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DEVELOPMENT AND VALIDATION OF AN *IN SITU* HYBRIDIZATION SYSTEM TO DETECT GENE EXPRESSION ALONG THE HPG-AXIS IN THE JAPANESE MEDAKA, *ORYZIAS LATIPES*

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DEVELOPMENT AND VALIDATION OF AN *IN SITU* HYBRIDIZATION SYSTEM TO DETECT GENE EXPRESSION ALONG THE HPG-AXIS IN THE JAPANESE MEDAKA, *ORYZIAS LATIPES*

By

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ABSTRACT

DEVELOPMENT AND VALIDATION OF AN *IN SITU* HYBRIDIZATION SYSTEM TO DETECT GENE EXPRESSION ALONG THE HPG-AXIS IN THE JAPANESE MEDAKA, *ORYZIAS LATIPES*

By

Amber Raye Tompsett

This project focused on the development of a whole-animal tissue section *in situ* hybridization system to detect aromatase (CYP19) gene expression along the HPG-axis in the Japanese medaka. The ISH system was then validated in a test exposure with a pharmaceutical aromatase inhibitor, fadrozole. The ISH method that was developed successfully detected gross changes in gene expression in the medaka. Fadrozole didn't have any statistically significant effects on male or female medaka aromatase gene expression, but female gonads exhibited a nonsignificant increase in gonadal aromatase expression at 100μg/L fadrozole treatment. The resolution and sensitivity of the ISH method developed contributed to the lack of significant results, and the system could be improved by using a fluorescent detection method. Histological evaluation revealed that females from the 100μg/L fadrozole treatment also lacked any mature oocytes while male gonadal morphology was normal across all treatments. The ISH method developed in this study allowed spatial resolution of gene expression in a whole animal model, as well as the ability to analyze morphological detail in the same organism.

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KEY TO ABBREVIATIONS AND SYMBOLS

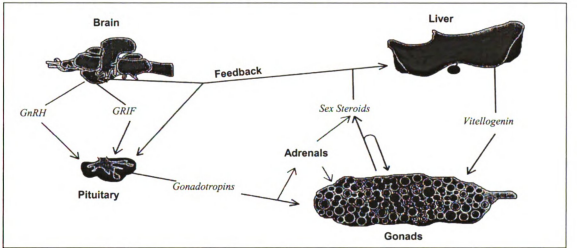
^{35}S	radioactive sulfur
ANOVA	analysis of variance
bp	basepair
C	Celsius
C18 estrogen	carbon-18 estrogen
C19 androgen	carbon-19 androgen
cAMP	3'-5'-cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CPM	counts per minute
CYP1A1	Cytochrome P450-1A1
CYP19	Cytochrome P450-19
CYP19a	Cytochrome P450-19a
CYP19b	Cytochrome P459-19b
DDT	dichloro-diphenyl-trichloroethane
DEPC	diethylpyrocarbonate
DIG	digoxygenin
DNA	deoxyribonucleic acid
EDC	endocrine disrupting chemical
ERE	estrogen response element
FETAX	embryo rearing solution
FPE	fixed and paraffin embedded
g	gram

HPG-axis	hypothalamic-pituitary-gonadal-axis
IHC	immunohistochemistry
ISH	*in situ* hybridization
L	Liter
LB	Luria-Bertani
mg	milligram
mL	milliliter
mRNA	messenger ribonucleic acid
NCBI	National Center for Biotechnology Information
PCB	polychlorinated biphenyl
PCR	polymerase chain reaction
ppm	parts per million
RT-PCR	real time polymerase chain reaction
RNA	ribonucleic acid
RO	reverse osmosis
SD	standard deviation
SF-1	steroidogenic factor-1
SSC	standard saline citrate buffer
UTP	uridine triphosphate
μCi	microCurie
μg	microgram
μm	micrometer or micron

Introduction

Project Overview

The overall objective of this project was to develop and validate a sensitive molecular whole-animal *in situ* hybridization (ISH) technique that could then be used to elucidate spatial and temporal changes in gene expression after chemical exposure. The technique that was developed, with some modifications, may allow for the evaluation of chemicals for their potential as disruptors of endocrine function, including the determination of mechanisms of toxic action of single chemicals or complex mixtures. The project focused on gene expression along the hypothalamic-pituitary-gonadal (HPG) axis, also referred to as the brain-pituitary-gonadal axis, with a special emphasis on genes involved in steroidogenic pathways and hormonal control mechanisms (Figure 1). The ISH method was validated by demonstrating that a gene expression endpoint was altered in the test organism, the Japanese medaka (*Oryzias latipes*), after chemical exposure to a potential endocrine-disrupting compound (EDC) with a known mode of action, the pharmaceutical aromatase inhibitor fadrozole. EDCs have been defined as exogenous agents that interfere with the "synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development, and/or behavior" (Kavlock et al., 1996). It has been hypothesized that such compounds may elicit a variety of adverse effects in both humans and wildlife, including promotion of hormone-dependent cancers, reproductive tract disorders, and reduction in reproductive fitness.



Tissue	Primary Gene Targets
Brain	CYP19B; GnRHs; ERα & β; AR
Pituitary	GnRH receptors; GtH I & II; ERα & β; AR; Activin/Inhibin
Adrenals	CYP11A & B; CYP17; 3β-HSD; 17β-HSD; StAR; AR; GtH receptors; Activin/Inhibin
Gonads	CYP11A & B; CYP17; CYP19A; 3β-HSD; 11β-HSD; 17β-HSD; StAR; ERα & β; AR; GtH receptors; Activin/Inhibin

Figure 1: Systems approach. Interactions among target organs and related genes of interest.

Background

To date, most studies employing biomarkers such as gene or protein expression as endpoints have assessed the expression of one to a few gene products that are known to be related with exposure to specific chemical classes. For example, increased Cytochrome P450-1A1 (CYP1A1) gene and protein expression have been linked convincingly with exposure to aryl hydrocarbons such as benzo[a]pyrene and polychlorinated biphenyls (PCBs) in mammals and fish (Carlson et al., 2004; Meucci and Arukwe, 2006), but less is known about effects on other genes and proteins, and their functional importance to organisms. While the expression of certain genes and proteins (i.e. receptors, enzymes and genes such as CYP1A1) has proven useful in assessing exposure to specific chemicals, the limited number of products assessed does not make it possible to distinguish overall patterns of response that may be used to differentiate exposure to and effects of different chemicals, chemical classes, and chemical mixtures. In addition, many of these studies lack the spatial and temporal resolution offered by some molecular techniques.

New techniques in molecular biology have made it possible to detect subtle alterations in the expression of genes and proteins in an organism as a result of exposure to chemicals or other environmental stressors. These techniques, which include quantitative real time polymerase chain reaction (Q RT-PCR), Western blotting, Northern blotting, immunohistochemistry (IHC) and *in situ* hybridization (ISH), have expanded the depth of knowledge about organismal response to chemical exposure. However, most studies utilizing these techniques have generally focused on one tissue at one specific time in the development of an organism and have only analyzed one or a few endpoints in

each tissue. The method employed in this study, whole-animal tissue section ISH, allowed the determination of effects on expression of a single gene in multiple tissues simultaneously, with the possibility to alter the system for analyzing the expression of multiple genes simultaneously (Jezzini et al., 2005). In addition, the system is amenable to the use of multiple probe labels, such as radionucleotides, biotin, digoxygenin, and fluorphores. The probe label can be changed depending on the objective of the experiment since each label has strengths and weaknesses depending on application. For example, radio isotopic probes offer a sensitive response that is easier to quantify than signal from biotin or digoxygenin probes (Moorman et al., 1997), but radioisotopic methods are more expensive and require special certifications in many institutions and extra precautions in the laboratory. Traditionally, fluorescent ISH (FISH) has been less sensitive than any of the other methods due to the labels used (Wilkinson, 1998), and the technique is also susceptible to autofluorescence from fixed tissues which confounds results. If desired, probes with different labels can also be used in concert to detect multiple genes in a single tissue (Peterson and McCrone, 1993; Lichter, 1997; Hrabovszky et al., 2004; Jezzini et al., 2005; Ijiri et al., 2006).

Some genes are only expressed in specific tissues at specific times in the development of an organism. For example, the expression of some components of neuroendocrine systems can vary greatly temporally during development (Sanderson et al., 2001). The ISH methods employed in this study allow analysis of the chosen animal model, Japanese medaka (*Oryzias latipes*), at any stage of development, from fry to adult. When using small laboratory model species, the limited amount of individual tissues available for study and the difficulty in excising them from the organism have limited the

efficacy of other techniques in determining effects during critical windows of time during ontogenesis. For example, both RT-PCR and Northern blotting require an amount of fresh tissue that cannot always be obtained from small animals. Whole animal tissue section ISH is a sensitive monitoring tool that allows for the analysis of gene expression in multiple tissues simultaneously at any stage of development, without the need to dissect out and process the small critical tissues.

Chemicals that disrupt the endocrine system can do so by direct and indirect mechanisms. Some chemicals are direct acting agonists or antagonists of a receptor while others act indirectly by modulating signal transduction systems. Direct acting chemicals can be screened using tests such as receptor binding assays while indirect actors cannot. For example, the triazine herbicide atrazine has a low affinity for the estrogen receptor (ER) (Roberge et al., 2004), but *in vitro* in the H295R mammalian cell system atrazine has been found to up-regulate the expression of aromatase, or Cytochrome P450-19 (CYP19), the enzyme that transforms androgens into estrogens (Sanderson et al., 2000). Although atrazine does not act like a typical estrogen via binding the ER, in the H295R cell system it is likely to result in an estrogenic effect by increasing endogenous estradiol production, most likely via a cAMP-mediated pathway (Sanderson et al., 2001). Simple, targeted screening methods such as receptor binding assays or even receptor mediated functional assays may not identify these types of effects. Since ISH can be used to detect the mRNA of any gene of interest, including those along the entire steroidogenic pathway, this technique can be used to reveal indirect effects that other assays may miss.

Chemicals can be grouped into classes based on their chemical structure and subsequent environmental fate and effects (Karcher and Devillers, 1990; Zeeman et al., 1995). Also, some chemical classes express their adverse effects through specific modes of action that lead ultimately to their toxic properties. However, organisms are usually exposed to complex mixtures of chemicals with different modes of actions, and there is still a significant lack of information regarding the mechanism or mode of action for the risk assessment of these mixtures. To be able to assess the potential effects of mixtures of compounds, knowledge of the critical mechanism of action is needed. The methods that were developed in this research project have the potential to be used to establish a better understanding of mechanisms of action of individual compounds and to help classify compounds so that assessments of complex mixtures can be made. These methods could be used to determine characteristic alterations in gene expression patterns that are indicators of toxic activity along the HPG-axis. By determining such patterns, assessment of whether chemicals currently grouped in the same "class" based on physiochemical properties can also be classified together based on their modes of action would be possible. This will be an important aspect to improving human and ecological risk assessment of chemicals and chemical mixtures, especially those that are considered to be endocrine disruptors.

*Japanese medaka (*Oryzias latipes*)*

Japanese medaka are a small teleost fish native to areas of Asia. They are a commonly used species in laboratory research. Medaka are easy to maintain and culture in captivity under the appropriate lighting and temperature conditions. In addition, male

and female fish can be separated by sex fairly reliably using secondary sexual characteristics, mainly fin morphology (Figure 2).

The physiology, embryology and genetics of the medaka have been extensively studied for more than 100 years (Wittbrodt et al., 2002). The medaka has clearly defined sex chromosomes, and sex determination has been extensively studied (summarized in Wittbrodt et al., 2002). Medaka are gonochoristic; fish are either genetically male (XY) or female (XX). Chemical exposure during sexual differentiation may reverse or alter phenotypic sex (Scholz and Gutziet, 2000), but genetic sex remains constant.

Medaka were chosen as test organisms because of their favorable attributes and the availability of gene sequence information for almost all of their genome. 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). Therefore, cloning and sequencing the genes of interest was unnecessary.

In situ *Hybridization*

In situ hybridization is a sensitive method that can be used to detect as few as 10-100 nucleic acid molecules in a single cell and can provide information relative to temporal and spatial expression of genes (Innis et al., 1990). The methods entail the specific annealing of labeled antisense nucleic acid probes to their complementary sequences in fixed or frozen tissue samples followed by a visualization method to reveal the location and quantity of the probe (Wilkinson, 1998). The major advantage of this method is that it provides a sensitive means to localize and potentially quantify the

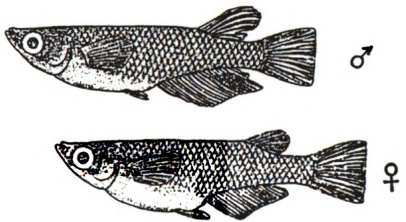


Figure 2: Male and female medaka fish fin morphology. The male medaka (top) has a fan-shaped anal fin and a notch in the dorsal fin while the female (bottom) has a smaller anal fin and no notch in the dorsal fin.

mRNA for specific genes in organs and tissues of interest in a manner that is consistent with other methods that are used to detect lesions, including histopathology and immunohistochemistry (Streight and Stern, 2001). This method has been successfully applied to a variety of tissue types including whole animal embryos, cells in culture, and individual organs in species as diverse as humans, mice, and fish (Wilkinson, 1998). In this study, whole medaka were fixed, sectioned and probed with RNA probes. This allowed analysis of gene expression in every tissue of the fish at once. The use of whole animal tissue sections also made it possible to examine tissue morphology via traditional histological techniques when desired. ISH hasn't traditionally been a popular technique outside of laboratories specializing in histopathology because it is very labor-intensive, time-consuming, costly, and requires specialized training and knowledge in multiple disciplines (i.e. molecular biology, histology, pathology). As molecular techniques become more commonplace through standardized methodology and more gene sequences are made available to the public, ISH will become attainable to more laboratories.

Histopathology

Since this study focused on effects of endocrine active chemicals along the HPG-axis, the histopathology of the gonads was a potentially important endpoint to evaluate. Exposure to endocrine disruptors has been documented to cause a number of gonadal abnormalities in fish. Potential abnormalities include testis-ova (Yamamoto, 1968; Jobling et al., 1998; Kiparissis et al., 2003; Kidd et al., 2007) and altered or delayed spermatogenesis (Kidd et al., 2007) in males and oocyte atresia (Kidd et al., 2007) and

alteration of oocyte maturation (Kim et al., 2002; Suzuki et al., 2004) in females and intersex in both sexes (Jobling et al., 1998).

Cytochrome P450 Aromatase – CYP19

The two isoforms of the aromatase gene, CYP19a and CYP19b, were selected as initial target genes to develop and optimize the ISH system for medaka due to the fact that their expression is highly tissue specific and responsive to endocrine disruptors in teleost fish (Callard et al., 2001; Villeneuve et al., 2006). Measuring the expression of these two genes was the main objective of this study.

Cytochrome P450 aromatase, or CYP19, is a member of a superfamily of heme-containing proteins. The CYP19 gene encodes for the enzyme that converts C19 androgens into C18 estrogens with a phenolic A ring (Lephart and Simpson, 1991; Simpson et al., 1994; Simpson and Davis, 2001). This conversion has been implicated as the rate limiting step in estrogen biosynthesis (Simpson et al., 1994). Aromatase expression is likely to be an integral part of maintaining homeostatic balance in the levels of circulating androgens and estrogens (Villeneuve et al., 2006). It is active in both the hypothalamic/pituitary and gonadal compartments of the HPG-axis. Therefore, it is an ideal candidate for determining the effects of endocrine active chemicals along the HPG-axis.

Teleost fish, including the Japanese medaka, express two separate aromatase isozymes; mammals, including humans, express only one form of aromatase (Kazeto et al., 2001). The CYP19a form of aromatase is expressed mainly in the gonad of teleost fish, while CYP19b is expressed mainly in the brain, specifically in the hypothalamus

and pituitary (Callard et al., 2001). CYP19b enzyme activity is much higher in the brain than CYP19a activity is in the gonad in teleosts (Callard et al., 2001; Zhao et al., 2001; Villeneuve et al., 2006). CYP19b has been determined to be critical for sexual differentiation (Kuhl et al., 2005) and normal sexual behavior (reviewed in Hecker et al., 2006), and modulation of CYP19a expression can lead to abnormal sexual differentiation in zebrafish (*Danio rerio*) (Fenske and Segner, 2004). In addition, aromatase enzyme activity in bream (*Abramis brama*) is positively correlated with general measures of reproductive health, such as gonadosomatic index and maturation stage (Hecker et al., 2006) Thus, both enzymes must function normally for teleost fish to develop properly and reproduce maximally.

The two aromatase isozymes in Japanese medaka are 60% homologous in amino acid sequence, with the greatest homology in regions responsible for substrate binding, heme binding, and androgen binding (Kuhl et al., 2005). All of these regions are integral to the functionality of the enzymes. Using phylogenetic analysis, CYP19a and b of the medaka were determined to be more closely related to their complementary forms in other teleosts than they are to one another (Kuhl et al., 2005). Therefore, it is assumed that the CYP19a and CYP19b isozymes have different functions within a single organism, but also that the two isozymes function in the same ways in the various teleosts.

The expression of CYP19a and b are under the control of different response elements in their promoter regions. The CYP19a promoter region contains an SF-1 (steroidogenic factor-1) binding site while the CYP19b promoter contains an ERE (estrogen response element) (Callard et al., 2001, Kazeto et al., 2001). The presence of an SF-1 site suggests that CYP19a is sensitive to circulating cAMP levels (Carlone and

Richards, 1999). Villeneuve et al. (2006) proposed that CYP19a gene expression is influenced by gonadotropins that regulate via cAMP-mediated signal transduction at the SF-1 site.

The ERE in the promoter of the CYP19b gene indicates that it is sensitive to circulating estrogens that interact with the estrogen receptor (Kuhl et al., 2005). Specifically, the nuclear estrogen receptor (ER) homodimerizes when bound by estrogen, then associates with an ERE in the DNA. The presence of the receptor complex at the ERE of the gene leads to upregulation of that gene. Therefore, higher levels of endogenous E2 would lead to increased transcription of CYP19b since it contains a complete and active ERE (Kuhl et al., 2005). Estrogen produced via CYP19b activity is important for proper neuronal differentiation and growth, as well as neurogenesis and homeostasis in the teleost brain (Kuhl et al., 2005) and is also responsible for stimulating the release of gonadotropins from the pituitary (Callard et al., 2001). CYP19b is essential in the regulation of reproduction (Gonzalez and Piferrer, 2003).

Aromatase activity is inhibited *in vitro* by numerous chemicals of environmental concern including polychlorinateddibenzo-p-dioxins, polychlorinated biphenyls, DDT and its metabolites, a number of azoles, and the herbicide atrazine (Drenth et al., 1998; Letcher et al., 1999; Vinggaard et al., 2000; Sanderson et al., 2001; Sanderson et al., 2002; Heneweer et al.; 2004, Trosken et al., 2006; Sun et al., 2007). A recent study (Villeneuve et al., 2006) added valuable information on *in vivo* response to pharmaceutical aromatase inhibitors, specifically fadrozole.

Fadrozole

Fadrozole (4-(5,6,7,8-tetrahydroimadazo[1,5-a]-pyridin-5-yl)benzonitrile monohydrochloride) is a potent pharmaceutical competitive inhibitor of the aromatase enzyme (Steele et al., 1987). It is not a chemical of great environmental concern due to its limited use and low presence in the environment. Studies suggest that it binds at a site different from the active site of the enzyme, perhaps thereby causing a conformational change at the active site and blocking the binding of androgen to the active site (Yue and Brodie, 1997). The effects of fadrozole are partially irreversible in the JEG-3 cell line (Yue and Brodie, 1997). Villeneuve et al. (2006) found that treatment of female fathead minnows (*Pimephales promelas*) with fadrozole resulted in a dose-dependent decrease in CYP19b while it increased CYP19a expression in a dose-dependent fashion.

Aromatase gene expression after fadrozole exposure isn't as well studied in males as in females. Thus, the aim of this study was to determine the effects of fadrozole exposure on gene expression in male and female Japanese medaka that were held together to simulate normal breeding conditions. Specifically, methods were developed and validated for examining the effects of fadrozole at the level of gene expression by measuring the quantity of mRNA present in the medaka by *in situ* hybridization (ISH) on whole animal tissue sections, including designing and synthesizing RNA probes of interest. These procedures were designed to be amenable to evaluating both the direct effects of chemicals on receptors and proteins as well as indirect effects on steroidogenic pathways, such as compensation and feedback responses, through the ability to analyze any gene for which sequence information was available. The techniques were applied in

a model that allowed analysis of gene expression in multiple target tissues simultaneously in the same organism.

The specific objectives of this project were:

1. To develop methods that permitted the identification of changes in the expression profiles of genes that are associated with key aspects along the HPG-axis such as hormone receptors, gonadotropins, steroidogenic enzymes, etc. in the Japanese medaka, a small animal model.
2. Application of these techniques to develop a gene expression profile for a "model" compound with a specific mode of endocrine action (e.g. enzyme inhibitor, estrogenic, or androgenic). Fadrozole, a potent competitive inhibitor of the aromatase enzyme, was chosen as a model chemical because of its specific targeted action, and its tissue specific effects.

Materials and Methods

Test chemical

The fadrozole (CGS016949A; MW: 259.74g) used in this research was provided as a gift from Novartis Pharma AG (Basel, CH). For the chemical stock, 5mg of fadrozole was dissolved in 1L of ultrapure water. The stock was allowed to mix on a stir plate overnight before being used in the chemical exposure. The stock was stored in the dark at 4°C over the course of the exposure to minimize degradation of the fadrozole.

*Culturing of Japanese medaka (*Oryzias latipes*)*

Six month-old male and female wild-type Japanese medaka (*Oryzias 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). The natural sex ratio was about 60% males and 40% females. The fish were held at a density of approximately 1 fish/L. Tanks were siphoned daily to remove solid waste products and other debris. Fish were fed Aquatox flake food (Aquatic Ecosystems, Apopka, FL, USA) and newly hatched *Artemia* (GSL, Ogden, UT, USA) twice per day.

Fertilized eggs were collected manually from female fish daily by removing the fish from the breeding tanks and gently removing the eggs from behind the anal fin. Eggs were separated, counted, and placed into sterile Petri dishes containing FETAX medium (ASTM, 1991; 0.625 g/L NaCl, 0.030 g/L KCl, 0.015 g/L $CaCl_2$, 0.096 g/L $NaHCO_3$, 0.060 g/L $CaSO_4 \cdot 2H_20$, 0.075 g/L $MgSO_4$) supplemented with 0.001% methylene blue (Sigma, St. Louis, MO) to discourage fungus growth. Eggs were held in an incubator at

25°C until hatch (7-14 days). Eggs were monitored daily for newly hatched fry and for the presence of fungus growth. Eggs that were unfertilized and/or infected with fungus were discarded on a daily basis during the hatching period.

Newly hatched fry were placed into a static holding tank. All the fry were from a 7 day long hatching period. The tank was aerated and kept at 24-25°C with a 16:8 light/dark cycle. Fry were fed *Spirulina* algae (Earthrise, Petaluma, CA) ad libidum every other day. When the medaka fry reached 8 weeks of age, the fry algal diet was supplemented once a day with flake food. Fish were kept under these conditions for 14 weeks, at which time they were acclimated for chemical exposure.

Acclimation and fadrozole exposure

Fourteen week-old medaka were placed into 10L tanks filled with 6L of carbon-filtered water. Each tank contained 5 male and 5 female fish. Fish were fed flake food ad libidum daily and held at 24°C with a 16:8 light/dark cycle. One half of the water in each tank (3L) was replaced daily with fresh carbon-filtered water. Temperature was monitored daily. Water quality (pH, hardness, dissolved oxygen, ammonia nitrogen, and nitrate nitrogen) were monitored once every 3-4 days. The acclimation period lasted 12 days. Overall mortality during this period was one.

After the acclimation period, fish were exposed to fadrozole in a 7 day static renewal exposure scenario. The treatments were control (carbon-filtered water), 1 μg/L, 10 μg/L, and 100 μg/L fadrozole. One half of the water in each tank (3L) was replaced with fresh carbon-filtered water dosed with the appropriate amount of fadrozole diluted from the 5 mg/L stock each day. Water quality parameters (temperature, pH, hardness,

dissolved oxygen, ammonia nitrogen and nitrate nitrogen) were measured daily. Fish were held in the same conditions as during the acclimation period (24°C, 16:8, fed flake food daily). No mortalities were observed at any treatment during the exposure period.

Exposure Termination and Processing of Samples for ISH

The fadrozole exposure was terminated after 7 days. Fish were euthanized in Tricaine S solution (Western Chemical, Ferndale, WA, USA). Total weight and snout-vent length were recorded for each fish. Medaka were then separated into two groups, one for this study and another for RT-PCR analysis performed by another graduate student. Two fish of each sex were processed for the ISH procedures from each tank.

Fish were first gross dissected by removing the fins, tail, skull roof, otoliths, and opercula. The body cavity was opened to allow for better penetration of the fixative into the tissues. Fish were then immersed in individual vials that contained a fixative cocktail (80% Histochoice MB [EMS, Hatfield, PA, USA], 2% paraformaldehyde [EMS, Hatfield, PA, USA], 0.05% glutaraldehyde [EMS, Hatfield, PA, USA]) and were allowed to fix for approximately 22 hours at room temperature. Whole-fish samples were then removed from the fixative cocktail, washed and dehydrated through a graded methanol series (80%, 3 washes 95%, and 3 washes 100%), and cleared in chloroform (3 washes). Dehydration and clearing took place at 4°C and each solvent change was 20 minutes in length. Fish were then infiltrated with melted Paraplast Plus paraffin (McCormick Scientific, St. Louis, MO, USA) for 4 hours with 4 paraffin changes at 60°C. Fish were embedded in Paraplast Plus paraffin in Tissue Tek base molds (Miles, Elkhart, IN, USA) with Tissue Tek embedding rings (Sakura Finetek, Torrance, CA, USA). The paraffin was allowed to

harden overnight at room temperature. Samples were then removed from base molds and stored at 4°C until sectioned.

Fish samples were sectioned on an AO-820 rotary microtome (American Optical, Buffalo, NY, USA) that had been cleaned and decontaminated with absolute ethanol and RNase-Zap (Sigma, St. Louis, MO, USA). Briefly, the tissue blocks were rough cut until the desired part of the fish was exposed. Then, serial 7μm sections were cut and the ribbon was floated out onto a 40°C water bath to remove wrinkles. The sections were then picked up on Superfrost Plus slides (Erie Scientific, Portsmouth, NH, USA) and the slides were placed into a 40°C oven and allowed to dry overnight. Slides were stored in clean, dust-free boxes at room temperature until used for ISH or hematoxylin and eosin staining.

RNA probe synthesis for In Situ *Hybridization*

Total RNA was extracted from whole Japanese medaka using an SV Total RNA Isolation kit (Promega, Madison, WI, USA). Total RNA was reverse transcribed to cDNA with a Superscript III first strand synthesis system (Invitrogen, Carlsbad, CA, USA). RNA probe sequences were designed using information cataloged in the NCBI database cDNA library for Japanese medaka. Using Beacon Designer version 2.06 (Premier Biosoft, Palo Alto, CA), primers were developed flanking a 496bp region of the CYP19a cDNA and a 495bp region of the CYP19b cDNA. Forward and reverse primers were then ordered from a manufacturer (IDT, Coralville, IA, USA). The primers and medaka total cDNA were used to synthesize a large amount of the cDNA fragments of interest using a SYBR Green kit (Applied Biosystems, Warrington, UK) to assure only

one PCR product was being obtained. The cDNA was purified using a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) and cloned into *Escherichia coli* JM109 competent cells (Promega, Madison, WI, USA) according to manufacturer's directions. The *E. coli* were grown out on LB plates (10 g/L Bacto Tryptone, 5 g/L Bacto Yeast extract, 15 g/L agar; pH 7.5) supplemented with 100 μg/mL ampicillin (Roche, Indianapolis, IN, USA) to allow for blue/white colony screening of transfected cells. A single transfected *E. coli* colony was removed from the agar plate and placed into liquid LB medium and allowed to grow for 48 hours. Plasmid DNA was then purified from the *E.coli* with the Wizard Plus SV Minipreps DNA Purification System (Promega, Madison, WI). Plasmid was quantified and purity was checked by agarose gel electrophoresis. Plasmid DNA was then linearized with an appropriate restriction enzyme. In this case, the restriction enzymes used were Sal1 (antisense probes) or Nco1 (sense probes) (Invitrogen, Carlsbad, CA, USA). The linearized plasmid was purified and quantified and run out on an agarose gel to check quality.

In order to minimize the volume in the transcription tubes, 125μCi of ^{35}S-labeled UTP (Perkin Elmer, Boston, MA, USA) was dried down under a vacuum prior to transcription. The following reaction was set up in the same tube, all components are included in the Riboprobe Combination System-SP6/T7 (Promega, Madison, WI, USA): 4μL 5x transcription buffer; 1μL RNasin RNase inhibitor; 1μL 100mM DTT; 2.5mM each ATP, CTP, GTP, and UTP; 1μg linearized plasmid DNA; 1μL of SP6 (sense) or T7 (antisense) RNA polymerase; plus nuclease-free water to bring the total reaction volume to 20μL. The reaction tubes were incubated at 37°C for 1 hour. RNase-free DNase solution was added to digest the template DNA. The tubes were then incubated for 15

minutes at 37°C. DNase-stop solution (Promega, Madison, WI, USA) was then added to the tubes and they were incubated for 15 minutes at 65°C.

Probes were purified by lithium chloride (Ambion, Austin, TX, USA) precipitation, reconstituted in nuclease-free water, and then unincorporated nucleotides were removed from the mixture using Quick Spin Columns for Radiolabeled RNA Purification (Roche, Indianapolis, IN). Probe quality and size were evaluated on a MOPS/formaldehyde gel. Probe activity was determined in counts per minute/μL in a multi-purpose scintillation counter (Beckman-Coulter, Fullerton, CA, USA). Probe specific activity ranged from approximately 3-5x10^7 CPM/μL. Probes were then quantified, separated into aliquots to avoid contamination with RNases, and stored at -80°C until use. Probes were stored no longer than 7d before being used in hybridization experiments to avoid both radioactive decay and contamination/RNA degradation.

In situ *hybridization procedures*

Prior to hybridization, slides with fish sections were first placed into an incubator at 60°C for 60 minutes to allow the paraffin to melt and to completely fuse the sections to the slides. Slides were then removed from the incubator and allowed to cool to room temperature. Sections were then treated as follows to remove paraffin and rehydrate the tissues: Wash with xylene, 2 times for 5 minutes; 100% ethanol, 2 times for 5 minutes; 95% ethanol for 5 minutes; 70% ethanol, 5 minutes; diethylpyrocarbonate-treated water (DEPC-water) for 5 minutes. Sections were then permeabilized in 0.1N HCl for 30 minutes, rinsed in DEPC-water, acetylated in triethanolamine-hydrochloride buffer plus 0.25% acetic anhydride (on a stir plate) for 10 minutes, and then rinsed in DEPC-water.

Sections were dehydrated in 70% ethanol for 5 minutes and then allowed to air dry completely before hybridization.

Hybridization buffer (50% de-ionized formamide, 10% dextran sulfate, 0.1% sodium pyrophosphate, 2xSSC (0.3M sodium chloride, 0.03M sodium citrate, pH 7.0), 1xDenhardt's solution, 500 μg/mL yeast tRNA) was prepared for use by the addition of dithiothreitol (Sigma, St. Louis, MO, USA) to a concentration of 0.5 M. To decrease background signal, slides were allowed to pre-hybridize with just buffer for 1 hour at 55°C. At the end of this period, excess buffer was blotted from the slides prior to hybridization. Prepared ^{35}S-labeled RNA sense (negative control) and antisense riboprobes were then diluted to approximately 60,000 CPM/μL with hybridization buffer. Diluted probes were placed onto fish tissue sections in an amount great enough to cover the tissue. Slides were placed into individual slide mailers (EMS, Hatfield, PA, USA) and these were then transferred to a humid box so slides did not dry out. The humid box was constructed with moistened paper towels placed in a Tupperware container. The humid box was placed into an incubator at 55°C and hybridization was allowed to proceed overnight for 16 hours.

At the end of the hybridization period, slides were removed from the humid box and slide mailers and placed into 1xSSC (0.15M sodium chloride, 0.015M sodium citrate, pH 7.0) to facilitate removal of the excess hybridization buffer. Slides were then placed into slide racks and rinsed 3 more times in 1xSSC. Once the excess hybridization buffer was removed, the following post-hybridization washes were performed on a stir plate: 2xSSC for 10 minutes at room temperature; 2xSSC/50% formamide 2 times for 30 minutes (at 52°C); 2xSSC for 10 minutes; 50μg/mL RNase A (Roche, Indianapolis, IN,

USA) in buffer (0.5M NaCl, 10mM Tris-HCl, 1mM EDTA; pH 7.8) for 30 minutes at 37°C; 2xSSC for 10 minutes; 2xSSC/50% formamide for 30 minutes (at 52°C); 2xSSC for 10 minutes at room temperature. Slides were then rinsed in RO water and dehydrated in 70% ethanol for 5 minutes. Slides were allowed to air dry completely.

Dried slides were arranged in a light-tight exposure chamber face up on top of a piece of cellulose paper. In a darkroom under safelight, a sheet of Kodak Biomax MR film (Kodak, Rochester, NY, USA) was placed on top of the slides, and an additional piece of cellulose paper was placed on top of the film. The exposure chamber was tightly sealed, then placed at 4°C for 5 days. After the 5-day holding period, the film was then developed in an X-OMAT M43A Processor (Kodak, Rochester, NY). Each tissue section on the developed film was digitized individually using a desktop ScanJet ADF (Hewlett Packard, Palo Alto, CA, USA) and saved as a JPG file.

Image Analysis

Digital files for each fish tissue section were edited and analyzed using Image J software (NIH, http://rsb.info.nih.gov/ij/index.html). Images were corrected for general background by subtracting the gray color of the film and sections were all oriented in the same direction. The tissue of interest, the entire gonad, was selected from each image using a tracing tool. A histogram was then constructed for the selected gonadal tissue. To complete the histogram, the program assigned a numerical value to each pixel in the selected area based on its color (from white to gray to black). The average value from each histogram was recorded for every gonad. Each fish had 2-3 sense and 5-6 antisense gonad tissue sections analyzed. The mean sense and antisense histogram values were

calculated for each fish by averaging the histograms for that fish. The sense value (background) was subtracted from the antisense value for data analysis.

Hematoxylin and Eosin (H&E) Staining and Slide Image Analysis

Slides were de-paraffinized in xylene and rehydrated through a graded ethanol series (100% ethanol three times for five minutes each, 95% ethanol twice for five minutes each, 70% ethanol for five minutes, and water for 5 minutes). Slides were then stained in Harris' Hematoxylin (EMS, Hatfield, PA, USA) for 3 minutes, processed through acid alcohol, ammonia, and ethanol washes, and then stained in 1% Eosin Y (EMS, Hatfield, PA, USA) in 80% ethanol for 1 minute. Slides were then dehydrated through an ethanol series (reverse of the rehydration series) and cleared in xylene. Slides were preserved under glass cover slips using Entellan mounting medium (EMS, Hatfield, PA, USA) and allowed to dry.

Images of the gonad on each slide were recorded using a Camedia C-3040ZOOM digital camera (Olympus, Center Valley, PA, USA) attached to an Olympus BX41 microscope (Optical Analysis Corporation, Nashua, NH, USA). Male images were examined for normal spermatogenesis and the presence of testis-ova. The oocytes in each female image were developmentally staged according to the system set forth in Iwasmatsu et al. (1988).

Statistics

Weight and length data were averaged and expressed as arithmetic means ± standard deviation for each sex by treatment. CYP19a gene expression data were also

expressed as arithmetic treatment means ± standard deviation by sex. The normality of the weight, length, and CYP19a expression data was determined using Shapiro-Wilk tests. Data that were normally distributed were analyzed by ANOVA, non-normally distributed data were analyzed by non-parametric Kruskal-Wallis tests. Statistical significance was defined as $p<0.05$.

Results

Water Quality

During the course of the exposure, water quality parameters ranged as follows in all tanks: temperature (24°C); pH (7.89-8.13); ammonia nitrogen (<0.02-0.04 ppm); nitrite nitrogen (<0.02-0.3 ppm); dissolved oxygen (4.3-6.9 ppm); and hardness (370-480 ppm $CaCO_3$). All values were within a normal range for water quality.

Weight and Length at Exposure Termination

Mean weight and length values were calculated for males and females separately for each treatment (Table 1). The weight and length data were normally distributed. There were no significant differences by treatment in weight (males p=0.350; females p=0.679) or length (males p=0.236; females p=0.640). Male weight increased slightly with increasing fadrozole concentration, and was between 0.133 ± 0.032g and 0.177 ± 0.050g in the controls and 100 μg/L fadrozole treatments, respectively. For females no such trend was observed, and fish weights were between 0.159 ± 0.006g (1 μg/L fadrozole) and 0.176 ± 0.022g (100 μg/L fadrozole). There were no noticeable trends in length.

In situ *Hybridization*

CYP19a gene expression was approximated as the mean expression value from histograms constructed for each tissue section autoradiograph (Figures 3, 4, and 5). The expression values are therefore semi-quantitative and unitless. Mean expression values are given as an arithmetic mean ± SD (Table 2). The data were not normally distributed,

and so were analyzed with Kruskal-Wallis tests. While the raw data showed an increase in CYP19a gene expression in females with increasing fadrozole treatment, the variability in the data masked any treatment effects when the data were statistically analyzed. There were no significant differences between treatments for males ($p=0.263$) or females ($p=0.376$) (Figures 6 and 7).

Histopathology

Hematoxylin and eosin stained tissue sections from each fish were examined for histopathological abnormalities. All male fish from the control and fadrozole treatments were classified as having normal spermatogenesis; the lumina were filled with mature spermatozoa and the lobules contained spermatogenic cysts (Figure 8). No fish exhibited testicular oocytes. Female fish from the control, 1μg/L, and 10μg/L fadrozole treatments were also classified as normal in terms of oocyte development. Fish from those treatments exhibited oocytes in all stages of development, which is expected in asynchronous spawners (Figure 9). Female fish from the 100μg/L fadrozole treatment exhibited many oocytes in late vitellogenesis that were all similar in size and lacked a distinct yolk globule (stage VII and VIII of development as classified by Iwamatsu et al. (1988)) as well as a number of early vitellogenic oocytes. None of the oocytes from these females was classified as stage IX, or mature with a pink-staining yolk globule inside the oocyte. (Figure 9). Thus, while the presence of early vitellogenic oocytes was similar across all treatments, females from the 100μg/L fadrozole treatment had more late vitellogenic oocytes and less maturing oocytes than fish in the other treatments.

Table 1: Weight and length of medaka at exposure termination

Treatment (μg/L fad)	Male weight (g)	Male length (mm)	Female weight (g)	Female length (mm)
0	0.133 ± 0.032	19.195 ± 1.389	0.163 ± 0.033	20.033 ± 1.387
1	0.150 ± 0.030	20.348 ± 1.344	0.159 ± 0.006	20.273 ± 0.426
10	0.172 ± 0.021	21.337 ± 1.481	0.176 ± 0.032	20.312 ± 0.973
100	0.177 ± 0.050	21.230 ± 1.648	0.176 ± 0.022	20.794 ± 0.569

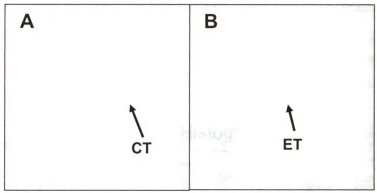
Values expressed as mean ± SD in grams for weight and millimeters for length. No significant treatment differences in weight (males p=0.350, females p=0.679) or length (males p=0.236, females p=0.640) were found.

Table 2: CYP19a gene expression in medaka after fadrozole exposure

Treatment (μg/L fadrozole)	Male CYP19a expression	Female CYP19a expression
0	4.642 ± 2.459	3.570 ± 3.166
1	2.441 ± 2.545	5.366 ± 2.775
10	6.408 ± 2.567	4.399 ± 1.590
100	2.715 ± 0.067	8.230 ± 5.453

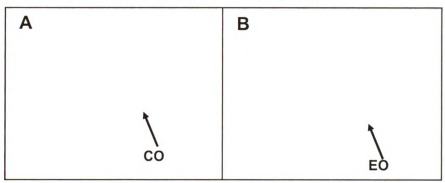
Values are expressed as mean ± SD. The gene expression values are representative of the pixel color of autoradiographic images as assigned by a computer program and are semi-quantitative and unitless. No significant treatment differences in gene expression for males (p=0.263) or females (p=0.376) were found.

Figure 3: Autoradiographs of male fish



Autoradiographs of male fish from control (A) and 100 ug/L fadrozole (B) treatments. Both the control testis (CT) and exposed testis (ET) show similar levels of CYP19a signal.

Figure 4: Autoradiographs of female fish

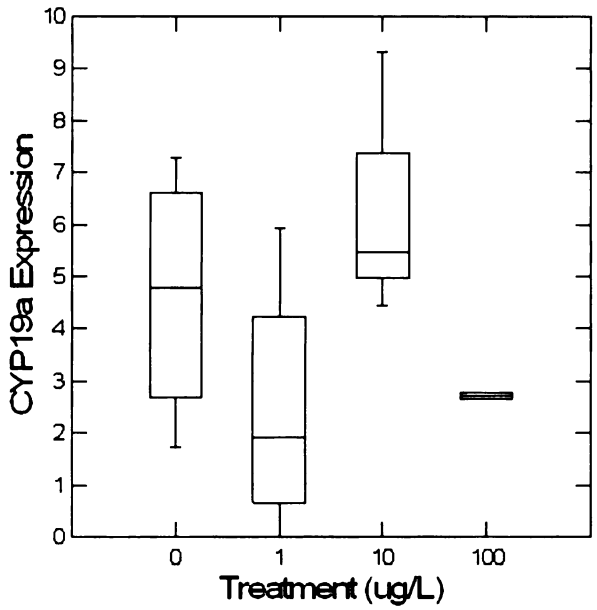


Autoradiographs of female fish from control (A) and 100 ug/L fadrozole (B) treatments. The control ovary (CO) shows no CYP19a signal; the exposed ovary (EO) shows distinct CYP19a signal.

Figure 5: Example Histogram

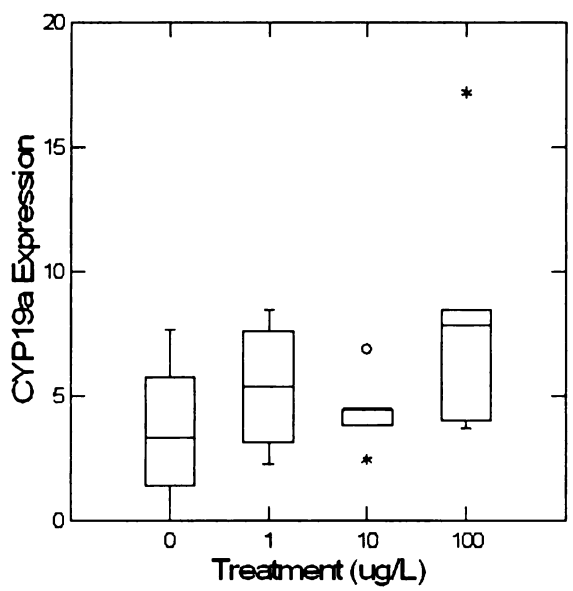
An example histogram output from the Image J software used for gonad image analysis. The software assigned a numeric value based on color to each pixel of the gonad area. The mean value from each histogram was used for statistical analysis.

Figure 6: Male CYP19a Expression



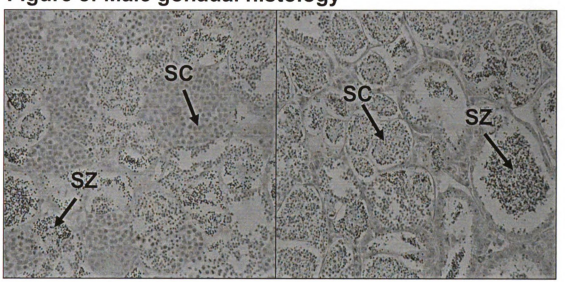
Boxplots of male CYP19a expression showing treatment means and interquartile ranges. CYP19a expression values (y-axis) are representative of autoradiograph pixel color and are therefore semi-quantitative and unitless. There were no significant treatment differences (p=0.263). The shape of the boxplot for the 100 ug/L treatment is due to the loss of samples from that treatment leaving sample size at 2 fish.

Figure 7: Female CYP19a Expression



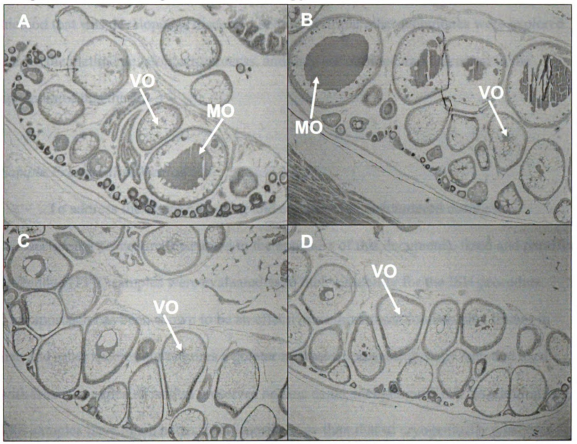
Boxplots of female CYP19a expression showing treatment means and interquartile ranges. CYP19a expression values (y-axis) are representative of autoradiograph pixel color and are therefore semi-quantitative and unitless. The * and ° symbols denote statistical outliers. These data points were included in statistical analysis. There were no significant treatment differences (p=0.376).

Figure 8: Male gonadal histology



A control male (left) and exposed male (right) both exhibited normal morphology and spermatogenesis with mature spermatozoa (SZ) in the lumina and spermatogenic cysts (SC) in the lobules.

Figure 9: Female gonadal histology



Normal gametogenesis is exhibited in females from the control (A) and 1 ug/L (B) treatments. Females from the 100ug/L treatment (C & D) have no mature oocytes (MO), but many vitellogenic oocytes (VO).

Discussion

This project resulted in the development and validation of a semi-quantitative radioisotopic *in situ* hybridization system to measure expression of genes of interest for which sequence information is available in the Japanese medaka. The utility of the ISH method that was developed is discussed below. Multiple other techniques were explored during the method development process, and they are outlined and discussed in the appendix of this thesis.

Sample Preservation Method

To address the issues of tearing and compression encountered during the cyrosectioning procedure (discussed in the appendix of this document), fixed and paraffin embedded (FPE) samples were evaluated as to their suitability for the ISH procedure. This approach has been shown to be an effective method to section complex tissues in fish and other species but requires a greater number of processing steps than that used with frozen samples. Fixation preserves nucleic acids, including mRNA. In addition, FPE samples have much better tissue morphology than that of cryogenically preserved samples, even in single organ samples. In this project, the processing and paraffin matrix completely overcame the presence of the bones and the oils in the body cavity. Since FPE samples offered better morphology and tissue preservation that cryogenically preserved samples, they were determined to be optimal for the applications of this project.

Detection Method – Radiolabeled RNA Probes

As a consequence of the problems encountered with DIG-labeled riboprobes (discussed in the appendix), an approach was undertaken that involved the development of probes labeled with ^{35}S (radioisotope). Radioisotopes are used extensively in ISH and blotting procedures due to their sensitivity and reliability. Moorman et al. (1997) preferred radioisotopic ISH applications because DIG-systems tend to have probe-dependent sensitivity issues and the signal obtained is sometimes difficult to quantify. While the preferred detection method to use with radio-labeled probes is silver grain emulsion exposure that detects the probe in the tissue on the slide (Hrabovszky et al., 2004; Moorman et al., 1997), this method is prohibitively expensive and as a result is not typically used to process large numbers of samples. Therefore, the slides in the current study were exposed to sheets of radiographic film that would show areas of the tissue that contained hybridized labeled probe when they were developed. While these films produce a reliable signal, it was difficult to digitize the images after the films were developed. The loss of resolution during the digitization process undoubtedly increased the uncertainty in the signal quantification.

While radio-labeled probes can be a good option for ISH, they are expensive to synthesize and detect and require extra training and laboratory certifications. As an alternative that could potentially avoid these issues, fluorophore-labeled probes were briefly evaluated in this study. Fluorescent images are simple to collect and manipulate while offering high picture quality and resolution and the fluorescent signal is also easily quantifiable (Neri et al., 2000). In addition, a wide range of fluorophores are commercially available for labeling nucleic acids. The major drawback to using

fluorophore-labeled probes on fixed tissues is autofluorescence that occurs at nearly all wavelengths associated with the fluorescent dyes used the production of the riboprobes. The methodology used in the correction of tissue autofluorescence is very tedious and requires knowledge of and extensive access to a confocal fluorescence microscope and a specialized computer imaging system (Szöllösi et al., 1995; van de Lest et al., 1995) that was beyond the scope of this project.

Gonadal histology and CYP19a gene expression after exposure to fadrozole

To investigate the effects of fadrozole on the reproductive health of medaka, measures of reproductive physiology were evaluated in the male and female fish from this exposure. In a study with genetically female Japanese medaka exposed to fadrozole in their food from hatch until sexual maturation (90 days post-hatch), females exhibited normal oogenesis and folliculogenesis prior to the vitellogenic phase but lacked any post-vitellogenic oocytes (Suzuki et al. 2004). Similar results have been reported in female fish exposed to a different aromatase inhibitor, letrozole (Sun et al., 2007). Based on these studies, it can be hypothesized that oocyte maturation may have been retarded in mature female medaka in the short-term waterborne exposure to fadrozole conducted in this study. In a post-exposure histological examination, female fish from the 0, 1, and 10μg/L treatments had oocytes in all stages of development present in the ovary and were in a normal reproductive state. However, the female fish from the 100μg/L treatment had stage VII and VIII vitellogenic oocytes but no mature stage IX oocytes (stages from Iwamatsu et al., 1988). Thus, while early oocyte development was normal in these fish they lacked mature oocytes and it is doubtful that they would have successfully spawned.

This hypothesis is supported by the observation that medaka exposed to letrozole for 21 days ceased to spawn (Sun et al., 2007). This study is the first documented case of changes in histological structure in female medaka being linked to short term (7d) fadrozole exposure. Little is known about whether female medaka could recover normal morphology and reproductive abilities if aromatase inhibitor exposure were halted.

The effects of aromatase inhibitors on male gonadal histology are more subtle than those in females. Suzuki et al. (2004) found that food borne exposure to fadrozole had no effect on testicular histology. However, letrozole exposure in male fish resulted in an enlargement of the lumina and seminiferous tubules and increased density of spermatozoa, but this only occurred at high (625μg/L) concentrations of letrozole (Sun et al., 2007). In this study, short term (7 days) exposure to fadrozole up to concentrations of 100μg/L had no effect on the gonadal histology of male fish. Even where effects are observed (Sun et al., 2007), it is difficult to decipher whether fertility and fecundity are actually altered, and gonadal aromatase probably doesn't play a pivotal role spermatogenesis.

The *in vivo* reproductive effects of fadrozole are diverse and, depending on the exposure regime, sometimes severe in teleost fish. Fadrozole treatment during development induces masculinization in a variety of species, including Chinook salmon (*Oncorhynchus tshawytscha*), zebrafish, Nile tilapia (*Oreochromis niloticus*), golden rabbitfish (*Siganus guttatus*), and Japanese flounder (*Paralichthys olivaceus*) (Komatsu et al., 2006; Fenske and Segner, 2004; Uchida et al., 2004; Afonso et al., 2001; Kitano et al., 2000; Piferrer et al., 1994). The male Endler guppy (*Poecilia reticulata*) displays fewer courtship behaviors after fadrozole exposure (Hallgren et al., 2006). Fadrozole has also

been shown to inhibit ovarian growth and induce testis growth in fathead minnows (Panter et al., 2004; Ankley et al., 2002). It can increase oocyte apoptosis in female zebrafish (Uchida et al., 2004). In fathead minnows, fadrozole reduces fecundity and decreases plasma concentrations of estradiol and vitellogenin in females and testosterone and 11-ketotestosterone in males; male fathead minnows also tend to accumulate mature sperm in the testis (Ankley et al., 2002). Fadrozole injection lowered plasma estradiol levels in female Coho salmon (*Oncorhynchus kisutch*) and induced ovulation (Afonso et al., 1999) and induced spermiation in males (Afonso et al., 2000). Fadrozole increased plasma testosterone levels in Eurasian perch (*Perca fluviatilis*) (Mandiki et al., 2005) and increased plasma gonadotropin II levels in black porgy (*Acanthopagrus schlegeli*) (Lee et al., 2001). Medaka embryos, though, show only a small percentage of sex reversal even at embryo-toxic concentrations (Kawahara and Yamashita, 2000), probably due to their strict genetic sex determination (Schartl, 2004). However, treatment of female medaka fry with fadrozole impedes the formation of the ovarian cavity (Suzuki et al., 2004), so it isn't without reproductive effects. Letrozole exposure does not affect medaka embryonic development, but does have distinct effects on oocyte maturation (Sun et al., 2007). Exposure of adult medaka to fadrozole and other similarly acting aromatase inhibitors such as letrozole, elicits adverse reproductive effects depending on exposure timing and concentration. The myriad effects of fadrozole on reproductive parameters in teleosts led to the hypothesis that there would be effects on aromatase gene expression in this study.

Fadrozole has been shown to affect aromatase gene expression and enzyme activity in the gonads of teleost fish previously. For instance, in fish exposed to fadrozole during sexual differentiation, aromatase gene expression was suppressed in the ovary of

genetically female flounder (Kitano et al., 2000) and zebrafish (Fenske and Segner, 2004) and resulted in masculinization. Exposure of adult female fathead minnows to fadrozole increased measurable aromatase activity in ovarian microsomes as well as the expression of the CYP19a gene in the ovary (Villeneuve et al., 2006). Overall, there is very little other information in the literature regarding gonadal aromatase activity or gene expression after fadrozole exposure. To my knowledge, no other studies have determined the effects of fadrozole on gene expression using *in situ* hybridization as a detection method.

There are no data in the literature on the effects of fadrozole exposure on CYP19a gene expression in mature male medaka, so it was hard to hypothesize possible effects. In the current study, exposure of male medaka to fadrozole up to 100μg/L had no significant effect on CYP19a expression after a 7-d waterborne exposure. The lack of measurable effects in this study could possibly be attributed to the ISH detection method used, but RT-PCR analysis on male fish from the same exposure confirmed that fadrozole treatment had no significant effects on CYP19a gene expression at the concentrations used (June Woo Park, unpublished data). It was expected that CYP19b (brain aromatase) would be much more responsive to fadrozole exposure than CYP19a in males, but this working hypothesis could not be evaluated due to the fact that a workable probe for CYP19b expression was not developed in this study.

Based on RT-PCR data collected from fathead minnows by Villeneuve et al. (2006), it was hypothesized that exposure to fadrozole in concentrations as small as 1.85μg/L would significantly increase expression of the CYP19a gene in female medaka gonads. No significant increases in ovarian aromatase gene expression were observed in

fish exposed to 10 or 100μg/L fadrozole in the current study. However, there was an increasing but not statistically significant trend in CYP19a expression that was most apparent in fish from the 100μg/L fadrozole treatment. Analysis of ovarian CYP19a gene expression in fish from the same exposure using RT-PCR confirmed this apparent increase. Female fish ovaries from the 10 and 100μg/L fadrozole treatments both had significantly higher gene expression, 7 and 14-fold respectively, than those from the control and 1μg/L treatments (June Woo Park, unpublished data). Therefore, the lack of a significant trend in the CYP19a gene expression in females exposed to fadrozole was most likely due to small sample size, large variability in the data, and, mostly, the lack of resolution associated with the ISH detection method used in this study. While the method wasn't optimal for quantification or resolution in relation to other techniques, it did provide a first look at spatial gene expression in the medaka gonad after fadrozole exposure. A method that had better resolution, such as silver gain emulsion radioisotopic or fluorophore ISH, may have captured the increase in gene expression in a quantifiable way that was more amenable to statistical analysis. In addition, an ISH method with better resolution would have also allowed for the classification of the types of oocytes that were expressing CYP19a. Only a few studies have evaluated the relationship between oocyte maturity and CYP19a gene expression in teleosts, specifically killifish (*Fundulus heteroclitus*), zebrafish, and gobiids (*Trimma okinawe*), using ISH. In these studies, CYP19a gene expression was highly specific to oocytes of certain maturation stages: there was no measurable expression in mature follicles, and the greatest expression was observed in vitellogenic follicles, thecal cells, granulosa cells, and pre-vitellogenic and vitellogenic oocytes (Goto-Kazeto et al., 2004; Kobayashi et al., 2004;

Rodriguez-Mari et al., 2005; Dong and Willet, 2007). A method that would provide a more detailed picture of cell types expressing CYP19a in medaka gonads would offer more insight into the potential effects of fadrozole.

The increase in CYP19a expression after fadrozole exposure in this study and prior studies (Villeneuve et al., 2006) can probably be linked to the promoter control and signal transduction pathway of the CYP19a gene. CYP19a contains a complete SF-1 site in its promoter (Callard et al., 2001; Kazeto et al., 2001). Villeneuve and coworkers suggested that CYP19a gene expression increased via a gonadotropin-mediated signal that altered levels of circulating cAMP that in turn interacted with the SF-1 promoter and altered expression of steroidogenic genes. The involvement of this type of signal transduction pathway makes it difficult to predict the effects of chemicals that act indirectly like fadrozole. Fadrozole's mechanism of action, inhibition of the aromatase enzyme, and the subsequent drop in levels of circulating estradiol are not directly linked to the increase the expression of the gonadal aromatase gene. These types of effects underscore the need for laboratory techniques that allow the quantification and spatial resolution of multiple gene products in the same animal, like ISH. ISH can offer a powerful tool to search out the indirect effects of chemical exposure *in vivo*.

Conclusions

Fadrozole increased expression of CYP19a in female gonads and induced changes in female gonadal morphology, by retarding final maturation of oocytes. It had no detectable effect on male gene expression or testicular histology. With some modifications, the ISH method developed in this thesis project will be able to aid in detecting patterns of gene expression along the HPG-axis in the Japanese medaka. While the method is not perfect, it does work well enough to distinguish gross changes in spatial gene expression at the tissue scale. With refinement, it will more reliably quantify changes in expression at the cellular level. ISH is a very useful tool for looking at the spatial and temporal aspects of gene expression. It can also give insight into the indirect effects of chemical exposure on signal transduction pathways.

Appendix

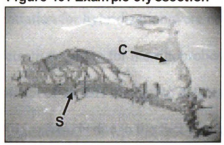
Cryosectioning – Methods, Results and Discussion

For cryosectioning procedures, Japanese medaka were euthanized in Tricane S solution (Western Chemical, Ferndale, WA, USA) and then embedded in Tissue Tek OCT compound (Miles, Elkhart, IN, USA) in Tissue Tek base molds (Miles, Elkhart, IN, USA). Samples were then frozen in isopentane that was held in a beaker in a liquid nitrogen bath. When the fish were completely frozen, they were either stored at -80°C or placed directly into a cryostat (Tissue Tek, Elkhart, IN, USA) that had been pre-cooled to -20°C. For sectioning, frozen samples were affixed to object holders (EMS, Hatfield, PA, USA) with OCT compound and clamped into the cryostat. Sections were cut serially at 10-20μm and thaw-mounted on Superfrost Plus slides (Erie Scientific, Portsmouth, NH, USA). Samples were then evaluated for their suitability for use in ISH procedures by hematoxylin and eosin staining (process detailed previously in the methods section of this thesis).

After some experimentation in conjunction with a pathology facility at Michigan State University, it was determined that whole Japanese medaka were not amenable to being flash-frozen and cryosectioned using the procedures available at Michigan State University. The two major problems that were encountered with the use of frozen whole-fish sections were shredding and compressing (Figure 10). The shredding was a result of the presence of calcified bone in the fish samples that chipped the cryostat blade and resulted in tearing of the fish sections. This was especially apparent in the caudal and spinal regions of the fish sections. Compression of the internal organs of the body cavity was also observed and resulted in the loss of tissue structure and morphology of all

organs. This loss of structure was most likely due to the presence of oils in the body cavity that retarded the freezing process and compromised the integrity of the frozen matrix which is integral to maintaining section morphology. As a result, even in the best examples of cryosections obtained in this study, no internal organs were identifiable.

Figure 10: Example cryosection



A medaka cryosection showing shredding (S) of the muscle tissue and compression (C) of the internal organs. Overall morphology of the cryosections was very poor.

Probe Labeling and Detection Systems – Methods, Results and Discussion

Digoxygenin-labeled probes were transcribed using the same methods as described in the methods section of this thesis for radio-labeled probes, except that DIG-labeling mix (Roche, Indianapolis, IN, USA) was used in the transcription reaction instead of ^{35}S-UTP. Probes were then purified by lithium chloride extraction and unincorporated nucleotides were removed with Centri-Sep spin columns (Princeton Separations, Princeton, NJ, USA). Probes were then quantified and used in hybridization trials. Optimal conditions and probe concentration for hybridization were never determined.

To synthesize fluorophore-labeled probes, RNA was synthesized exactly as for radio-labeled probes except no ^{35}S-UTP was used in the transcription reaction. Probes were then purified by lithium chloride extraction and unincorporated nucleotides were removed with Centri-Sep spin columns. Probes were quantified and labeled with Alexa-Fluor dyes (Invitrogen – Molecular Probes, Carlsbad, CA, USA) according to the manufacturer's protocol. Probes were then used in hybridization trials, but optimal conditions and probe concentration for hybridization were never determined.

The selection of a detection system for the riboprobes was a critical area of investigation to the development of an ISH method to detect altered gene expression in medaka. The importance of this phase is due to the fact that as sample complexity increases there is a greater likelihood that non-specific hybridization will occur resulting in an increase in false positives relative to the detection of gene expression. As a result, several labeling approaches were investigated and used to develop the riboprobes for CYP19a. Digoxygenin (DIG) is one of the most commonly used non-radioisotopic methods for labeling nucleic acid probes. DIG-linked reporter systems have been shown to work well in mice and humans (Wilkinson, 1998) and have also been used with some success in fish (Ijiri et al., 2006), including Japanese medaka (Suzuki et al., 2004; Quiring et al., 2004). However, in the current study, the implementation of a DIG-labeled riboprobe system was never successful with the paraffin-embedded medaka samples. In addition, several other researchers with ISH experience recommended not using DIG-systems on fish tissues (Sandra Peterson and Erich Ottem, personal communications). Thus, while it was possible to synthesize and label appropriate riboprobes for CYP19a with DIG, the results were highly variable between runs and the background signal was

much greater than desired based on the hybridization signal measured in the sense samples and the DIG-system was abandoned.

CYP19b Probe – Methods, Results and Discussion

The original intent of this study was to evaluate the expression of two genes, the two isoforms of CYP19 aromatase, in the development of the ISH method. However, there were unexpected difficulties in developing a functioning CYP19b (brain aromatase) probe. This was due to several factors including the use of inappropriate gene sequences that were not completely specific to the CYP19b gene. In addition, some sequences cloned into the plasmid vector in the improper orientation and resulted in riboprobes that were not usable in the ISH procedures. Finally, while one riboprobe sequence was properly incorporated into the vector, it failed hybridization trials because of high levels of background signal. ISH is still a fairly new technique in our laboratory, so the chosen sequences may not have been amenable to hybridization or hybridization conditions could have been unsuitable for detecting signal in brain tissue. Future studies experimenting with different probe sequences and hybridization conditions will further explore the reasons for the issues encountered with the CYP19b probe.

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