka HPG axis RT-PCR array provides a powerful tool to not only delineate chemical-induced modes of action, but also to quantitatively evaluate chemical induced adverse effects on reproduction.

To My Parents:

Lanxin Zhang (张兰欣) and Xiaohuan Chen (陈晓焕)

To My Wife:

Jiali Miao (苗佳丽)

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My research for this dissertation was made more efficient through the use of several genomic research facilities. Thus I gladly express my gratitude to Dr. Annette Thelen and Dr. Jeff Landgraf at the Research Technology Support Facility (RTSF) in Michigan State University, especially for the training and kind assistance.

My family deserves a great deal of credit for my development and achievement. I thank my parents for all their love and support. Finally, I offer my most genuine thanks to my wife Jiali Miao, whose unwavering compassion, faith and love carries me through life.

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KEY TO ABBREVIATIONS

ANOVA: Analysis of variance

AR: androgen receptors

BSI: brain-somatic index

CHG: choriogenin

CHG H: choriogenin H

CHG HM: choriogenin Hminor

CHG L: choriogenin L

Ct: cycle threshold

CYP: cytochrome P450

CYP17: cytochrome P450 c17α hydroxylase, 17,20-lyase

DMSO: dimethyl sulfoxide

E2: 17β-estradiol

EDCs: Endocrine disrupting chemicals

EE2: 17α -ethinylestradiol

ELISA: enzyme-linked immunosorbent assay

ER: estrogen receptors

FAD: fadrozole

FSH: follicle-stimulating hormone

GSI: gonadal-somatic index

HDL: high-density lipid

HI: hepatic index

HIS: hepatic-somatic index

KEY TO ABBREVIATIONS (Cont'd)

HPG: hypothalamic-pituitary-gonadal

IACUC: Institutional Animal Care and Use Committee

KT: 11-ketotesterone

KTC: ketoconazole

LDL: low-density lipid

LH: lutinizing hormone

MOA: modes of action

MSU: Michigan State University

OECD: Organization of Economic Cooperation and Development

PC1: first principle component

PCR: polymerase chain reaction

PCZ: prochloraz

RACE: rapid amplification of cDNA end

RT-PCR: real-time polymerase chain reaction

T: testosterone

ThR: thyroid hormone receptors

TRB: 17β-trenbolone

VTG: vitellogenin

UNITS OF MEASURE

g:	gram
h:	hour
L:	liter
µg:	microgram
µL:	microliter
min:	minutes
mg:	milligram
ml:	milliliter
ng:	nanogram
pg:	picogram
yr:	year

Chapter I

Introduction

The issue of potential endocrine disruption by chemicals was highlighted by recent legislation mandating that chemicals and formulations be screened for their potential to modulate the endocrine system before they are manufactured or used in certain processes (Safe Drinking Water Act Amendments of 1995 - Bill Number S.1316; Food Quality Protection Act of 1996 - Bill Number P.L. 104-170). Currently knowledge of chemical-induced endocrine disruption is largely limited to the pathways mediated through several classical steroid hormone receptors, including *estrogen receptors (ER), androgen receptors (AR)* and *thyroid hormone receptors (ThR)*. Our recent studies have evaluated the chemicals induced effect on steriodogenesis which alter the rates as well as absolute and relative concentrations of hormones produced by steroidogenic cells by altering the expression of steroidogenic enzymes (Gracia *et al.* 2006; Hecker *et al.* 2006; Hilscherova *et al.* 2004; Zhang *et al.* 2005). Endocrine disrupting chemicals (EDCs) could alter normal patterns of gene expression either by direct (steroid hormone receptor mediated pathways) or compensatory effects (Villeneuve *et al.* 2007). Historically, studies on endocrine disrupting induced chemicals have generally focused on several endpoints and one tissue at one specific time in the development of an organism. However, considering the complicated endocrine system in humans and wildlife, current chemical screening tools are limited to either narrow molecular targets or restricted methods. What is needed is a sensitive, flexible monitoring tool that allows for the screening of a multiple molecular targets genes in multiple tissues simultaneously at any stage of development and allow for mechanism based toxicity prediction and assessment.

A significant degree of conservation has been shown in the basic aspects of the hypothalamic-pituitary-gonadal (HPG) axis among vertebrates (Ankley and Johnson

2005; Danger *et al.* 1990). Teleost fish, such as the Japanese medaka (*Oryzias latipes*), zebrafish (*Danio rerio*) and fathead minnow (*Pimephales promelas*), have been suggested to be appropriate models for testing EDCs in terms of both ecological impacts and species extrapolation (Ankley and Villeneuve 2006; Villeneuve *et al.* 2007). The medaka is a small, oviparous (egg-laying) freshwater fish native to Asia. Its physiology, embryology and genetics have been extensively studied for more than 100 y (Wittbrodt *et al.* 2002). The medaka represents an important test system for environmental research and is widely used for testing endocrine disrupters in ecotoxicology (Pastva *et al.* 2001; Villalobos *et al.* 2003). The medaka has several major advantages compared to the zebrafish (*Danio rerio*), another commonly used model organism that are important for the proposed study. First, to date nothing is known about the exact mechanism of sex determination in zebrafish, an aspect that is highly relevant when testing EDC effects on reproductive endocrinology. Conversely, the Japanese medaka has clearly defined sex chromosomes, and sex determination system is the same as human: XX-XY (summarized in Wittbrodt et al., 2002). Thus, it has been suggested that the Japanese medaka may provide a valuable model to study the disruption of sex differentiation caused by chemical in human. Secondly, medaka is hardier and less susceptible to disease than the zebrafish (Wittbrodt et al., 2002). Another advantage of the Japanese medaka is its rapid development and ease of breeding, producing eggs on a regular schedule under the appropriate conditions of lighting and temperature. Also, there are closely related marine and freshwater species of medaka. Because of these advantages the medaka has been recognized by the international scientific community as an attractive complementary model system to the zebrafish. Furthermore, a draft genome sequence of the freshwater

Japanese medaka has been assembled and over 20 thousands genes have been predicted (Kasahara *et al.* 2007), which allow the application of toxicogenomics to evaluate toxicity of different chemicals across the genomes of these fish and extrapolate the observed toxicity in these models to humans and other species.

Transcriptional profiling methods, like microarray and real- time PCR, are powerful tools for examining chemical mechanisms or modes of action (MOA) and could potentially be used to support aspects of regulatory decision making in ecotoxicology (Ankley *et al* 2007). Microarray technology can scan expression profiles of multiple genes. The medaka has a relatively well-characterized genome. However, it lacks robust annotation for many gene products. Therefore, because of absence of baseline information of a large proportion of array spots, full interpretation of data collected by medaka microarray is impossible. On the other hand, real-time polymerase chain reaction (Real time -PCR) is a sensitive and reliable technique enabling quantitative quantification of mRNA in biological samples. Real-time PCR methods have greater precision for quantification of changes in gene expression than does the microarray. In addition, the lesser expense of the real time PCR technique relative to that of the microarray technique allows robust investigation on the studied chemical by examining more concentrations, organs and replicates. Therefore, we have developed a medaka HPG PCR array system that combines the quantitative performance of SYBR® Green-based real-time PCR with the multiple gene profiling capabilities of a microarray to examine chemical-induced gene expression profiles along the HPG axis. In the present study, we have selected a suite of the functionally relevant genes associated with the pathways of concern (HPG) based on literatures. All the genes investigated here either

have a cDNA sequence that is characterized in the NCBI database or have been sequenced by our group using rapid amplification of cDNA end (RACE) techniques in this study.

To evaluate the performance of the medaka HPG PCR array system and to develop the associated data analysis and visualization tools, five chemicals were selected as model chemicals to which medaka were exposed. The selected model chemicals included estrogen 17α-ethinylestradiol (EE2), anabolic androgen 17β-trenbolone (TRB), potent aromatase inhibitor fadrozole, fungicide prochloraz, and ketoconazole. These chemicals have displayed different MOAs, but all have been shown to adversely affect animal reproduction through the HPG axis. To elucidate the molecular mechanism associated with the adverse effects by these chemicals, concentration /time –dependent experiment design were applied.

I have included three closely related studies as chapters of my dissertation. However, during my program of graduate, I have also conducted research in the field of *in vitro* Endocrine Toxicology and Dioxin/PCB Toxicology. Thus, in addition to the three chapters included in my dissertation, each of which has been submitted as a manuscript to a journal, I have completed several other collateral studies and been involved as a co-investigator in several additional studies in the general area of molecular toxicology of endocrine disrupting chemicals that have resulted in publications in peer-reviewed journals. In particular, I have been involved with the development of the H295R steroidogenesis assay, which currently in the final phases of global validation by the Organization of Economic Cooperation and Development (OECD). In addition to completing the Ph.D. degree in the Toxicology track of the Environmental Toxicology

and Zoology, while at MSU I have simultaneously completed an MS in Statistics, for which I concentrated on techniques appropriate for toxico-genomics. The manuscripts that I have authored or co-authored while at MSU include the following.

In vitro Endocrine Toxicology

Zhang, X., Yu, R.M., Jones, P.D., Lam, G.K., Newsted, J.L., Gracia, T., Hecker M., Hilscherova K, Sanderson T, Wu R.S., Giesy JP. (2005). Quantitative RT-PCR methods for evaluating toxicant-induced effects on steroidogenesis using the H295R cell line. *Environ Sci Technol.* **39**(8):2777-85.

Hilscherova, K., Jones, P.D., Gracia, T., Newsted, J.L., **Zhang, X.**, Sanderson, J.T., Yu, R.M.K., Wu, R.S.S., Giesy, J.P. (2004). Assessment of the effects of chemicals on the expression of ten steroidogenic genes in the H295R cell line using real-time PCR. *Toxicol Sci.* **81**, 78-8.

Gracia, T., Hilscherova, K., Jones, P.D., Newsted, J.L., Higley, E.B., **Zhang, X.**, Hecker, M., Murphy, M.B., Yu, R.M., Lam, P.K., Wu, R.S., Giesy, J.P. (2007). Modulation of steroidogenic gene expression and hormone production of H295R cells by pharmaceuticals and other environmentally active compounds. *Toxicol Appl Pharmacol.* **225**(2), 142-153.

Gracia, T., Hilscherova, K., Jones, P.D., Newsted, J.L., **Zhang, X.**, Hecker, M., Higley, E.B., Sanderson, J.T., Yu, R.M.K., Wu, R.S.S., Giesy, J.P. (2006). The H295R system for evaluation of endocrine-disrupting effects. *Ecotoxicol Environ Saf.* **65**(3), 293-305.

Zhang, X., Jones, P.D., Newsted, J.L, Hecker, M., Gracia, T., Hilscherova, K., Yu, R.M.K., Wu, R.S.S. Giesy, J.P. (2008). Comparison of Human H295R, JEG-3 and Rat R2C Cell lines for Determining Effects on Steroidogenesis, *Toxicol Appl Pharmacol.* (Submitted)

Zhang, X., Newsted, J.L, Jones, P.D. Giesy, J.P. (2008). "ToxClust" --- a new data mining method for the analysis of concentration and/or time-dependent biological data. *J Chem Inf Model* (Submitted)

Dioxin/PCB Toxicology

Fung, C.N., Zheng, G.J., **Zhang, X.**, Wong, H.L., Giesy, J.P., Fang Z., Lam. P.K.S. (2005). Risk Posed by Concentrations of Trace Organic Contaminants in Coastal Sediments in the Pearl River Delta, China. *Mar Pollut Bull.* **50**, 1036-1049.

So, M.K., **Zhang, X.**, Giesy, J.P., Wong, H.L., Zheng J.G., Lam. P.K.S. (2005). Organochlorines, and dioxin-like compounds in green-lipped mussels Perna viridis from Hong Kong mariculture zones. *Mar Pollut Bull.* **51**, 677-87.

Xu, Y., Yu, R.M., **Zhang, X.**, Murphy, M.B., Giesy, J.P., Lam, M.H., Lam, P.K.S., Wu, R.S.S., Yu, H. (2006). Effects of PCBs and MeSO2-PCBs on adrenocortical steroidogenesis in H295R human adrenocortical carcinoma cells. *Chemosphere* **63**(5), 772-784.

Zhang, X., Moore, J.N., Zwiernik, M.J., Hecker, M., Newsted, J.L., Jones, P.D., Bursian, S.J., Giesy, J.P. (2007). Sequencing, and characterization of mixed function monooxygenase genes *CYP1A1* and *CYP1A2* of Mink (*Mustela vison*) to facilitate study on dioxin-like compounds. *Toxicol Appl Pharmacol.* (Submitted)

Reproductive and Endocrine Toxicology of Fish

Zhang, X., Park, J., Tompsett, A.R., Hecker,M., Jones, P.D., Newsted, J.L., Giesy, J.P. (2007). Development and validation of a medaka brain-gonadal-liver axis model and a real time-PCR array method to facilitate the mechanistic classification of endocrine-disrupting chemicals (EDCs). *Aquat Toxicol.* (Accepted)

Zhang, X., Park, J., Tompsett, A.R., Hecker,M., Jones, P.D., Newsted, J.L., Giesy, J.P. (2008). Time-dependent transcriptional profiles of genes of the hypothalamic-pituitary-gonadal (HPG) axis in medaka (*O. latipes*) exposed to fadrozole and 17β-trenbolone. *Environ Toxicol Chem.* (Accepted)

Zhang, X., Hecker,M., Jones, P.D., Newsted, J.L., Giesy, J.P. (2008). Responses of the Medaka HPG axis PCR array and reproduction to prochloraz and ketoconazole. *Environ Sci Technol.* (Submitted)

REFERENCES

Ankley, G.T., Johnson, R.D. (2005). Small Fish Models for Identifying and Assessing the Effects of Endocrine-disrupting Chemicals. *ILAR J* **45**(4), 469-483.

Ankley, G.T., Villeneuve, D.L. (2006). The fathead minnow in aquatic toxicology: past, present and future. *Aquat. Toxicol.* **78**(1), 91-102.

Danger, J.M., Tonon, M.C., Jenks, B.G., Saint-Pierre, S., Martel, J.C., Fasolo, A., Breton, B., Quirion, R., Pelletier, G., Vaudry, H. (1990). Neuropeptide Y: localization in the central nervous system and neuroendocrine functions. *Fundam Clin Pharmacol* **4**(3), 307-340.

Gracia, T., Hilscherova, K., Jones, P.D., Newsted, J.L., Zhang, X., Hecker, M., Higley, E.B., Sanderson, J.T., Yu, R.M.K., Wu, R.S.S., Giesy, J.P. (2006). The H295R system for evaluation of endocrine-disrupting effects. *Ecotoxicol Environ Saf* **65**(3), 293-305.

Hecker, M., Newsted, J.L., Murphy, M.B., Higley, E.B., Jones, P.D., Wu, R., Giesy, J.P. (2006). Human adrenocarcinoma (H295R) cells for rapid in vitro determination of effects on steroidogenesis: hormone production. *Toxicol Appl Pharmacol* **217**(1), 114-124.

Hilscherova, K., Jones, P.D., Gracia, T., Newsted, J.L., Zhang, X., Sanderson, J.T., Yu, R.M.K., Wu, R.S.S., Giesy, J.P. (2004). Assessment of the effects of chemicals on the expression of ten steroidogenic genes in the H295R cell line using real-time PCR. *Toxicol Sci.* **81**(1), 78-89.

Kasahara, M., Naruse, K., Sasaki, S., Nakatani, Y., Qu, W., Ahsan, B., Yamada, T., Nagayasu, Y., Doi, K., Kasai, Y., Jindo, T., Kobayashi, D., Shimada, A., Toyoda, A., Kuroki, Y., Fujiyama, A., Sasaki, T., Shimizu, A., Asakawa, S., Shimizu, N., Hashimoto, S., Yang, J., Lee, Y., Matsushima, K., Sugano, S., Sakaizumi, M., Narita, T., Ohishi, K., Haga, S., Ohta, F., Nomoto, H., Nogata, K., Morishita, T., Endo, T., Shin, I., Takeda, H., Morishita, S., Kohara, Y. (2007). The medaka draft genome and insights into vertebrate genome evolution. *Nature* **447**(7145), 714-719.

Pastva, S.D., Villalobos, S.A., Kannan, K., Giesy, J.P. (2001). Morphological effects of Bisphenol-A on the early life stages of medaka (Oryzias latipes). *Chemosphere* **45**(4-5), 535-541.

Villalobos, S.A., Papoulias, D.M., Pastva, S.D., Blankenship, A.L., Meadows, J., Tillitt, D.E., Giesy, J.P. (2003). Toxicity of o,p'-DDE to medaka d-rR strain after a one-time embryonic exposure by in ovo nanoinjection: an early through juvenile life cycle assessment. *Chemosphere* **53**(8), 819-826.

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. (2007). A graphical systems model to facilitate hypothesis-driven ecotoxicogenomics research on the teleost brain-pituitary-gonadal axis. *Environ Sci Technol.* **41**(1), 321-330.

Wittbrodt, J., Shima, A., Schartl, M. (2002). Medaka--a model organism from the far East. *Nat Rev Genet.* **3**(1), 53-64.

Zhang, X., Yu, R.M., Jones, P.D., Lam, G.K., Newsted, J.L., Gracia, T., Hecker, M., Hilscherova, K., Sanderson, T., Wu, R.S., Giesy, J.P. (2005). Quantitative RT-PCR methods for evaluating toxicant-induced effects on steroidogenesis using the H295R cell line. *Environ Sci Technol.* **39**(8), 2777-2785.

Chapter 2

Real time PCR array to study effects of chemicals on the Hypothalamic-Pituitary-Gonadal axis of the Japanese medaka

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ABSTRACT

System models utilizing genomic approaches can be powerful tools for mechanistic toxicological research. This paper describes the development and validation of a PCR array for studying chemical-induced effects on gene expression of selected endocrine pathways along the hypothalamic-pituitary-gonadal (HPG) axis of the small, oviparous fish, the Japanese medaka (*Oryzias latipes*). The Japanese medaka HPG PCR array combines the quantitative performance of SYBR® Green-based real-time PCR with the multiple gene profiling capabilities of a microarray to examine expression profiles of 36 genes associated with endocrine pathways in brain, liver and gonad. The performance of the Japanese medaka HPG PCR array was evaluated by examining effects of two model compounds, the synthetic estrogen, 17α-ethinylestradiol (EE2) and the anabolic androgen, 17β-trenbolone (TRB) on the HPG axis of the Japanese medaka. Four-month-old medaka were exposed to three concentrations of EE2 (5, 50, 500 ng/L) or TRB (50, 500, 5000 ng/L) for 7 d in a static renewal exposure system. A pathway-based approach was implemented to analyze and visualize concentration-dependent mRNA expression in the HPG axis of Japanese medaka. The compensatory response to EE2 exposure included the down-regulation of male brain *GnRH R I* and testicular *CYP17*. The down-regulation of *AR-α* expression in brain of EE2-exposed males was associated with suppression of male sexual behavior. Compensatory responses to TRB in the female HPG axis included up-regulation of brain *GnRH R II* and ovary steroidogenic *CYP19A*. Overall, the results suggested that the Japanese medaka HPG PCR array has potential not only as a screening

tool of potential endocrine disrupting chemicals but also in elucidating mechanisms of action.

Keywords: 17α-ethinylestradiol, 17β-trenbolone, expression profile, steroidogenesis, fecundity, endocrine, fish

INTRODUCTION

Knowledge of chemical-induced endocrine disruption is largely limited to the pathways mediated through several steroid hormone receptors, including estrogen receptors (ER) androgen receptors (AR) and thyroid hormone receptors (ThR). More recently, increasing efforts have been underway to evaluate alternate pathways of chemical interaction with the endocrine system such as effects on steroidogenesis, which can alter the rates as well as absolute and relative concentrations of hormones produced by an organism by altering the expression of steroidogenic enzymes (Ankley *et al.* 2005; Gracia *et al.* 2006; Hecker *et al.* 2006; Hilscherova *et al.* 2004; Villeneuve *et al.* 2007a; Zhang *et al.* 2005).

Endocrine-Disrupting Chemicals (EDCs) can alter normal patterns of gene expression either by direct (steroid hormone receptor-mediated pathways) or compensatory effects (Villeneuve *et al.* 2007b). Historically, studies of interactions of EDCs with organisms have focused primarily on a few endpoints in one tissue at one specific time in the development of an organism. However, considering the complicated nature of endocrine systems in humans and wildlife, current chemical screening tools are often limited to a few molecular targets. What is needed is a sensitive, flexible monitoring tool that allows for the screening of a multiple molecular target genes in multiple tissues simultaneously at any stage of development and allows for mechanism-based toxicity prediction and assessment.

A significant degree of evolutionary conservation has been found to occur in the basic aspects of the hypothalamic-pituitary-gonadal (HPG) axis among vertebrates (Ankley and Johnson 2005). Teleost fish, such as the Japanese medaka (*Oryzias latipes*),

zebrafish (*Danio rerio*) and fathead minnow (*Pimephales promelas*), have been suggested to be appropriate models for testing EDCs relative to both ecological relevance and species extrapolation (Ankley and Villeneuve 2006). The Japanese medaka is a small, oviparous, freshwater fish, native to Asia, for which extensive information on physiology, embryology and genetics has been developed (Wittbrodt *et al.* 2002; Pastava *et al.* 2001; Villalobos *et al.* 2003). Recently a marine medaka model (*Oryzias melastigma*) has also been developed for ecotoxicological study (Kong *et al.* 2007).

Transcriptional profiling methods, like microarray and real-time (quantitative) polymerase chain reaction (RT-PCR or Q-PCR), are powerful tools for examining chemical mechanisms or modes of action (MOA) and could potentially be used to support aspects of regulatory decision making in ecotoxicology (Ankley *et al* 2007). In the present study a Japanese medaka hypothalamic-pituitary-gonadal (HPG) PCR array system was developed that combines the quantitative performance of SYBR® Green-based RT-PCR with the multiple gene profiling capabilities of a microarray to examine chemical-induced gene expression profiles along the HPG axis. Based on literature, a suite of functionally relevant genes associated with the pathways of concern (HPG) were selected. All the genes investigated here either have a cDNA sequence that has been characterized in the NCBI database or have been sequenced using rapid amplification of cDNA end (RACE) techniques in our laboratory.

To evaluate the performance of the Japanese medaka HPG PCR array and to develop the associated data analysis and visualization tools, fish were exposed to two model chemicals, the synthetic estrogen, 17α-ethinylestradiol (EE2) and the anabolic androgen, 17β-trenbolone (TRB). The aims of the study were: 1) to select a suite of

functionally relevant genes to develop a Japanese medaka HPG model; 2) to clone and sequence the selected genes that lack cDNA evidence; 3) to develop SYBR Green based RT-PCR methods for the selected genes; 4) to conduct 7-d exposures with EE2 and TRB to examine the gene expression patterns in brain, liver and gonad; 5) to test the hypothesis that the chemicals can induce concentration-dependent, organ-specific gene expression response patterns; and 6) to compare gene changes and effects at other biologically relevant levels such as fecundity.

MATERIALS AND METHODS

Chemicals and regents

17 α-ethinyl estradiol (EE2), 17β-trenbolone (TRB) and dimethyl sulfoxide (DMSO) were obtained from Sigma (St. Louis, MO).

Animals

Male and female wild-type *O. latipes* were obtained from the aquatic culture unit at the US Environmental Protection Agency Mid-Continent Ecology Division (Duluth, MN, USA). The fish were cultured in flow-through tanks in conditions that facilitated breeding (23-24°C; 16:8 light/dark cycle), and that were in accordance with protocols approved by the Michigan State University Institutional Animal Care and Use Committee (MSU-IACUC).

Total RNA isolation and reverse-transcription PCR

Total RNA was extracted from tissue samples using the Agilent Technologies Total RNA Isolation Mini Kit (Agilent Technologies, Palo Alto, CA) according to the manufacturer's protocol. Purified RNA was stored at -80 °C until analysis. First-strand cDNA synthesis was performed using Superscript III first-strand synthesis SuperMix and Oligo-dT primers (Invitrogen, Carlsbad, CA). Briefly, a 0.5 to 2 μg aliquot of total RNA was combined with 1 μL of 50 μM Oligo$(dT)_{20}$, 1 μL of annealing buffer, and RNase-free water to a final volume of 8 μL. Mixes were denatured at 65 °C for 5 min and then quickly cooled on ice for 2 min. Reverse transcription was performed after adding 10 μL

2X first-stand reaction mix, and 2 μL SuperScript III/RNaseOUT enzyme mix. Reactions were incubated at 50 °C for 50 min and, on completion, were inactivated at 85 °C for 5 min. To digest RNA, 1.25 μL RNase H (Invitrogen, Inc., Carlsbad, CA) was added before incubation at 37 °C for 30 min. The cDNA synthesis reactions were stored at -20 °C until further analysis.

Gene selection and model development

A total of 36 genes representing key signaling pathways and functional processes within the Japanese medaka HPG axis were selected for study based on the teleost "Graphical Systems Model" previously proposed by (Villeneuve *et al.* 2007b) and Japanese medaka species-specific literature (Table 2.1). In addition, reference genes, β-actin, 16S rRNA and RPL-7, were selected as internal quantitative controls. The Japanese medaka HPG transcriptional model was constructed and visualized using GenMAPP 2.1 (Salomonis *et al.* 2007).

Cloning and sequencing

Of the 36 selected HPG genes, 14 genes did not have cDNA sequences available in the public NCBI Genbank database and cDNA cloning was conducted based on predicted transcript sequences. Briefly, the corresponding homologous genes were identified from the ensembl Japanese medaka genome (http://www.ensembl.org/Oryzias_latipes/index.html). Gene-specific primers were designed based on the predicted sequences for each of the studied genes. 5'-RACE and 3'-RACE PCR reactions were performed using a BD SMART RACE cDNA amplification kit (BD-Biosciences Clontech, Palo Alto CA)

according to the manufacturer's protocol. Purified PCR products were cloned into a plasmid vector or directly sequenced using the corresponding primers by an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Real-time PCR reaction

Real-time Q-RT-PCR was performed by using an ABI 7900 high throughput real time PCR System in 384-well PCR plates (Applied Biosystems, Foster City, CA) (Table 2.2). PCR reaction mixtures for one hundred reactions contained 500 μL of SYBR Green master mix (Applied Biosystems, Foster City, CA), 2 μL of 10 μM sense/anti-sense gene-specific primers, and 380 μL of nuclease-free distilled water (Invitrogen). A final reaction volume of 10 μL was made up with 2 μL of diluted cDNA and 8 μL of PCR reaction mixtures using a Biomek automation system (Beckman Coulter, Inc., Fullerton, CA). The PCR reaction mix was denatured at 95 °C for 10 min before the first PCR cycle. The thermal cycle profile was: denaturizing for 15 s at 95 °C; annealing for 30 s at 60 °C; and extension for 30 s at 72 °C. A total of 40 PCR cycles were used. PCR efficiency, uniformity, and linear dynamic range of each Q-RT-PCR assay were assessed by the construction of standard curves using DNA standards.

Chemical exposure

Japanese medaka (14 wk old) were acclimated in 10-L tanks filled with 6 L of carbon-filtered water for a period of 12 d prior to initiation of experiments. Fish were held at 24°C with a 16:8 light/dark cycle. Females were first separated from males by visual morphological determination and 5 female and 5 male fish were put into each tank.

Half of the water in each tank (3 L) was replaced daily with fresh carbon-filtered water. Overall mortality for all fish during the acclimation period was one. After the acclimation period, fish were exposed to EE2 or TRB in a 7-d static renewal exposure scenario. Each treatment was replicated in triplicate tanks and consisted of a vehicle control (DMSO with a final concentration of 1:10000 v/v water), 5, 50, and 500 ng/L EE2 and 50, 500, and 5000 ng/L TRB. Half of the water in each tank (3L) was replaced daily with fresh carbon-filtered water dosed with the appropriate amount of chemicals. Water quality parameters (temperature, pH, hardness, dissolved oxygen, ammonia-nitrogen and nitrate-nitrogen) were measured daily. Eggs produced during the previous day were counted and recorded before the replacement of water. No mortalities were observed in any treatment during the exposure period. At the end of the 7-d exposure period fish were euthanized in Tricaine S solution (Western Chemical, Ferndale, WA, USA), and total weight and snout-vent length were recorded for each fish. For gene expression analysis, 4-6 males and females were randomly sampled from the three replicate tanks of each treatment. Tissues from brain, liver, and gonads were collected and preserved in RNA*later* storage solution (Sigma, St Louis, MO) at -20°C until analysis. The quantification of target gene expression was based on the comparative cycle threshold (*Ct*) method with adjustment of PCR efficiency according to the procedures described previously (Zhang *et al.* 2005). To increase the reliability of comparative *Ct* method –based gene expression quantification, the average *Ct* value of multiple reference genes was used as reference Ct. *β-actin, 16S rRNA* and *RPL-7* were used as reference genes in brain and gonad tissues. Only *16S rRNA* and *RPL-7* were used

as reference genes in liver tissue because the hepatic expression of *β-actin* has been shown to be responsive to estrogenic chemicals (Zhang and Hu 2007).

Data analysis

Gene expression was calculated as fold-change relative to the average expression in the vehicle control. Statistical analyses were conducted using the R project language (http://www.r-project.org/). Prior to conducting statistical comparisons, normality was evaluated by Shapiro-Wilks test and if necessary, data were log-transformed to approximate normality. Differences were evaluated by ANOVA followed by a pair-wise t-test. Levels of statistical significance are $p < 0.05$ unless specified. To examine the relationship between the expression level of steroid hormone receptors and other HPG genes in different tissues, Spearman rank correlation analysis was conducted independently for females and males. Because each receptor gene was compared with multiple genes, the chance of false correlation increased. Therefore correlations with $p < 0.01$ were considered to be statistically significant.

Table 2.1 Gene list of the medaka HPG PCR array system

Abbreviation	Gene Name	Category	Sequence
CYP3A	Cytochrome P450 3A	Catabolic gene	AF105018
Annexin max2	Annexin max2	Estrogen response	Y11253
CHG H	Choriogenin H	Estrogen response	D89609
CHG HM	Choriogenin Hminor	Estrogen response	AB025967
CHG L	Choriogenin L	Estrogen response	AF500194
cGnRH II	Chicken-type gonadotropin-releasing hormone II	Hormone	AB041330
mfGnRH	Medaka-type gonadotropin-releasing hormone	Hormone	AB041336
sGnRH	Salmon-type gonadotropin-releasing hormone	Hormone	AB041332
AR-α	Androgen receptor	Steroid receptor	AB076399
ER-α	Estrogen receptor alpha	Steroid receptor	D28954
ER-β	Estrogen receptor beta	Steroid receptor	AB070901
GnRH RI	GnRH receptor type I	Peptide receptor	AB057675
GnRH RII	GnRH receptor type II	Peptide receptor	AB057674
GnRH RIII	GnRH receptor type III	Peptide receptor	AB083363
CYP11B	Cytochrome P450 11B	Steroidogenesis	AB105880
CYP19A	Cytochrome P450 19A	Steroidogenesis	D82968
CYP19B	Cytochrome P450 19B	Steroidogenesis	AY319970
CYP17	Cytochrome P450 17A1	Steroidogenesis	D87121
VTG I	Vitellogenin 1	yolk precursor	AB064320
VTG II	Vitellogenin II	yolk precursor	AB074891
StAR	Steroidogenic acute regulatory protein	Steroidogenesis	DQ988930
HMGR	Hydroxymethylglutaryl CoA reductase	Steroidogenesis	EU159456*
CYP21	Cytochrome P450c21 steroid 21-hydroxylase	Steroidogenesis	EU159457*
CYP11A	Desmolase (20,22 desmolase)	Steroidogenesis	EU159458*
3β-HSD	3β-hydroxysteroid dehydrogenase	Steroidogenesis	EU159459*

Table 2.1. Con't

FSHR	Follicle stimulating hormone receptor	Peptide receptor	EU159460*
LHR	Luetenizing hormone receptor	Peptide receptor	EF535803
LDLR	Low density lipoprotein receptor	Lipid receptor	EU159461*
GTHa	Glycoprotein hormone alpha chain	Hormone	EU047760*
NeuropepY	Neuropeptide Y	Hormone	EU047761*
LH-β	Luteinizing hormone, beta polypeptide	Hormone	EU047762*
HDLR	High density lipoprotein receptor	Lipid receptor	EU159466*
20β-HSD	20-beta-hydroxysteroid dehydrogenase	Steroidogenesis	EU159462*
Activin BA	Activin beta A chain	Hormone	EU159463*
Activin BB	inhibin, beta B	Hormone	EU159464*
Inhibin A	Inhibin alpha chain precursor	Hormone	EU159465*
16S	16S rRNA	Reference gene	AP008946
β actin	Beta actin	Reference gene	S74868
RPL-7	Ribosomal protein L7	Reference gene	DQ118296

* partial cDNA sequence collected in this study

Table 2.2. primer sequences of selected medaka HPG axis genes

Abbreviation	Sense	Antisense
CYP3A	GAGATAGACGCCACCTTCC	ACCTCCACAGTTGCCTTG
Annexin max2	CTGATCGTGGCTCTGATGAC	CTGCTGAGGTGTTCTGGAAG
CHG H	TGGCAAGGCACTGGAGTATCAC	CTGAGGCTTCGGCTGTGGATAG
CHG HM	GGAGCCATTACCAGGGACAG	AAGTTCCACACGCAAGATTCC
CHG L	TCCTGTCTCTGACTCTGAATGG	GCTTGGCTCGTCCTCACC
cGnRH II	TGTCTCGGCTGGTTCTAC	GAGTCTAGCTCCCTCTTCC
mfGnRH	GTGTCGCAGCTCTGTGTTC	AGTATTTCAGTTCTCGCTTCCC
sGnRH	GATGATGGGCACAGGAAGAGtG	GGGCACTTGCATCTTCAGGA
AR-α	ACCTGGCTCACTTCGGACAC	TCTGACGCCGTACTGCTCTG
ER-α	GAGGAGGAGGAGGAGGAGGAG	GTGTACGGTCGGCTCAACTTC
ER-β	GCTGGAGGTGCTGATGATGG	CGAAGCCCTGGACACAACTG
GnRH RI	TCCGACGAGCCGCATCTG	GATGAAGCCGACGACGATGAC
GnRH RII	GCAGCGGCACAGACATCATC	GGACAGCACAATGACCACAGAC
GnRH RIII	ACTTCCAGAGGAGCCAGTTGAG	GCCAGCCAAGAGTCGTTGTC
CYP11B	CTAGACGACGTGGCGAAAGACT	CCTCTGCTCCTCTTCCTTCTcG
CYP19A	CTCTTCCTGGGTGTTCCTGTTG	GCTGCTGTCTTGTGCCTCTG
CYP19B	TCCTGATAACCCTGCTGTCTCG	GTTGGTCTGCCTGATGCTGTTC
CYP17	CGACCACCACCGTACTCAAATG	TCTGGATAATGGATCAGGTAGGtG
VTG I	ACTCTGCTGCTGTGGCTGTAG	AAGGCGTGGGAGAGGAAAGTC
VTG II	TCGCCGCAAGAGCAAGAC	CTGGAGGAGCTGGAAGAACTG
StAR	TGACAGGTTTGAGAAAGAATG	CAATGCGAGAACTTAGAAGG
HMGR	CTGCTGCTGGCTGTCAAG	GCTGGCGGCTGCTTTATG
CYP21	CAGCCAGCAATGTCATCAC	TCAGCAGTGGGAAGGAATC
CYP11A	GCTGCATCCAGAACATCTATcG	GACAGCTTGTCCAACATCAGGA
3β-HSD	GGGCGGGACGAAACTCAG	GGAGGCGGTGTGGAAGAC
FSHR	TTCAGGCCACTGATGATGTTATcG	CCTTCGTGGGTTCCAGTGAGT
LHR	GTCCTGGTCATCCTGCTCGTtAG	AACCGGGAGATGGTCAGTTTGT
LDLR	GTGCTACGAAGGCTACGAGATG	AGGTCAATGCGGCGGATTTC
GTHa	GCAGAACGGAGGGATGAAGGAG	ATTGGAGTAGGTGTCGGCTGTG
NeuropepY	CTTCCACAGTCAAGTTACAAC	TGATCTGCAAGGACGAATG
LHbeta	GCCAGCCAGTCAAGCAGAAG	TCCACCGTATGACAGCCAGAG
HDLR	TCTGCCGAACTGTCACTGTC	CCACCTGGTCGTCGATGATG
20β-HSD	CAAAGGCATCGGCCTGGCCATTGT	GGCGGCGTTGTTGATAAGGACATC
Activin BA	GATGGTGGAAGCAGTGAAG	TTCTTGATGGCGTTGAGTAG
Activin BB	GGCTAATCGGCTGGAATG	CATGCGGTACTGGTTCAC
Inhibin A	CGTTTCCCTTCCAGCCTTC	AAGAGCGTTGCGGATGAG
16S	CGATCAACGGACCGAGTTACC	AATAGCGGCTGCACCATTAGG
Beta actin	GATGAAGCCCAGAGCAAGAGG	CATCCCAGTTGGTAACAATACcG
RPL-7	GTC GCC TCC CTC CAC AAA G	AAC TTC AAG CCT GCC AAC AAC

Figure 2.1 Cumulative fecundity in medaka exposed to EE2 (A) or TRB (B) in a 7-**d** test. Data represent the mean cumulative number of eggs per female collected from 3 replicate tanks, each containing 6 pairs of fish. The asterisks indicate a significant different (p-value < 0.05) from control group.

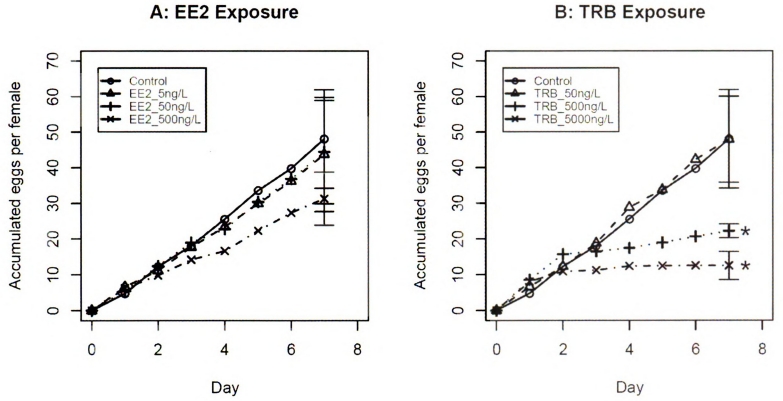


Table 2.3. Chemical induced effects on medaka gonadal-somatic index (GSI), hepatic-somatic index (HSI) and brain-somatic index (BSI) [a, b, c].

	Female			Male		
Treatment	**BSI**	**GSI**	**HSI**	**BSI**	**GSI**	**HSI**
Control	1.61	10.85	4.18	1.64	1.28	2.14
5 ng EE2/L	1.67	9.40	6.40	1.70	1.68	3.80*
50 ng EE2/L	1.43	10.08	3.67	1.84	1.62	3.69
500 ng EE2/L	1.64	10.01	5.48	1.44	1.96	3.86*
50 ng TRB /L	1.00	14.51	5.70	1.46	1.43	2.27
500 ng TRB /L	1.32	15.72*	4.87	1.48	1.73	3.90*
5000 ng TRB /L	1.51	11.96	3.66	1.66	1.34	2.38

[a] n = 4 ~6,

[b] BSI = brain weight x 100 / body weight;

GSI = gonad weight x 100 / body weight;

HSI = liver weight x 100 / body weight

[c] * p-value < 0. 05 comparing control by pairwise Wilcox test.

Figure 2.2. Volcano plots of chemically induced changes in gene expression pattern in males and females. Data are from medaka exposed to 500 ng EE2/L or 5000 ng TRB/L. Genes plotted farther from the either the x or y- axis have larger changes in gene expression. Thresholds for fold-change (vertical lines, 2-fold) and significant difference (horizontal line, $p < 0.01$) were used in this display.

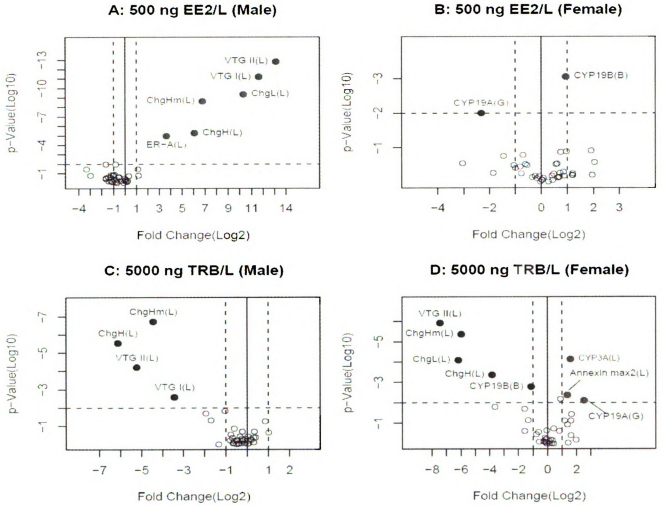

A: 500 ng EE2/L (Male)
p-Value(Log10)
Fold Change(Log2)
VTG II(L)
VTG I(L)
ChgL(L)
ChgHm(L)
ChgH(L)
ER-A(L)
B: 500 ng EE2/L (Female)
p-Value(Log10)
Fold Change(Log2)
CYP19B(B)
CYP19A(G)
C: 5000 ng TRB/L (Male)
p-Value(Log10)
Fold Change(Log2)
ChgHm(L)
ChgH(L)
VTG II(L)
VTG I(L)
D: 5000 ng TRB/L (Female)
p-Value(Log10)
Fold Change(Log2)
VTG II(L)
ChgHm(L)
ChgL(L)
ChgH(L)
CYP19B(B)
CYP3A(L)
Annexin max2(L)
CYP19A(G)

Table 2.4. Changes in expression for BHG genes in EE2-exposed medaka fish. Genes exhibited an over two-fold or significant change (p-value < 0.05) in expression between control and exposed medaka are listed. [a,b]

		EE2/Male			EE2/Female		
Tissue	Gene	5ng/L	50ng/L	500ng/L	5ng/L	50ng/L	500ng/L
Brain	*ER-α*	-1.14	-2.13	-1.72	1.93	2.19	1.62
	ER-β	1.09	-1.52	-2.1	2.26	2.08	1.83
	AR-α	-2.27*	-2.29*	-2.08*	1.26	1.51	1.54
	NeuropepY	1.94*	1.19	-1.47	1.26	1.47	1.67
	mfGnRH	-1.23	1.12	-1.4	1.12	1.13	1.02
	sGnRH	1.15	1.29	-1.89	-1.29	1.39	-1.18
	cGnRH II	-1.14	-1.57	1.05	1.09	1.24	1.95
	GnRH RI	-1.39	-3.98	-10*	3.06	2.78	2.31
	GnRH RII	-1.2	-1.45	-1.61	1.65	1.81	1.34
	GnRH RIII	1.15	-1.74	-1.98	2.09	2.83	1.81
	GTHa	-1.52	-13.33	-2.62	1.37	-2.29	-8.26
	LH beta	1.18	1.11	-1.02	1.6	-2.26	-3.58
	CYP19B	1.2	1.18	2.17***	1.16	1.11	1.92***
Gonad	*ER-α*	-1.18	-1.15	-1.26	-1.35	-1.08	-1.69
	ER-β	-1.13	1.06	-1	1.33	-1.14	1.47
	AR-α	-1.25	1.24	1.27	1.06	-1.02	-1.62
	FSHR	-1.02	1.1	-2.31	1.11	1.02	-1.27
	LHR	-1.04	1.28	-1.09	-1.78	1.14	-1.76
	HDLR	-1.86	1.03	-3.13	1.17	-1.23	-1.54
	LDLR	1.46	1.07	-1.87	1.13	-1.09	1.05
	HMGR	-1.59	1.06	-2.31	1.12	2.62	3.77
	StAR	-1.42	1.06	-1.47	-1.22	1.14	1.4
	CYP11A	1.01	1.25	-2.36	-1.14	1.12	-2.72
	CYP11B	-1.52	1.01	-3.11	1.32	3.48	4.11
	CYP17	-1.09	-1.22	-8*	1.42	1.25	-1.98
	CYP19A	-1.1	1.6	1.2	-1.31	-1.07	-5*
	CYP21	-1.39	1.22	1.15	1.04	3.48	3.86
	20β-HSD	-1.28	1.66	-2.77	1.52	1.01	-1.06
	3β-HSD	-1.17	1.3	-2.15	-1.17	-1.17	-2.08
	ActinBA	-1.22	1.45	1.06	1.18	3.28	4.14
	ActinBB	-1.51	1.04	-1.01	1.06	-1.08	1.41
	Inhibin A	-1.29	1.1	-2.27	-1.55	-1.16	-2.08
Liver	*ER-α*	-1.15	4.16**	12.33***	-1.35	1.32	1.59
	ER-β	-2.17	-1.57	-1.65	1.18	-1.17	1.63
	AR-α	2.94	5.49	2.3	9.88	1.23	2.29
	VTG I	-1.04	164***	3205***	1.29	1.27	1.33
	VTG II	1.78	325***	9004***	-1.52	1.02	1.21
	CHG H	-2.19	2.65	67.8***	-1.28	1.27	2.28
	CHG HM	-1.74	10.8***	111***	-1.36	-1.13	-1.03
	CHG L	2.83	11.5***	1270***	-1.47	-1.19	1.02
	CYP3A	-1.53	1.6*	-3.13*	-1.1	-1.18	-1.12
	Annexin max2	1.22	-1.08	-1.76*	1.68	-1.22	-1.46

[a] animal replicate (n= 4-6).
[b] * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

RESULTS

1. Construction of medaka HPG pathways focused PCR array.

The 36 HPG genes selected in this study included peptide hormones, steroid receptors, peptide hormone receptors, steroidogenic genes, selected estrogen responsive genes, catabolic gene, lipid receptors, and yolk precursor genes (Table 2.1). For the 14 genes that lacked transcript evidence, their cDNAs were cloned, sequenced, and submitted to GenBank/DDBJ/EMBL. The 14 gene partial transcript sequences and their Accession Numbers are *HMGR* (**EU159456**), *CYP21* (**EU159457**), *CYP11A* (**EU159458**), *3β-HSD* (**EU159459**), *FSHR* (**EU159460**), *LDLR* (**EU159461**), *GTHa* (**EU047760**), *NeuropepY* (**EU047761**), *LH-β* (**EU047762**), HDLR (**EU159466**), *20β-HSD* (**EU159462**), *activin BA* (**EU159463**), *activin BB* (**EU159464**), and *inhibin A* (**EU159465**). In addition, three house keeping genes including *β-actin*, *16S* and *RPL-7* were also measured as references of gene expression calculation. Of the 39 genes targeted in this study, 16, 15, and 24 genes were measured in the brain, liver and gonad tissue, respectively.

The Japanese medaka HPG real-time PCR array system was designed and optimized based on the SYBR Green detection method, which makes this PCR array system flexible and widely applicable. The generation of single, gene-specific amplicons of each primer set was confirmed by the real time PCR dissociation curve of each gene and by agarose gel electrophoresis characterization. The RT-PCR results demonstrate the high degree of plate-to-plate, run-to-run and replicate-to-replicate reproducibility inherent in the technology.

2. Chemical-induced effects on fish fecundity and indices of body condition

Exposure to EE2 did not affect fecundity, but exposure to TRB significantly reduced fecundity (Figure 2.1). There was no statistically significant effect on the accumulated egg production for any Japanese medaka exposed to EE2. No statistically significant differences in fecundity were observed between the control and 50 ng TRB/L for any durations of exposure. However, after 3 d of exposure, egg production by fish exposed to 500 or 5000 ng TRB/L was significantly less than that of the unexposed fish. Furthermore, the rate of egg production in the 5000 ng TRB/L exposure almost completely inhibited egg production by the end of the study.

Exposure to either TRB or EE2 resulted in statistically significant increases of the gonad-somatic index (GSI) and hepatic-somatic index (HSI) determined at the end of study (Table 2.3). Exposure to 5 or 500 ng EE2/L resulted in significantly greater HSI values in male Japanese medaka. No treatment-related effects of EE2 were observed for females. The only statistically significant effects of TRB were greater HSI in males and GSI in females exposed to 500 ng TRB/L.

3. Chemical-induced effects on HPG gene expression

To evaluate overall changes in gene expression patterns for all tissues in males and females exposed to 500 ng EE2/L and 5000 ng TRB/L were first examined by 'volcano plot' where Log_2-transformed fold-changes in gene expression were plotted against t-test p-values (Figure 2.2). Genes plotted farther from the central axis's have greater fold-changes and p-values. In male Japanese medaka exposed to 500 ng EE2/L (Figure 2.2A), 7 genes were significantly up-regulated in liver. The magnitudes of change for different

genes ranged from 3- to 9000-fold, and genes displaying such an increase in abundance included *VTG I*, *VTG II*, *choriogenin L* (*CHG L*), *choriogenin Hminor* (*CHG HM*), *choriogenin H* (*CHG H*) and *ER-α*. Only one gene, *CYP3A*, was down-regulated by EE2 in the liver. EE2 caused no statistically significant alteration in genes in the liver of females, but gonadal *CYP19A* was significantly less (-5 fold) and brain *CYP19B* was significantly greater (+1.92 fold) relative to that of the controls (Figure 2.2.B).

Exposure of TRB caused different gene expression profiles from that of EE2 in both males and females (Figure 2.2.C, D). Expression of *CHG L*, *CHG HM*, *VTG I* and *VTG II* in liver were significantly down-regulated by TRB in both males and females. Furthermore, greater expression of gonadal aromatase (*CYP19A*) and less expression of brain aromatase (*CYP19B*) was observed in females exposed to TRB, but not in males. Other effects on gene expression of females included a greater expression of *CHG L*, and lesser expression of both *CYP3* and *HMGR* in liver.

3.1 EE2- induced gene expression profile

Exposure to EE2 resulted in concentration-dependent alterations in gene expressions in livers of Japanese medaka that were specific to males (Figure 2.3; Table 2.4). Statistically significant up-regulation was observed for *VTG I*, *VTG II*, *CHG L*, and *CHG HM* in males exposed to 50 or 5000 ng EE2/L. *CHG H* was less sensitive to EE2 exposure than for *CHG HM* and *CHG L* for which significant changes were only observed in fish exposed to 500 ng EE2/L. Expression of *ER-α* was in liver was directly proportional to EE2 concentration, and were up-regulated 4.5-fold and 12-fold by

exposure to 50 and 500 ng EE2/L, respectively. Expression of *CYP3A* was significantly down-regulated in livers of males exposed to 500 ng EE2/L.

Exposure to EE2 for 7 d affected fewer genes and caused lesser fold changes of gene expression in testes, ovaries and brains than in livers of male Japanese medaka. Exposure to 500 ng EE2/L caused significant down-regulation of *CYP17* and *CYP19A* expression in testes and ovaries, respectively. In brains of males, exposure to EE2 resulted in significant down-regulation of expression of *AR-α* at all concentrations and concentration –dependently decreased the expression of *GnRH RI* with a 10-fold change at 500 ng EE2/L. Furthermore, EE2 exposure down regulated *neuropep Y* at 500 ng EE2/L, and up-regulated the expression of *CYP19B* at 500 ng EE2/L. Expression of *CYP19B* was significantly up-regulated (approximately 1.9- fold) in brains of females exposed to 500 ng EE2/L.

3.2 TRB- induced gene expression profile

TRB differentially regulated gene expression along the HPG axis in Japanese medaka (Figure 2.4; Table 2.5). In livers of females, exposed to 5000 ng TRB/L the expression of *VTG I*, *VTG II*, *CHG L*, *CHG H*, or *CHG HM* was significantly down-regulated. Conversely, the expression of *CYP3A* and *annexin max2* in liver of females was up-regulated in a concentration –dependent manner. In livers of males, TRB exposure significantly down- regulated *VTG I*, *VTGII*, *CHG H*, and *CHG HM* expression when they were exposed to 500 or 5000 ng TRB/L. A bimodal response was observed for *CHG L* where a statistically significant up-regulation in expression was observed in males exposed to 50 or 500 ng TRB/L and a statistically significant down-regulation of

expression when exposed to 5000 ng TRB/L. Exposure to 50 ng TRB/L did not affect the expression of the selected genes in testis or ovaries. However TRB down- regulated *HDLR* and up-regulated *CYP19A* in ovaries at 500 and 5000 ng TRB/L. In testis, TRB exposure down regulated expression of *HMGR*, *StAR* and *CYP11B*. In brain, exposure to TRB did not affect the gene expression in males, but up-regulated *cGnRH II* and down-regulated *CYP19* in females.

4. Correlations between expression of receptors and other genes

Expression of receptor genes and other genes in liver of both males and females were inter-correlated. Liver *ER-α* did not correlate to either *ER-β* or *AR-α* in either males or females (Table 2.6). However, *ER-α* was significantly correlated with *CHG HM*, *CHG H*, *CHG L*, *VTG I* and *VTG II* in both sexes. In male Japanese medaka, hepatic *ER-β* was significantly correlated with *CYP3A*.

Furthermore, the expression of *CYP19B* in brain was significantly and negatively correlated with the ovary expression of *CYP19A* (Figure 2.5). But this relationship was not significant in males.

Figure 2.3 Striped view of concentration dependent response profile in EE2 exposure of male Japanese medaka. Gene expression data from medaka treated by 5, 50 and 500 ng EE2/L are shown as striped color sets on the selected endocrine pathways along the medaka BHG axis. The legend listed in the upper right corner of the graph describes the order of the three EE2 concentration and the eight colors designating different fold thresholds. LH, lutinizing hormone; FSH, follicle-stimulating hormone; E2, 17β-estradiol; T, testosterone; HDL, high-density lipid; LDL, low-density lipid.

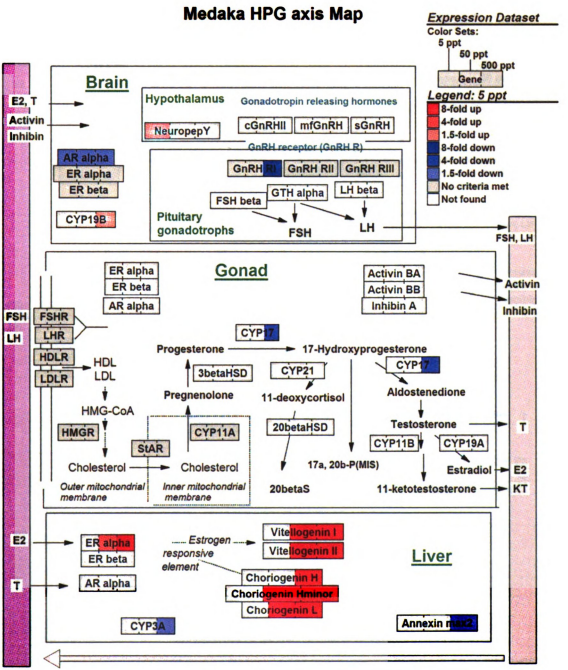


Figure 2.4 Striped view of concentration dependent response profile in TRB exposure of female Japanese medaka. Gene expression data from medaka treated by 50, 500 and 5000 ng TRB/L are shown as striped color sets on the selected endocrine pathways along the medaka HPG axis. The legend listed in the upper right corner of the graph describes the order of the three TRB concentration and the eight colors designating different fold thresholds. LH, lutinizing hormone; FSH, follicle-stimulating hormone; E2, 17β-estradiol; T, testosterone; HDL, high-density lipid; LDL, low-density lipid.

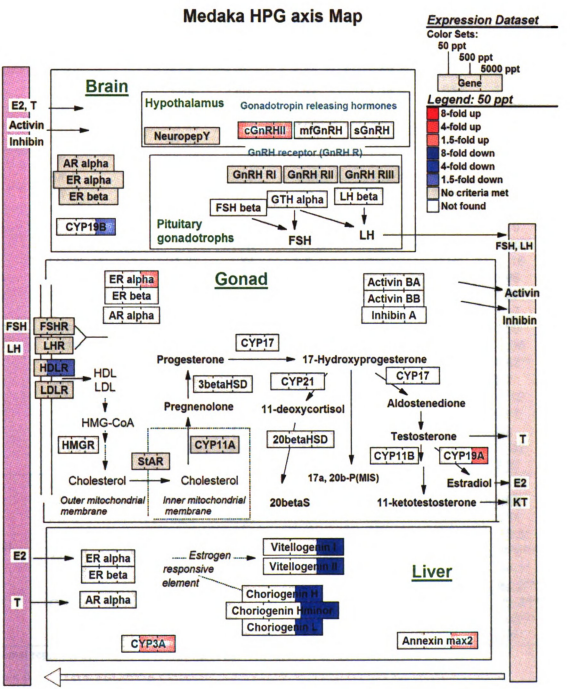


Table 2.5. Changes in expression for BHG genes in TRB-exposed medaka fish. Genes exhibited an over two-fold or significant change (p-value < 0.05) in expression between control and exposed medaka are listed. [a,b]

		Male			Female		
Tissue	Gene	50ng/L	500ng/L	5000ng/L	50ng/L	500ng/L	5000ng/L
Brain	*ER-α*	1.61	2.86	1.14	-1.77	-1.12	1.11
	ER-β	1.18	1.68	1.01	-2.5	-1.01	-1.11
	AR-α	-1.52	-1.12	-1.17	1.03	-1.12	-1.07
	NeuropepY	1.11	1.66	1.1	1.21	1.97	-1.19
	mfGnRH	-1.04	-1.17	-1.17	1.07	1.36	-1.03
	sGnRH	1.26	1.62	1.03	-1.06	1.31	-1.52
	cGnRH II	-1.46	-1.41	-1.54	2.21**	2.19**	1.88**
	GnRH RI	-1.21	1.9	-1.27	-1.44	-1.04	1.27
	GnRH RII	1.06	1.08	1.05	-1.07	-1.03	-1.48
	GnRH RIII	1.34	2.69	1.02	-1.15	1.05	1.34
	GTH alpha	-4.07	-13.2	-2.53	-333	2.36	-1.03
	LH beta	-3.34	-3.23	-1.75	-100	2.05	-2.98
	CYP19B	-1.08	1.04	-1.43	-1.07	1.06	-2.14**
Gonad	*ER-α*	-1.36	-1.48	-1.15	-1.1	2.24	3.13*
	ER-β	-1.09	-1.59	-1.08	-1.15	1.2	1.34
	AR-α	-1.13	-1.63	-1.31	-1.02	1.25	-1.02
	FSHR	-1.29	-1.57	1.27	1.09	1.38	2.26
	LHR	-1.04	-1.4	-1.22	1.01	1.88	1.79
	HDLR	-1.77	-3.73	-1.58	-1.4	-2.59*	-2.98*
	LDLR	-1.27	-1.74	1.3	-1.05	-1.87	-1.18
	HMGR	-1.4	-2.79**	-1.72	-1.26	3.73	3.06
	StAR	-1.34	-2.18**	-2.06*	-1.51	1.36	1.23
	CYP11A	1.08	-1.43	-1.34	-1.16	1.72	1.17
	CYP11B	-1.61	-2.58*	-1.56	-1.06	5.15	3.89
	CYP17	-2.22	-2.49	-1.19	-1.15	1.23	-1.06
	CYP19A	1.27	-1.17	-1.54	-1.19	4.14	5.82**
	CYP21	1.05	-1.73	-1.63	-1.96	4.49	2.7
	20β-HSD	-1.13	-2.3	-1.42	-1.17	-1.34	-1.12
	3β-HSD	-1.25	-1.69	-1.07	-1.32	2.1	3.07
	ActinBA	1.17	-1.1	-1.36	-1.32	4.73	4.4
	ActinBB	-1.29	-1.34	-1.31	-1	-1.52	-1.92
	InhibinA	-2.02	-2.5	-1.19	-1.55	1.36	2.37
Liver	*ER-α*	-1.75	-4.94**	-3.26*	1.02	1.29	-2.51
	ER-β	1.58	-1.26	1.25	1.06	2.22	2.56
	AR-α	2.25	-1.51	2.03	1.65	1.22	1.11
	VTG I	-2.3	-3.9**	-10.7**	-1.62	-2.48	-12.5*
	VTG II	1.28	-19.1***	-37.0***	-2.01	-2.7	-173***
	CHG H	-2.13	-21.1***	-69.1***	-1.31	1.04	-14.1*
	CHG HM	-2.27	-4.75***	-21.5***	-2.38	-2.01	-62.2***
	CHG L	3.14*	7.3*	-3.81*	-2.26	-1.29	-72.3***
	CYP3A	1.66	-1.04	1.8	-1.58	1.92**	3.03***
	Annexin max2	1.28	-1.03	1.23	-1.27	1.41	2.6**

[a] animal replicate in each treatment group (n=4-6).

[b] * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 2.6. Spearman rank correlation coefficients (numbers) and probabilities (*) between hepatic expression levels of ER-α , ER-β, AR-α mRNA and other genes.[a,b,c]

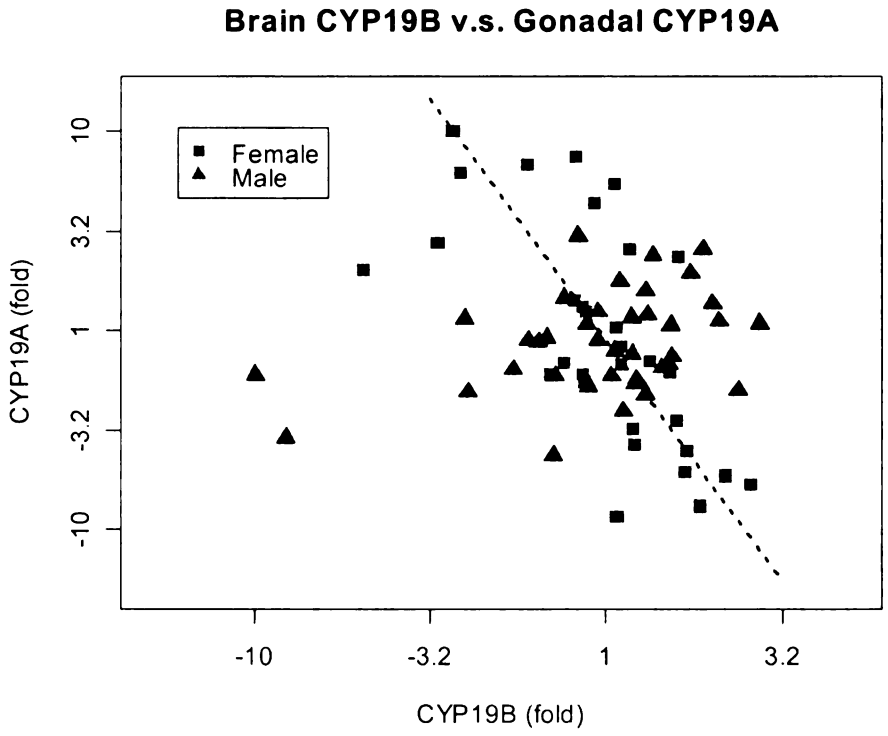
	Female			Male		
	ER-α	***ER-β***	***AR-α***	***ER-α***	***ER-β***	***AR-α***
ER-β	0.08			-0.014		
AR-α	0.132	0.512 **		0.12	0.156	
CHG HM	0.798 ***	-0.209	0.132	0.76 ***	-0.011	0.213
CHG H	0.887 ***	0.098	0.152	0.747 ***	0.19	0.251
CHG L	0.834 ***	-0.201	0.22	0.596 ***	-0.043	0.212
VTG I	0.69 ***	-0.207	0.094	0.817 ***	0.043	0.146
VTG II	0.813 ***	-0.207	0.141	0.848 ***	-0.073	0.217
CYP3A	0.035	0.281	0.055	-0.146	0.521 ***	0.072
Annexin max2	-0.124	0.246	-0.007	-0.17	0.315	-0.144

[a] Gene expressed level in each animal was calculated as fold change comparing to the average expression level in control group.

[b] Analyses were conducted separately within female (n = 31) and male (n = 40) groups

[c] ** $p < 0.01$, *** $p < 0.001$.

Figure 2.5 Correlation of brain expression level of brain *CYP19B* v.s. ovary *CYP19A*. Spearman rank correlation coefficients for female -0.676 (p-value < 0.001) male 0.266 (p-value = 0.102).



DISCUSSION

Japanese medaka HPG axis RT-PCR array

The Japanese medaka HPG real-time PCR array developed for assessing chemical induced effects on the endocrine pathways of brain, liver, and gonad was found to be reliable for transcriptional profiling and the results can provide mechanistic knowledge of chemical induced-effects in a systematic manner. Most of the 36 genes selected in the Japanese medaka HPG real-time PCR array haven't been previously examined in teleost exposures to model chemical EE2 or TRB by the time of study. However, for the genes previously studied in Japanese medaka and other fishes during similar exposures, their reported changes in transcription are consistent with the results reported here in terms of both magnitudes and directions of change (Lee *et al.* 2002, Martyniuk *et al.* 2007, Miracle *et al.* 2006). For example, *VTG I*, one of yolk precursor genes, was up-regulated by 164-fold and 3205-fold in male Japanese medaka from 7 d exposures to 50 and 500 ng EE2 /L, respectively in this study. An about 700-fold up-regulation for the homologous *VTG I* were reported in zebrafish exposed for 21 d to 10 ng EE2/L (Martyniuk *et al.* 2007). In fathead minnows, exposure to EE2 for 24 h also resulted in up-regulation of *VTG1* (Miracle *et al.* 2006). In the present study, exposure to 50 ng EE2/L significantly up-regulated expression of *CHG HM* and *CHG L*, but not *CHG H*, which is consistent with the report that estrogenic chemicals dose-dependently up-regulated mRNA expression of *CHG* subunits and that *CHG L* was more responsive than *CHG H* (Lee *et al.* 2002). Furthermore, up-regulation of *ER-α* in livers of fish exposed to EE2 similar to that observed in our study has been previously reported for Japanese medaka (Yamaguchi

et al. 2005), zebrafish (Martyniuk *et al.* 2007) and fathead minnow (Filby *et al.* 2007). Finally, the statistically significant up-regulation (2-fold) of brain *neuropeptide Y* gene expression in Japanese medaka exposed to 5 ng EE2/L observed in this study was consistent with the 3-fold increase reported to occur in zebrafish exposed to 10 ng EE2/L for 21 d (Martyniuk *et al.* 2007). These comparisons have not only verified the reliability of the measurement by the Japanese medaka HPG axis PCR array, but also demonstrated that the responses of the HPG axis pathway were similar among teleosts.

Quantification of changes in expression of genes in the HPG axis of Japanese medaka by RT-PCR array provides unique information to develop hypothesises about the transcriptional machinery. For example, expression of the *VTG* and *CHG* genes were significantly correlated to expression of *ER-α* expression but not to either *ER-β* or *AR-α* in liver of both sexes exposed to EE2 or TRB (Table 2.6). This indicates that the *VTG* and *CHG* genes were both primarily regulated by *ER-α* in the liver of Japanese medaka exposed to the two model chemicals. Although TRB is considered to be an *AR* agonist (Ankley *et al.* 2003), the hepatic ER mediated pathway is also responsive to TRB exposure. Teleost fish such as Japanese medaka, have two distinct aromatase genes, the gonadal (*CYP19A*) and brain (*CYP19B*) forms (Kuhl *et al.* 2005). The estradiol synthesized by gonadal aromatase has critical impacts on reproductive and sexual functioning, and brain aromatase activity can modulate neurogenic activity in the brain (Callard *et al.* 1995). However, the relationship between the transcriptional regulation of brain *CYP19A* and that of gonadal *CYP19B* has not been examined previously. The negative correlation between the transcription of brain *CYP19B* and ovary *CYP19A* indicates different physiological roles of brain and ovary aromatase activity.

17α-ethinylestradiol exposure

Exposure to EE2 had been reported to elevate VTG concentration and cause feminization of male Japanese medaka and zebrafish (Orn *et al.* 2006). Recent toxico-genomic investigations have improved understanding of the molecular mechanisms of EE2 effects in fish (Martyniuk *et al.* 2007; Moens *et al.* 2007; Santos *et al.* 2007). However, these studies only focused on single tissue types, such as liver or gonad. Transcriptional responses observed in the Japanese medaka HPG axis RT-PCR array provide systematic information on EE2-induced mechanisms on gene expression. In this study, the concentration dependent up-regulation of *ER-α*, *VTG* and *CHG* genes in livers of males exposed to EE2 suggests that exposure to estrogen has resulted in increase of endogenous estrogen concentration in Japanese medaka. The greater expression of *VTG* and *CHG* genes in EE2 exposed males could produce extra yolk and envelop protein in liver and explains the greater HSI in EE2 exposed male Japanese medaka (Table 2.3). The HPG axis pathway in Japanese medaka displayed compensatory feedback mechanism to EE2 exposure. In male brain, the expression *GnRH R I* and *GTHa* were down-regulated. If less expression of these genes can be translated into lower protein expression, it could lead to insufficient *GnRH* signaling and a fall in gonadotrophin secretion, which could consequently down-regulate gonadal steroidgenesis. The observed gonadal gene expression profile further confirms this hypothesis. EE2 exposure down-regulated the testicular expression of *CYP17*, which is one of the key enzymes involved in estrogen synthesis. In ovary, expression of *CYP19A* also displayed

significant down-regulation (5-fold), which is in favor of decreased estrogen synthesis to compensate for redundant endogenous estrogen.

Expression responses of other HPG axis genes have also characterized the mechanisms induced by EE2 exposure. For example, expression of *AR-α* was down-regulated in brains of males exposed to EE2. The agonist of *AR*, androgen is well known to act on the brain to modify male sexual behavior and other brain functions. Therefore in brains of EE2 exposed Japanese medaka, the lesser expression of *AR-α* may be associated with some alterations in sexual behavior and olfactory preference for receptive females. This hypothesis has been confirmed by the previous observation that exposure to another form of estrogen, 17β-estradiol, suppressed sexual behavior (following, dancing, floating, and crossing) in male Japanese medaka (Oryzias latipes) (Oshima *et al.* 2003). In another example, the down-regulation of the expression of *CYP3A* in liver of males exposed to 500 EE2 ng/L is consistent with reduced production of P450 isoforms in E2 exposed male fish (Kashiwada *et al.* 2007). In fish, *CYP3A* plays a major role in the metabolism of endogenous compounds, including steroids (Miranda et al., 1989). The less expression of *CYP3A* is related to the greater ER transcript that may down-regulate *CYP3A* in favor of maintaining high endogenous hormone concentrations during reproduction (Kashiwada *et al.* 2007).

17β-trenbolone exposure

TRB is an active metabolite of trenbolone acetate which is used as a growth promoter for farm animals. It has been reported to cause adverse effects on immune responses and reproduction (Hotchkiss and Nelson 2007; Miller *et al.* 2007). In fish,

TRB has been reported to cause masculinization of female zebrafish and decrease *VTG* production in both zebrafish and Japanese medaka (Masanori *et al.* 2006; Orn *et al.* 2006). TRB is an androgen receptor agonist and it has a greater affinity for the fish androgen receptor than that of the endogenous ligand, testosterone (Ankley *et al.* 2003). Nevertheless, TRB exposure has also been related to alterations of estrogen related pathways in recent literature. Down-regulation of *VTG* mRNA (*Vt1* and *Vt3*) were observed in female fathead minnows in a 21 d exposure to 50 and 500 ng TRB/L (Miracle *et al.* 2006). In Japanese medaka, TRB exposure for 7 d down-regulated expression of *VTG-I*, *VTG-II*, *CHG H*, *CHG L* and *CHG HM* in liver of both males and females. The reduced expression of *VTG* and *CHG* genes in TRB exposed females could produce less yolk and envelop protein and explains the reduced fecundity in TRB exposed Japanese medaka (Figure 2.1). These results suggest a lower concentration of endogenous estrogen (17β-estradiol) in liver of TRB exposed Japanese medaka because hepatic *VTG* and *CHG* genes were primarily regulated by estrogen. The down-regulation of *ER-α* in liver of TRB exposed Japanese medaka further supports this hypothesis. Furthermore, TRB exposure reduced concentrations of plasma 17β-estradiol and testosterone In female fathead minnows (Ankley *et al.* 2003).

The mechanism by which the androgen TRB inhibits the production of 17β-estradiol is still unclear. It has been hypothesized that decreased testosterone production is likely to represent a compensatory response to exposure to exogenous androgen, TRB. Consequently, less endogenous testosterone results in less 17β-estradiol because 17β-estradiol is produced by conversion of testosterone by aromatase (Miracle *et al.* 2006). The expression response of the Japanese medaka HPG axis supports this hypothesis. In

male brain, the down-regulation of the expression of *GTHα* and *LH-β* could result in decreased luteinizing hormone concentrations. The brain gonadotrophin LH plays a major role in the regulation of gonadal steroidogenesis. In accordance with the decreased number of *LH* transcripts in brain, the expression of testicular steroidogenic genes involved in the synthesis of testosterone and estrogen, including *HMGR*, *StAR* and *CYP11B*, showed down-regulation in TRB exposed Japanese medaka.

TRB exposure induced gender-specific responses along the HPG axis in Japanese medaka. Previous reports on the adverse effects by TRB have mainly focused on masculinization of females (Ankley *et al.* 2003; Miracle *et al.* 2006). Nevertheless, males of Japanese medaka were more sensitive to TRB exposure than were females to the reduction of expression of estrogen receptor, *VTG* and *CHG* genes in liver. Although the physiological function of the expression of the *VTG* and *CHG* genes in males is largely unknown, these genes have been shown to rapidly respond in male fish exposed to estrogens and anti-estrogens in both field and laboratory studies (Hutchinson *et al.* 2006). The higher susceptibility of estrogen-responsive pathways to fluctuations in the concentration of endogenous estrogen in males than in females may be due to the lower basal estrogen concentration and estrogen metabolizing capability in males. Conversely females showed a greater compensatory response to TRB exposure to reduce endogenous estrogen than males, which includes the up-regulation of brain *GnRH R II* and ovarian *CYP19A*. These gender-specific transcriptional response profiles in the HPG axis of TRB exposed Japanese medaka provide a unique signature of TRB exposure.

CONCLUSIONS

The HPG -PCR array system developed in this study represents a sensitive, reliable and flexible monitoring tool to research chemical-induced effects along the HPG axis in Japanese medaka. The Japanese medaka HPG PCR array developed in the study combines the quantitative performance of SYBR Green-based real-time PCR with the multiple gene profiling capabilities of a microarray to examine chemical-induced gene expression profiles along the HPG axis. The pathway-based approach implemented in this study provides a valuable tool to analyze and visualize concentration-dependent responses induced by chemical in the HPG axis of Japanese medaka.

Overall, this study demonstrated that profiling of HPG transcripts by RT-PCR array methods can discriminate estrogenic and androgenic EDCs, and represents a useful tool to systematically evaluate chemical induced molecular responses in multiple pathways and multiple organs. Mechanistic information derived from the results can be used in diagnostic and predictive assessments of the risk of EDCs.

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REFERENCE

Ankley, G. T., Jensen, K. M., Makynen, E. A., Kahl, M. D., Korte, J. J., Hornung, M. W., Henry, T. R., Denny, J. S., Leino, R. L., Wilson, V. S., Cardon, M. C., Hartig, P. C., Gray, L. E., 2003. Effects of the androgenic growth promoter 17-beta-trenbolone on fecundity and reproductive endocrinology of the fathead minnow. Environ. Toxicol. Chem. 22, 1350-1360.

Ankley, G. T., Johnson, R. D., 2005. Small Fish Models for Identifying and Assessing the Effects of Endocrine-disrupting Chemicals. ILAR J 45, 469-483.

Ankley, G. T., Villeneuve, D. L., 2006. The fathead minnow in aquatic toxicology: past, present and future. Aquat. Toxicol. 78, 91-102.

Ankley, G. T., Kahl, M. D., Jensen, K. M., Hornung, M. W., Korte, J. J., Makynen, E. A., Leino, R. L., 2002. Evaluation of the aromatase inhibitor fadrozole in a short-term reproduction assay with the fathead minnow (Pimephales promelas). Toxicol. Sci. 67, 121-130.

Callard, G. V., Kruger, A., Betka, M., 1995. The goldfish as a model for studying neuroestrogen synthesis, localization, and action in the brain and visual system. Environ. Health Perspect. 103, 51-57.

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.

Gracia, T., Hilscherova, K., Jones, P. D., Newsted, J. L., Zhang, X., Hecker, M., Higley, E. B., Sanderson, J. T., Yu, R. M. K., Wu, R. S. S., Giesy, J. P., 2006. The

H295R system for evaluation of endocrine-disrupting effects. Ecotoxicol. Environ. Saf. 65, 293-305.

Hecker, M., Newsted, J. L., Murphy, M. B., Higley, E. B., Jones, P. D., Wu, R., Giesy, J. P., 2006. Human adrenocarcinoma (H295R) cells for rapid in vitro determination of effects on steroidogenesis: hormone production. Toxicol. Appl. Pharmacol 217, 114-124.

Hilscherova, K., Jones, P. D., Gracia, T., Newsted, J. L., Zhang, X. W., Sanderson, J. T., Yu, R. M. K., Wu, R. S. S., Giesy, J. P., 2004. Assessment of the effects of chemicals on the expression of ten steroidogenic genes in the H295R cell line using real-time PCR. Toxicol. Sci. 81, 78-89.

Hutchinson, T. H., Ankley, G. T., Segner, H., Tyler, C. R., 2006. Screening and testing for endocrine disruption in fish-biomarkers as "signposts," not "traffic lights," in risk assessment. Environ. Health Perspect. 114, 106-114.

Kashiwada, S., Kameshiro, M., Tatsuta, H., Sugaya, Y., Kullman, S. W., Hinton, D. E., Goka, K., 2007. Estrogenic modulation of CYP3A38, CYP3A40, and CYP19 in mature male medaka (Oryzias latipes). Compar. Biochem. Phys. Part C: Toxicol. Pharmacol. 145, 370-378.

Kuhl, A. J., Manning, S., Brouwer, M., 2005. Brain aromatase in Japanese medaka (Oryzias latipes): Molecular characterization and role in xenoestrogen-induced sex reversal. J Steroid Biochem. Mol Biol. 96, 67-77.

Lee, C., Na, J. G., Lee, K. C., Park, K., 2002. Choriogenin mRNA induction in male medaka, Oryzias latipes as a biomarker of endocrine disruption. Aquat. Toxicol. 61, 233-241.

Martyniuk, C. J., Gerrie, E. R., Popesku, J. T., Ekker, M., Trudeau, V. L., 2007. Microarray analysis in the zebrafish (Danio rerio) liver and telencephalon after exposure to low concentration of 17alpha-ethinylestradiol. Aquat. Toxicol. 84, 38-49.

Miracle, A., Ankley, G., Lattier, D., 2006. Expression of two vitellogenin genes (vg1 and vg3) in fathead minnow (Pimephales promelas) liver in response to exposure to steroidal estrogens and androgens. Ecotoxicol. Environ. Saf. 63, 337-342.

Miranda, C.L., Wang, J.L., Henderson, M.C., Buhler, D.R., 1989. Purification and characterization of hepatic steroid hydroxylases from untreated rainbow trout. Arch. Biochem. Biophys. 268, 227–238

Orn, S., Yamani, S., Norrgren, L.,2006. Comparison of vitellogenin induction, sex ratio, and gonad morphology between zebrafish and Japanese medaka after exposure to 17alpha-ethinylestradiol and 17beta-trenbolone. Arch. Environ. Contam. Toxicol. 51, 237-243.

Pastva, S. D., Villalobos, S. A., Kannan, K., Giesy, J. P., 2001. Morphological effects of Bisphenol-A on the early life stages of medaka (Oryzias latipes). Chemosphere 45, 535-541.

Salomonis, N., Hanspers, K., Zambon, A.C., Vranizan, K., Lawlor, S.C., Dahlquist, K.D., Doniger, S.W., Stuart, J., Conklin, B.R., Pico. A.R., 2007. GenMAPP 2: new features and resources for pathway analysis. BMC Bioinformatics, 8: 217.

Villalobos, S. A., Papoulias, D. M., Pastva, S. D., Blankenship, A. L., Meadows, J., Tillitt, D. E., Giesy, J. P. (2003). Toxicity of o,p'-DDE to medaka d-rR strain after a one-

time embryonic exposure by in ovo nanoinjection: an early through juvenile life cycle assessment. Chemosphere 53, 819-826.

Villeneuve, D. L., Ankley, G. T., Makynen, E. A., Blake, L. S., Greene, K. J., Higley, E. B., Newsted, J. L., Giesy, J. P., Hecker, M., 2007. Comparison of fathead minnow ovary explant and H295R cell-based steroidogenesis assays for identifying endocrine-active chemicals. Ecotoxicol. Environ. Saf. 68, 20-32.

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., 2007. A graphical systems model to facilitate hypothesis-driven ecotoxicogenomics research on the teleost brain-pituitary-gonadal axis. Environ. Sci. Technol. 41, 321-330.

Wittbrodt, J., Shima, A., Schartl, M., 2002. Medaka--a model organism from the far East. Nat. Rev. Genet. 3, 53-64.

Yamaguchi, A., Ishibashi, H., Kohra, S., Arizono, K., Tominaga, N., 2005. Short-term effects of endocrine-disrupting chemicals on the expression of estrogen-responsive genes in male medaka (Oryzias latipes). Aquat. Toxicol. 72, 239-249.

Zhang, X., Yu, R. M., Jones, P. D., Lam, G. K., Newsted, J. L., Gracia, T., Hecker, M., Hilscherova, K., Sanderson, T., Wu, R. S., Giesy, J. P., 2005. Quantitative RT-PCR methods for evaluating toxicant-induced effects on steroidogenesis using the H295R cell line. Environ. Sci. Technol. 39, 2777-2785.

Zhang, Z., Hu, J., 2007. Development and validation of endogenous reference genes for expression profiling of medaka (Oryzias latipes) exposed to endocrine disrupting chemicals by quantitative real-time RT-PCR. Toxicol. Sci. 95, 356-368.

Chapter 3

Time-dependent transcriptional profiles of genes of the hypothalamic-pituitary-gonadal (HPG) axis in medaka (O. latipes) exposed to fadrozole and 17β-trenbolone

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ABSTRACT

Both the anabolic androgen 17β-trenbolone (TRB) and the aromatase inhibitor fadrozole (FAD) can cause decreased plasma concentrations of estrogen (E2) and reduce fecundity of fish. However, the underlying mechanisms and the molecular pathways involved are largely unknown. This study was designed to assess time-dependent effects of FAD and TRB on the transcriptional responses of the hypothalamic-pituitary-gonadal (HPG) axis of Japanese medaka (*Oryzias latipes*). Fourteen wk-old Japanese medaka were exposed to 50μg FAD/L or 2μg TRB/L in a 7-d static renewal and expression profile of 36 HPG axis genes were measured by means of a medaka HPG real time PCR (RT-PCR) array after 8 h, 32 h or 7 d of exposure. Exposure to TRB or FAD caused lesser fecundity of Japanese medaka and down-regulated transcription of *vitellogenin* (*VTG*) and *choriogenin (CHG)* genes in the liver of females. Exposure to FAD for 8 h resulted in an 8-fold and 71-fold down-regulation of expression of estrogen receptor alpha (*ER-α*) and *CHG L*, respectively in female liver. 17β-trenbolone caused similar down-regulation of these genes but the effects were not observed until 32 h of exposure. These results support the hypothesis that FAD reduces plasma E2 more quickly by inhibiting aromatase enzyme activity than does TRB, which inhibits production of the E2 precursor testosterone. Exposure to FAD and TRB resulted in rapid (after 8 h) down-regulation of *LHR* and *LDLR* in the testis to compensate excessive androgen level. Overall, the molecular responses observed in the present study differentiate the mechanisms of the reduced fecundity by TRB and FAD.

Keyword- fecundity, activin, vitellogenin, HPG axis, Real time PCR array

INTRODUCTION

Over the last decade, molecular biomarkers have been successfully applied in the screening and testing of endocrine disrupting chemicals in fish [1]. While molecular biomarkers have potential to aid in the elucidation of causative modes of action (MOA) and may in some cases allow for prediction of possible adverse effects of chemicals at higher organizational levels, to date this potential has not been realized [2, 3]. It is still difficult to relate changes in expression of single genes to population-level fitness. One example of a successful biomarker has been the yolk protein precursor *vitellogenin* (*VTG*) which has been shown to rapidly respond in fish exposed to estrogens and anti-estrogens in both filed and laboratory studies [1]. Recent studies have demonstrated that changes in VTG caused by several model chemicals can be quantitatively translated into adverse health effects at apical and population-level in fathead minnow (*Pimephales promelas*) [4,5]. Measurement of the VTG in plasma using immunology-based detection methods has been applied in various fish species [6]. Measurements of VTG mRNA transcripts using real time PCR methods have been demonstrated to be advantageous over protein determination due to the rapid induction of the gene (early warning) and less susceptibility to cleavage [1]. Additional molecular biomarkers that have been shown to serve as useful indicators for the interaction of EDCs with the teleost hypothalamic-pituitary-gonadal (HPG) axis include nuclear hormone receptors [7], steroidogenic enzymes (e.g. aromatase) [8], and gonadotropins (follicle-stimulating hormone and luteinizing hormone) [9]. These studies have demonstrated that molecular responses, specifically gene profiling, of one or two functional-relevant biomarkers, can facilitate a

better understanding of the mechanisms of estrogenic disruption in fish. However, chemicals can cause similar responses of biomarkers at certain target organs by acting through different mechanisms. For example, both the specific inhibitor of aromatase fadrozole (FAD) and the androgen receptor agonist 17 β-trenbolone (TRB) inhibit hepatic *estrogen receptor* (*ER*) and *VTG* transcripts, and consequently impair fecundity in fathead minnow [10, 11] and Japanese medaka (*Oryzias latipes*) [12, 13]. To better understand these types of interactions, more comprehensive and integrated molecular approaches are needed to evaluate potential endocrine disrupting chemicals (EDCs) with unknown mechanisms.

Recently, Villeneuve *et al.* summarized the current understanding of the reproduction-related molecular pathways within the teleost HPG-axis [14]. Using the Japanese medaka as a small fish model, we have developed and validated a reliable, sensitive and flexible real time PCR (RT-PCR) array to systematically study chemical-induced effects at multiple endocrine pathways in brain, gonad and liver [13]. 36 genes has been selected from the four modules representing (1) gonadotropin synthesis and release in the hypothalamus and pituitary, (2) cholesterol transport, (3) steroidogenesis in the gonads, and (4) production of egg precursors in the liver [13]. Through systematic monitoring of key genes along the HPG-axis in fish exposed to EDC of concern, it is anticipated that a better understanding of chemical induced mechanisms of actions (MOA) can be achieved that ultimately will aid in improving chemical risk assessment.

In the present study, we used FAD and TRB as model compounds to investigate different MOAs resulting in the same net effect. Because of the direct inhibitory effect of estrogen production by FAD, we hypothesized that FAD exposure would elicit responses

more rapidly on gonadal steroidogenesis –related genes and hepatic estrogen-responsive genes than would TRB. Time–dependent transcriptional responses of key genes along the HPG axis were examined after exposure to FAD and TRB using the medaka HPG RT-PCR array [13].

MATERIALS AND METHODS

Compounds and reagents

Fadrozole was provided by Novartis, Inc. (Summit, NJ, USA). 17β-trenbolone (TRB) and dimethyl sulfoxide (DMSO) was obtained from Sigma (St. Louis, MO).

Animals

Male and female wild-type Japanese medaka (*Oryzias latipes*) used in this study originated from the aquatic culture unit at the US Environmental Protection Agency Mid-Continent Ecology Division (Duluth, MN, USA). The fish were maintained in flow-through tanks in conditions that facilitated breeding (23-24 °C; 16:8 light/dark cycle) in accordance with protocols approved by the Michigan State University Institutional Animal Care and Use Committee (MSU-IACUC).

Chemical exposure

Chemical exposure was carried out by a static renewable system as previously described [13]. Briefly, studies were conducted in 10-L tanks filled with 6 L of carbon-filtered water. Each day during the exposure half of the water in each tank (3 L) was replaced with fresh carbon-filtered water containing the appropriate amount of chemical or solvent. Each tank contained 5 male and 5 female 14 wk -old medaka as determined by secondary sexual characteristics of the fins. Each treatment had 2 replicate tanks that were sampled at each time point. After the acclimation period, medaka were exposed to one of three treatments: vehicle control (DMSO with a final concentration of 1:10000 v/v

water), 50 μg FAD/L or 2 μg TRB/L. Concentrations of the chemicals were selected based on the results of previous studies [12, 13] so that the selected concentrations would inhibit fecundity of medaka without causing mortality. Exposures started at midnight (12:30 AM) of the first day. Eggs produced during the previous 24 h period were counted and recorded before the replacement of water. No mortality of adult medaka was observed in any treatment. The three sampling times were 8:30 AM of the fist day (8 h), day 2 (32 h) and day 7 (152 h) of exposure. When sampling fish were euthanized in Tricaine S solution (Western Chemical, Ferndale, WA, USA), and total weight and snout-vent length were recorded for each fish. For gene expression analysis, 4-6 males and females were randomly sampled from the two replicate tanks of each treatment. Tissues from brain, liver, and gonads were collected and preserved in RNA*later* storage solution (Sigma, St Louis, MO) at -20°C until analysis.

Total RNA isolation and reverse transcription PCR

Total RNA was extracted from individually tissues and first strand cDNA was separately made for further quantification of transcripts [13]. Total RNA was extracted by use of the Agilent Total RNA Isolation Mini Kit (Agilent Technologies, Palo Alto, CA) according to the manufacture's protocol. Purified RNA was stored at -80 °C until analysis. First-strand cDNA synthesis was performed using Superscript III first-strand synthesis SuperMix and Oligo-dT primers (Invitrogen, Carlsbad, CA). Briefly, a 0.5 to 2 μg aliquot of total RNA was combined with 1 μL of 50 μM of Oligo$(dT)_{20}$, 1 μL of annealing buffer, and RNase-free water to a final volume of 8 μL. Mixes were denatured at 65 °C for 5 min and then quickly cooled on ice for 2 min. Reverse transcription was

performed after adding 10 µL 2X first-stand reaction mix, and 2 µL SuperScript III/RNaseOUT enzyme mix. Reactions were incubated at 50 °C for 50 min and, on completion, were inactivated at 85 °C for 5 min. RNA was digested by adding 1.25 µL RNase H (Invitrogen, Carlsbad, CA) then incubated at 37 °C for 30 min. cDNA was stored at -20 °C until further analysis.

Real time –PCR array measurement

Gene expression in brain, liver and gonad was quantified by use of the medaka HPG axis PCR array described previously (Table 2.1) [13]. Briefly, real time, quantitative polymerase chain reaction (Q-RT-PCR) was performed by using a 384-well ABI 7900 high throughput real time PCR System (Applied Biosystems, Foster City, CA). PCR reaction mixtures sufficient for one hundred reactions contained 500 µL of SYBR Green master mix (Applied Biosystems, Foster City, CA), 2 µL of 10 µM sense/anti-sense gene-specific primers, and 380 µL of nuclease-free distilled water (Invitrogen). A final reaction volume of 10 µL was made up with 2 µL of diluted cDNA and 8 µL of PCR reaction mixtures using a Biomek automation system (Beckman Coulter, Inc., Fullerton, CA). Expression of target genes was quantified by use of the comparative cycle threshold (*Ct*) method according to methods reported elsewhere [15]. The average *Ct* value of the three reference genes (*ß-actin, RPL-7* and *16s*) was used as reference for the expression calculation of target genes

Statistical analysis

Statistical analyses were conducted using the R project language (http://www.r-project.org/). Analysis of fecundity data was using analysis of variance (ANOVA) model, in which the effects of time (day), chemical (TRB or FAD) and their interaction on the daily recorded egg production were examined. Prior to conducting statistical comparisons of gene expression, the assumption of normality of distributions of data was evaluated by the Shapiro-Wilk's test. If necessary, data was log-transformed to approximate the normal probability function. Differences in magnitudes of expression among genes were evaluated by use of ANOVA followed by pair-wise t-test. Differences with $p < 0.05$ were considered to be statistically significant.

Figure 3.1 Cumulative fecundity in medaka exposed to 2.0 µg TRB/L or 50 µg FAD/L in a 7-d test. Data represent the mean and standard deviation of cumulative number of eggs per female collected from 2 replicate tanks, each containing 5 pairs of fish. The asterisks indicate a significant difference (p-value < 0.05) from vehicle control group.

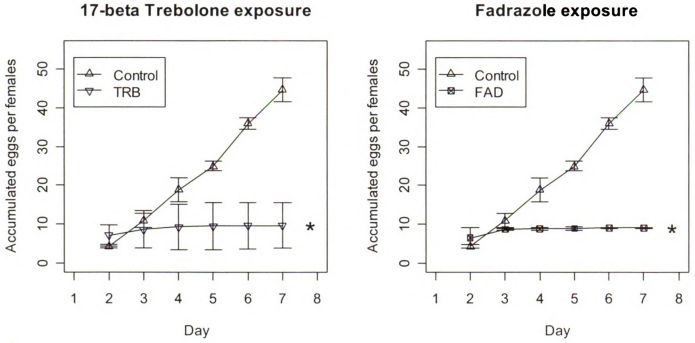


Table 3.1 Transcriptional response profiles of HPG axis pathways in medaka fish exposed to 50 μg FAD /L. Gene expression was expressed as the fold change comparing to the corresponding solvent controls [a,b]

		Female			Male		
Tissue	gene	8 h	32 h	7 d	8 h	32 h	7 d
Brain	*ER-α*	1.22	-1.12	1.15	1.13	-1.03	-1.14
	ER-β	1.25	1.2	1.48	1.13	2.27*	-1.52
	NeuropepY	1.15	-1.6	1.16	1.46	-1.06	-1.21
	mfGnRH	1.61	1.13	1.15	1.43	-1.07	-1.08
	sGnRH	1.17	-1.08	-1.01	1.16	-1.05	-1.13
	cGnRH II	1.65	-1.34	1.76	1.05	-1.34	1.25
	GnRH RI	1.62	-1.04	-1.34	-1.16	1.18	-1.29
	GnRH RII	1.05	1.01	-1.04	1.13	1.11	-1.18
	GnRH RIII	1.06	-1.19	-1.02	1.03	1.21	-1.08
	GTHa	-1.44	1.71	3.84***	-1.57	-1.3	-1.2
	LH-β	-1.44	-1.62	1.47	-2.33	-2.67	-3.26
	CYP19B	-1.20	-3.11***	-2.90***	-1.50*	-2.92***	-3.11***
Gonad	*ER-α*	1.32	1.84	-1.18	1.05	2.56***	-1.01
	ER-β	1.52	-1.67	-1.76	-1.06	1.78*	1.17
	AR-α	1.19	-1.07	-1.15	-1.15	1.49*	1.06
	FSHR	1.19	1.77	1.97	1.02	2.23*	-1.10
	LHR	-1.07	1.10	-1.36	-6.71*	-3.33	-1.19
	HDLR	-1.21	-2.29***	-2.56*	-1.06	1.82*	-1.03
	LDLR	1.12	1.11	-1.04	-2.09*	1.01	-1.44
	HMGR	1.14	-3.55	-3.23	-5.15	1.90*	-1.02
	StAR	1.04	1.52	1.48	-1.04	2.17*	2.80*
	CYP11A	-1.15	1.19	-1.08	1.45	1.70	1.34
	CYP11B	1.50	-5.62*	-3.28	-1.16	2.29*	1.92*
	CYP17	1.22	1.19	1.45	2.03	3.13*	1.56
	CYP19A	2.02	4.19*	3.99	1.80	-1.56	1.11
	CYP21	1.06	-2.64*	-2.84	-1.30	-2.37*	1.06
	20β-HSD	1.09	-1.11	-1.11	1.11	1.05	-1.12
	3β-HSD	1.21	2.26	1.25	1.27	2.49**	1.44
	CYP3A	-1.47	-13.28	-9.17**	-1.09	-1.75	1.30
	Inhibin A	1.78	1.15	1.16	1.11	1.86*	1.34
	Activin BA	1.50	-5.54*	-3.81	1.17	-2.59*	1.53
	Activin BB	1.20	-1.61*	-2.81*	-1.21	1.33	1.01
Liver	*ER-α*	-8.46**	-4.23*	-8.47**	1.09	-2.42***	-5.16***
	ER-β	1.30	-1.74	1.11	1.12	1.09	1.20
	AR-α	-2.04	-1.27	-1.36	-1.08	-1.07	-1.16
	VTG I	1.96	-2.54	-63.2*	-5.23	5.68	-125**
	VTG II	-1.81	-20.3***	-355***	-2.47	14.8	-41.1***
	CHG H	1.22	-1.31	-19.6**	-2.56	5.57	-178***
	CHG HM	-2.19	-17.0***	-61.2***	-2.37	-2.37*	-52.0***
	CHG L	-71.4***	-62.1***	-32.1***	-1.17	-1.34	-34.8***
	Annexin max2	1.41	3.27**	1.68	-1.17	1.43	1.39

[a] animal replicate (n= 4-6).

[b] * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 3.2. Transcriptional response profiles of HPG axis pathways in medaka fish exposed to 2.0 μg TRB /L. Gene expression was expressed as the fold change comparing to the corresponding solvent controls [a,b,c]

		Female			Male		
Tissue	gene	8 h	32 h	7 d	8 h	32 h	7 d
Brain	*ER-α*	-1.14	1.08	-1.12	1.01	-1.05	-1.13
	ER-β	-1.93*	-1.11	1.63	1.14	1.71	1.07
	NeuropepY	-1.19	-1.18	1.03	1.84	-1.21	1.13
	mfGnRH	-1.8	1.08	-1.16	2.15**	-1.07	1.45
	sGnRH	-1.27	-1.46	1.35	1.39	1.08	1.09
	cGnRH II	1.1	1.37	1.56	-1.12	1.1	1.04
	GnRH RI	-1.48	1.34	-2.12**	1.16	1.03	1.44
	GnRH RII	-1.16	1.06	-1.07	1.04	1.13	-1.36**
	GnRH RIII	-1.22	1.04	-1.32*	-1.00	1.42**	-1.06
	GTHa	-1.28	-1.52	-2.85	-1.13	-1.66	-1.33
	LH-β	1.01	-2.15	-3.68	1.03	-1.59	-2.36
	CYP19B	1.04	-1.75*	-1.95**	1.17	-1.41	-1.16
Gonad	*ER-α*	2.51*	1.01	1.20	-1.15	2.72***	-1.22
	ER-β	1.53	-1.73	-1.75	-1.35	1.75*	-1.11
	AR-α	2.16	-2.31***	-1.58	-1.31	1.56*	-1.27
	FSHR	3.69***	1.73	1.86	-1.16	2.22*	-1.4
	LHR	7.61	1.1	1.07	-13.6**	-2.07	1.02
	HDLR	1.42	-2.57***	-3.45*	-1.14	2.37*	-1.48
	LDLR	2.21**	-1.09	2.00	-2.53**	1.02	-1.17
	HMGR	-2.8	-3.59	-4.32	-14.9*	1.80*	-1.34
	StAR	-1.16	-2.99*	-1.09	-1.34	-1.51	-1.34
	CYP11A	-2.2	-3.17**	-1.81	1.10	-1.09	-1.61*
	CYP11B	-3.04	-3.76	-3.67	-1.46	1.02	-1.47
	CYP17	-1.58	-4.05***	-2.48	-1.02	1.62	-2.12*
	CYP19A	2.97*	1.35	3.05	1.29	-1.39	-1.20
	CYP21	-2.03	-2.51	-2.16	-2.12	-1.31	-1.22
	20 β-HSD	1.13	-1.11	1.08	1.28	1.25	-1.31
	3β-HSD	3.18**	1.24	-1.01	-1.46	1.89	-1.47
	CYP3A	-11.9	-10.7	-8.14*	-1.12	5.09	-1.28
	Inhibin A	3*	-1.27	-1.14	-1.16	2.06*	-1.68
	Activin BA	-3.17	-4.86	-4.49	-1.09	-2.04	1.10
	Activin BB	-1.11	-2.17***	-3.56*	-1.68	1.46*	-1.08
Liver	*ER-α*	1.57	-8.08*	-5.8*	2.74*	-2.72***	-2.19*
	ER-β	2.09	-3.09	-1.71	-1.07	-1.00	2.09
	AR-α	1.8	-3.25	-2.35	-1.06	-1.73	1.04
	VTG I	3.41*	-4.45	-52.7*	6.91	1.92	-115***
	VTG II	1.03	-29.8***	-145***	13.9	1.19	-16.9***
	CHG H	1.98	-5.97***	-73.7***	1.66	1.43	-86.7***
	CHG HM	1.23	-24.1***	-84.4***	2.84	-7.51***	-29.8***
	CHG L	-1.28	-119***	-93.0***	1.76	-2.17	-25.0***
	Annexin max2	-1.04	1.73	1.47	1.09	1.16	1.66

[a] animal replicate (n= 4-6).

[b] * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 3.2 Striped view of Time dependent response profile in female Japanese medaka exposed to 50 μg FAD/L. The legend listed in the upper right corner of the graph describes the order of the three sampling time points and the eight colors designating different fold thresholds. LH, lutinizing hormone; FSH, follicle-stimulating hormone; E2, 17β-estradiol; T, testosterone; HDL, high-density lipid; LDL, low-density lipid.

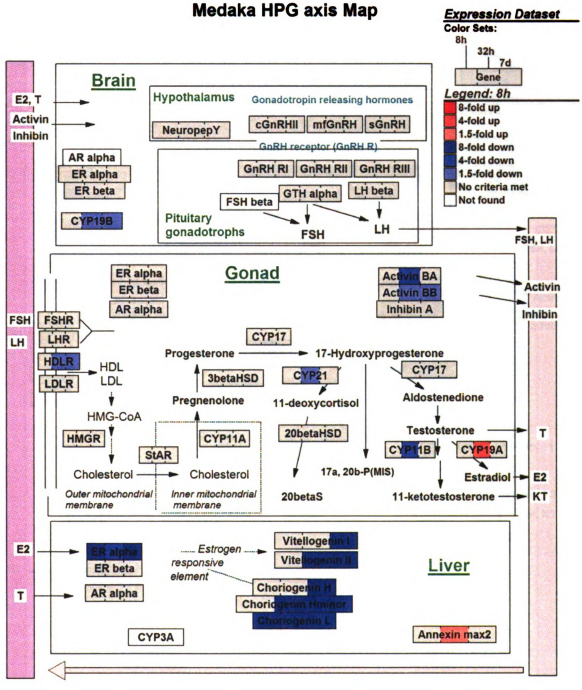
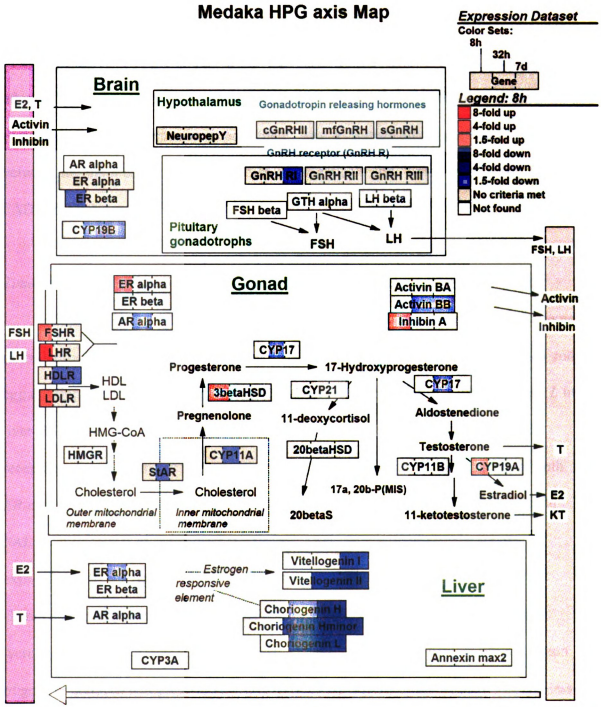


Figure 3.3 Striped view of Time dependent response profile in female Japanese medaka exposed to 2.0 µg TRB/L. The legend listed in the upper right corner of the graph describes the order of the three sampling time points and the eight colors designating different fold thresholds. LH, lutinizing hormone; FSH, follicle-stimulating hormone; E2, 17β-estradiol; T, testosterone; HDL, high-density lipid; LDL, low-density lipid.



RESULTS

Chemical induced effects on medaka fecundity

Both exposures to TRB and to FAD reduced the egg production of Japanese medaka in a time-dependent manner. There were no differences among daily production of eggs in the control group during the exposure. However, fewer eggs were produced after exposure to 2 μg TRB /L or 50 μg FAD /L for 3 or more days (Figure 3.1). Fecundity was time-dependent; and there was a statistically significant interaction between the main classification variables of "chemical" and "time" ($p < 0.001$) for both FAD and TRB.

Time course of HPG gene expression profiles: FAD

Exposure to 50 μg FAD /L caused time-dependent changes in expression of some of the genes studied. Exposure to FAD down-regulated expression of yolk precursor and egg envelop precursor genes, including *VTG I, VTG II, CHG H, CHG HM and CHG L* in liver of both sexes (Table 3.1, Figure 3.2). Expression of *ER-α* was the only steroid receptor in liver of females that was significantly affected by FAD. Expression of both *ER-α* and *CHG L* were significantly down-regulated in the liver of females exposed to FAD relative to that of unexposed females after 8 h. While down-regulation of expression of most genes in the liver of females, was like that of *CHG H/HM, VTG* changed only slightly as a function of time while for some genes such as ER-α, there were no changes in effects as a function of time. In the case of *CHG L* down-regulation of gene expression was greatest after 8 h, with the magnitude of the effect becoming less

as a function of exposure duration. Annexin max2 was 3.3-fold greater than that of the control after 32 h. Down-regulation of gene expression of genes in the liver occurred earlier in females than in males. *ER-α* in liver of males was not affected after 8 h. The effect of FAD on *VTG I* in liver of males was greater than that in females while the effect on *VTG II* was less than that in females.

Changes in gene expression in the gonads of male and female medaka exposed to FAD were generally less responsive than those observed in the liver. Exposure to FAD for 8 h caused no statistically significant alteration in expression of any of the genes analyzed in ovary. However, *HDLR, CYP21, CYP11B* and the *activin*s were down-, and *CYP19A* was up-regulated in ovaries after 32 h of exposure. After 7 d exposure to FAD, ovarian *CYP3A, HDLR* and *Activin BB* were significantly down-regulated while *CYP19A* was up-regulated 4-fold (p-value < 0.068). Effects of FAD on the testis were broader and more evident than those observed in ovaries. *LDLR* and *LHR* were significantly down-regulated in testis after 8 h. After 32 h *CYP21* and *Activin BA* were significantly down-regulated. Genes that were up-regulated after 32 h of exposure included the testicular hormone receptors *ER-α, ER-β, AR-α, FSHR*, steroidogenic genes, including *HMGR, StAR, CYP17, CYP11B, and 3β-HSD, HDLR*, and inhibin A. After 7 d *StAR* and *CYP11B* were the two genes for which significant up-regulation in expression occurred in the testis.

Exposure to 50 μg FAD/L caused a time-dependent down-regulation in expression of *CYP19B* in brains of both males and females. The other gene for which expression was significantly altered in the female brain was *GTHα* with a 3.84-fold up-regulation after 7 d. *ER-β* was the only other gene that was significantly affected in male

brain (2.3-fold up-regulation). This effect, however, occurred only after 32 h of exposure to FAD.

Time course of HPG gene expression profiles: TRB

TRB affected genes in the liver and gonad of both male and female medaka. 17β-trenbolone down-regulated expression of genes coding for yolk precursor and egg envelope in liver of both male and female medaka in a time-dependent fashion (Table 3.2, Figure 3.3). However, down-regulation in TRB exposure only occurred after 32 h. After 8 h, *VTG I* in females and *ER-α* in males were slightly up-regulated in liver. In ovary, significant up-regulation after 8h was observed for the receptors (*ER-α, FSHR* and *LDLR*), some steroidogenic enzymes (*3β-HSD, CYP19A*) and Inhibin alpha. In contrast, after 32 h down-regulation was observed for ovarian expression of *AR, CYP11A, CYP17, HDLR, StAR* and *Activin BB*. After 7 d, *CYP3A, HDLR* and *Activin BB* were significantly down-regulated in liver of TRB exposed females. In testis, *LHR, LDLR* and *HMGR* were down-regulated by TRB after 8 h, while the up-regulated genes at 32 h included *ERA, HDLR* and *inhibin-α*.

Some genes were affected by TRB exposure in the brain of both male and female medaka. 17β-trenbolone caused time-dependent down-regulation of *CYP19B* transcripts in brain of females but not males. 17β-trenbolone also down-regulated female *ER-β* at 8 h, and *GnRH RI* and *GnRH RIII* at 7 d. *GnRH RII* was down-regulated in male brain at 7 d, but *mfGnRH* and *GnRH RIII* were down-regulated at 8 h and 32 h, respectively, though to a less extent.

DISCUSSION

Fadrozole exposure

FAD is a potent aromatase inhibitor and has been shown to suppress production of estrogen, 17β-estradiol (E2), in different *in vitro* and *in vivo* systems including the human H295R cell line and gonad tissues of different fishes [8, 10, 16]. The decreased E2 production caused by exposure to FAD is due to its inhibition of aromatase enzyme activity, which catalyzes the conversion of C19 androgens to C18 estrogens such as E2. In a study with adult fathead minnow that were exposed to increasing concentrations of FAD between 2 and 50 μg /L in a short-term (21 d) study, a concentration-dependent reduction in fecundity was observed [10]. Similarly, exposure to 50 μg FAD /L significantly reduced fecundity in Japanese medaka. In accordance with the reduced egg production, time–dependent down-regulation of egg precursor genes including VTGs and CHGs was observed in liver of FAD exposed females (Figure 3.2). Prior to the decrease of *VTG I/II* and *CHG H/HM*, FAD exposure first down- regulated the expression of *ER-α* in liver of females after 8 h. These results not only confirm the primary role of *ER-α* in the transcriptional regulation of egg precursor in Japanese medaka [13], but also suggest that exposure to 50 μg FAD /L could decrease the endogenous E2 concentration as early as within 8 h. Furthermore, males and females display different patterns of gene expression in the exposure of FAD. Down-regulation of *ER-α* and egg precursor genes in males were slower than for females to FAD-caused decrease of E2 concentration, which is consistent to the key function of E2 in female reproduction. In FAD exposed females expression of *CHG L* decreased earlier than *VTG*s. Conversely, *CHG HM* in liver of

male medaka responded more rapidly than the other *VTG* and *CHG* genes. These results suggest that the regulatory mechanisms of *VTG*s and *CHG*s were different in male and female medaka.

Ovary response of gene expression compensates the inhibitory effect of FAD on E2 production in Japanese medaka. The up-regulation of *CYP19A* transcription is apparently a compensatory response to cope with reduced E2 synthesis. Because FAD inhibits the conversion of C19 androgens to C18 E2, it may result in excess production of androgen, which is confirmed by the down-regulation of ovarian *HDLR* and *CYP11B* transcripts in FAD exposed Japanese medaka at 32 h. CYP11B mRNA encodes for the key enzyme involved in synthesis of 11β-hydro-testosterone, the direct precursor of the active non-aromatizable teleost androgen 11-ketotesterone. *HDLR* is one of the key transport proteins for cholesterol, precursor for all steroids. If the down-regulated transcription of *HDLR* and *CYP11B* in ovary by FAD lead to a correspondingly less production of functional proteins, the activity of 11-ketotesterone would be expected to be less.

Gonadal transcriptional responses also explain the other adverse effects observed in FAD exposed fish. For example, transcription of *CYP11B* and the cholesterol transferring protein, *StAR* were up-regulated at 7 d, which could potentially lead to the increased production of 11-ketotesterone (KT) and/or testosterone (T). If the testicular response to FAD is similar between the Japanese medaka and fathead minnow, this result explains the observed increase of plasma T and KT concentrations and marked accumulation of sperm in the testes of FAD exposed fathead minnow [10]. In female, the down-regulation of ovarian *activin BA* and *activin BB* might be connected to the retarded

oocyte maturation observed in FAD-exposed Japanese medaka [12]. Activins are dimeric proteins consisting of two *inhibin β* subunits, *BA* and *BB*. And the three forms of activins, *activin-A*, *-B* and *-AB*, are produced by homo- and hetero-dimerization of the two inhibin β subunits. In vertebrates, activins have been identified as important regulators of the reproductive axis [17]. In fish, the activin system has also been indicated to be involved in gonadotropin-regulated ovarian functions, such as oocyte maturation. Specifically, it has been suggested that *activin-A* mediates gonadotropin-induced oocyte maturation in zebra fish [17]. Our results support the hypothesis that the retarded oocyte maturation by FAD exposure could be related to the inhibition of *activin* gene expression.

Brain response is also consistent to the inhibitory effect of E2 production by FAD. Fadrozole exposure reduced the expression of brain aromatase (*CYP19B*) transcript in both males and females. *CYP19B* mRNA reduction has also been observed in FAD treated fathead minnow [8]. Different from *CYP19A*, teleost brain *CYP19B* is regulated by estrogen responsive element (ERE) [18]. Therefore, FAD exposure reduces the local E2 concentration in brain by both inhibiting brain aromatase activity and less circulating E2 level due to inhibited ovarian aromatase activity.

17-β-trenbolone exposure

17-beta-trenbolone is the primary metabolite of trenbolone acetate, which is used to promote growth in cattle. 17β-trenbolone has been characterized as a potent androgen in both in vitro and in vivo studies with mammals and fish [11]. Although TRB has different biochemical properties from FAD, it induced similar response in Japanese medaka, such as less fecundity and down-regulation of egg precursor genes in liver.

These observations can be explained by decreased endogenous E2 production by TRB exposure. Reduced plasma steroid (T and E2) and *VTG* concentrations have been observed in females of TRB treated fathead minnow, [11]. It has been postulated that exposure to exogenous androgen such as TRB leads to the compensatory response of decreased endogenous androgen (T and KT) production and in turn, the decreased E2 production since E2 is converted from T by *CYP19* aromatase in vertebrates [19]. Similar to FAD, TRB eventually elicits less E2 level and greater concentration of 'functional' androgen. In TRB exposed females, the less E2 not only resulted in the down-regulation of brain aromatase (*CYP19B*) and ovarian activin BB, but also elicited compensatory responses, including greater ovarian *CYP19A* and less *CYP3A*. While testicular *LHR, LDLR* and *HMGR* were down- regulated at 8 h to compensate androgenic TRB exposure by slowing down steroidogenesis.

However, TRB treated Japanese medaka also displayed gene expression patterns different from what were observed in FAD exposure, which can be explained by the different dynamic change of E2 level in FAD or TRB exposed fish (Figure 3.3). Because of the direct inhibition of aromatase by FAD, we hypothesize that FAD -induced estrogen reduction can be more instant than that of TRB, which indirectly repress estrogen production by inhibited its precursor testosterone. This subtle difference between the two chemicals can be observed at the time –dependent gene expression changes in Japanese medaka. For example, TRB exposure decreased expression of *ER-α*, *VTG*s and *CHG*s transcripts in liver of females after 32h, instead of 8 h in case of FAD. Nevertheless, significant increases of *ER-α* in males and *VTG I* in females were observed in TRB treatment at 8 h. This leads to the hypothesis that TRB exposure might initially cause a

temporary increase in the availability of aromatizable androgen, which in turn leads a slight increase of E2 production. The temporary E2 production by TRB exposure up-regulated the expression of ovarian *ER-α, FSHR, LHR* and *inhibin A* at 8 h. *CYP19B* transcripts in brain are highly sensitive in response to estrogen exposure [20]. But *CYP19B* did not respond to TRB exposure in brain of males, but was down-regulated by FAD, which suggests that the tempered decrease of estrogen by TRB exposure might not affect the endogenous E2 level in brain. On the other hand, TRB exposure might cause androgen 'surge' more rapidly than that of FAD. Compensatory response to the exposure of androgenic TRB could be found in the reduction of gonadal *CYP17*, the key enzyme synthesizing androstenedione, the direct precursor of testosterone. Decreased plasma concentrations of 11-ketotestosterone has been observed in TRB exposed male fathead minnows after 21 d exposure [11]. However, such alteration could not be seen in the FAD treatment.

Overall, the present study examined the molecular responses in the medaka HPG axis to exposure of anabolic androgen TRB and aromatase inhibitor FAD. Fadrozole induced a more rapid reduction of hepatic estrogen-responsive pathways than did TRB, while exposure to FAD and TRB resulted in rapid (after 8 h) down-regulation of *LHR* and *LDLR* in the testis to compensate excessive androgen level. The time-dependent molecular responses by FAD and TRB developed in the present study not only help to elucidate the mechanism of the reduced fecundity, but also present unique signatures for the two model chemicals. Overall the results from this study demonstrated the great utility of systematic approach of HPG axis real time PCR array in the testing of potential EDCs using Japanese medaka.

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REFERENCES

[1] Hutchinson TH, Ankley GT, Segner H, Tyler CR. 2006. Screening and testing for endocrine disruption in fish-biomarkers as ""signposts"" not ""traffic lights"" in risk assessment. *Environ Health Perspect* 114: 106-114.

[2] Ankley GT, Daston GP, Degitz SJ, Denslow ND, Hoke RA, Kennedy SW, Miracle AL, Perkins EJ, Snape J, Tillitt DE, Tyler CR, Versteeg D. 2006. Toxicogenomics in regulatory ecotoxicology. *Environ Sci Technol* 40: 4055-4065."

[3] Fielden MR, Kolaja KL. 2006. The state-of-the-art in predictive toxicogenomics. *Curr Opin Drug Discov Devel* 9: 84-91.

[4] Miller DH, Jensen KM, Villeneuve DL, Kahl MD, Makynen EA, Durhan EJ, Ankley GT. 2007. Linkage of biochemical responses to population-level effects: a case study with vitellogenin in the fathead minnow Pimephales promelas. *Environ Toxicol Chem* 26: 521-527.

[5] Thorpe KL, Benstead R, Hutchinson TH, Tyler CR. 2007. Associations between altered vitellogenin concentrations and adverse health effects in fathead minnow Pimephales promelas. *Aquat Toxicol* 85: 176-183.

[6] Jones PD, Coen WD, Tremblay L, Giesy JP. 2000. Vitellogenin as a biomarker for environmental estrogens. *Wat Sci Technol* 42. 1-14.

[7] Yamaguchi A, Ishibashi H, Kohra S, Arizono K, Tominaga N. 2005. Short-term effects of endocrine-disrupting chemicals on the expression of estrogen-responsive genes in male medaka Oryzias latipes. *Aquat Toxicol* 72: 239-249.

[8] Villeneuve DL, Knoebl I, Kahl MD Jensen KM Hammermeister DE Greene KJ Blake LS Ankley GT. 2006. Relationship between brain and ovary aromatase activity and isoform-specific aromatase mRNA expression in the fathead minnow Pimephales promelas. *Aquat Toxicol* 76: 353-368.

[9] Villeneuve DL, Miracle AL, Jensen KM, Degitz SJ, Kahl MD, Korte JJ, Greene KJ, Blake LS, Linnum AL, Ankley GT. 2007. Development of quantitative real-time PCR assays for fathead minnow Pimephales promelas. gonadotropin beta subunit mRNAs to support endocrine disruptor research. *Comp Biochem Physiol C Toxicol Pharmacol* 145: 171-183.

[10] Ankley GT, Kahl MD, Jensen KM, Hornung MW, Korte JJ, Makynen EA, Leino RL. 2002. Evaluation of the aromatase inhibitor fadrozole in a short-term reproduction assay with the fathead minnow Pimephales promelas. *Toxicol Sci* 67: 121-130.

[11] Ankley GT, Jensen KM, Makynen EA, Kahl MD, Korte JJ, Hornung MW, Henry TR, Denny JS, Leino RL, Wilson VS, Cardon MC, Hartig PC, Gray LE. 2003. Effects of the androgenic growth promoter 17-beta-trenbolone on fecundity and reproductive endocrinology of the fathead minnow. *Environ Toxicol Chem* 22: 1350-1360.

[12] Tompsett AR, Park J, Zhang X, Jones PD. Newsted JL, Au DWT, Chen EXH, Yu RMK, Wu RSS, Kong RYC, Giesy JP, Hecker M. 2007. Development and validation of an in situ hybridization system to detect gene expression along the HPG-axis in Japanese medaka Oryzias latipes. *Aquat Toxicol* (submitted).

[13] Zhang X, Hecker M, Park J, Tompsett AR, Nakazawa K, Jones PD, Newsted JL, Au DWT, Kong RYC, Wu RSS, Giesy JP. 2008. Development and validation of a PCR array method to study chemical induced effects on Brain-Hepatic-Gonadal axis of the Japanese medaka. *Aquat Toxicol* (Accepted).

[14] Villeneuve DL, Larkin P, Knoebl I, Miracle AL, Kahl MD, Jensen KM, Makynen EA, Durhan EJ, Carter BJ, Denslow ND, Ankley GT. 2007. A graphical systems model to facilitate hypothesis-driven ecotoxicogenomics research on the teleost brain-pituitary-gonadal axis. *Environ Sci Technol* 41: 321-330.

[15] Zhang X, Yu RM, Jones PD, Lam GK, Newsted JL, Gracia T, Hecker M, Hilscherova K, Sanderson T, Wu RS, Giesy JP. 2005. Quantitative RT-PCR methods for evaluating toxicant-induced effects on steroidogenesis using the H295R cell line. *Environ Sci Technol* 39: 2777-2785

[16] Hecker M, Newsted JL, Murphy MB, Higley EB, Jones PD, Wu R, Giesy JP. 2006. Human adrenocarcinoma H295R. cells for rapid in vitro determination of effects on steroidogenesis: hormone production. *Toxicol Appl Pharmacol* 217: 114-124

[17] DiMuccio T, Mukai ST, Clelland E, Kohli G, Cuartero M, Wu T, Peng C. 2005. Cloning of a second form of activin-betaA cDNA and regulation of activin-betaA subunits and activin type II receptor mRNA expression by gonadotropin in the zebrafish ovary. *Gen Comp Endocrinol* 143: 287-299.

[18] Pellegrini E, Menuet A, Lethimonier C, Adrio F, Gueguen MM, Tascon C, Anglade I, Pakdel F, Kah O. 2005. Relationships between aromatase and estrogen receptors in the brain of teleost fish. *Gen Comp Endocrinol* 142: 60-66.

[19] Miracle A, Ankley G, Lattier D. 2006. Expression of two vitellogenin genes vg1 and vg3. in fathead minnow Pimephales promelas. liver in response to exposure to steroidal estrogens and androgens. *Ecotoxicol Environ Saf* 63: 337-342.

[20] Kuhl AJ, Manning S, Brouwer M. 2005. Brain aromatase in Japanese medaka (Oryzias latipes): Molecular characterization and role in xenoestrogen-induced sex reversal. *J Steroid Biochem Mol Biol* 96: 67-77.

Chapter 4

Responses of the Medaka HPG axis PCR array and reproduction to prochloraz and ketoconazole

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ABSTRACT

Effects of two model imidozole-type fungicides, prochloraz (PCZ) and ketoconazole (KTC), on the hypothalamic-pituitary-gonadal (HPG) axis of the Japanese medaka (*Oryzias latipe*) were examined by use of real time PCR (RT-PCR) array. Fourteen-wk-old Japanese medaka were exposed for 7 d to concentrations of PCZ or KTC from 3.0 to 300 μg /L. Exposure to KTC or PCZ caused significantly less fecundity of Japanese medaka and down-regulated expression of *estrogen receptor (ER)-α* and egg precursors in livers of males and females. These effects are consistent with inhibition of gonadal 17-β estradiol (E2) and testosterone (T) production by both KTC and PCZ. However, PCZ was more potent than KTC both in modulating transcription and causing lesser fecundity. Exposure to 30 μg PCZ/L resulted in 50% less fecundity and significant down-regulation of *VTG II* expression, but KTC did not cause such effects at this concentration. Exposure to PCZ caused a compensatory up-regulation in *CYP19A* and *CYP17* expression in the ovary, while KTC did not. The ecologically relevant endpoint, fecundity was log-log related to mRNA level of six genes in livers of females.

Keywords: imidozole, vitellogenin, fecundity, HPG axis RT-PCR array, potency.

INTRODUCTION

Concerns about possible links between natural and man-made substances on endocrine and reproductive systems in humans and wildlife (1,2) have resulted in various international agencies initiating projects to develop new guidelines for the screening and testing of potential endocrine disrupting chemicals in vertebrates (3,4,5). These assays include: 1) structure activity relationships and *in vitro* assays that could be used to identify potential chemical candidates; 2) short-term *in vivo* assays to demonstrate relevant activity in intact animal models; and 3) long-term assays that evaluate different reproductive and developmental stages of animals (6). Small teleost fish, such as the Japanese medaka (*Oryzias latipes*) and fathead minnow (*Pimephales promelas*), are appropriate models for testing endocrine disrupting chemicals (EDCs), not only from the perspective of existing ecological impacts, but also in terms of among-species extrapolation. The Japanese medaka is a promising small fish model test organism because individuals are small and can be easily reared and brought into reproductive condition. Large numbers of fish can be cultured and tested in a small area. The physiology, embryology, and genetics of the medaka are well known because they have been extensively studied for more than 100 yr (7). The Japanese medaka has clearly defined sex chromosomes and sex determination (7). In addition, all mRNA/cDNA sequences used for this project, which were necessary to design appropriate RNA probes, are available online in the NCBI database (www.ncbi.nlm.nih.gov). Finally, there is a marine species of medaka (*Oryzias melastigma*) that is very similar to the freshwater

species, such that these two species simultaneously provides a test system that can be applied to freshwater, marine, and brackish ecosystems (8).

System models utilizing genomic approaches can be useful tools for mechanistic toxicological studies of EDCs. It has been suggested that mechanistic information derived from changes in molecular or biochemical biomarkers can be used to aid in extrapolation of effects among species and chemicals (9,10). However, it is important to understand linkages between alteration at the molecular and biochemical levels and ecological relevance of adverse effects at the individual and population levels that might relate to fitness. We have previously developed an HPG-axis-based real time PCR (RT-PCR) array system using the Japanese medaka (11). To evaluate the chemical-induced effects on reproductive endocrinology, the HPG axis-based RT-PCR array systematically examines the transcriptional expression of 36 functionally relevant genes in brain, gonad and liver of Japanese medaka. In addition, reproductive performance, including fertility and fecundity are observed during the exposure. This method not only furthers our understanding of chemical modes of action along the HPG axis, but also provides opportunity to examine the relationship between transcriptional responses of HPG axis and fecundity in Japanese medaka.

Two imidozole fungicides ketoconazole (KTC) and prochloraz (PCZ) were chosen as model chemicals to assess the utility of the HPG-axis-based real time PCR array system and reproductive performance of the Japanese medaka. These commonly used fungicides have been reported to affect reproduction and development in fish and wildlife (12,13). Imidazole fungicides were designed to inhibit a cytochrome P450 (*CYP*) enzyme involved in ergosterol synthesis of fungi (14). However, it has also been shown

that these fungicides can inhibit other *CYP* genes, including steroidogenic cytochrome P450 c17αhydroxylase, 17,20-lyase (*CYP17*) and aromatase (*CYP19*) in mammals and fish (15,16). Recently, KTC and PCZ have been shown to reduce both 17β-estradiol (E2) and/or testosterone (T) production in vitro in H295R human adenocarcinoma cells and fathead minnow ovary explants (17,18). Although these chemicals are structurally similar, they have also displayed different modes of action (MOA) on other biological pathways within the reproductive tract and the HPG axis. For example, PCZ, but not KTC, has been identified as an *androgen receptor* (*AR*) antagonist in rat and fathead minnow (19,20).

The objective of this study was to evaluate the effects of the two fungicides, KTC and PCZ, during a short-term exposure, on the transcriptional profiles of the key pathways within the medaka HPG axis. It was hypothesized that the observed different transcriptional responses by the two chemicals are due to different modes of action on the HPG axis of Japanese medaka. In the present study, the quantitative relationship between hepatic transcriptional responses and egg reproduction of medaka was investigated during 7-d laboratory exposures.

MATERIALS AND METHODS

Animals and Exposure

Male and female wild-type *O. latipes* were maintained in flow-through tanks in conditions that facilitated breeding (23-24 °C; 16:8 light/dark cycle) using the protocol previously described (11,21,22). Before exposure, 14-wk-old, Japanese medaka were acclimated in 10-L tanks filled with 6 L of carbon-filtered water for a period of 7 d prior to initiation of experiments. Each tank contained 5 male and 5 female fish. One half of the water in each tank (3 L) was replaced daily with fresh carbon-filtered water. Ketoconazole (KTC) and prochloraz (PCZ) were obtained from Sigma-Aldrich (St. Louis, MO) and dissolved in the least amount of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO) possible to produce a stock solution of known concentration. After the acclimation period, fish were exposed to vehicle control (DMSO with a final concentration of 1:10000 v/v water), 3.0, 30 and 300 μg KTC/L, or 3.0, 30 and 300 μg PCZ/L in a 7-d static renewal exposure scenario. Exposures started at 8:30 AM and one half of the water in each tank (3L) was replaced with fresh carbon-filtered water dosed with the appropriate amount of chemicals at 8:30 AM each day during exposure. Eggs produced during the previous 24 hr period were counted and recorded before the replacement of water. No mortalities were observed at any treatment during the exposure period. Fish were euthanized in Tricaine S solution (Western Chemical, Ferndale, WA, USA), and total weight and snout-vent length were recorded for each fish. Samples of brain, liver, and gonads were collected and preserved in RNA*later* storage solution (Sigma, St Louis, MO) at -20 °C until analysis of gene expression. Masses of body, brain,

liver and gonad of each fish were recorded. Indices including, hepatic-somatic index (HSI), gonadal-somatic index (GSI), and brain-somatic index (BSI) were calculated.

Real time –PCR array measurement

Processing of tissues followed the previously reported protocol (11). Briefly, total RNA was individually extracted from tissues according to the manufacture's protocol with Agilent Total RNA Isolation Mini Kit (Agilent Technologies, Palo Alto, CA). First-strand cDNA synthesis was performed using Superscript III first-strand synthesis SuperMix and Oligo-dT primers (Invitrogen, Carlsbad, CA). The measurement of gene expression in brain, liver and gonad tissues was conducted using the medaka HPG axis PCR array system described previously (11). Briefly, real-time Q-RT-PCR was performed by using a 384-well ABI 7900 high throughput real time PCR System (Applied Biosystems, Foster City, CA). PCR reaction mixtures for one hundred reactions contained 500 μL of SYBR Green master mix (Applied Biosystems, Foster City, CA), 2 μL of 10 μM sense/anti-sense gene-specific primers, and 380 μL of nuclease-free distilled water (Invitrogen). A final reaction volume of 10 μL was made with 2 μL of diluted cDNA and 8 μL of PCR reaction mixtures using a Biomek automation system (Beckman Coulter, Inc., Fullerton, CA). Quantification of target gene expression was based comparative cycle threshold (*C*t) method with adjustment of PCR efficiency according to a previous study (23). The average ct value of the three reference genes (*beta-actin*, RPL-7 and 16s) was used as reference for the expression calculation of target genes

Statistical analyses

Statistical analyses were conducted using the R project language (http://www.r-project.org/). Fecundity data was analyzed using analysis of variance (ANOVA), in which the effects of time (day) and chemicals on the daily recorded egg production were examined. Levels of expression of genes in tissues were expressed as the fold change relative to the average value of the average of the vehicle control. Prior to conducting statistical comparisons of gene expression value Bartlett Test were performed to check homogeneity of variances of data. Normality of the distributions of data was evaluated by Shapiro-Wilk's test. If necessary, data was log-transformed to approximate normality. Differences of relative gene expressions among treatments were evaluated by ANOVA followed by pair-wise t-test. Differences with $p < 0.01$ were considered to be significant.

Table 4.1 Transcriptional response profiles of HPG axis pathways in medaka fish exposed to prochloraz (PCZ). Gene expression was expressed as the fold change comparing to the corresponding vehicle controls [a,b]

		Female			Male		
Tissue	gene	3 μg /L	30 μg /L	300 μg /L	3 μg /L	30 μg /L	300 μg /L
	ER-α	-1.06	1.79	-1.26	1.78	2.97*	1.06
	ER-β	1.15	1.05	1.15	-1.07	1.17	-1.17
	AR-α	1.03	-1.22	-1.08	1.16	1.14	1.13
	NPY	-1.58	-1.2	-1.42	-1.06	1.03	-1.35
Brain	*cGnRH II*	1.96*	-1.35	1.35	1.22	-1.1	1.06
	mfGnRH	-1.19	1.85	-1.69	1.7	3.66*	1.71
	sGnRH	-1.8*	-1.38	-1.26	1.32	1.46	-1.02
	GnRH RI	1.07	1.63	-1.46	1.65	2.6	1.47
	GnRH RII	-1.29	-1.49**	-1.31	1.14	1.02	1.09
	GnRH RIII	-1.17	-1.27	-1.53*	1.21	1.17	-1.07
	CYP19B	-1.09	-1.05	-2.69***	-1.19	-1.61**	-2.76***
	ER-α	-1.26	-1.5	-1.09	1.25	-1.02	1.22
	ER-β	-1.39	-1.68	-2.26*	1.25	1.06	1.35
	AR-α	-1.29	1.01	1.02	1.3	1.14	1.81**
	FSHR	-1.19	1.1	1.39	2.12	1.43	1.34
	LHR	-1.74	-1.46	1.01	1.54	1.31	-1.11
	HDLR	-1.05	-1.02	-2.74*	1.59	1.33	1.12
	LDLR	1.01	1.05	1.04	1.25	1.53*	1.28
	HMGR	-1.76	-2.39	-3.73*	-1.19	-1.5	-1.62*
Gonad	*StAR*	-1.36	-1.0	-1.04	1.53	-1.03	1.4
	CYP11A	-1.04	1.34	1.33	1.62	1.46	2.66*
	CYP11B	-1.74	-2.57	-4.0*	1.65	1.75	3.02**
	CYP17	1.19	1.8*	3.46***	1.51	1.58	3.48**
	CYP19A	-1.39	2.46**	2.38**	2.48*	2.76	1.84
	CYP3A	-1.02	-1.4	-1.86	-3.64	-2.83	-2.34
	3βHSD	-1.67	-1.01	1.42	1.41	1.29	2.39**
	Inhibin A	1.07	1.11	1.28	1.53	-1.05	1.22
	ActivinBA	-2.12	-3.09*	-8.33*	2.57*	4.41**	2.04
	ActivinBB	-1.09	-1.2	-1.03	1.66	1.53	1.09
	ER-α	-1.0	-1.27	-54.5***	1.34	-1.09	-1.72
	ER-β	1.32	2.0	2.02	1.2	1.41	1.88**
	AR-α	1.3	1.69	-1.78	1.01	-1.11	-1.33
	VTG I	1.07	-2.07	-58.7***	3.66*	2.85	-4.37
Liver	*VTG II*	-1.37*	-3.35***	-4000***	2.72	1.12	-9.11*
	CHG H	1.07	-1.93	-113***	3.49**	2.32*	-5.8*
	CHG HM	-1.32	-2.62**	-877***	1.55	1.3	-10.5***
	CHG L	-1.01	-1.78	-1020***	4.27	2.78	-4.87*
	Annexin.max2	1.6	1.8	2.21*	1.01	-1.32	1.02

[a] animal replicate (n= 4-6).

[b] * $p < 0.01$, ** $p < 0.001$.

Table 4.2 Transcriptional response profiles of HPG axis pathways in medaka fish exposed to ketoconazole (KTC). Gene expression was expressed as the fold change comparing to the corresponding vehicle controls [a,b]

		KTC / Female			KTC /Male		
Tissue	Gene	3 μg /L	30 μg /L	300 μg /L	3 μg /L	30 μg /L	300 μg /L
Brain	*ER-α*	-1.1	-1.27	-1.07	1.52	1.34	1.83
	ER-β	-1.01	1.26	-1.11	1.16	-1.06	-1.01
	AR-α	-1.02	-1.09	-1.12	1.15	-1.13	-1.16
	NPY	1.01	-1.08	-1.04	-2.03	-1.37	-1.05
	cGnRH II	-1.21	1.23	-1.01	-1.46	1.01	1.07
	mfGnRH	-1.52	-1.84	-1.33	2.44	1.33	2.98
	sGnRH	-1.44	-1.68	-1.39	-1.55	-1.08	-1.15
	GnRH RI	-1.03	-1.18	-1.59	1.97	1.39	2.54
	GnRH RII	-1.54*	-1.31	-1.63**	-1.01	-1.06	-1.25
	GnRH RIII	-1.48*	-1.44	-1.67**	1.1	-1.15	-1.19
	CYP19B	1.06	1.14	-1.39	-1.4	-1.33	-1.7*
Gonad	*ER-α*	1.29	-1.61	-1.49	-1.27	-1.21	1.65
	ER-β	-1.78	-1.59	-2.35**	-1.05	-1.15	1.53**
	AR-α	-1.45	-1.32	-2.36**	-1.12	1.2	1.86*
	FSHR	1.31	-1.05	1.21	1.03	1.4	1.61
	LHR	-1.6	-2.25	-3.18**	1.17	1.31	2.09**
	HDLR	-1.17	-1.83	-1.29	1.04	1.38	1.3
	LDLR	-1.06	1.22	-1.45	1.11	1.41	1.75**
	HMGR	-1.75	-2.89*	-3.17*	-1.86*	1.31	-1.31
	StAR	-1.13	-2.03*	-3.14***	-1.78	1.46	1.51
	CYP11A	-1.03	-1.61*	-1.87**	-1.29	-1.43	1.34
	CYP11B	-2.38	-3.17	-7.3**	-1.11	1.22	2.28*
	CYP17	1.17	-1.2	1.25	-1.44	-1.61	1.56
	CYP19A	1.18	-1.39	1.08	-1.07	1.91*	2.17*
	CYP3A	-2.96	-2.47	1.43	-1.15	-13.6	-18.4
	3βHSD	-1.16	-1.63	-2.04*	-1.18	-1.17	1.59
	Inhibin A	1.03	-1.69	-1.55	1.21	1.14	1.56
	ActivinBA	-2.67	-3.01	-8.18**	1.07	1.83	2.82*
	ActivinBB	-1.31	-1.12	-2.01**	1.08	2.13	1.37
Liver	*ER-α*	-1.77*	-1.21	-3.24***	-1.5	-1.96	6.2***
	ER-β	-1.02	1.14	1.28	-1.04	-1.45	1.19
	AR-α	-1.04	1.3*	-2.71***	-1.23	-2.12**	-3.76***
	VTG I	-1.38	-1.49	-54.0***	28.1	3.31	-6.46
	VTG II	-1.46*	-1.08	-79.6***	2.97	1.13	-1.12
	CHG H	-1.6	-1.64	-35.6**	1.3	1.39	-22.1**
	CHG HM	-1.09	1.23	-83.0***	1.39	-1.08	-19.6***
	CHG L	1.11	1.21	-77.8**	2.52	5.96	-1.38
	Annexin max2	-1.17	1.48	12.1***	-1.55	-1.92	6.45***

[a] animal replicate (n= 4-6).

[b] * $p < 0.01$, ** $p < 0.001$.

Figure 4.1 Cumulative fecundity of Japanese medaka exposed to PCZ or KTC for 7 d. In the PCZ exposure, values are means (n=2) of the cumulative number of eggs, expressed on a per female basis. In the KTC exposure, n = 1. Asterisks indicate a significant different from control group: *, $p < 0.05$.

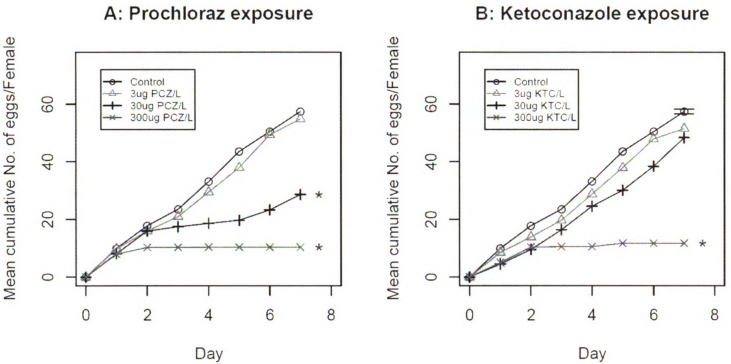


Figure 4.2. Striped view of concentration dependent response profile in PCZ exposure of female Japanese medaka. Gene expression data from medaka treated by 3.0, 30 and 300 µg PCZ/L are shown as striped color sets on the selected endocrine pathways along the medaka HPG axis. The legend listed in the upper right corner of the graph describes the order of the three PCZ concentrations and the eight colors designating different fold thresholds. *LH*, lutinizing hormone; *FSH*, follicle-stimulating hormone; E2, 17β-estradiol; T, testosterone; *HDL*, high-density lipid; *LDL*, low-density lipid.

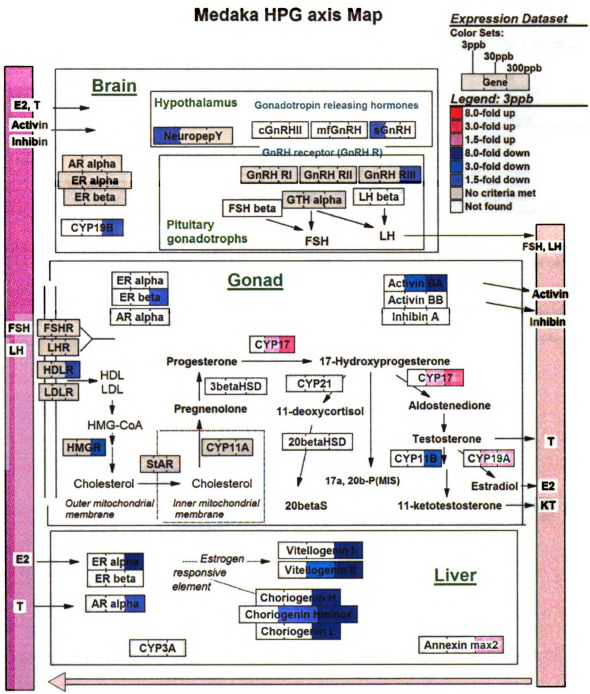
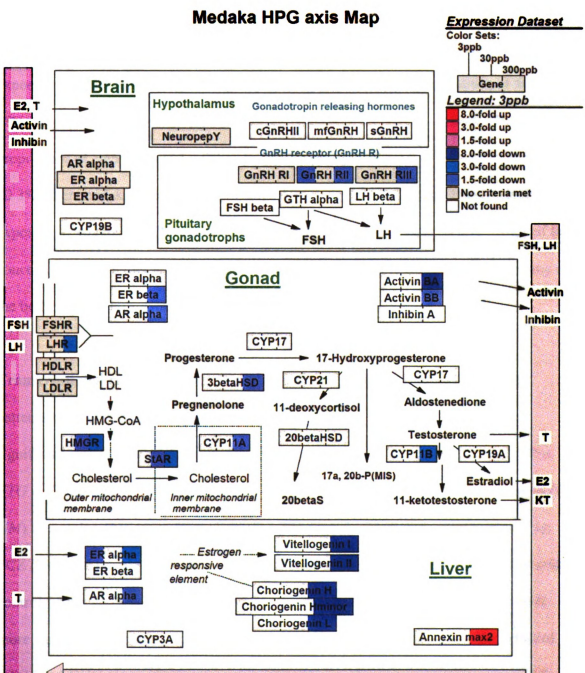


Figure 4.3. Striped view of concentration dependent response profile in KTC exposure of female Japanese medaka. Gene expression data from medaka treated by 3.0, 30 and 300 μg KTC/L are shown as striped color sets on the selected endocrine pathways along the medaka HPG axis. The legend listed in the upper right corner of the graph describes the order of the three KTC concentrations and the eight colors designating different fold thresholds. *LH*, lutinizing hormone; *FSH*, follicle-stimulating hormone; E2, 17β-estradiol; T, testosterone; *HDL*, high-density lipid; *LDL*, low-density lipid.



RESULTS

Fecundity. Statistically significant differences were observed on the cumulative egg productions by medaka from different treatments (Figure 4.1). Both PCZ exposure and KTC exposure induced concentration-dependent decrease of fish fecundity in a time-dependent manner. There was no statistically significant difference between cumulative egg production by fish exposed to 3.0 μg PCZ/L and that of unexposed fish. However, exposure to 30 μg and 300 μg PCZ/L resulted in significantly less fecundities of 50% and 18%, respectively, relative to that of medaka exposed to the vehicle control. The proportions of eggs produced, relative to the solvent control were 89%, 84.2%, and 20.3% when medaka were exposed to 3.0, 30, or 300 μg KTC/L, respectively. Only exposure to 300 μg KTC/L resulted in statistically significant less fecundity. None of the concentrations of either KTC or PCZ caused any statistically significant effects on any of the body indices, HIS, GSI or BSI.

Transcriptional response to prochloraz. Sex- and organ-specific transcriptional patterns were observed in the PCZ treated medaka fish. There was a statistically significant, concentration-dependent down-regulation of hepatic genes including *ER-α, VTG I, VTG II, choriogenin L (CHGL), choriogenin H (CHG H)* and *CHG Hminor* (*CHG HM*) in females exposed to PCZ (Table 4.1, Figure 4.2). Exposure of males to 300 μg PCZ /L caused the greatest (54-fold) less *ER-α* expression, relative to that of the vehicle control. Alternatively, responses of genes in the livers of males exposed to PCZ were more variable and of lesser magnitude than that of the females. Slight up-regulation of *VTG I* and *CHG H* transcript were observed in liver of males exposed to 3.0 μg PCZ/L.

However, exposure to 300 μg PCZ/L significantly down-regulated expression of *VTG II, CHG H*, and *CHG HM* in livers of males. In contrast, exposure of males to 300 μg PCZ/L caused a statistically significant and concentration dependent up-regulation in expression of *ER-β* in livers.

Exposure to PCZ caused differences in transcriptional responses of genes in both ovaries and testis. There were no statistically significant differences between levels of transcription in ovaries from fish exposed to 3.0 μg PCZ /L and that of the vehicle control. Concentration-dependent up-regulation of *CYP17* and *CYP19A* were observed in ovaries of females exposed to PCZ with the significant differences observed in females exposed to 300 μg PCZ /L. Conversely, expression of ovarian *activin BA* was inversely proportional to PCZ exposure concentration. PCZ exposure caused concentration-dependent up-regulation expression of *AR-α* and steroidogenic *CYP11B, CYP11A, CYP17* and *3β HSD* in testes. Expression to 30 μg PCZ /L caused statistically significant up-regulation of *Activin BA* in liver of males.

Exposure to PCZ caused only minor effects in the brains of medaka. PCZ caused dose-dependent down-regulation of brain-type aromatase (*CYP19B*) in both male and female after 7 d of exposure. *GnRH RII* and *GnRH RIII* were also down-regulated in brains of PCZ exposed females, but not in males.

Transcriptional response to ketoconazole. Exposure to KTC altered the transcriptional expression of E2 responsive genes in livers of males and females. Consistent with the lesser fecundity, exposure to KTC caused less mRNA of *ER-α, VTG I, VTG II, CHG L, CHG H* and *CHG HM* in liver of females (Table 4.2). Choriogenin H and CHG HM

were decreased, while *ER-α* was increased by 6.2-fold in livers of males exposed to 300 μg KTC /L. KTC caused reduction of hepatic *AR-α* mRNA and an increase in annexin max2 transcript in both males and females exposed to 300 μg KTC /L.

Changes in opposite direction were observed in testis and ovaries of KTC exposed Japanese medaka. KTC exposure caused concentration-dependent down-regulation of ovarian receptors *ER-β*, *AR-α*, *LHR*, steroidogenic genes *HMGR*, *StAR*, *CYP11A*, *CYP11B*, and activin subunits, *activin BA* and *activin BB*. Conversely, exposure to 300 μg KTC /L caused a statistically significantly up-regulated the *ER-β*, *LHR*, and *LDLR* genes in testes. In addition, transcription of *CYP19A* (aromastase) and *activin BA* genes were up-regulated in a dose-dependent manner, with significant alteration caused by exposure to 300 μg KTC /L.

KTC caused down-regulation of gene expression in brains of both males and females. Concentrations of *GnRH RII* and *GnRH RIII* were both less in brains of females exposed to 300 μg KTC /L. *CYP19B* was slightly down-regulated (-1.7-fold change; $p = 0.022$) in brains of males exposed to 300 μg KTC /L.

DISCUSSION

Prochloraz. PCZ is a fungicide that can inhibit other *CYP* enzymes including steroidogenic *CYP17* and CYP19 in mammals and fish (15,16). Exposure of rats to PCZ resulted in less production of T in the testes, due to inhibition of *CYP17* activity (15). In fathead minnows, plasma concentrations of T and 11-ketotestosterone were less in PCZ exposed males and plasma E2 was less in PCZ exposed females (19). While PCZ was a more potent suppressor of E2 production than T production as observed in the H295R and fathead minnow ovary explant assays (18). The inhibitory effects on production of E2 and T elicited comprehensive transcriptional responses in gonads, livers, and brains of Japanese medaka exposed to PCZ. Because the plasma sex steroid hormones are primarily secreted by the gonad, the concentration-dependent up-regulation of gonadal *CYP19A* and *CYP17* in males and females can compensate for inhibition of gonadal E2 and T production by PCZ exposure. Nevertheless, the increased mRNA level of gonadal *CYP19A* and *CYP17* did not change the decrease of circulating concentration of E2. In liver, expression of *ER-α* mRNA and *VTG* as well as *CHG* genes were down-regulated in PCZ exposed females and males, which suggests the local E2 concentration was decreased in livers. As a consequence, egg production of PCZ exposed medaka was reduced in a time and concentration –dependent manner. In brain, PCZ exposure down-regulated brain CYP19B in both males and females, which suggests that the local E2 concentration was also decreased by PCZ exposure because brain *CYP19B* is primarily regulated by E2 through the estrogen responsive element (ERE) on its promoter sequence (24). These results demonstrate that transcriptions of genes along the HPG axis react to

EDCs in an organized manner among organs, and systematic investigation of the HPG axis can help elucidate chemical-induced MOAs

Changes in transcription of other gonadal genes are also consistent with the effects on fish previously ascribed to PCZ. The activin system is involved in gonadotropin-regulated ovarian functions, such as oocyte maturation thus, the concentration-dependent decrease in activin BA transcript would be expected to result in decreased fecundity. Activin-A, a homo-dimerization of two activin BA subunits, has been suggested to mediate gonadotropin-induced oocyte maturation in zebra fish (25). Although the transcriptional regulation mechanism of activin BA is unknown in medaka, its reduced expression is consistent with lesser fecundity caused by PCZ. Conversely, transcription of activin BA in the testes was increased by exposure of Japanese medaka to 30 μg PCZ/L for 7 d. In Japanese eels stimulation of activin B mRNA is accompanied by spermatogonia proliferation in after gonadotropin treatment (26). While the relative number of spermatogonia was increased when fathead minnows were exposed to concentrations of 30 to 300 μg PCZ/L exposed (19). Those results suggest a role of activins in the onset of spermatogenesis in Japanese medaka and activin transcripts can be used as a biomarker of chemical induced -effects on reproduction in males.

Ketoconazole exposure Similar to PCZ, KTC can also reduce E2 and/ or T production in mammals and fish (17,18), and exposure to KTC induced similar response profiles to those of PCZ, including inhibition of hepatic VTG and CHG transcripts, the reduction of fecundity. Although the underlying mechanism has not been identified, PCZ and KTC both down-regulated the expression of *GnRH R II/ III* in brains of females. The

inhibition of GnRH R was possibly a part of negative feedback mechanism that would reduce the responsiveness of the pituitary to GnRH stimulation and lead to less secretion of gonadatrophins in females. This hypothesis was supported by the down-regulation of steroidogenic pathways except *CYP17* and *CYP19A* in ovaries of Japanese medaka exposed to either PCZ or KTC.

Previous studies suggested that KTC is a more potent inhibitor of one or more upstream steroidogenesis enzymes than it is an inhibitor of aromatase, while the aromatase enzyme is more sensitive to inhibition by PCZ than the upstream targets (18). These differences can be reflected by the different transcriptional responses observed in Japanese medaka. For example, exposure to 30 μg PCZ /L caused a 50% less fecundity and less expression of *VTG II* and *CHG HM* relative to controls, while the same concentration of KTC did not cause such effects. In addition, expression of *CYP19B* was down-regulated in brains of females exposed to 300 μg PCZ/L but not of KTC. These results are consistent with the previous observation that PCZ was more than 10-fold more potent than KTC on the reduction of E2 production by fathead minnow ovary explants (18). However, KTC was a more potent suppressor of T production than E2 production. The expression of *AR-α* was significantly down-regulated in livers of KTC exposed males and females, but was unaffected in the PCZ exposure.

In summary, the present study demonstrated that transcriptional profiling with the Japanese medaka HPG axis RT-PCR array provides a systematic understanding of PCZ or KTC induced effect on the HPG axis of Japanese medaka. The medaka HPG PCR array system combines the quantitative performance of real-time PCR with the multiple gene profiling capabilities of a microarray to examine expression profiles of over 30

genes along the endocrine pathways in brain, liver and gonad. The organ- gender- and concentration –specific gene expression profiles derived by the Japanese medaka HPG axis RT-PCR array provides a powerful tool to not only delineate chemical-induced modes of action, but also to quantitatively evaluate chemical induced adverse effects

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REFERENCES

1 Kavlock, R. J.; Daston, G. P.; DeRosa, C.; Fenner-Crisp, P.; Gray, L. E.; Kaattari, S.; Lucier, G.; Luster, M.; Mac, M. J.; Maczka, C.; Miller, R.; Moore, J.; Rolland, R.; Scott, G.; Sheehan, D. M.; Sinks, T.; Tilson, H. A. Research needs for the risk assessment of health and environmental effects of endocrine disruptors: a report of the U.S. EPA-sponsored workshop. *Environ. Health Perspect.* **1996,** 104 (Suppl 4), 715-740.

2 Ankley, G.; Mihaich, E.; Stahl, R.; Tillitt, D.; Colborn, T.; McMaster, D.; Miller, R.; Bantlem, J.; Campbell, P.; Denslow, N.; Dickerson, R.; Folmar, L.; Fry, M.; Giesy, J.; Gray, L.E.; Guiney, P.; Hutchinson, T.; Kennedy, S.; Kramer, V.; LeBlanc, G.; Mayes, M.; Nimrod, A.; Patino, R.; Peterson, R.; Purdy, R.; Ringer, R.; Thomas, P.; Touart, L.; Van Der Kraak, G.; Zacharewski, T. Overview of a workshop on screening methods for detecting potential (anti-) estrogenic/androgenic chemicals in wildlife. *Environ. Toxicol. Chem.* **1998,** 17, 68-87.

3 OECD. Report of the First Meeting of the OECD Endocrine Disrupter Testing and Assessment (EDTA) Working Group. **1998,** OECD Paris.

4 OECD. The Fourth Meeting of the OECD Validation Management Group (VMG) for the Screening and Testing of Endocrine Disrupters. **2003,** OECD, Paris.

5 EDSTAC. Endocrine Disruptor Screening and Testing Advisory Committee Final Report. 1998.

6 Kanno, J.; Onyon, L.; Haseman, J.; Fenner-Crisp, P.; Ashby, J.; Owens, W. The OECD program to validate the rat uterotrophic bioassay to screen compounds for in vivo estrogenic responses: phase 1. *Environ. Health Perspect.* **2001,** 109(8), 785-794.

7 Wittbrodt, J.; Shima, A.; Schartl, M. Medaka--a model organism from the far East. *Nat. Rev Genet.* **2002,** 3(1), 53-64.

8 Kong, R.Y.; Giesy, J.P.; Wu, R.S.; Chen, E.X.; Chiang, M.W.; Lim, P.L.; Yuen, B.B.; Yip, B.W.; Mok, H.O.; Au, D.W. Development of a marine fish model for studying in vivo molecular responses in ecotoxicology. *Aquat. Toxicol.* **2007,** 86(2), 131-41.

9 Ankley, G.T.; Daston, G.P.; Degitz, S.J.; Denslow, N.D.; Hoke, R.A.; Kennedy, S.W.; Miracle, A.L.; Perkins, E.J.; Snape, J.; Tillitt, D.E.; Tyler, C.R.; Versteeg, D. Toxicogenomics in regulatory ecotoxicology. *Environ. Sci. Technol.* **2006,** 40(13), 4055-4065.

10 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. A

graphical systems model to facilitate hypothesis-driven ecotoxicogenomics research on the teleost brain-pituitary-gonadal axis. *Environ. Sci. Technol.* **2007**, 41(1), 321-330.

11 Zhang, X.; Hecker, M.; Park, J.; Tompsett, A.R.; Nakazawa, K.; Jones, P.D.; Newsted, J.L.; Au, D.W.T.; Kong, R.Y.C.; Wu, R.S.S.; Giesy, J. P. Development and validation of a PCR array method to study chemical induced effects on Brain-Hepatic-Gonadal axis of the Japanese medaka. *Aquat. Toxicol.* **2008 (Accepted)**

12 Gray, L.E.; Kelce, W.R.; Wiese, T.; Tyl, R.; Gaido, K.; Cook, J.; Klinefelter, G.; Desaulniers, D.; Wilson, E.; Zacharewski, T.; Waller, C.; Foster, P.; Laskey, J.; Reel, J.; Giesy, J.; Laws, S.; McLachlan, J.; Breslin, W.; Cooper, R.; Di Giulio, R.; Johnson, R.; Purdy, R.; Mihaich, E.; Safe, S.; Sonnenschein, C.; Welshons, W.; Miller, R.; McMaster, S.; Colborn, T. Endocrine screening methods workshop report: Detection of estrogenic and androgenic hormonal and antihormonal activity for chemicals that act via receptor or steroidogenic enzyme mechanisms. *Reprod. Toxicol.* **1997,** 11(5), 719-750.

13 Raun Andersen, H.; Vinggaard, A.M.; Hoj Rasmussen, T.; Gjermandsen, I.M.; Cecilie Bonefeld-Jorgensen, E. Effects of Currently Used Pesticides in Assays for Estrogenicity, Androgenicity, and Aromatase Activity in Vitro. *Toxicol. Appl. Pharmacol.* **2002,** 179(1), 1-12.

14 Henry, M.J.; and Sisler, H.D. Effects of sterol biosynthesis-inhibiting (SBI) fungicides on cytochrome P-450 oxygenations in fungi. *Pestic. Biochem. Physiol.* **1984,** 22(3), 262-275.

15 Blystone, C.R.; Lambright, C.S.; Howdeshell, K.L.; Furr, J.; Sternberg, R.M.; Butterworth, B.C.; Durhan, E.J.; Makynen, E.A.; Ankley, G.T.; Wilson, V.S.; LeBlanc, G.A.; Gray, L.E.Jr. Sensitivity of Fetal Rat Testicular Steroidogenesis to Maternal Prochloraz Exposure and the Underlying Mechanism of Inhibition. *Toxicol. Sci.* **2007,** 97(2), 512-519.

16 Kan, P.B.; Hirst, M.A.; Feldman, D. Inhibition of steroidogenic cytochrome P-450 enzymes in rat testis by ketoconazole and related imidazole anti-fungal drugs. *J. Steroid Biochem.* **1985,** 23(6), 1023-1029.

17 Hecker, M.; Newsted, J.L.; Murphy, M.B.; Higley, E.B.; Jones, P.D.; Wu, R.; Giesy, J.P. Human adrenocarcinoma (H295R) cells for rapid in vitro determination of effects on steroidogenesis: hormone production. *Toxicol. Appl. Pharmacol.* **2006,** 217(1), 114-124.

18 Villeneuve, D.L.; Ankley, G.T.; Makynen, E.A.; Blake, L.S.; Greene, K.J.; Higley, E.B.; Newsted, J.L.; Giesy, J.P.; Hecker, M. Comparison of fathead minnow ovary explant and H295R cell-based steroidogenesis assays for identifying endocrine-active chemicals. *Ecotoxicol. Environ. Saf.* **2007,** 68(1), 20-32.

19 Ankley, G.T.; Jensen, K.M.; Durhan, E.J.; Makynen, E.A.; Butterworth, B.C.; Kahl, M.D.; Villeneuve, D.L.; Linnum, A.; Gray, L.E.; Cardon, M.; Wilson, V.S. Effects of two fungicides with multiple modes of action on reproductive endocrine function in the fathead minnow (Pimephales promelas). *Toxicol. Sci.* **2005,** 86(2), 300-308.

20 Earl Gray, L.; Wilson, V.S.; Stoker, T.; Lambright, C.; Furr, J.; Noriega, N.; Howdeshell, K.; Ankley, G.T.; Guillette, L. Adverse effects of environmental antiandrogens and androgens on reproductive development in mammals. *Int. J. Androl.* **2006,** 29(1), 96-104.

21 Tompsett, A.R.; Park, J.; Zhang, X.; Jones, P.D.; Newsted, J.L.; Au, D.W.T.; Chen, E.X.H.; Yu, R.M.K.; Wu, R.S.S.; Kong, R.Y.C.; Giesy, J.P.; Hecker, M. Development and validation of an in situ hybridization system to detect gene expression along the HPG-axis in Japanese medaka, Oryzias latipes. *Aquat. Toxicol.* **2008, (sumitted)**

22 Zhang, X.; Park, J.; Hecker, M.; Tompsett, A.R.; Jones, P.D.; Newsted, J.L.; Au, D.W.T.; Kong, R.Y.C.; Wu, R.; Giesy, J.P. Time-dependent transcriptional profiles of hypothalamic-pituitary-gonadal (HPG) axis to fadrozole and 17beta-trenbolone in medaka (O. latipes). *Environ. Toxicol. Chem.* **2008, (Accepted)**

23 Zhang, X, Yu, R.M.; Jones, P.D.; Lam,G.K.; Newsted, J.L.; Gracia, T.; Hecker, M.; Hilscherova, K.; Sanderson, T.; Wu, R.S.; Giesy, J.P. Quantitative RT-PCR methods for evaluating toxicant-induced effects on steroidogenesis using the H295R cell line. *Environ. Sci. Technol.* **2005,** 39: 2777-2785

24 Pellegrini, E.; Menuet, A.; Lethimonier, C.; Adrio, F.; Gueguen, M.M.; Tascon, C.; Anglade, I.; Pakdel, F.; Kah, O. Relationships between aromatase and estrogen receptors in the brain of teleost fish. *Gen.Comp.Endocrinol.* **2005,** 142(1-2), 60-66.

25 DiMuccio, T.; Mukai, S.T.; Clelland, E.; Kohli, G.; Cuartero, M.; Wu, T.; Peng, C. Cloning of a second form of activin-betaA cDNA and regulation of activin-betaA subunits and activin type II receptor mRNA expression by gonadotropin in the zebrafish ovary. *Gen. Comp. Endocrinol.* **2005,** 143(3), 287-299.

26 Nagahama, Y.; Miura, T.; Kobayashi, T. The onset of spermatogenesis in fish. *Ciba Found. Symp.* **1994,**182, 255-267.

27 Miller, D.H.; Jensen, K.M.; Villeneuve, D.L.; Kahl, M.D.; Makynen, E.A.; Durhan, E.J.; Ankley, G.T. Linkage of biochemical responses to population-level effects: a case study with vitellogenin in the fathead minnow (Pimephales promelas). *Environ. Toxicol. Chem.* **2007,** 26(3), 521-527.

Chapter 5

Conclusion

The HPG -PCR array system developed in this study represents a sensitive, reliable and flexible monitoring tool to research chemical-induced effects along the HPG axis in Japanese medaka. Microarray and real-time PCR are currently the two most popular transcriptional profiling techniques. While the Japanese medaka has a relatively well-characterized genome, it lacks robust annotation for many gene products. Therefore, because of absence of baseline information of a large proportion of array spots, full interpretation of data collected by a Japanese medaka microarray is not yet possible. Alternatively, real-time polymerase chain reaction (real-time PCR) is a sensitive and reliable technique enabling reliable quantification of mRNA in biological samples. Real-time PCR methods have greater precision for quantification of changes in gene expression than do the microarray techniques. In addition, real time PCR techniques are relatively less expensive than the microarray methodologies. Thus, the real-time PCR techniques are more powerful for investigation of chemical effects because they allow higher throughputs at lesser cost. The Japanese medaka HPG PCR array developed in this study combines the quantitative performance of SYBR Green-based real-time PCR with the multiple gene profiling capabilities of a microarray to examine chemical-induced gene expression profiles along the HPG axis. Furthermore, the pathway-focused PCR array for Japanese medaka is an open system, in which primer sets for new biomarker genes are ready to be added when new information is available.

The results of the study demonstrated that profiling of HPG transcripts by RT-PCR array method represents a powerful tool for mechanistic toxicological studies of EDCs. It has been suggested that mechanistic information derived from changes in molecular or biochemical biomarkers can be used to aid in extrapolation of effects among

species and chemicals (1,2). However, it is important to understand linkages between alteration at the molecular and biochemical levels and ecological relevance of adverse effects at the individual and population levels that might relate to fitness. Fecundity represents the potential reproductive capacity of an organism or population. In the present study, four out of five selected model chemicals significantly reduced fecundity of Japanese medaka, in a concentration-dependent manner (Table 5.1) (3-5). A similar pattern was identified in the gene expression of liver tissue of females(Figure 5.1). Four of the five chemicals studied, including KTC, PCZ, TRB and FAD, induced concentration dependent down-regulation of six hepatic genes, which consists of *ER-α, VTG I, VTG II, CHG L, CHG H* and *CHG HM*. Principal component analysis on the transcript expression of the selected hepatic genes among chemical treatments confirmed that expression of these genes was correlated, with the first principle component (PC1) explaining 96.3% of the variance among expression of the six genes (Figure 5.2). To reduce the dimension of gene expression data and to simplify their relationship with fish fecundity, we further developed a hepatic index (HI; Equation 1) for each treatment by multiplying the fold change in gene expression by the PC1.

$$HI = 0.236 * \log_{10}(ER\text{-}\alpha) + 0.326 * \log_{10}(VTG\ I) + 0.537 * \log_{10}(VTG\ II) + 0.472 * \log_{10}(CHG\ L) + 0.343 * \log_{10}(CHG\ H) + 0.457 * \log_{10}(CHG\ HM) \quad (1)$$

The HI is a sum of log-transformed expression levels of the six hepatic genes with similar weighting, which represents the overall expression level of this cluster of gene. Plotting log-fecundity as a function of HI revealed that fecundity was directly

proportional to HI (Figure 5.3A). Furthermore, a log-log, relationship was developed for the log–fecundity as a function log-HI (Equation 2) (Figure 5.3B).

$$\log_{10} \text{fecundity} = 1.616 - 0.4493 * \log_{10} \text{HI} \qquad (2)$$

The coefficient of determination for this relationship (r^2) was 0.864 and the analysis of variance test indicated that the linear relationship was statistically significant (n =11, F= 56.5, $p < 0.001$)

A major challenge in the emerging field of ecotoxicogenomics is to define the relationships between chemically induced changes in gene expression and alterations in conventional toxicological parameters (1,6). The result, for the first time, quantitatively linked the alteration of gene expression to ecologically-relevant endpoints such as fecundity. All of the pre-selected hepatic genes are functionally relevant to fish fecundity. Of the 6 selected hepatic genes, *VTG I* and *VTG II* are yolk precursor while *CHG L, CHG H* and *CHG HM* are egg envelop precursors, which all are regulated by E2 through *ER-α*. A similar linear relationship had also been found between the production of vitellogenin (VTG), and the reproductive success of fish (7). However, the mRNA measurement by the RT-PCR method offers many advantages over the *VTG* assay based on enzyme-linked immunosorbent assay (ELISA). First, alterations in *VTG* mRNA would precede changes of VTG protein, which makes the mRNA response a more rapid response. Second, the inherent amplification of RT-PCR method makes the measure more sensitive than ELISA *VTG* assay and needs only a small amount of tissue, which would reduce the number of fish for screening of chemical. This is especially true when small fish species is used.

Finally since the hepatic index, integrates the responses of six genes, and thus circumvents the variation of any single gene, it provides a more reliable prediction of effects of chemicals on fecundity. Therefore, the hepatic index has a potential to be used in the quantitative assessment of chemical induced effects on reproduction of Japanese medaka in short-term exposure.

In summary, the present study demonstrated that transcriptional profiling with the Japanese medaka HPG axis RT-PCR array provides a systematic understanding of PCZ or KTC induced effect on the HPG axis of Japanese medaka. The medaka HPG PCR array system combines the quantitative performance of real-time PCR with the multiple gene profiling capabilities of a microarray to examine expression profiles of over 30 genes along the endocrine pathways in brain, liver and gonad. The organ- gender- and concentration –specific gene expression profiles derived by the Japanese medaka HPG axis RT-PCR array provides a powerful tool to not only delineate chemical-induced modes of action, but also to quantitatively evaluate chemical induced adverse effects.

Table 5.1. Effects of different chemicals on fecundity of Japanese medaka in 7 d exposure.

Chemical	Conc.	Fecundity (%)
	5 ng/L	91.0%
EE2	50 ng/L	92.4%
	500 ng/L	65.1%
	50 ng/L	99.8%
TRB	500 ng/L	46.1% (*)
	5000 ng/L	26.0% (*)
	3 ug/L	95.5%
Prochloraz	30 ug/L	49.8% (*)
	300 ug/L	18.0% (*)
	3 ug/L	89.7%
Ketoconazole	30 ug/L	84.2%
	300 ug/L	20.3% (*)
	1 ug/L	N.A.
	10 ug/L	N.A.
Fadrozole	50 ug/L	20.4% (*)
	100 ug/L	N.A.

*, $p < 0.05$

Figure 5.1 Heatmap of the concentration-dependent gene expression profiles in livers of chemical exposed females. Gene tree was constructed by pearson correlation metric. Chemical tree was constructed by 'ToxClust" method, where the dissimilarity between any two chemicals was calculated by the distance between the concentration–dependent response curves in the exposure of both chemical.

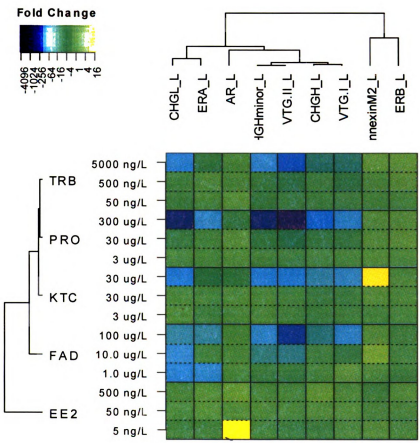
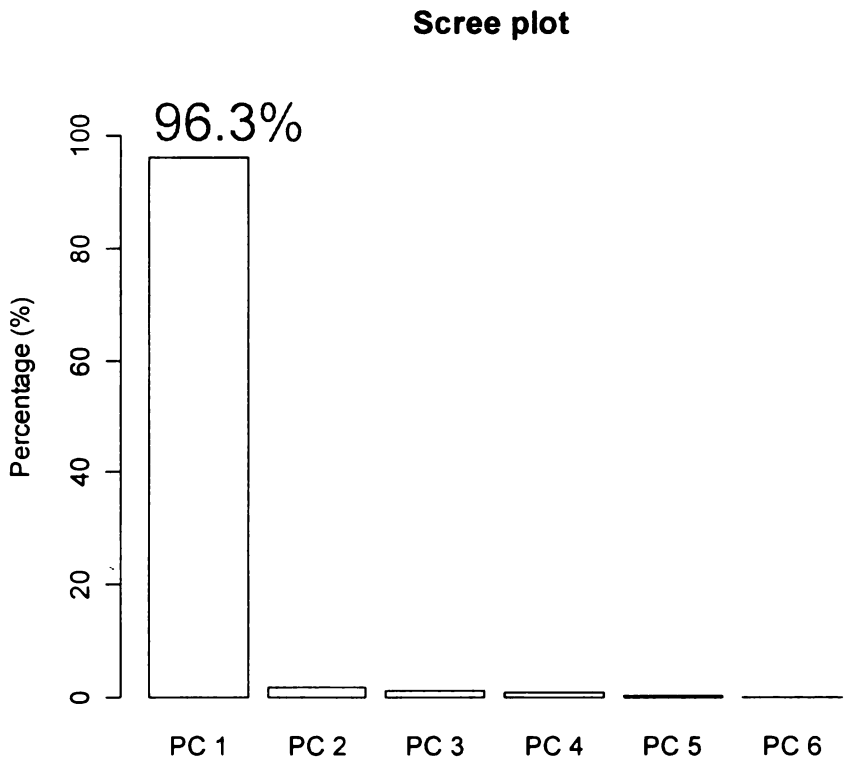
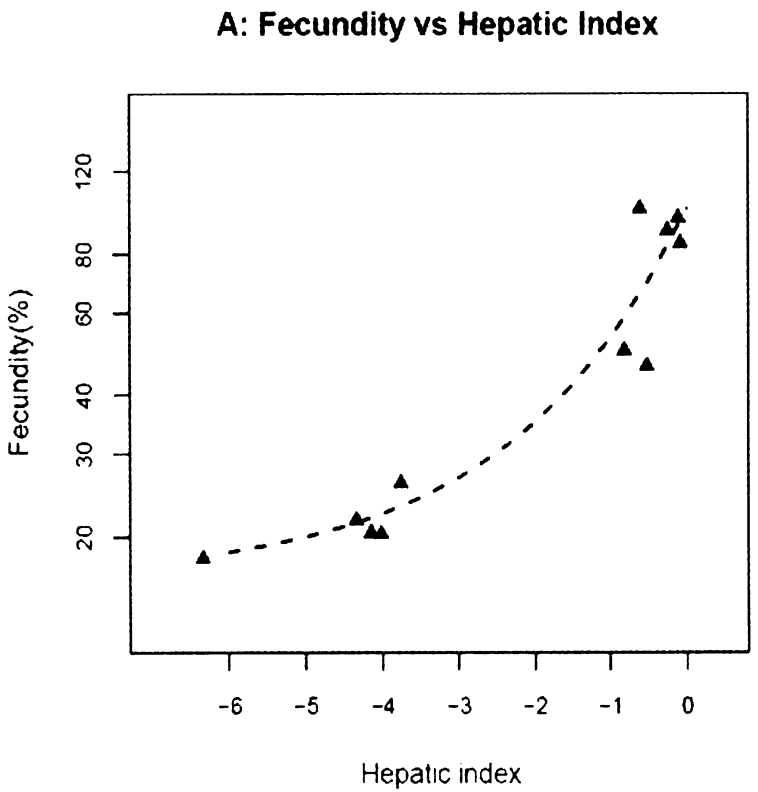
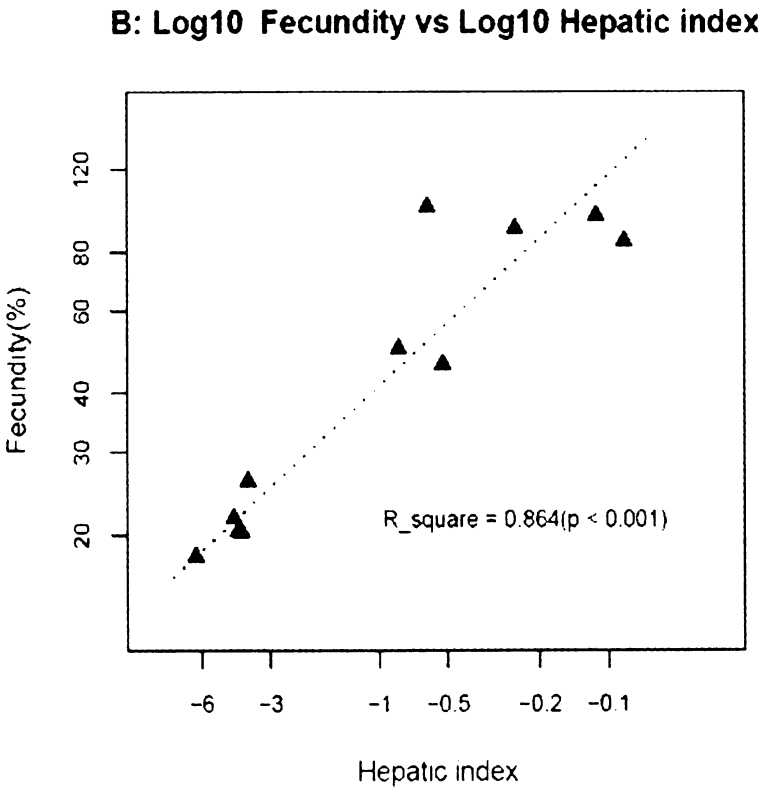


Figure 5.3 Relationship between fecundity and gene expression in livers of females. A: fecundity vs hepatic index, the broken line shows the trend of data. B: Simple linear regression of log10-transformed fecundity and hepatic index. The functions describing the relationship are: Hepatic index = 0.236 *log10 (ER-α)+ 0.326 *log10 (VTG I)+ 0.537 *log10 (VTG II) + 0.472 *log10 (CHG L) + 0.343 *log10 (CHG H) + 0.457 *log10 (CHG HM). The formula for the regression model was: log10 (fecundity) = 1.616 – 0.4493 * log10(-hepatic index).

Figure 5.2 Scree plot of the percentage of variance explained by each of the Principal Components (PC) as a percentage of the total variance of the gene expression. The six hepatic genes included in the analysis were *ER-α, VTG I, VTG II, CHG L, CHG H* and *CHG HM.*




A: Fecundity vs Hepatic Index
Fecundity(%)
20
30
40
60
80
120
-6
-5
-4
-3
-2
-1
0
Hepatic index


B: Log10 Fecundity vs Log10 Hepatic index
Fecundity(%)
20
30
40
60
80
120
R_square = 0.864(p < 0.001)
-6
-3
-1
-0.5
-0.2
-0.1
Hepatic index

REFERENCES

1 Ankley, G.T.; Daston, G.P.; Degitz, S.J.; Denslow, N.D.; Hoke, R.A.; Kennedy, S.W.; Miracle, A.L.; Perkins, E.J.; Snape, J.; Tillitt, D.E.; Tyler, C.R.; Versteeg, D. Toxicogenomics in regulatory ecotoxicology. *Environ. Sci. Technol.* **2006,** 40(13), 4055-4065.

2 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. A graphical systems model to facilitate hypothesis-driven ecotoxicogenomics research on the teleost brain-pituitary-gonadal axis. *Environ. Sci. Technol.* **2007**, 41(1), 321-330.

3 Zhang, X., Park, J., Tompsett, A.R., Hecker,M., Jones, P.D., Newsted, J.L., Giesy, J.P. Development and validation of a medaka brain-gonadal-liver axis model and a real time-PCR array method to facilitate the mechanistic classification of endocrine-disrupting chemicals (EDCs). *Aquat Toxicol.* **2008**. (Accepted)

4. Zhang, X., Park, J., Tompsett, A.R., Hecker,M., Jones, P.D., Newsted, J.L., Giesy, J.P. Time-dependent transcriptional profiles of genes of the hypothalamic-pituitary-gonadal (HPG) axis in medaka (*O. latipes*) exposed to fadrozole and 17β-trenbolone. *Environ Toxicol Chem.* **2008**. (Accepted)

5. Zhang, X., Hecker,M., Jones, P.D., Newsted, J.L., Giesy, J.P. Responses of the Medaka HPG axis PCR array and reproduction to prochloraz and ketoconazole. *Environ Sci Technol.* **2008**. (Submitted)

6. Moggs, J. G. Molecular responses to xenoestrogens: mechanistic insights from toxicogenomics. *Toxicol.* **2005**, 213(3), 177-193.

7 Miller, D.H.; Jensen, K.M.; Villeneuve, D.L.; Kahl, M.D.; Makynen, E.A.; Durhan, E.J.; Ankley, G.T. Linkage of biochemical responses to population-level effects: a case study with vitellogenin in the fathead minnow (Pimephales promelas). *Environ. Toxicol. Chem.* **2007**, 26(3), 521-527.