Molecular Responses of the Murray River rainbowfish, *Melanotaenia fluviatilis* Exposed to Endocrine Disrupting Chemicals

A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

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M.F.Sc

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Declaration

I certify that except where the acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date for the approved research program; and any editorial work, paid or unpaid, carried out by a third party is acknowledged.

______________________________  ________________
Admane Holeyappa Shanthanagouda  Date
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I dedicate my PhD thesis to my beloved parents.
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<tbody>
<tr>
<td>BPA</td>
<td>Bisphenol A</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>cyp19a1a</td>
<td>Gene coding for Ovarian aromatase protein</td>
</tr>
<tr>
<td>Cyp19a1a</td>
<td>Ovarian aromatase protein (enzyme)</td>
</tr>
<tr>
<td>cyp19a1b</td>
<td>Gene coding for brain aromatase protein</td>
</tr>
<tr>
<td>Cyp19a1b</td>
<td>Brain aromatase protein (enzyme)</td>
</tr>
<tr>
<td>d</td>
<td>day old embryo</td>
</tr>
<tr>
<td>dd</td>
<td>Double distilled water</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DNase</td>
<td>An enzyme to eliminate DNA</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxyribonucleoside triphosphate</td>
</tr>
<tr>
<td>dph</td>
<td>days post hatch</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>E2</td>
<td>17β-estradiol</td>
</tr>
<tr>
<td>EDCs</td>
<td>Endocrine Disruptive Chemicals</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen Receptor</td>
</tr>
<tr>
<td>ERE</td>
<td>Estrogen Receptor Element</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>gapdh</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase (gene)</td>
</tr>
<tr>
<td>gDNA</td>
<td>genomic DNA</td>
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<tr>
<td>GSP</td>
<td>Gene Specific Primer</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>kb</td>
<td>Kilobase</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
</tbody>
</table>
L  Litre
LB  Luria Bertoni media
MgCl₂  Magnesium Chloride
mL  Millilitre
mM  Millimolar or millimole per litre
MMLV-RT  Moloney Murine Leukemia Virus Reverse Transcriptase
mRNA  Messenger RNA
ng  Nanogram
nM  Nanomolar
NP  Nonylphenol
NUP  Nested Universal Primer
OD  Optical Density
ORF  Open Reading Frame
PCR  Polymerase Chain Reaction
ppm  parts per million
qPCR  quantitative Real-Time PCR
RACE  Rapid amplification of cDNA ends
RNA  Ribonucleic Acid
RNase  An enzyme to eliminate RNA
rpm  Rotation per minute
RT  Room Temperature
RT-PCR  Reverse-Transcription Polymerase Chain Reaction
s  Second
S.O.C  Super Optimal Broth
scc  side chain cleavage
UPM  Universal Primer (Adaptor primer)
Vtg  Vtg (Vitellogenin) is a female specific protein
vtg  Gene coding for vitellogenin protein
µg  Microgram
µL  Microlitre
SUMMARY

Endocrine Disrupting Chemicals (EDCs) are those that interfere with the endocrine system of animals, primarily impacting the steroidogenic hormone pathways. Sources of these chemicals include sewage, industrial waste, urban and agricultural runoff. These reach the aquatic environment via human activities and impact negatively on non-target species, such as fish which play a key role in the aquatic food web and more importantly are also a source of protein for humans. In Australia, there is little information available on the effects of environmental pollutants including EDCs on Australian native fish species, and even less information on the effects of EDCs at the proteomic or genomic level in Australian fish. Therefore, this PhD project investigated the effects of selected EDCs on aromatase and vitellogenin at the molecular level in the Australian native Murray River rainbowfish, *M. fluviatilis*.

The ovarian (*cyp19a1a*) and brain (*cyp19a1b*) aromatase gene isoforms were isolated and characterised from *M. fluviatilis* for the first time in this study. The sequence information enabled the construction of a Phylogenetic tree and it was observed that the aromatase gene isoforms of the Murray River rainbowfish showed a high identity with pejerrey, a closely related athernid species. The observation confirmed the close taxonomic relationship of these two species, in spite of their current geographical separation. The tissue specific expression of aromatase isoforms in both sexes of adult fish was also studied using quantitative Real-Time PCR (qPCR). The *cyp19a1a* was exclusively expressed in the ovarian tissue and *cyp19a1b* was predominantly expressed in the brain of both sexes. A low level of expression was also observed in other tissues including liver, eyes and gonads, with no measurable expression in the spleen of
both sexes. In addition, the aromatase gene expression was also studied during ontogeny. \textit{cyp19a1a} was not detected in any of the developmental stages studied. On the contrary \textit{cyp19a1b} was expressed in all selected developmental stages. It was concluded that the gonadal differentiation has not yet begun in the selected developmental stages, since \textit{cyp19a1a} was not detected. Maternal inheritance and onset of zygotic expression of \textit{cyp19a1b} in early larval stages suggests a significant role for the isoform during early larval growth and development, including in the brain.

Next, a series of aqueous exposure experiments were conducted for 96 h on both sexes of adult \textit{M. fluviatilis} to evaluate the effects of selected EDCs including 17β-estradiol (E2) at 1, 3 and 5 µg/L, nonylphenol (NP), Bisphenol A and the aromatase inhibitor fadrozole at 100 and 500 µg/L on aromatase gene expression. In addition, the effect of E2 and NP on vitellogenin gene and protein expression in male \textit{M. fluviatilis} was also studied. With exposure to E2 and NP, a significant reduction in the expression of \textit{cyp19a1a} in ovarian tissues at the two lowest exposure concentrations was observed. At higher concentrations of E2 and NP, \textit{cyp19a1a} was completely inhibited in ovarian tissues. Similarly, \textit{cyp19a1b} was significantly downregulated in the brain of male fish exposed to E2 and NP. However, \textit{cyp19a1b} in testicular, ovarian and brain tissues of female fish exposed to E2 was initially upregulated until 72 h of exposure and then downregulated at 72 and 96 h exposure. Expression of \textit{cyp19a1b} in female fish brain was upregulated at both exposure concentrations of NP.

With exposure to BPA, \textit{cyp19a1a} in the gonads of the rainbowfish was not significantly affected, whereas with exposure to fadrozole the expression of
cyp19a1a was increased at high exposure concentration. However, the expression of cyp19a1b in the brain and gonads of both sexes contrasted with each other with exposure to both BPA and fadrozole.

The vitellogenin was analysed in testis and liver of male *M. fluviatilis* and showed significantly high expression in male fish exposed to E2 and NP compared to that of the controls.

Collectively the results of this study suggested that the tested EDCs can have a disruptive effect on the steroidogenic pathways of *M. fluviatilis* and hence sex differentiation, sexual behaviour and reproductive cycles in this fish. The results of this study indicated that *M. fluviatilis* is sensitive to EDCs and both aromatase isoforms and vitellogenin have potential as biomarkers of exposure to such chemicals. Based on the results obtained in this investigation, the Murray River rainbowfish can be used as a model species to assess the impact of the EDCs in the Australian freshwater ecosystem.
CHAPTER 1

General Introduction

1.0. Introduction

Freshwater is one of the most valuable resources on earth and its availability is often limited. Therefore, its protection is a major challenge to mankind. At present, more than 200,000 (Tyler et al., 2008) chemicals are widely used and many of them are potential contaminants to our water resources. Both agricultural and industrial chemicals often enter water ways through agricultural runoff or the release of effluents. Many of these chemicals are persistent and hazardous to non target organisms especially those that are sensitive to these chemicals. They become toxic at various levels and eventually may reduce biodiversity in the environment. Some of these chemicals act as estrogens, antiestrogens or androgens; since they mimic or replace the endogenous hormones, in turn affecting the physiology, behaviour, growth, metabolism, reproduction and even the sex ratio of aquatic organisms (Abinawanto et al., 1996; Kazeto et al., 2004). Such effects can lead to biased sex ratios, with the potential to lead to the extinction of the affected species. Fish species in their aquatic habitat are in direct contact with the surrounding environment, with several tissues and internal compartments exposed to water, potentially inducing a high sensitivity to water-borne parameters such as temperature, oxygen levels, salinity, and more importantly to toxic chemicals.
Hence, there is a strong need to assess the risks that may originate from these water borne chemical compounds to fish.

There is little information on the lethal and sublethal effects of environmental pollutants on Australian freshwater fish species, and as a result, many of the Australian water quality guidelines are based on data from exotic fish species (Harford et al., 2005). Sublethal responses to toxicant exposure can be estimated using a suite of biomarkers and this project is focussed on evaluating potential biomarkers of exposure to EDCs in an Australian native fish species.

1.1. Endocrine Disrupting Chemicals (EDCs)

The USEPA (United States Environmental Protection Agency) defines endocrine disrupting chemicals as “exogenous chemicals that interfere with the production, release, transport, metabolism, binding, action, or elimination of endogenous hormones responsible for the maintenance of homeostasis and the regulation of developmental processes” (Kavlock et al., 1996). These compounds are also known as hormone mimics, estrogen mimics or xenoestrogens (Zhou et al., 2009). They affect reproduction, the immune system and the endocrine system (Jobling et al., 1996; Desbrow et al., 1998; Hale et al., 2000; Lee and Peart, 2000; Oshima et al., 2003; Milla et al., 2011). The effects of exposures to EDCs on aquatic organisms include permanent or irreversible sex reversal (Colborn et al., 1993; Kuhl and Brouwer, 2006). Basically they interfere with the function of the endocrine system in different ways, including mimicking endogenous hormones like estrogen or testosterone.
and lead to similar chemical and physiological stimulation. Their mechanism of action includes blocking receptors in cells receiving these hormones and thereby preventing the actions of endogenous hormones (Barcelo and Kettrup, 2004; Burger and Moolman, 2006); affecting the synthesis, transport, metabolism and excretion of hormones and thereby altering the concentrations of natural hormones (Soto et al., 1995). This study focussed on three commonly encountered endocrine mimics (E2, NP and BPA) and an aromatase inhibitor (fadrozole).

1.2. Selected Endocrine Disrupting Chemicals (EDCs)

1.2.1. 17β-estradiol (E2):

E2 (Fig. 1.1) is an exogenous estrogen and a highly potent estrogenic chemical. The concentrations of E2 in the aquatic environment vary from nanograms to hundreds of nanograms per litre (Shore et al., 1993; Desbrow et al., 1998). E2 is used in large quantities by humans being in contraceptive pills and in dairy farming. This has resulted in the discharge of huge quantities to the natural environment by industries and sewage treatment plants, finally reaching water bodies and disrupting the endocrine system of aquatic organisms (Oshima et al., 2003). Structurally the exogenous E2 is similar to that of endogenous estrogen (Fig. 1.1); therefore many researchers have found that this acts as a highly potent chemical in the aquatic environment and that it disrupts the endocrine function of fish at very low levels (Kishida et al., 2001; Hinfray et al., 2006; Jin et al., 2009; Woods et al., 2007).
1.2.2. Nonylphenol (NP):

NP (Fig. 1.2) in the aquatic environment originates from decomposition of Nonylphenol Ethoxylates (NPEs). It is an important source of surfactant which is widely used in the production of paints, detergents, rubber auxiliaries, antioxidants, in corrosion inhibitors and wetting agents (Soto et al., 1991; Kortner et al., 2009). In natural water bodies it is detected at microgram levels ranging from 0.1 to 336 µg/L (Ahel et al., 1994; Kvestak et al., 1994; Blackburn and Waldock, 1995; Kang et al., 2003).

Several studies show that NP mimics an estrogen (Soto et al., 1991; Knudsen and Pottinger, 1999; Laws et al., 2000; Madigou et al., 2001; Yang et al., 2006) and adversely affects the sexual behaviour, sex differentiation and reproductive physiology in teleosts (Jobling, 1996; Hill and Janz, 2003; Seki et al., 2003).
1.2.3. Bisphenol (BPA):

BPA (Fig. 1.3) is a widely used chemical in the plastic industry and has been shown to leach out of plastic containers and affect aquatic biota (Magdalena et al., 2010; Wang et al., 2010). BPA is known to be a weak estrogen mimic, and appears to accumulate in lipophilic fish tissues, leading to growth and developmental defects (Krishnan et al., 1993; Wetherill et al., 2007). This chemical is used primarily in the manufacture of polycarbonate plastic, epoxy resins and as a non-polymer additive to other plastics. Every year, more than 6 billion pounds of BPA are used in the manufacture of epoxy resins and polycarbonate plastics (Ranjit et al., 2010). Because of BPA’s huge application in the manufacture of consumer products, including polycarbonate food containers and utensils, dental sealants, protective coatings, flame retardants, and water supply pipes, there is a widespread and well-documented human exposure to BPA (Kuo and Ding, 2004; Munguía-LÓpez et al., 2005; Le et al., 2008; Vandenberg et al., 2009). Recent discoveries regarding the environmental distribution and presence of BPA in humans and wildlife have generated persistent scientific, regulatory, and public interest in assessing the potential health risks associated with BPA exposure. The detection of adverse health effects in a number of laboratory animal models upon exposure to environmentally relevant doses of BPA, as well as potential effects on human reproduction and development, have fueled additional concern (Wetherill et al., 2007).
1.2.4. Fadrozole:

Fadrozole (Fig. 1.4) is a non steroidal aromatase inhibitor. Recently these aromatase inhibitors are used to inhibit high aromatase activity which causes breast cancer, especially in post-menopausal patients (Bonnefoi et al., 1996). However, in fish the aromatase inhibitors are used to manipulate sex in fish by inhibiting the aromatase enzyme (Piferrer et al., 1994; Afonso et al., 2000; Fenske and Segner, 2004). It is possible to achieve single sex population through hormonal manipulations in fish (Piferrer et al., 1994). Piferrer et al. (1994) observed that fadrozole treatment showed effect on coho salmon when genetic females were treated with fadrozole they developed into males and these males had indistinguishable testes compared to unexposed males both in size and structure. Hong and Donaldson (1998) observed gonads with atretic oocytes of the fadrozole treated coho salmon. Afonso et al. (2001) showed the increased maturation and ovulation in coho salmon by using fadrozole. However in fish, high level of dopamine affects LH (Luteinizing Hormone) secretion, which is released by the influence of circulating estrogen (Saligaut et al., 1998). Lee et al. (2001) observed increased LH secretion with fadrozole treatment in Black porgy; on the contrary it is possible to achieve sex reversal in tilapia with aromatase inhibitors (Afonso et al., 2001). Therefore, in
this project we used fadrozole to understand the effect of fadrozole on aromatase gene expression in both sexes of *M. fluviatilis*.

![Figure 1.4. Chemical Structure of fadrozole](image)

### 1.3. Biomarkers

A biomarker is defined as “the changes in biological responses of the organisms when they are exposed to environmental toxicants or chemicals” (Peakall, 1994). Therefore, biomarkers in fish can be used as tools to evaluate any shift from normal physiological or biochemical levels as a result of exposure to environmental pollutants or EDCs. The most important reason for using biomarkers is that they give information on the effects of pollutants at different biological levels. These may also provide insight into the potential mechanisms of contaminant effects. By screening multiple biomarker responses, important information will be obtained about an organism’s response to toxicant exposure and stress or effect (Oost *et al.*, 2003).

### 1.4. Common biomarkers in ecotoxicology

#### 1.4.1. Metallothionin (MT)

Metallothionins are low molecular weight proteins rich in cysteine amino acids (Chan, 1995). These are used extensively as heavy metal indicators in many species including crab, oysters, fish and mammals. It has been widely stated
that these proteins protect cells against toxicants like heavy metals by chelation. Metallothionins are produced by cells to detoxify metals upon exposure to elevated levels of metals in the environment and the level of mRNA and protein synthesis is used as a bioindicator of metal pollution. A study by John et al. (1987) on brook trout exposed to cadmium reported that the concentration of MT increased significantly in all fish exposed to cadmium and it led to mortality of the fish and they stated that not only was the MT concentration a biomarker, the level of cadmium in the whole body tissue could also be used as a bioindicator. The increased cadmium content was correlated with the increased MT concentration in Perch (Perca fluviatilis) in a study by Olsson and Haux (1986). Hogstrand and Haux (1990) reported that the hepatic metallothionin levels in fish increased dose dependently after injection of cadmium and exposure to zinc and copper. Knapen et al. (2007) studied the use of hepatic metal and metallothionin levels as a biomarker of exposure before and after the cleanup of contaminated sites in Flanders in Belgium. Their data showed a significant correlation between metal concentration and both metallothionin protein and gene expression. They also observed a significant correlation between metallothionin and mRNA levels.

1.4.2. Ethoxyresorufin-O-deethylase (EROD)

EROD activity in fish is a well established biomarker of exposure to halogenated and polycyclic aromatic hydrocarbons (PAHs) and structurally related compounds. EROD is a highly sensitive indicator of contaminant exposure in fish livers. A large number of laboratory studies and field studies
have examined EROD activity in various fish species and correlated this with exposure to PAHs or other similar xenobiotics (Whyte et al., 2000).

1.4.3. Vitellogenin (Vtg)

Vitellogenin is the major female specific egg-yolk precursor protein in oviparous vertebrates. This protein provides energy for embryonic development. In mature females, it is synthesised in response to endogenous estrogens. In males, the \textit{vtg} gene, although present, is normally silent. However, it is activated by xenoestrogens. Much research is being conducted on many fish species using vitellogenin as a biomarker of estrogens and estrogen like chemicals (Purdom et al., 1994; Sumpter and Jobling, 1995; Folmar et al., 1996; Harries et al., 1997; Arukwe et al., 1998; Denslow et al., 1999; Kime et al., 1999; Porter and Janz, 2003; Nakari, 2004; Barucca et al., 2006; Henry et al., 2009; Woods et al., 2009). The effect of the estrogen mimic nonylphenol on the induction of Vtg and EROD in juveniles and adult grey mullet were reported by Cionna et al. (2006). In their experiment, they observed that exposure of juveniles to NP failed to induce measurable Vtg and in male adults the Vtg level was measurable only at the highest dose of NP injected.

1.4.4. Spiggin

Spiggin is a glue protein produced by male sticklebacks (\textit{Gasterosteus aculeatus}) in the kidney during the breeding period; this protein is used to build a nest for the female stickleback to lay eggs (Jones et al., 2001; Kawahara and Nishida, 2006). In females the spiggin gene although present, is normally
silent, however when females are exposed to androgens or androgenic chemicals they will produce spiggin protein. This protein can be used as a biomarker exposure to androgens in sticklebacks. The majority of studies on endocrine disruption in European waters are based on exotic species and none of them were found to be suitable for detection of androgenic chemicals. Therefore, Katsiadaki et al. (2002) developed a biomarker model using stickleback in European waters. This is the only fish species in which it is possible to test the effect of estrogens and androgens simultaneously (Katsiadaki et al., 2002). Sanchez et al. (2008) exposed male and female sticklebacks to 0.1 and 1 µg/L of methyltestosterone and reported a significant dose dependent increase in the spiggin protein in female fish exposed to methyltestosterone.

1.5. Ecotoxicogenomics

Ecotoxicogenomics is the study of gene and protein expression in non-target organisms as a response of exposure to environmental toxicants (Snape et al., 2004; Iguchi et al., 2007). This field of study provides information on the pollution of the aquatic environment well in advance to deleterious effects occurring at the population or community level (Fedorenkova et al., 2010). To understand the effect of toxicants at the molecular level various techniques have been used including In situ hybridization, Northern blotting, Southern blotting, Western blotting and quantitative real-time assay. These techniques are used to quantify the effects of pollution on different end points including RNA, DNA, protein and metabolomics. Figure 1.5 explains how the chemicals act at genome level through to the population level. The effect of these
chemicals at the population level could be on reproduction, growth or sex ratio of the next generation.

1.5.1. The advantages of ecotoxicogenomics studies are

1. Provides a better understanding of the mechanism of action of the chemicals and also gives information about the specific molecular targets. Also helps to understand the effects of exposure to multiple stressors at low levels.

2. They provide a molecular basis for the extrapolation of laboratory data to natural populations.

3. Provides an opportunity to measure sub-lethal molecular responses of the effects of pollutants and help better manage their release.
4. Through the identification of the molecular targets of pollution it may be possible to increase our knowledge of possible effects on sensitive or threatened species.

5. Has the potential to reduce uncertainties in risk assessment and facilitate a more rapid evaluation of a chemical’s toxic potential.

1.6. Molecular biomarkers in fish

Fish are considered the best organisms to evaluate biomarkers and for biomonitoring, since they are often exposed to sources of EDCs including sewage, industrial effluents, urban and agricultural runoff (Yan et al., 2010). In addition their endocrine physiology is well understood (Matthiessen, 2003).

Presently biomarkers have been used to understand the health status of fish and also as early warning signals of environment contamination (Di Giulio et al., 1995; Pretty and Cognetti-Varriaile, 2001). Biomarkers that have been employed for quantifying chemical exposure and in biomonitoring represent a variety of different biochemical or metabolic processes (Viarengo et al., 2007). Several biomarkers are being used for different groups of chemicals including Metallothionins (MTs) for heavy metals; vitellogenin, vitelline envelope proteins (VEPs), zona radiate proteins (ZRPs) and P450 aromatase (P450arom) for estrogenic chemicals; cholinesterases for neurotoxic chemicals and measures of DNA damage for genotoxic chemicals (Chan, 1995; Sumpter and Jobling, 1995; Hylland et al., 1996; Arukwe et al., 1997). Some of these biomarkers are specific to certain groups of toxicants, for example MTs for metals, cholinesterase activity for organophosphates and carbamates, vitellogenin and
zona radiate protein for estrogenic endocrine active chemicals (EACs) and CYPA1 activity (EROD activity) for planar aromatic compounds (Arukwe et al., 1998).

Changes in the gene expression of some of these specific biomarkers are becoming more popular for rapidly assessing the toxicity of individual chemicals in the laboratory and for monitoring of exposure to toxicants in fish (Tyler et al., 2008). Methods used to measure changes in the expression of individual genes in fish have included in situ hybridization, Northern hybridisation, Southern hybridization, Western blot (immunodetection) and quantitative real-time PCR. The latter technique is now the most prevalent and widely used.

In this project, aromatase (cyp19a) at mRNA and vitellogenin (vtg) at mRNA and protein levels were used as biomarkers to test the effect of selected chemicals (EDCs) on M. fluviatilis. These biomarkers are usually used as early warning signals of aquatic pollution and they also inform us of the possible outcomes of the exposures well in advance (Adams and Greeley, 2000; Lam and Wu, 2003).

1.6.1. Aromatase

Aromatase (estrogen synthetase) is a member of the cytochrome P450 family. It is an enzyme which aromatizes androgens including testosterone and androstenedione to estrogens (Lephart, 1996; Lubzens et al., 2010; Schulz et al., 2010). The biosynthesis of aromatase is catalyzed by the cytochrome P450 superfamily, namely aromatase cytochrome P450 (P450arom, the product of
the *cyp19a* gene). The P450 gene superfamily is very large, containing over 480 members in 74 families, of which cytochrome P450arom is the sole member of family 19 (Simpson *et al.*, 2002). This is a heme protein responsible for the binding of the C19 androgenic steroid substrate and catalysing the series of reactions leading to formation of the phenolic A ring characteristic of estrogens (Fig. 1.6). The aromatase reaction employs 3 moles of oxygen and 3 moles of NADPH for every mole of steroid substrate metabolized (Simpson *et al.*, 2002). These oxygen molecules are utilized to oxidize the C19 angular methyl group to formic acid, which occurs concomitantly with aromatization of the A ring to give the phenolic A ring characteristic of estrogens. The reducing equivalents for this reaction are supplied from NADPH via a ubiquitous microsomal flavoprotein, NADPH-cytochrome P450 reductase (Simpson *et al.*, 1997). In humans, a number of tissues have the capacity to express aromatase and hence synthesize estrogens. These include the ovaries and testes, the placenta and foetal (but not adult) liver, adipose tissue, chondrocytes and osteoblasts of bone, the vasculature smooth muscle, and numerous sites in the brain, including several areas of the hypothalamus, limbic system, and cerebral cortex (Simpson *et al.*, 2003).

![Figure 1.6. Biosynthesis of estrogens from androgens](http://themedicalbiochemistrypage.org/steroid-hormones.html)

**Figure 1.6. Biosynthesis of estrogens from androgens**

(Reference: http://themedicalbiochemistrypage.org/steroid-hormones.html)
1.6.2. Aromatase isoforms (cyp19a)

The cyp19a gene exists as a single copy per haploid genome in mammals with the exception of porcines (Means et al., 1989; Harada et al., 1990). In contrast, teleosts have two aromatase isoforms including cyp19a1a and cyp19a1b and they encode two structurally and functionally different proteins with similar catalytic activities. Among these two genes, cyp19a1a is predominantly expressed in the ovary and cyp19a1b in the brain; however they are also expressed in different key tissues like the pituitary, liver, spleen and kidney at lower levels (Piferrer and Blázquez, 2005).

1.6.3. Tissue Localization of aromatase activity

The aromatase enzyme activity is restricted to the smooth endoplasmic membrane of steroidogenic cells (Piferrer and Blázquez, 2005). Various techniques including real-time PCR and In-Situ hybridizations are used to detect the aromatase activities in various fish species (Piferrer and Blázquez, 2005). The predominant expression sites for cyp19a1a and cyp19a1b are the gonads and brain respectively. In the majority of species studied to date (see summary of Table 3.1, chapter 3), cyp19a1a was predominantly expressed in the ovary, with very low or no expression in the testis. The brain appears to be the primary organ of aromatisation in both males and females, although the level of expression depends on age, reproductive stage and sex of the fish (Blázquez and Piferrer, 2004).

As presented in Fig. 1.7, the precursor for all the steroids is cholesterol. Cholesterol located on the outer mitochondrial membrane in lipid droplets or
the plasma membrane of the steroidogenic cells, gets transported to the inner membrane of the mitochondria following side chain cleavage by the P450scc enzyme, which converts cholesterol to pregnenolone (androgen) and this is the first steroid produced in all steroidogenic cells (Stocco, 2000).

Various androgens (steroids) are synthesised in the theca cells of females and leydig cells of males with the help of a number of enzymes under the stimulation of Luteinizing Hormone (LH). The androgens (testosterone and androstenedione) then get diffused into the granulosa cells in females and sertoli cells in males, after which the granulosa/serloli cells convert the final steroids of the androgens (androstenedione or testosterone) to estrogens (Lubzens et al., 2010; Schulz et al., 2010) by P450 aromatase (P450 cytochrome Cyp19a) under the influence of Follicle Stimulating Hormone (FSH) as showed in Fig.1.7.

Figure 1.7. Biosynthesis of aromatase in two different cells. Adapted and modified from Lubzens et al., 2010; Schulz et al., 2010
1.7. Fish as test organisms

The objective of this study was to assess the effect of exposure to EDCs in fish and potential implication on fish populations (being a key food source for humans) using a laboratory model. It is important to assess the potential harmful effects of pollutants on animals that are a food source. There are sufficient physiological differences, similarities between fish and mammals to warrant this research and it is a requirement that data should be generated in the target animal species. Moreover, the endocrine system and physiology of the fish is well understood (Matthiessen, 2003) and they play a key role in aquatic food chains or ecosystems.

1.8. Aromatase as a biomarker in fish

1.8.1. Zebrafish (*Danio rerio*):

Zebrafish has been used as a model test organism in various laboratories all over the world and it is considered an “aquatic mouse”. This species is used in biomedical research for various purposes and it is one of the ideal model organisms for vertebrate development because it appears to combine the best features of all other models (www.zfin.org).

As a model organism for the study of vertebrate development, disease, biological pathways, and toxicological mechanisms, the zebrafish (*Danio rerio*) has a number of advantageous features. It is a vertebrate with organs similar to those of man. In particular, the zebrafish is being used to understand the effect of exposure to estrogens and xenoestrogens in many laboratories.
Sawyer et al. (2006) used zebrafish to clarify the effects of estrogen regulation on aromatase expression in different key tissues, the effect of estrogens on sex difference and developmental physiology. Meanwhile, Fenske and Segner (2004) carried out experiments on zebrafish by exposing them to 17α-methyltestosterone and fadrozole; they observed effect of these on aromatase modulation and the alteration of gonadal difference in developing zebrafish. Brain and gonadal aromatase levels were studied as potential biomarkers of exposure to endocrine disruption in zebrafish by Hinfray et al. (2006).

As far as zebrafish embryos are concerned, these develop outside the mother and without an eggshell, since they are transparent, the embryo development and movement of individual cells can be observed easily, and followed by the development of organs including bone, muscle, heart, and the circulatory, hematopoietic, and central nervous systems (Stainier and Fishman, 1994; Kimmel et al., 1995; Lele and Krone, 1996) hence the effect of EDCs on embryonic and larval development has been intensively investigated by several researchers (Trant et al., 2001; Sawyer et al., 2006; Jin et al., 2009).

It has been widely stated that estrogens and estrogen like chemicals alter the expression of aromatase genes encoding for steroidogenesis at transcriptional level (Kazeto et al., 2003; Hinfray et al., 2006; Lee et al., 2006; Kortner et al., 2009). Hinfray et al. (2006) showed that aromatase activities were higher in the brain than in the ovary. E2 exposure in adult zebrafish showed no effect on aromatase expression in brain, whereas at the larval stage E2 increased cyp19a1b expression. Meanwhile exposure to E2 completely suppressed cyp19a1a and aromatase activities in adult zebrafish whereas, androstatrienedione (ATD) completely inhibited both brain and ovarian
aromatase enzyme activities but did not affect the *cyp19a* at transcript levels in this study.

Some studies reported that over expression of aromatase leads to increased tissue estrogenic activity and induction of neoplastic lesions (Keshava *et al.*, 2001). The mechanism is thought to involve an increase in certain cell cycle genes and decrease in others (Kirma *et al.*, 2001). In the aquatic environment, downregulation of aromatase activity is the likely scenario since laboratory investigations using fish have reported a decrease in aromatase enzyme activities following exposure to exogenous estradiol (Tsai *et al.*, 2001).

Cheshenko *et al.* (2007) studied the effects of 17β-estradiol and 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) in zebrafish on expression of *cyp19a1a* and *cyp19a1b* genes. In their study, mRNA quantification in zebrafish larvae exposed to E2 and TCDD and they reported that none of the used chemicals affected *cyp19a* (except for a slight upregulation of *cyp19a1a* by E2 observed in *in vitro* studies).

Zebrafish, exposed to the xenoestrogens predominantly upregulated the brain isoform, implying that xenoestrogens could act as neurodevelopmental toxicants by altering *cyp19a1b* (Kishida *et al.*, 2001). To test this hypothesis, they exposed zebrafish embryos to 17β-estradiol (E2), diethylstilbestrol (DES) and bisphenol A (BPA) and reported that E2 (0.01-10 µM) upregulated *cyp19a1b* in a dose response manner and the effect of DES was similar to 1 µM of E2, but BPA was less effective. They concluded that *cyp19a1b* mRNA is a sensitive marker of exposure to xenoestrogens during embryogenesis and
that further studies are required to understand neural aromatase expression and biosynthesis of estrogens which could have consequences on the development of the central nervous system (CNS).

Fenske and Segner (2004) investigated whether the gonadal sex differentiation of zebrafish is susceptible to compounds that interfere with cytochrome P450 aromatase. They exposed zebrafish to non-steroidal aromatase inhibitors including fadrozole and 17α-methyltestosterone. In their study they observed that application of fadrozole (500 µg/g of food) between 35 and 71 days post-fertilization resulted in downregulation of gonadal cyp19a1a mRNA expression and 100% masculinisation. Interestingly, despite downregulation of cyp19a1a, MT treated (10 µg/L) zebrafish during gonadal differentiation (days 35 to 71 post fertilization) showed phenological feminisation. They concluded that EDCs interfere with the P450arom systems and are able to disrupt gonadal sex differentiation in the zebrafish.

Hinfray et al. (2006) were the first to develop a method to measure brain and ovarian aromatase activities in zebrafish. They exposed zebrafish to E2 and androstatrienedione (ATD) and reported that cyp19a1b was predominantly expressed in the brain. Moreover aromatase activity was higher in the brain than in the ovary. In their study, E2 exposure had nil effects on aromatase activity in the brain of adult fish. However, it strongly triggered cyp19a1b expression during larval development. In contrast, E2 completely inhibited both cyp19a1a expression and aromatase enzyme activity in ovary. Exposure to ATD in their studies completely inhibited both brain and ovarian aromatase activity but it had no effect on cyp19a transcript abundance. Finally they
concluded that brain and ovarian aromatase are promising biomarkers of endocrine disrupting chemicals in fish.

Anderson et al. (2003b), observed an induction of vitellogenin in male and brain aromatase activity in adult zebrafish when adult zebrafish were exposed to either 25 ng/L of ethynylestradiol, 25 ng/L of methyltestosterone or 100 µg/L of futamid. They did not find any difference in brain aromatase activity between the two sexes and also no change was observed in brain aromatase activity in males exposed to Ethynylestradiol (EE2), methyltestosterone and futamid. However females exposed to EE2 and futamid showed significantly increased brain aromatase activity compared to controls. Females exposed to methyltestosterone also showed significant activity, not as significant as EE2 and futamid. They also observed significantly increased vitellogenin in males exposed to EE2 and MT. However, the induction of vitellogenin was unexpected in MT exposed males.

1.8.2. Japanese Medaka (*Oryzias latipes*):

Medaka is a small bony fish, similar to zebrafish, which is particularly popular as a model test organism in Japan for studies on development. Its genome is being sequenced and resources are being allocated to bring it up to speed with zebrafish as a model test organism particularly in Japan and South East Asia (Tong and Chu, 2002). Medaka is the only fish in which the sex determining gene (*DMY*) has been sequenced to date, and the sex determining gene has been utilised in many laboratories to understand the effects of pollutants on individual animal before their sexes are differentiated phenotypically. The draft genome sequence of medaka is published (Kasahara et al., 2007). This is
being used as a model test organism in ecotoxicology to study the effects of estrogens, xenoestrogens and androgens on aromatase and vitellogenin at the transcript as well as the protein level (Min et al., 2003; Sun et al., 2007). For example, the toxicity of the aromatase inhibitor letrozole on Japanese medaka eggs, larvae and breeding adults was studied by Sun et al. (2007) while; the effect of endocrine disrupting chemicals on distinct expression patterns of estrogen receptor, cytochrome P450 aromatase and p53 genes in medaka was studied by Min et al. (2003).

Aromatase gene expression has been used as a biomarker of endocrine disruption in medaka (Oryzias latipes) exposed to 17α-ethinylestradiol (Scholz and Gutzeit, 2000). They reported that, aromatase is normally expressed in ovaries, but when males were exposed to low level of 17α-ethinylestradiol (10 ng/L), aromatase was detectable in the testis of these males. These laboratory studies showed that a change in aromatase gene expression precedes histological changes in exposed fish making it a reliable early biomarker of biological effect (Guiguen et al., 1999).

Japanese Medaka exposed to 17β-estradiol showed increased activity of aromatase in brain in a concentration dependent manner at 2.5 µg/L and 25 µg/L, but the increase was lower with exposure to higher concentrations of E2 (Melo and Ramsdell, 2001). These researchers also determined that the effect was time dependent i.e. activity of brain aromatase in brain increased until the fifth day of the exposure and after that it reached a plateau. They concluded that the positive feedback mechanism regulates the brain aromatase.
Effects of EDCs on expression patterns of estrogen receptors (ERs), P450 aromatase and p53 genes in Japanese medaka liver were reported by Min et al. (2003). These researchers exposed adult fish to E2 for 10 days and observed the expression of the listed genes in the liver. The expression of the ER gene increased rapidly for the first 2 days, then levelled out and maintained a stable expression level. Meanwhile, the *cyp19a* gene expression slowly increased after exposure. In contrast to these two genes, the p53 gene increased rapidly within 2 days and declined during the remainder of the experiment. They concluded that, in response to the EDCs different gene expression mechanisms may exist in medaka.

The toxicity of the aromatase inhibitor letrozole to adult Japanese medaka was studied by Sun et al. (2007). In their experiment, they exposed adult fish to varying concentrations of letrozole (5 µg/L to 625 µg/L). They observed a dose dependent decrease in fecundity (>25µg/L) and fertility (>5 µg/L) as well as changes in the histology of oocyte growth. At higher concentration (625 µg/L), fish ceased spawning ability during the last week of exposure. The plasma vitellogenin level was reduced in a dose dependent manner with letrozole (>5->25 µg/L) exposure in Japanese medaka.
1.8.3. Other teleost models

1.8.3.1. Fathead minnows

Fathead minnows (FHM) are widely studied freshwater cyprinid species for aquatic toxicology studies (Kramer et al., 1998) and it is endorsed by the United States Environmental Protection Agency for reproductive assay studies for screening of EDCs. These (FHMs) are sexually dimorphic fish and reach adult stage within 5 months. They are fractional spawners and produce 50-100 eggs for every 3-5 days (Jensen et al., 2001). In addition to these advantages, Kahl et al. (2001) observed that the reproductive biology including fecundity, egg hatching rate, fertilization, and condition of the gonads were not significantly affected by repeated handling in laboratory conditions making them the laboratory model of choice for the USEPA.

1.8.3.2. Sticklebacks

Sticklebacks are mostly studied in Canada, USA and some European countries, from the point of view of ecology and behaviour because of their variable body shapes (Katsiadaki et al., 2002). However, not all is easy with using sticklebacks as laboratory model fish species. Whereas zebrafish do not exhibit much dramatic behaviour, sticklebacks can be very aggressive, territorial, individual fish (Bell, 2004). It is considered a nightmare to keep large families of sticklebacks in the same tank, since they fight continuously. On the positive side, however they may end up becoming a model for human
social interactions given that the draft genome is sequenced (Sarropoulou et al., 2008).

In mammals there is ample of evidence that estrogens enhance many aspects of cellular metabolism and proliferation by upregulating the expression of gene of interest (Ing and O’Malley, 1995: Ing et al., 1996). Similar evidence of effects of estrogens on the regulation of several genes is available in teleosts including regulation of vitellogenin production in liver during the period of reproduction (Devlin and Nagahama, 2002).

Greytak et al. (2005) studied the differential expression of cytochrome P450 aromatase forms in killifish, Fundulus heteroclitus from polluted and unpolluted environments. In their study, they determined whether long term, multigenerational exposures to environmental contaminants has affected reproductively relevant genes and biological processes. They compared expression of aromatase from the highly polluted New Bedford Harbour (NBH) to unpolluted Scorton Creek (SC) and reported that cyp19a1b was two fold higher in the brain of fish NBH than in SC fish when both reproductively active and inactive males and females were examined. To strengthen the study they also observed induction of vtg mRNA and protein in seasonally active and inactive males, and reproductively inactive females from NBH and compared them to SC fish. During the reproductively active period, females were observed to have low GSI and low plasma estrogen, a lower hepatosomatic index and lower vitellogenin expression than SC female fish. Therefore, they concluded that killifish population at NBH maintains a level of reproductive competence in the face of exposure to estrogen like pollutants and EDCs indicating possible adaptation to pollutant exposures.
Ankley et al. (2002) assessed the effects of fadrozole on cyp19a in fathead minnows (Pimephales promelas). They exposed fathead minnows to fadrozole ranging from 2 to 50 µg/L for 3 weeks and observed significant inhibition of brain aromatase activity in both male and female fathead minnows. In females they observed that the decrease in plasma E2 and vitellogenin levels were concentration dependent. Added to the above effects their histological assessment shows a decrease in mature oocytes and increased preovulatory atretic follicles. Exposure of male fathead minnows to fadrozole significantly increased the level of androgens including testosterone and 11-ketotestosterone (KT) in the plasma and led to more accumulation of sperm in the testes.

Kroon et al. (2005) demonstrated for the first time that experimental manipulation of E2 levels via the aromatase pathway induces adult sex change in each direction in coral goby (Gobiodon erythrostilus) that naturally exhibits bidirectional sex change and they reported that a single enzymatic pathway can regulate both female and male sexual differentiation. They concluded that aromatase may be the key enzyme that transduces environmental including social cues to functional sex determination.

Most of the studies reveal that the cyp19a expression levels in the majority of the species studied are higher in female gonads in comparison to male gonads (Piferrer and Blázquez, 2005), and also the level of mRNA expression depends on the sex of the fish as well as the age and their reproductive status (Blázquez and Piferrer, 2004). In this respect the difference in mRNA
expression of \textit{cyp19a} during oogenesis is also observed in many other fish species (Gen \textit{et al.}, 2001; Chang \textit{et al.}, 2005).

Piferrer and Blázquez (2005) in a review discussed that the Estrogen Responsive Elements (EREs) are only present within the promoter region of \textit{cyp19a1b} and not in \textit{cyp19a1a} in the promoter regions of all fish species sequenced to date. This is a plausible explanation for the non responsiveness of \textit{cyp19a1a} to xenoestrogens. This could indicate that the disruption of the reproductive axis may be mediated by \textit{cyp19a1b} and it could provide evidence that ERE is a functional transcription factor.

1.9. Vitellogenin as a biomarker in fish

As stated earlier, Vtg is a female specific egg yolk protein and it is used as a biomarker of exposure to estrogenic chemicals in males. Many studies have shown that exposure to endocrine disrupting chemicals can alter the physiology of the fish including metabolism, growth and reproduction by altering the hormonal level and induction of vitellogenin in males (Jobling \textit{et al.}, 1996; Ashfield \textit{et al.}, 1998; Denslow, 1999; Cheek \textit{et al.}, 2001).

Sumpter and Jobling (1995) observed induction of Vtg in male fish that were maintained in the effluent from treated sewage works. Later they identified the treated sewage discharge and it contained mixture of chemicals that induced vitellogenin in males indicating the effluent was estrogenic. They concluded that discharge of chemicals and their degradation are being as estrogenic. Anderson \textit{et al.} (2003a) showed induction of Vtg in male zebrafish which were exposed to very low concentration (25 ng/L) of EE2 for one week. It clearly
explains that vitellogenin could be used as a biomarker to know the presence of estrogen like chemicals in the environment.

Meucci and Arukwe (2005) exposed male Atlantic salmon to NP from 5-50 µg/L and observed induction of vitellogenin in the plasma as well as in the mucus in a dose dependent manner. They concluded that detection of the vitellogenin in the mucus of male represents a sensitive non-invasive method detecting endocrine disruptors. Recently the vitellogenin induction is measured at mRNA level. For example Yamaguchi et al. (2005) observed induction of \( vtg \) mRNA in medaka exposed to E2 from 0.1 to 10 µg/L, NP at 500 µg/L, 1-adamantyl phenol (AdP) at 220 µg/L and 4,4'-dihydroxy-α-methylstilbene (DHMA) at 40 and 400 µg/L. Henry et al. (2009) observed \( vtg \) mRNA expression in male and juvenile zebrafish exposed to 17β-estradiol (1 µg/L) and 17α-ethinylestradiol (0.1 µg/L).

1.10. Biology and habitat of the Murray River rainbowfish (Melanotaenia fluviatilis)

Melanotaenia fluviatilis is an Australian native teleost, belonging to the class Actinopterygii, order Athereniformes under the family Melanotaeniidae. Within this family there are seven genera and Murray River rainbowfish belongs to the genus Melanotaenia. \( M. \ fluviatilis \) is commonly called the “Murray River rainbowfish”. This fish is sexually dimorphic, males with typically elongated dorsal and anal fins with bright and blackish coloration at the edge when they are sexually matured and females have smaller rounded dorsal and anal clear fins (Fig. 1.8).
Figure 1.8. Adult Murray River rainbowfish, *Melanotaenia fluviatilis*. Male with elongated and dark colouration at the margin of fins, female with clear fins.

### 1.10.1 Taxonomy of the species:

- **Class**: Actinopterigii
- **Order**: Atheriniformes
- **Family**: Melanotaeniidae
- **Genus**: Melanotaenia
- **Species**: *Melanotaenia fluviatilis* (Castelnau, 1878)

### 1.10.2. Distribution of the Murray River rainbowfish

*M. fluviatilis* is an omnivorous fish the most southward-ranging rainbowfish and the only species adapted to low winter temperatures (normally about 10-15 °C). There is evidence that numbers are drastically reduced during winter droughts when water temperatures drop below 10 °C and it is suitable for outdoor pond culture in temperate climates (Allen, 1991; Pollino *et al.*, 2007).

Murray River rainbowfish is mainly found in Murray-Darling basin, rivers, creeks, drains, ponds and reservoirs (Fig.1.9). It occurs usually in still or slow-flowing conditions and also it is found in streams, backwaters of larger rivers,
drainage ditches, overflow ponds and reservoirs. This fish usually congregates along grassy banks or around submerged logs and branches (Linternans, 2007)

Figure 1.9. Geographic distribution of Murray River rainbowfish (highlighted in green)

1.10.3. Genome of *Melanotaenia*

Currently there is no initiative focussed on sequencing the genome of *M. fluviatilis*. Nonetheless, there are 210 gene sequence entries for the entire genus *Melanotaenia* in the NCBI database as of 30/03/2011. Amongst these, there are only 15 sequences entries from *M. fluviatilis* (including two that were sequenced in this study). Table 1.1 lists the genes which have been reported in *Melanotaenia fluviatilis* to date.

The aromatase cyp19a isoforms and vtg genes were not isolated or studied in this species prior to initiation of this research project. However part way through the study the vtg gene sequences were published (Woods, 2007),

33
Table 1.1. List of genes reported for Murray River rainbowfish as of March 2011

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Genes</th>
<th>No. of Entries</th>
<th>Accession Numbers</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Melanotaenia fluviatilis</em> Mitochondrial tRNA-Pro gene (partial) and D-loop (partial), country Australia</td>
<td>1</td>
<td>AJ400670</td>
<td>McGuigan et al., 2000</td>
</tr>
<tr>
<td>2</td>
<td><em>Melanotaenia fluviatilis</em> DEAD box RNA helicase Vasa mRNA complete cds</td>
<td>1</td>
<td>AF479824</td>
<td>Knaut et al., 2002</td>
</tr>
<tr>
<td>3</td>
<td><em>Melanotaenia fluviatilis</em> Mitochondrial partial subunit B gene (cyb gene), exon1, various isolate</td>
<td>1</td>
<td>AJ401676</td>
<td>McGuigan et al., 2006</td>
</tr>
<tr>
<td>4</td>
<td><em>Melanotaenia fluviatilis</em> clone 93 18S ribosomal RNA gene, partial sequence</td>
<td>1</td>
<td>EU873123</td>
<td>Hardy et al., 2008</td>
</tr>
<tr>
<td>5</td>
<td><em>Melanotaenia fluviatilis</em> estrogen receptor alpha (ESR1) mRNA, partial cds</td>
<td>1</td>
<td>GU319956</td>
<td>Woods et al., 2010</td>
</tr>
<tr>
<td>6</td>
<td><em>Melanotaenia fluviatilis</em> estrogen receptor beta (ERS2) mRNA, partial cds</td>
<td>1</td>
<td>GU319957</td>
<td>Woods et al., 2010</td>
</tr>
<tr>
<td>7</td>
<td><em>Melanotaenia fluviatilis</em> beta-actin mRNA, partial cds</td>
<td>1</td>
<td>GU319958</td>
<td>Woods et al., 2010</td>
</tr>
<tr>
<td>8</td>
<td><em>Melanotaenia fluviatilis</em> vitellogenin 1 mRNA, partial cds</td>
<td>1</td>
<td>GU319959</td>
<td>Woods et al., 2010</td>
</tr>
<tr>
<td>9</td>
<td><em>Melanotaenia fluviatilis</em> 18S ribosomal RNA gene</td>
<td>1</td>
<td>GU319960</td>
<td>Woods et al., 2010</td>
</tr>
<tr>
<td>10</td>
<td><em>Melanotaenia fluviatilis</em> voucher GU_CS2 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial</td>
<td>1</td>
<td>HM006966</td>
<td>Page and Hughes, 2010</td>
</tr>
<tr>
<td>11</td>
<td><em>Melanotaenia fluviatilis</em> voucher GU_CS2 control region, partial sequence; mitochondrial</td>
<td>1</td>
<td>HM006931</td>
<td>Page and Hughes, 2010</td>
</tr>
<tr>
<td>12</td>
<td><em>Melanotaenia fluviatilis</em> voucher GU_CS2 ATP synthase F0 subunit 6 (ATP6) gene, partial cds; mitochondrial</td>
<td>1</td>
<td>HM007007</td>
<td>Page and Hughes, 2010</td>
</tr>
<tr>
<td>13</td>
<td><em>Melanotaenia fluviatilis</em> voucher GU_CS2 cytochrome b (CYTB) gene, partial cds; mitochondrial</td>
<td>1</td>
<td>HM007048</td>
<td>Page and Hughes, 2010</td>
</tr>
<tr>
<td>14</td>
<td><em>Melanotaenia fluviatilis</em> ovarian Aromatase mRNA (cyp19a1a), complete cds</td>
<td>1</td>
<td>GU723457-Appendix-B</td>
<td>This study</td>
</tr>
<tr>
<td>15</td>
<td><em>Melanotaenia fluviatilis</em> brain Aromatase mRNA (cyp19a1b), complete cds</td>
<td>1</td>
<td>GU723458-Appendix-B</td>
<td>This Study</td>
</tr>
<tr>
<td>16</td>
<td><em>Melanotaenia fluviatilis</em> gapdh partial cds</td>
<td>1</td>
<td></td>
<td>Appendix-B</td>
</tr>
</tbody>
</table>

1.10.4. Criteria for selection of Murray River rainbow fish as a model test organism

1.10.4.1. Regional and Taxonomical relevance

- It is an Australian native fish, distributed throughout the Murray Darling basin, regarded as the food bowl of Australia.
- It is an ideal species for laboratory and field studies
- It is a key indicator species under the order Atheriniformes, since it is considered a threatened species due to high agricultural pressure (Ingram et al., 1990)
- It has been used for the last 20 yrs for laboratory ecotoxicological studies and it is known to be sensitive to toxicants (Pollino et al., 2007)

1.10.4.2 Relevant biological characteristics

- It is easy to maintain and breed in laboratory conditions
- All life stages are traceable in the laboratory
- The entire life cycle can be studied within a short period; they reach the adult stage in 7-8 months (210-240 days).

The genus Melanotaenidae is one of Australia’s most threatened native freshwater genera. Ingram et al. (1990) state that it is a genus of great interest to conservationist and freshwater ecologists. Therefore, *M. fluviatilis* was used as a test organism in this project. In addition Australian native species have been isolated in their habitats for centuries. Therefore, it was of interest to investigate the evolutionary context of the *cyp19a* genes in a native species.
1.11. Objectives of the project

A. Overall aim:
To assess the potential of *M. fluviatilis* as a bioindicator of exposure to the EDCs

B. Key questions to be addressed in the research project:
1. To which of the selected EDCs are the fish most sensitive?
2. Can *M. fluviatilis* serve as a model biomonitor for the detection of EDCs in aquatic systems?

C. Specific aims of the project:
1. Isolation and characterisation of cDNA for aromatase genes in *M. fluviatilis*
2. Expression analysis of aromatase genes in the developmental stages of *M. fluviatilis*
3. Expression analysis of aromatase genes in different key tissues of adult fish
4. Expression of aromatase genes in adult fish exposed to 17β-estradiol (E2), Nonylphenol (NP), Bisphenol A (BPA) and fadrozole
5. Investigate vitellogenin mRNA and protein expression in male adult fish exposed to E2 and NP

This project was aimed at examining the molecular responses in *M. fluviatilis* following exposure to selected EDCs including exogenous 17β-estradiol, nonylphenol, Bisphenol A and an aromatase inhibitor fadrozole. The project isolated and characterised aromatase gene isoforms from *M. fluviatilis* and established their responses as well as Vtg responses following exposure to these chemicals.
CHAPTER 2

Materials and Methods

2.1. Fish used in experiments

Murray River rainbowfish were purchased from a commercial aquarium fish wholesaler (Aquarium Industries, Epping, Melbourne, Victoria, Australia) and reared at 25±1 °C in 16:8 h light: dark regime in flowthrough aquaria with carbon filtered aerated water (Fig. 2.1). Water quality parameters including temperature, dissolved oxygen, pH and conductivity were monitored in all holding tanks and the fish were fed commercial fish pellets (Tetra colour™, Tetra Holding (US). Inc. 3001, Commerce street, Blacksburg, VA 24060, Germany) twice daily. For tissue sampling all adult fish were anaesthetised in AQUI-S (Isoeugenol (2-Methoxy-4-propenylphenol) and decapitated. Samples of brain, gonad, liver, spleen, eyes and body tissues were collected. Samples were stored in RNA<sub>later</sub> (Sigma-Aldrich Pty. Ltd. Australia) according to the manufacturer’s instruction for RNA extraction. Muscle tissues were used for genomic DNA extraction. All procedures were conducted as approved in the RMIT Animal Ethics Committee (AEC) project number 0732.
2.2. Extraction of genomic DNA using CTAB Method

Extraction of genomic DNA (gDNA) was carried out following the procedure described (Doyle and Doyle, 1987). Briefly, where possible muscle tissues (~200 mg) from individual fish were collected in separate microcentrifuge tube and 200 µL of 2x cetyltrimethylammonium bromide (CTAB) was added. The tissue was homogenised using a sterile 1 mL syringe followed by addition of 5 µL of proteinase K enzyme, mixed 5-10 times by inverting the microcentrifuge tubes and incubated at 65 °C for 1 h for digestion. Halfway through the digestion a second grinding was conducted to improve the efficiency of
digestion. After 1 h, 600 µL of chloroform: isoamyl (24:1) was added, mixed thoroughly and centrifuged for 15 mins at room temperature to separate the aqueous and the organic phases. The upper aqueous phase (containing DNA) was transferred to a new microcentrifuge tube and extracted with 600 µL of phenol: chloroform: isoamyl (25:24:1) at 14,000 rpm for 15 mins. The aqueous phase was subject to a second extraction with 600 µL of chloroform: isoamyl (24:1) for 15 mins at 14,000 rpm. The supernatant was transferred to sterile microfuge tubes, mixed with 600 µL of cold isopropanol and the DNA allowed to precipitate at 4 °C for 1 h. The tubes were spun for 30 mins at room temperature at 14,000 rpm to pellet the gDNA. Supernatant was discarded, pellet washed with 1 mL of 70 % cold ethanol and spun for 5 mins at 14,000 rpm. Resulting supernatant (70% ethanol) was discarded and the excess removed using 200 µL yellow tips. The pellets were air dried in the fume hood for 25 mins to remove traces of ethanol. After complete evaporation of ethanol, 50 µL of autoclaved Milli-Q water was added to the pellets to dissolve the DNA overnight at 4 °C. Multiple tissues samples from each of the fish were processed to obtain adequate amounts of genomic DNA for subsequent use. Extracted genomic DNA was stored at -20 °C.

2.3. Quantification of gDNA

The gDNA sample from each individual fish was measured separately. One µL of stock DNA was added to 99 µL of double distilled water in separate microfuge tube, mixed thoroughly by vortexing and transferred to a mini cuvette and the DNA quantified by reading the absorbance at 260 nm
spectrophotometer (Varian Cary50 Bio™, UV-visible spectrophotometer). Absorbance ratio at 260 and 280 nm was used to measure the purity of gDNA.

2.4. PCR amplification of gDNA

To assist cloning of the *M. fluviatilis cyp19a* transcripts (cDNA), partial amplification of the genes was carried out using gDNA as template.

The PCR reaction consisted of 10x buffer, 10mM dNTP, 25mM MgCl2, 0.65 units *AmpliTaq* Gold, 0.2 µm of the respective gene specific primer (Table 2.1) and ~ 15ng of genomic DNA template in a final volume of 25 µL. The 30 cycles of PCR amplification procedure consisted of denaturation at 95 °C for 5 mins, amplification steps including 95 °C for 15 s, gradient annealing temperatures (49.0 °C, 49.9 °C, 50.8 °C, 51.9 °C, and 53.0 °C) for 30 s and extension at 72 °C for 1 min, with final extension at 72 °C for 5 min.

Table 2.1. List of degenerate primers used for amplification of gDNA for aromatase genes in the Murray River rainbowfish

<table>
<thead>
<tr>
<th>Primers</th>
<th>Forward</th>
<th>Reverse</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Set 1</td>
<td>gArom980Fdeg</td>
<td>gArom1250Rdeg</td>
<td>Patil (unpublished)</td>
</tr>
<tr>
<td>Set 2</td>
<td>cBRArom1F</td>
<td>cBRAromAR</td>
<td>Barney <em>et al.</em>, 2008</td>
</tr>
<tr>
<td>Set 3</td>
<td>Arom1F</td>
<td>Carp270R</td>
<td>Barney <em>et al.</em>, 2008</td>
</tr>
</tbody>
</table>

Amplified products were analysed by gel electrophoresed on 1% agarose gel in 1xTAE buffer at 120 volts for 70 min (or until the loading dye reached near the end of the gel). DNA molecular marker (2-log ladder, New England, Biolabs. Inc) was used as size standards. After completion of the
electrophoresis, the gel was stained in ethidium bromide (EtBr) for 5-7 min and destained in running water for 30 min. Gel photographs were documented using the Gel Doc apparatus (Bio Rad Pty. Ltd. Australia).

2.5. Ligation

Following gel confirmation the potential amplicons were cloned using Topo cloning kit (Invitrogen Pty. Ltd. Australia). Typically 2 µL of the PCR amplicon was directly used in cloning and as recommended by the vendor. Details are presented below.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>TOPO TA (pCR2.1) Vector</td>
<td>0.5 µL</td>
</tr>
<tr>
<td>Salt solution (200mM NaCl+10 mM MgCl₂)</td>
<td>1.0 µL</td>
</tr>
<tr>
<td>DNA</td>
<td>2.0 µL (about 50 ng)</td>
</tr>
<tr>
<td>Water</td>
<td>2.5 µL</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>6.0 µL</strong></td>
</tr>
</tbody>
</table>

2.6. Bacterial transformation

Frozen chemically competent *E. coli* (DH-5α Top 10; Invitrogen, Pty. Ltd. Australia) was thawed on ice for 5-10 mins and 50 µL of the cells aliquoted into separate prechilled plastic vials (microfuge tubes). A total of 2 µL ligation reaction was dispensed to the pre-chilled plastic vial containing 50 µL of competent cells, mixed gently and chilled on ice. The cells were then subjected to heat shock at 42 °C for 30 s without shaking. Immediately after heat shock, the vials were chilled on ice for 2 mins and 250 µL of Super Optimal Broth (S.O.C) medium was added immediately. The cells were incubated at 37 °C on an orbital shaker (Ratek Pty. Ltd. Australia) at 200 rpm for 1 h.

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2.7. Blue and white colony selection

The transformants were grown in S.O.C medium for 2 h and then plated on LBA/IPTG/X-gal plates containing 50 mg/mL of ampicillin. A total of 70 µL was spread on five plates and plates were wrapped in aluminium foil and incubated at 37 °C for 12-16 h, generally until the colonies were big enough to pick. Between 5-10 white colonies representing each transformation reaction were picked using inoculating loop, inoculated into ~2 mL of LB media containing antibiotic ampicillin (50 mg/mL) and incubated for ~12 h at 37 °C, in a shaking incubator (200 rpm).

2.8. Plasmid DNA extraction—mini prep.

Extraction of plasmid DNA was carried out using alkaline lysis protocol using buffers supplied in plasmid maxi prep kit (Qiagen Pty. Ltd. Australia). Briefly, ~1 mL of culture was transferred into separate microcentrifuge tubes (1.5mL) with remaining saved for future use. The cells were then spun for 1 min at 8,000 rpm, after which the supernatant was discarded. Then 200 µL of buffer P1 (Resuspension buffer) was added to the harvested cells and resuspended, followed by addition of 200 µL of buffer P2 (Lysis buffer) and gently mixed by inverting the tubes 12-15 times. Immediately 200 µL of buffer P3 (Neutralization buffer) was then added and mixed and the tubes were kept on ice for 5 mins. The tubes were centrifuged for 10 mins at 14,000 rpm and the supernatants removed carefully without disturbing the pelleted tissue debris and transferred to clean microfuge tubes. Immediately 500 µL of 100% isopropanol was added to each tubes and spun for 15 mins at 14,000 rpm. The supernatant was again discarded and the DNA pellet washed with 400 µL 70%
ethanol and spun for 15 mins at 14,000 rpm. The supernatant discarded, excess ethanol removed using a pipette tip and the pellet dried at 37°C. The dry DNA pellet was resuspended in 30 µL of autoclaved Milli-Q water. All bacterial waste was chlorinated for 30 minutes and discarded.

2.9. Restriction enzyme digestion

The plasmids were subjected to EcoRI (New England Biolabs. Inc) restriction enzyme digestion for 2 h for confirmation of the cloned inserts. A typical restriction digestion reaction consisted of the following.

**Restriction enzyme reaction mix (for each sample)**

- *EcoRI* enzyme : 0.15 µL
- 10 x Restriction enzyme Buffer (NEB EcoRI Buffer) : 2.00 µL
- ddWater : 15.85 µL
- Plasmid DNA (~20-40ng / µL) : 2.00 µL
- **Total** : 20.00 µL

The digested samples were run on 1% agarose gel on 1xTAE buffer for confirmation of the cloned inserts.

2.10. Sequencing of genomic DNA

The extracted clones were sequenced using M13 forward and M13 reverse primers and ABI BigDye terminator v3.1 reaction mix. Reactions were then analysed on a ABI3730xl DNA (Department of Primary Industries, Latrobe
University, Victoria, Australia) analyser and their respective identity confirmed using NCBI blast returns. Gene specific primers were also used for sequencing as and when required. A list of these primers presented in Table 3.2, chapter 3.

2.11. Extraction of total RNA from brain, ovary and liver of *M. fluviatilis*

Female Murray River rainbowfish were anaesthetized using AQUI-S (Isoeugenol (2-Methoxy-4-propenylphenol)) and dissected to remove the tissues including brain, ovary and liver. The tissues were stored overnight at 4 °C in RNA*later* to stabilize the RNA. About ~30 mg each of the fixed tissues were taken for RNA extraction using sterilized forceps and scalpel. 1mL of QIAzol lysis buffer was added to each tube and homogenised followed by extracted with 200 µL of chloroform and centrifuged at 12,000g for 15 mins at 4 °C. Then the supernatant was removed and transferred to new clean tubes. Immediately one volume of 70% ethanol was added to the each lysate, mixed gently, transferred to the RNeasy spin column, placed in a 2 mL collection tube and centrifuged for 15 s at ≥10,000 rpm. The flow-through was discarded, spin column was loaded with 350 µL of wash buffer (RW1) and centrifuged for 15 s at ≥ 10,000 rpm and the flowthrough discarded. Then the columns were subjected for RNAase free-DNAase treatment for 15 mins at RT to eliminate the genomic DNA. A second wash was done with 350 µL of buffer (RW1) and centrifuged for 15 s at ≥ 10,000 rpm. Then 500 µL of buffer RPE (with ethanol) was added to wash, the buffer was removed by centrifugation (15 s at ≥ 10,000 rpm). The process was repeated and tubes centrifuged at ≥ 10,000 rpm for 2 mins to remove any traces of wash buffer. The RNeasy spin column was then
placed in new collection tube and 30 µL of RNase free water was added
directly to the spin column membrane and centrifuged at ≥ 10,000 rpm for 1
min to elute total RNA. A secondary elution was performed using 20 µL RNase
free water. All procedures were carried out in a laminar flow cabinet treated
with RNAseZap® (Ambion, Inc) to minimise RNase contamination.
Immediately after the elution of RNA, the samples were sub-aliquoted and
stored at -80 °C for future use. Before cDNA synthesis, 2 µL samples were
electrophoresed at 110V for 70 min, following which gels were stained with
ethidium bromide exposed under UV light and gel pictures were documented
using Gel Doc apparatus (Bio Rad Pty. Ltd. Australia). Only those samples
with intact 18S and 24S rRNA bands were used for RACE ready cDNA
synthesis (Fig. 2.2).

![RACE Ready cDNA synthesis](image)

*Figure 2.2. A representative gel picture showing intact 18s and 24s rRNA bands that were used for RACE ready cDNA synthesis. M: 2-Log DNA ladder, B: Brain tissue, O: Ovarian tissue, L: Liver tissue.*

### 2.12. RACE Ready cDNA synthesis for gene isolation

RACE Ready cDNA was synthesised from total RNA samples using the
SMART RACE cDNA Amplification kit, (Clontech, TaKaRa Bio, Japan), as
recommended by the kit manufacturer.
Both 5’ and 3’- RACE Ready cDNA were prepared separately from three different tissues—brain, ovary and liver. About ~1 µg of (3.0-4.0 µL) total RNA was placed separately in 200 µL volume microcentrifuge tubes. As recommended the first strand synthesis of 5’ RACE ready cDNA utilized 1.0 µL each of 5’ – CDS primer and SMART II A Oligo, whilst the 3’ RACE reaction used only the CDS primer. Typical first strand synthesis setup for 5’ and 3’ RACE is presented in Table 2.2.

<table>
<thead>
<tr>
<th>Components</th>
<th>5’ RACE Ready cDNA</th>
<th>3’ RACE Ready cDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA sample</td>
<td>~1.0 µg</td>
<td>~1.0 µg</td>
</tr>
<tr>
<td>5’-CDS primer</td>
<td>1.0 µL</td>
<td>-</td>
</tr>
<tr>
<td>3’-CDS primer A</td>
<td>-</td>
<td>1.0 µL</td>
</tr>
<tr>
<td>SMART II A oligo</td>
<td>1.0 µL</td>
<td>-</td>
</tr>
</tbody>
</table>

The primer and template were placed together in a total volume of 10 µL, incubated at 70 °C for 2 mins, chilled on ice and briefly spun to collect contents at the bottom of the microfuge tube. Then remaining reagents (Table 2.3) were added to each of the tubes, mixed gently, spun to collect the contents at the bottom of the tube and incubated at 42 °C for 1.5 h in a thermal cycler (PCRExpress, ThermoHybaid).

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 X First strand buffer</td>
<td>2.0 µL</td>
</tr>
<tr>
<td>DTT (20 mM)</td>
<td>1.0 µL</td>
</tr>
<tr>
<td>dNTP Mix (10 mM)</td>
<td>1.0 µL</td>
</tr>
<tr>
<td>MMLV Reverse Transcriptase (10 units)</td>
<td>1.0 µL</td>
</tr>
<tr>
<td>Total</td>
<td>5.0 µL</td>
</tr>
</tbody>
</table>
On completion of the reaction, 20 µL of Tricine -EDTA buffer was added to each of the tubes and then heated at 72 °C for 7 min. The samples were stored at -20 °C for further use.

Figure 2.3. Schematic diagram of the relationship of gene specific primers (GSP) to the cDNA template (adapted from BD SMART™ RACE cDNA Amplification Kit User manual).

2.13. cDNA synthesis for real-time PCR analysis

cDNA for real-time PCR analysis was synthesised using a High Capacity cDNA Reverse Transcription Kits (Applied Biosystems Pty. Ltd. Australia) according to the manufacturer’s instructions. Again the quality of each RNA sample was checked on agarose gel prior to cDNA synthesis. About ~1.0 µg of RNA was added to each reaction mix (final volume 10 µL) containing 2 µL of 10x RT buffer, 0.8 µL of 25x dNTP (100 mM) mix, 2 µL of 10 x RT Random Primers and 1.0 µL (10 units) of Multiscribe Reverse Transcriptase (Applied Biosystems. Pty. Ltd. Australia). A thermocycler program consisting of 25 °C for 10 mins, 37 °C for 120 mins and 85 °C for 5 min and a final holding step at 4°C was used. Resulting cDNA templates were quantified by spectrophotometer (Varian Cary50 Bio™, UV-visible spectrophotometer) and stored at -20 °C for real-time assay.
2.14. Expression analysis (qPCR)

Real-time assay was carried out on a MJ MiniOpticon system version 3.1 (Bio-Rad Pty. Ltd.) with SYBR green fluorescent label using gapdh as an endogenous control. Gene specific primers were designed for each gene (See details in Chapter 3, Table 3.2 for aromatase isoforms and Chapter 6, Table 6.1 for vtg) and tested for specificity and efficiency. In case of aromatase isoforms specificity of primers was tested by both standard and qPCR by pairing both primer combinations with cloned brain and ovarian aromatase cDNA as templates (Appendix C, Fig. 1).

Real-time PCR analysis conducted using a final reaction volume of 20 µL: using 50-55 ng/µL of cDNA, 1xSYBR green PCR master mix (Applied Biosystems Pty. Ltd. Australia) and primers at a final concentration of 100nM. Each sample was run in triplicate for each of the genes including gapdh as an internal control. This was selected based on previous literature and in addition we tested its expression before the start of the experiments (see chapter 3). The expression of this gene did not fluctuate significantly between the tissues in both sexes. Cycling parameters for the real-time qPCR were as follows: 50 °C for 2 mins, 95 °C for 10 mins, then 40 cycles of 95 °C for 15 s and 62 °C for 1 min. A melting curve analysis was performed at the end of the amplification phase with a minimum of 30 °C to a maximum of 95 °C, to test the specificity and identity of the qPCR products.
2.15. Data analysis

Real-time data was collected and compiled and the cycle threshold was calculated automatically using a CFX software package (Bio Rad Pty. Ltd. Australia). The data was analysed using the publicly available Q-gene Excel script package (Muller et al., 2002). The efficiency values for each aromatase primer and the average efficiency value for gapdh were used in subsequent analysis. Data from each sample was averaged and shown as Mean Normalized Expression (MNE± SE). The data was tested for homogeneity of variance before the ANOVA and subsequently analysed by two-way analysis of variance (ANOVA). The two-way ANOVA was selected because the experiments were conducted using various concentrations at different time points. Then Tukey’s post hoc test was applied for multiple comparisons and the analysed data was compared between the concentrations and time within the tissues using SPSS17.0 (SPSS Inc. 2008). Data means were considered significantly different from each other at P<0.05.
CHAPTER 3

Phylogenetic and expression analysis of aromatase isoforms in adult Murray River rainbowfish, *Melanotaenia fluviatilis* (Castelnau 1878)

Shanthanagouda A H, Patil J G and Nugegoda D

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Abstract

Cytochrome P450 aromatase is the key steroidogenic enzyme, which converts androgens to estrogens and plays a major role in neural development, sex differentiation and reproductive cycles. The present study describes the isolation of two distinct aromatase isoforms-ovarian (*cyp19a1a*) and brain (*cyp19a1b*) and their expression in different tissues including brain, gonads, liver, eyes and spleen in the Murray River rainbowfish (*Melanotaenia fluviatilis*), native to Australia. The cloned cDNA for *cyp19a1a* and *cyp19a1b* had an open reading frame (ORF) of 492 and 499 amino acid residues, with shared identity of up to 83% and 87% with the corresponding homologues of other teleosts respectively. In contrast the *cyp19a1a* and *cyp19a1b* of the Murray River rainbowfish had a shared identity of only 61%. Not surprisingly,
the phylogenetic analysis clustered the *M. fluviatilis* cyp19a1a and cyp19a1b genes with the corresponding isoforms of other teleosts, suggesting a shared evolutionary ancestry of the respective isoforms. We also studied the tissue specific expression of aromatase isoforms in both sexes of adult fish using quantitative real-time PCR (qPCR). The cyp19a1a was exclusively expressed in the ovarian tissue of female and completely absent in other tissues examined in either male or female fish. In contrast cyp19a1b was predominantly expressed in the brain of both males and females; however, it was also expressed in other tissues including gonads, liver and eyes at lower levels while no measurable levels were observed in the spleen of both sexes.

**Keywords**  : cyp19a isoforms, Murray River rainbowfish, qPCR

### 3.1. Introduction

Aromatase is the key steroidogenic heme-binding protein of the enzyme complex which converts androgens to estrogens. The conversion of androgens to estrogens is catalyzed by desaturating (aromatization) the ring A of C19 androgens and converts them to C18 estrogens (Blázquez and Piferrer, 2004). This enzyme is membrane-bound and is located in the endoplasmic reticulum of estrogen-producing cells of ovaries, placenta, testes, adipose and brain tissues (Simpson *et al*., 2003). It is expressed in different key tissues including brain, gonads, retina, spleen, kidney, liver and other tissues and is essential for gonad development and other physiological processes including growth, neurogenesis and reproductive behaviour. Although, several putative multiple functions for both ovarian and brain aromatases of teleost have been proposed...
(Barney et al., 2008; Penman and Piferrer, 2008; Guigen et al., 2010), a number of these as well as regulatory mechanisms largely remain unresolved.

In mammals, except in porcines, there is a single cyp19a gene which is expressed in different tissues. In contrast, two structurally and functionally different cyp19a isoforms have been found in teleosts which are products of different cyp19a gene loci—one preferentially expressed in the ovary and the other in the brain, officially designated cyp19a1a (CYP19a/P450AromA/CYP19A1) and cyp19a1b (CYP19b/P450AromB/CYP19A2) respectively (Tchoudakova and Callard, 1998; Blázquez and Piferrer, 2004, Chang et al., 2005; Guigen et al., 2010).

The cDNAs encoding P450Arom isoforms have been isolated from several (over twenty) teleost species including the Japanese medaka (Fukuda et al., 1996), goldfish (Gelinas et al., 1998, Halm et al., 2001), zebrafish (Kishida and Callard, 2001), pejerrey (Strobl-Mazzulla et al., 2005) and common carp (Barney et al., 2008). This gene is duplicated in all the investigated teleosts except the Japanese eel, Anguilla japonica (Jeng et al., 2005).

Strikingly teleosts are peculiar in that their aromatase activity is 100-1000 times higher than that of mammals and other vertebrates (Pasmanik and Callard, 1985). The significance of elevated aromatase is not yet clear at the biological level even though several hypotheses on neuroprotection (Garcia-Segura et al., 2001) and neurogenesis (Callard et al., 2001) have been proposed. Among cyp19a genes, cyp19a1a is predominantly expressed in the ovary and believed to play a role in sex differentiation and ovarian development; whereas cyp19a1b is thought to be involved in neural
development in brain, retina and pituitary as well as play a key role in sexual behaviour (Kishida and Callard, 2001).

In teleosts, brain is the primary organ expressing aromatase with both isoforms exhibiting subtle species and or sex-specific differences in their spatial and temporal expression patterns. For example the expression of cyp19a1a is strictly restricted to ovaries in some species, whilst in others albeit dominant in ovary, is also expressed in other tissues, including testes at low levels (See Table 3.1 for summary). Similar observations have been reported for cyp19a1b (Table 3.1), including within organ and between sex differences in the common carp (Barney et al., 2008). Further, cellular examination of cyp19a1b shows its expression to be restricted to radial glial cells in the brain of zebrafish (Goto-Kazeto et al., 2004; Pellegrini et al., 2005) and trout (Muller et al., 2002). However, there was a striking difference in that the cyp19a1b transcripts appear to be exported into the extensions of the radial glial cells in zebrafish but not in trout (Menuet et al., 2003). Significance of such within organ, between species and or sex specific differences, as well as mechanisms of their regulation remain poorly understood. In this context, the inherent diversity of teleost species combined with functional specialization of the two isoforms provide unique opportunities to dissect the cellular and molecular basis of the diverse roles of estrogen in vertebrates at large.

In the present study, aromatase gene expression in the Murray River rainbowfish was investigated. This species has been utilized in laboratory experiments for various reasons including; its ease of maintenance and handling in laboratory conditions. It also has a short life cycle which enables
the study of all life stages quickly (Pollino et al., 2007). The purpose of this study was to isolate the cDNA encoding the brain and ovarian aromatase and characterize their tissue expression in both sexes by qPCR as part of a larger study to investigate the role of aromatase in sexual differentiation of this species and to elucidate the influence of environmental effluents (thermal and chemical) in laboratory and their natural environment.

3.2. Materials and Methods

3.2.1. Animals

Murray River rainbowfish were purchased from a commercial aquarium fish wholesaler (Aquarium Industries, Epping, Melbourne, Victoria, Australia) and reared at 25±1 °C in 16:8 h light: dark regime in flow-through aquaria with carbon filtered aerated water. Throughout the maintenance, water quality parameters including temperature, dissolved oxygen, pH and conductivity were monitored and the fish were fed commercial fish pellets (Tetra colour™, Tetra Holding (US). Inc. 3001, Commerce street, Blacksburg, VA 24060, Germany) twice daily. Reproductively active and ready to breed male and female fish were anaesthetised in AQUI-S (Isoeugenol (2-Methoxy-4-propenylphenol) and decapitated. Samples of brain, gonad, liver, spleen, eyes and body tissues were collected. Samples were weighed and stored in RNA later (Sigma-Aldrich Pty. Ltd.) according to the manufacturer’s instruction for RNA extraction. Muscle tissues were used for genomic DNA extraction. All procedures were conducted under the RMIT AEC approved project number 0732.
3.2.2. Cloning, sequencing and sequence analysis of *M. fluviatilis* 

* cyp19a1a and cyp19a1b cDNA

Genomic DNA was extracted from muscle tissues of adult fish aged ~18 months using the Cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987), quantified by a spectrophotometer and used as template in a standard PCR reaction. To facilitate cloning the aromatase isoforms, partial genomic regions were amplified by a simple and rapid method previously developed for *Gambusia holbrooki* (Patil *et al.*, unpublished) using degenerate primers corresponding to highly conserved amino acid sequences amongst teleosts. Primers sets gArB980Fdeg, gArB1250Rdeg and gArO315Fdeg, gArO1400Rdeg (Table 3.2) were used to amplify the partial genomic fragments of brain and ovarian aromatase genes respectively. The PCR cocktail contained 0.4 µM of each primer, 0.125 mM dNTPs, 2.5 mM MgCl₂, 1X AmpliTaq Gold® buffer and 0.625 units AmpliTaq Gold® polymerase (Applied Biosystems Pty. Ltd. Australia). Cycling conditions were: 94 °C for 10 minutes then 30 cycles (94 °C, 30s/60 °C, 30s/72 °C, 1 minute), followed by 72 °C for 2 minutes. Amplified products were cloned using a pCR®2.1TOPO® vector (Invitrogen Pty. Ltd. Australia) according to the manufacturer’s instructions, resulting clones were sequenced using M13 forward and M13 reverse primers and ABI BigDye terminator v3.1 reaction mix. Reactions were then analysed on ABI3730xl DNA analyser and the respective identity confirmed using an NCBI blast search.

Total RNA was extracted from RNA/later preserved, brain and ovary tissue using a lipid tissue mini kit (Qiagen Pty. Ltd. Australia) according to the
manufacturer’s instructions. Total RNA was quantified using a spectrophotometer and the quality was confirmed on 1% agarose gel using 1X TAE buffer. Following which, the 5' and 3' RACE ready cDNA for brain and ovarian aromatase were synthesised using a BD SMART RACE cDNA amplification kit (Clonetech, TaKaRa Bio, Japan) according to the manufacturer’s instructions.

*M. fluviatilis* specific oligonucleotide primers were designed using primer express (ABI) software, based on the partial genomic DNA sequences obtained and synthesised by Sigma-Proligo (Sigma-Aldrich Pty. Ltd.) or GeneWorks (GeneWorks Pty. Ltd. SA, Australia). The 5' and 3' ovarian aromatase cDNA was amplified using the gene specific primers mfORACE817R and mfORACE616F (Table 3.2) respectively, each paired with the universal adapter primer (UPM) supplied in the RACE cDNA amplification kit. The PCR reaction consisted of 10x ExTaq buffer, 10mM dNTP and 0.65 units of TaKaRa ExTaq HS, 0.2 µm of the respective gene specific primer, 0.125 µm of UPM and ~10ng of RACE ready cDNA template in a final volume of 25 µL. The 30 cycles amplification procedure consisted of cycling at 98 °C for 10 s and 68 °C for 1 min. In case of the 5' RACE a secondary nested RACE PCR reaction was carried out using mfORACE594R and nested universal primer (NUP), with the diluted (1:100 Tricine EDTA buffer) primary PCR product as template and identical PCR cycling conditions. Similarly the 3' brain aromatase cDNA was amplified using mfBArGSPF with universal primer (UPM) as an adapter primer. It required seven different RACE amplifications to isolate the entire 5' region of brain aromatase cDNA as the synthesis truncated prematurely. All the seven primers used for the RACE reactions are listed in
Table 3.2. All other reaction and cycling conditions were identical to those used to amplify the ovarian isoform, with appropriate templates.

The amplified fragments were cloned, sequenced (using M13 and gene specific primers), identity confirmed and full cDNA contigs constructed. The deduced amino acid sequences were aligned with each other and those from select species of teleosts and Atlantic stingray, *Dasyatis sabina*. All alignments were carried out using the ClustalW (Thompson *et al*., 1994).

### 3.2.3. Sequence Alignment and Phylogenetic analysis.

The amino acid sequences were aligned using the SECentral Clone Manager V8.0 (Global DNA Alignment) to understand the homology between the brain and ovarian aromatase amino acid sequences of rainbowfish and also between sequences of select teleosts based on the NCBI blast returns. The top eight and seven returns (species) for brain and ovarian aromatase genes respectively were used for alignment (Fig. 3.2 and 3.3).

The aligned sequences were then used to construct phylogenetic trees using four different methods—neighbour joining, minimum evolution, maximum parsimony and UPGMA, with 1000 replicates in MEGA Version 4.0 (Tamura *et al*., 2007). Elasmobranches including sharks, rays and skates diverged from other vertebrates more than 350 million years ago. The P450arom of stingray might represent the most primitive form of this enzyme, while the stingray sequence may provide insights into the evolution of this key steroidogenic cytochrome P450 (Ijiri *et al*., 2000). Therefore, it was used as an out-group to root the Phylogenetic tree.
Table 3.1. Summary of cyp19a1a and acyp19a1b expression in brain and gonads of teleosts published as of January 2011

<table>
<thead>
<tr>
<th>Species</th>
<th>cyp19a1a</th>
<th>cyp19a1b</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Brain</td>
<td>Testis</td>
<td>Brain</td>
</tr>
<tr>
<td>Melanotaenia fluviatilis</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Odontesthes bonariensis</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Oreias latipes</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dicentrarchus labrax (Juveniles)</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Dicentrarchus labrax (Adults)</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Oreochromis niloticus</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Trinca okinawae</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Danio rerio</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cyprinus carpio</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Carassius auratus</td>
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<td>-</td>
<td>-</td>
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<tr>
<td>Oncorhynchus mykiss</td>
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<td>NA</td>
<td>+</td>
</tr>
<tr>
<td>Fundulus heteroclitus</td>
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<td>-</td>
<td>+*</td>
</tr>
<tr>
<td>Ictalurus punctatus</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Pimelichthys promelas</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pagrus major</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Paralichthys olivaceus</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Paralichthys lethostigma</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hippoglossus hippoglossus</td>
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<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Sparus aurata</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Perca flavescens</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Monopterus albus</td>
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<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Anguilla anguilla</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Protractus ephippus</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cynoglossus semilaevis</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Amphiprion clarkii</td>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Gobioscypris rarus</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* Only those species with known duplicated cyp19a are presented

+: Expression, -: No expression, +*: Restricted expression to either ovary or brain, NA: Not Analysed

References:
- Strobl-Mazzulla et al., 2005; Karube et al., 2007
- Fukuda et al., 1996; Patil and Gunasekera, 2008
- Blázquez and Pifferer, 2004
- Dalla Valle et al., 2002a
- Chang et al., 2005
- Kobayashi et al., 2004
- Trani et al., 2001; Kishida and Callard, 2001
- Barney et al., 2008
- Tchoudakov and Callard, 1998
- Dalla Valle et al., 2002b
- Gretyak et al., 2005
- Kazeto and Trant, 2005
- Halm et al., 2001; Villeneuve et al., 2006
- Gen et al., 2001
- Kitano et al., 1999
- Luckenbach et al., 2005
- Van Nes et al., 2005
- Wong et al., 2006
- Yu et al., 2008
- Tzchori et al., 2004
- Jin-Young et al., 2005
- Deng et al., 2009
- Kobayashi et al., 2010
- Wang et al., 2010
Table 3.2. Primers used for cloning and or quantification of *cyp19a1a*, *cyp19a1b* and *gapdh* expression in the Murray River rainbowfish

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5'- 3')</th>
<th>Amplicon</th>
</tr>
</thead>
<tbody>
<tr>
<td>gArB980Fdeg</td>
<td>ACTCTGTCCATCAGTCTGTTTCATGYT</td>
<td>gDNA</td>
</tr>
<tr>
<td>gArB1250Rdeg</td>
<td>GTGCATTGACCACGTTTCAGDATGATGTTT</td>
<td>gDNA</td>
</tr>
<tr>
<td>gArO315Fdeg</td>
<td>GTCGAGTCTGGATCAACCGGWGARGARAC</td>
<td>gDNA</td>
</tr>
<tr>
<td>gArO1400Rdeg</td>
<td>TACCACTACTACTTCAGGTAAAGGACACT</td>
<td>gDNA</td>
</tr>
<tr>
<td>mfBArGSPF</td>
<td>GCGTAAAGCTCTGGAGGATGATGACATTGGC</td>
<td>3' Brain RACE and qPCR</td>
</tr>
<tr>
<td>mfBArGSPR</td>
<td>CAAAgTCATCACCTCCAgGTTTACgC</td>
<td>5' Brain RACE</td>
</tr>
<tr>
<td>mfBArGSPR1</td>
<td>GAAGAAGCGATTGGGACTGTTTGG</td>
<td>5' Brain RACE and qPCR</td>
</tr>
<tr>
<td>mfBArGSPR2</td>
<td>TCTCTTCCCAGCATCCGCAGCTCCACTTCAG</td>
<td>Nested 5' Brain RACE</td>
</tr>
<tr>
<td>mfBArGSPR3</td>
<td>CCAACCAGGACACACTGCGCTGAGTAC</td>
<td>5' Brain RACE</td>
</tr>
<tr>
<td>mfBArGSPR4</td>
<td>GGTCTCGGGCAAAGATGAGCTCTGTGTGC</td>
<td>5' Brain RACE</td>
</tr>
<tr>
<td>mfBArGSPR5</td>
<td>TCACTGCTTGCAAGCTGCTCCAGGTAC</td>
<td>5' Brain RACE</td>
</tr>
<tr>
<td>mfBArGSPR6</td>
<td>CGACCTGCTCAGGATAAGGGGTCTCTTCTC</td>
<td>Nested 5' Brain RACE</td>
</tr>
<tr>
<td>mfORACE616F</td>
<td>ACGTAAAGCGATTGGAGATGATGCTGG</td>
<td>3' Ovarian RACE and qPCR</td>
</tr>
<tr>
<td>mfORACE817R</td>
<td>TCCACCAGTGGGTGAAAGGCCAGGCGATT</td>
<td>5' Ovarian RACE and qPCR</td>
</tr>
<tr>
<td>mfORACE594R</td>
<td>CTTACGTTTCAGCAGAAATGGC</td>
<td>5' Ovarian RACE (Nested)</td>
</tr>
<tr>
<td>GAPDHF</td>
<td>ACACCCACTCTCCACCATCTTTT</td>
<td>GAPDH</td>
</tr>
<tr>
<td>GAPDHR</td>
<td>GTTGCTGTAGGCGAACTCAT</td>
<td>GAPDH</td>
</tr>
</tbody>
</table>
3.2.4. Sample preparation from adult fish tissues for qPCR

Total RNA was extracted using RNeasy lipid tissue mini kits (Qiagen Pty. Ltd. Australia) according to the manufacturer’s instructions. For adult fish tissues, the whole organs/tissues were weighed and used for RNA extraction. The tissues were homogenised in 1mL QIAzol lysis buffer using a sterile disposable syringe head and extracted with 200 µL chloroform and centrifuged to separate RNA. RNeasy lipid tissue mini kits were then used according to the manufacturer’s recommendation and during the procedure RNase-Free DNase was also used to eliminate genomic DNA (gDNA) contamination according to the manufacturer’s (Qiagen Pty. Ltd. Australia) instructions. Gel electrophoresis and spectrophotometry were used to determine the quality and quantity of RNA.

Synthesis of cDNA for qPCR was carried out using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Pty. Ltd. Australia) according to manufacturer’s instructions. An equal volume of RNA (~1µg) was added to each 10 µL of master mix. The thermocycler program consisted of 25 ºC for 10 mins, 37 ºC for 120 mins and 85 ºC for 5 min with a final holding step at 4 ºC. Resulting cDNA templates were quantified by spectrophotometer and stored at -20 ºC for real-time assay.

3.2.5. Expression analysis (qPCR)

Real-time assay was carried out on a MJ MiniOpticon system version 3.1 (Bio Rad Pty. Ltd. Australia) with SYBR green fluorescent label using gapdh as an
endogenous control. Gene specific primers were designed for each isoform (Table 3.2) and tested for specificity and efficiency. Specificity of primers was tested by both standard and qPCR by pairing both primer combinations with cloned brain and ovarian aromatase cDNA as templates. To serve as internal control and normalize expressions in qPCR, a 100 bp cDNA coding for glyceraldehyde-3-phosphate dehydrogenase (gapdh) was obtained using previously published sequence (Ponza, 2006). The gapdh was selected as a reference gene due to its stability between the tissues (Rui-Xue et al., 2010) and its sequence for M. fluviatilis was readily available (Ponza, 2006) before the start of this study.

Throughout the study cDNA samples were standardized based on the quantity of the cDNA in each sample. The qPCR primers amplified 161bp, 229bp and 100bp for brain, ovarian and gapdh genes respectively and all primers had nearly equal GC content and annealing temperatures. No products were obtained when primer pairs and cyp19a containing plasmids were mismatched.

Real-time PCR analysis was carried out with a final reaction volume of 20 µL: using 50-55 ng/µL of cDNA, 1x SYBR green PCR master mix (Applied Biosystems Pty. Ltd. Australia) and primers at a final concentration of 100 nM. Each sample was run in triplicate for each of the genes including brain, ovarian aromatase and gapdh as an internal control. Cycling parameters for the qPCR were as follows: 50 ºC for 2 mins, 95 ºC for 10 mins, then 40 cycles of 95 ºC for 15 s and 62 ºC for 1 min. A melting curve analysis was performed at the end of the amplification phase with a minimum of 30 ºC to a maximum of 95 ºC, to test the specificity and identity of the qPCR products.
Since, \textit{cyp19a1a} was not transcribed in any males tissue tested; its presence in the male genome of \textit{M. fluviatilis} was tested using \textit{cyp19a1a} specific primers and male genomic DNA templates.

3.2.6. Data analysis

Real-time data was collected and compiled and the cycle threshold was calculated automatically using CFX software package (Bio Rad. Pty. Ltd. Australia). The data was analysed using the publicly available Q-gene excel script package (Muller \textit{et al.}, 2002). The efficiency values for each aromatase primer and the average efficiency value for \textit{gapdh} were used in subsequent analysis. These values were 2.0 for ovarian aromatase, 1.8 for brain aromatase and 1.9 for \textit{gapdh}, with correlation coefficients of 0.99, 0.97 and 0.98 for ovarian, brain aromatase and \textit{gapdh} respectively. Data from each sample was averaged and shown as Mean Normalized Expression (MNE) ± SE. The data was tested for normality and subsequently analysed by univariate analysis of variance (ANOVA) followed by the Tukey’s test for multiple comparisons and the analysed data was compared between and within the tissues using SPSS17.0 (SPSS Inc. 2008). The significance was set at P<0.05.
3.3. Results

3.3.1. Sequences and Phylogenetic analysis

Two distinctly different isoforms of aromatase genes were isolated from brain and ovary. The ovarian aromatase gene (GU723457) was 1763 bp in length, with a GC content of 46.7% and having an ORF of 492 amino acids. In contrast, the brain isoform (GU723458) was longer (2264 bp) with an overall GC content of 44.5%, with the 5' end (5' of 5' RACE primer) higher (47.4%) than the 3' end (40.2% —3' of 3' RACE primer) and an ORF of 499 amino acids. Although the coding regions were of comparable in length (492 and 499 bp for cy19a1a and cyp19a1b respectively), the brain isoform had significantly longer UTR’s both at 5' and 3' ends —189 bp 5'- and 575 bp 3' UTR compared with 44 bp 5'- and 240 bp 3' UTR of the ovarian isoform. The deduced protein of the brain derived aromatase comprises 499 amino acids with a molecular weight of 56.9 kDa whereas the ovarian derived aromatase has 492 amino acid residues with a molecular weight of 55.09 kDa.

The M. fluviatilis cyp19a1a and cyp19a1b shared an identity of 83-63% and 87-63% with corresponding teleostean isoforms respectively. The highest identity was with the corresponding isoforms of pejerrey (Odontesthes bonariensis) followed by Japanese medaka (Oryzias latipes). The least identity among the ovarian aromatase sequences was observed with that of zebrafish and goldfish (63%), where as the brain aromatase was with that of zebrafish, goldfish and common carp (63%). In contrast the total amino acid sequence identity between the brain and ovarian aromatase genes of Murray River
rainbowfish was only 61%. As seen in Fig. 3.1 the brain and ovarian aromatase genes of the rainbowfish (in bold) clustered into two distinct clades along with the respective teleostean isoforms. All four methods used to construct the tree exhibited identical results, therefore in the Fig. 3.1 only phylogenetic tree generated using neighbour-joining method is presented.
Figure 3.1. Phylogenetic tree of representative fish aromatase proteins. The tree was constructed using the clustalW multiple sequence neighbour joining alignment program (MEGA4). The deduced amino acid sequences of P450arom were used from Japanese medaka brain (AY319970) and ovary (D82968), pejerrey brain (AAQ88434) and ovary (ABK30807), European seabass brain (AY138522) and ovary (AJ298290), Nile tilapia brain (AF306786) and ovary (TNU72071), zebrafish brain (AF183908) and ovary (AF226620), common carp brain (EU375456) and ovary (EU375455), goldfish brain (CAU18974) and ovary (AB009336), seabream brain (ABC70868), with Atlantic Stingray, *Dasyatis Sabina* (AF091513) as an out-group. Numbers indicate values supporting the branching pattern from 1000 bootstraps. The markers of 0.05 differences between the two lines are the length that corresponds to 5% sequence difference.
3.3.2. Amino acid alignments and comparison of conserved domains.

The amino acid alignment of the ovarian (Fig. 3.2) and brain (Fig. 3.3) isoforms of *M. fluviatilis* with those of select teleosts reveals the presence of all the key conserved domains.

The conserved domains of the ovarian aromatase (*cyp19a1a*) of rainbowfish generally shared higher identity with the corresponding domains of all other teleosts compared—aromatase specific conserved domain (86-95%), I-helix (91-97%) and heme binding domain (83-100%), with the exception of membrane-spanning domain which was comparatively low (42-85%).

Similarly the conserved domains of the brain isoform (*cyp19a1b*), shared higher identity with the corresponding domains of other teleosts—aromatase specific conserved domain (63-90%), I-helix (85-97%), heme binding (88-100%) domain. The membrane-spanning region had relatively low identity (35-76%) with those of the other teleosts.
### Alignment of Amino Acid Sequence

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**Figure 3.2** Alignment of amino acid sequence of Murray River rainbowfish cyp19a1a (GU723457), Pejerrey fish cyp19a1a (AB1C30087), Medaka cyp19a1a (D28968), Common carp cyp19a1a (EU375455), Zebrafish cyp19a1a (AF226620), Goldfish cyp19a1a (AB099336), Seabass cyp19a1a (AJ298290), and Nile tilapia cyp19a1a (TNU72071). Identical amino acid residues and conserved domains are highlighted in dark colour. The four domains including membrane-spanning, I-helix region, aromatase specific conserved and heme-binding domains are indicated by thick lines.
domains including membrane-spanning, I-Helix region, aromatase specific conserved and heme-binding domains are indicated by thick lines.

Figure 3.3. Alignment of amino acid sequence of Murray River rainbowfish cyp19a1b (GU723458), Pejerrey fish cyp19a1b (AAQ88434), Medaka cyp19a1b (AY138522), Nile tilapia cyp19a1b (AF306786) and Seabream cyp19a1b (ABC70668). Identical amino acid residues and conserved domains are highlighted in dark colour. The four domains including membrane-spanning, I-Helix region, aromatase specific conserved and heme-binding domains are indicated by thick lines. * 5' end sequence is not available.
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Figure 3.4. Deduced amino acid sequence of Murray River rainbowfish brain and ovarian aromatases (shared 61 % identity). The four domains including membrane-spanning, I-Helix region, aromatase specific conserved and heme-binding domains are indicated by thick lines.
3.3.3. Tissue and sex specific distribution of *cyp19a1a* and *cyp19a1b* transcripts in adult Murray River rainbowfish

Tissue and sex-specific expression of *cyp19a* isoforms are summarised in Fig. 3.6. In this study the presence of *cyp19a* isoforms in different tissues was investigated; then the expression of both isoforms in different key tissues of reproductively active male and female rainbowfish were compared. Brain, gonad, eye, liver and spleen tissues were examined by qPCR assay. Results showed that the *cyp19a* genes were predominantly expressed in their respective tissue domains (*cyp19a1a* in ovary and *cyp19a1b* in brain). Expression of *cyp19a1a* was specific and restricted to ovarian tissue whereas *cyp19a1b* was expressed in multiple tissue but predominantly in the brain. Analysis of results by one-way variance (ANOVA) showed significant differences between tissue types and sex: *cyp19a1a* ($F_{9, 20} = 15.341, P = 0.05$), *cyp19a1b* ($F_{9, 20} = 88.318, P = 0.05$). Despite *cyp19a1a* not being expressed in any of the male tissues, its presence in the male genome was confirmed by genomic PCR (Fig. 3.7).

The expression of *cyp19a1b* was predominantly expressed in brains of both sexes, but the expression in male brain was ~3-fold higher than female brain tissue. A low level of *cyp19a1b* expression was observed in other tissues tested including gonad, eye, liver, however it was undetected in the spleen of both sexes. When we compared the expression levels of *cyp19a1a* and *cyp19a1b* in ovarian tissue, the expression of *cyp19a1a* was significantly higher than *cyp19a1b* (~1000-fold higher). There was no significant difference
observed in expression of cyp19a1b between eye, liver and gonad of both sexes.

Figure 3.5. Tissue specific expression of cyp19a1b, cyp19a1a and gapdh in different tissues of male and female Murray River rainbowfish in brain (B), testis (T), Liver (L), Eyes (E), spleen (S) and ovary (O). A 1000 bp molecular marker (M) was used to assess the size of the PCR products. The sizes of the brain aromatase (cyp19a1b, Panel A), ovarian aromatase (cyp19a1a, panel B) and gapdh (Panel C) were 161, 229 and 100 bp respectively. Expression of aromatase was analysed in triplicate. Real-time PCR reactions and amplicons were separated on 1% agarose gel.
Figure 3.6. Expression of both aromatase genes within tissues showing differential expression. Each bar represents the mean normalised expression (±SE; n = 3), X-axis labels; B - Brain, T-Testis, O - Ovarian, L - Liver. E - Eyes and S - Spleen. The means that are significantly different when tested using Tukey’s posthoc test for both aromatase genes in each tissue between male and females in adult Murray River rainbowfish. Values described by the same letter are not significantly different from each other (P<0.05, n=3).

Figure 3.7. Gel electrophoresis for ovarian aromatase genomic DNA in both sexes. M – 2- Log DNA Ladder, Lanes 1 to 2-Male genomic DNA + cyp19a1a specific primers, Lanes 3 to 4-Female genomic DNA+ cyp19a1a specific primers.
3.4. Discussion

In this study we cloned full-length *cyp19a* isoforms encoding aromatase genes in Australian native Murray River rainbowfish along with their quantitative expression in different key tissues for the first time.

The ovarian derived *cyp19a1a* comprises 1763 bp containing 1479 bp ORF and 492 amino acids; meanwhile the brain derived *cyp19a1b* comprises 2264 bp containing 1500 bp of ORF and 499 amino acid residues from the deduced protein. This is in agreement with what has been observed previously in other teleosts where generally the brain derived *cyp19a1b* is either of the same size (Chang *et al*., 1997, Kwon *et al*., 2001) or longer (Gelinas *et al*., 1998; Kishida and Callard, 2001; Barney *et al*., 2008) than the ovarian derived *cyp19a1a*.

3.4.1. Phylogenetics

The phylogenetic analysis demonstrates that the aromatase isoforms (*cyp19a1a* and *cyp19a1b*) of *M. fluviatilis* shared greater homology with corresponding isoforms of other teleosts, than between themselves. This is consistent with observation made in many teleosts species (Kishida and Callard, 2001; Blázquez and Piferrer, 2004; Zhang *et al*., 2004; Strobl-Mazzulla *et al*., 2005; Barney *et al*., 2008). In agreement with this observation, the *M. fluviatilis* aromatase isoforms clustered on separate branches comprising teleostean *cyp19a1a* (ovarian aromatase) and *cyp19a1b* (brain aromatase) respectively (Fig. 3.1). This suggests that the *M. fluviatilis* *cyp19a1a* and *cyp19a1b* are homologous to the corresponding teleostean
isoforms but are paralogous to one another as has been reported in other species of teleosts (Kishida and Callard, 2001; Blázquez and Piferrer, 2004; Zhang et al., 2004; Strobl-Mazzulla et al., 2005; Barney et al., 2008). These observations support the shared ancestry of the two genes and the hypothesis that the paralogous genes evolved as a result of genome duplication early in the teleost lineage (Taylor et al., 2003). Not surprisingly the phylogenetic analysis shows that aromatase isoforms of the Murray River rainbowfish clustered with pejerrey, a closely related athernid and thus reconfirming close relationship between the two species.

As seen in the amino acid sequence alignments (Fig. 3.2 and 3.3), the putative functional domains (I-helix, aromatase specific conserved and heme binding domains) are highly conserved with the exception of the membrane-spanning region. The relatively higher homology between the conserved domains of Murray River rainbowfish and pejerrey further confirm the evolutionary close relationship between the two species. A relatively lower identity between the conserved domains of cyp19a1a and cyp19a1b of M. fluviatilis compared to between corresponding domains of the respective isoforms of different teleosts support the hypothesis of duplication and paralogous evolution of the isoforms in teleosts. Not surprisingly conserved regions of both isoforms shared higher homology with members of the same taxonomic order compared to others.
3.4.2. Tissue specific and sexually dimorphic expression of aromatase isoforms in adults

Of all the tissues tested for *cyp19a* expressions in Murray River rainbowfish, the expression level of *cyp19a1b* was significantly higher in the brain tissues of both sexes in comparison to other tissues tested including gonads, eyes and liver, with no measurable expression in the spleen of both sexes. The preferential expression of *cyp19a1b* in brain is consistent with reports on zebrafish (Trant *et al.*, 2001; Sawyer *et al.*, 2006), European Seabass (Blázquez and Piferrer, 2004), Nile tilapia (Chang *et al.*, 2005), protogynous wrasse (Jin-young *et al.*, 2005), southern flounder (Luckenbach *et al.*, 2005), common carp (Barney *et al.*, 2008) and Japanese Medaka (Patil and Gunasekera, 2008).

The high level of *cyp19a1b* transcripts in the brain of teleosts has been implied to contribute towards neuroestrogen production in the brain (Chang *et al.*, 2005), which plays a role in continuous neurogenesis, sexual plasticity and regeneration of the brain (Forlano *et al.*, 2001). Further observations that *cyp19a1b* expression in the brain of adult fish is restricted to radial glial cells-abundant in preoptic area, hypothalamus and central nervous system (including spinal cord), supports a direct role for *cyp19a1b* in neurogenesis and brain regeneration (Forlano *et al.*, 2001; Pellegrini *et al.*, 2005; Diotel *et al.*, 2010).

Surprisingly, the expression of *cyp19a1b* in *M. fluviatilis*, male brain was ~3-fold higher than in female fish brain. However, this result is consistent with
observations in seabass (González and Piferrer, 2003), pejerrey (Strobl-Mazzulla et al., 2005), Atlantic halibut (Matsuoka et al., 2006) and common carp (Barney et al., 2008). In contrast, significantly higher expression of cyp19a1b in adult female brain than male has been reported in zebrafish (Sawyer et al., 2006) and Japanese medaka (Patil and Gunasekera, 2008). These differences across species and between sexes could be associated with the reproductive status of the individual fish or sex linked seasonal variations of the cyp19a1b in the brain tissues associated with reproduction (Kazeto et al., 2003; Nunez and Applebaum, 2006) and or could be related to mating and sexual behaviour (Patil and Gunasekera, 2008).

The expression of the ovarian isoform (cyp19a1a) in M. fluviatilis was restricted to ovaries and no measurable expression of cyp19a1a was observed in other tissues or organs examined in both males and females. This pattern of specific and restricted expression of cyp19a1a in ovaries is consistent with the well-known high estrogenic activity of the ovaries and the role of estrogens in ovarian development and growth (Devlin and Nagahama, 2002). Similar specific ovarian expression of cyp19a1a was observed in other teleosts including Nile tilapia (Chang et al., 2005), killifish (Greytak et al., 2005) and pejerrey (Karube et al., 2007). Whereas in other species including Japanese flounder (Kitano et al., 1999), orange-spotted grouper (Zhang et al., 2004), zebrafish (Sawyer et al., 2006), common carp (Barney et al., 2008) and medaka (Patil and Gunasekera, 2008) low level overlapping expression of cyp19a1a has been observed in tissues other than ovary. Such restricted expression of the aromatase isoform in ovaries of some teleosts compared to
others may reflect subtle evolutionary differences in their regulation and hence their subfunction following genome wide duplication of teleosts.

The subtle differences in expression pattern of the aromatase genes between species are likely to reflect the diverse reproductive and behavioural diversity observed in teleosts. It is therefore likely that a comparative gene structure and function studies in a wide range of species with diverse reproductive strategies will assist in dissection of multiple roles the estrogen and aromatase play. The duplication and sub functional specialization of the aromatase in teleosts is particularly useful in dissection of the multiple functions the gene plays in vertebrate development, reproduction and behaviour. However it must be noted that contradictory results in zebrafish—one reporting restricted expression of *cyp19a1a* to ovary (Kishida and Callard, 2001) and the other with low level expression in tissues other than ovary (Chiang *et al.*, 2001) have been reported. This discrepancy though appears to reflect the differences in sensitivity and type of the expression assays employed.

The observed lower levels of *cyp19a1b* in the ovarian tissue relative to *cyp19a1a* in this species is consistent with the hypothesis that the *cyp19a1a* plays a specific and primary role in the ovarian differentiation and development in teleosts (Patil and Gunasekera, 2008).

In the current study expression of brain isoform (*cyp19a1b*) was observed in the eyes of both sexes albeit at low levels. Similar expression (only *cyp19a1b* in eyes of both sexes) has also been observed in zebrafish (Kishida Callard, 2001). In slight contrast, expression of both isoforms (*cyp19a1a and
*cyp19a1b* in eyes of both sexes has been observed in goldfish (Callard *et al*., 1993), common carp (Barney *et al*., 2008) and half-smooth tongue-sole (Deng *et al*., 2009). Expression of aromatase in eyes could be related to the function of neuroestrogens in transmission, integration and processing of optic cues. The lower level expression of aromatase in eyes has been suggested to act in an intracrine, wherein it produces estrogens from the circulating androgens for localized action (Barney *et al*., 2008). Interestingly no measurable levels of *cyp19a* isoforms were observed in the eyes of killifish (Greytak *et al*., 2005) and pejerrey (Strobl-Mazzulla *et al*., 2005; Karube *et al*., 2007).

Low level *cyp19a1b* expression in liver of both sexes was observed in the rainbowfish. This is consistent with the reports on some oviparous species including frog (Di Fore *et al*., 1998; Assisi *et al*., 2000), European sea bass (Goto-Kazeto *et al*., 2004), sex changing goby (Kobayashi *et al*., 2004), protogynous wrasse (Jin-young *et al*., 2005), , wrasse, *Pseudolabrus sieboldi* (Sundaray *et al*., 2005) and common carp (Barney *et al*., 2008). The expression of *cyp19a1b* in hepatic tissue could be related to need for extra sources for estrogen stimulation during vitellogenesis (Assisi *et al*., 2000; González and Piferrer, 2003). However, the expression of *cyp19a1b* in male fish liver suggests that it may be required for an as yet unknown liver function or is a consequence of ectopic expression in the liver of both sexes. This study also observed the expression of brain isoform in the testis. Testicular expression of aromatase has been previously led to the suggestion that estrogen could be required for males during testes development and function (Barney *et al*., 2008).
3.5. Conclusions

In conclusion, the brain and the ovarian derived aromatase isoforms were isolated and their expression characterized in the Murray River rainbowfish for the first time. As in other teleosts, expression of the isoforms occurs predominantly in their respective tissue domains (i.e. brain and ovary) and interestingly, expression of cyp19a1a is specific and restricted to ovarian tissue in this species. The observed data is consistent with the teleost genome duplication millions of years ago and these isoforms have acquired specialized roles; cyp19a1a performing the role in ovarian differentiation and development, whereas cyp19a1b playing a role in the brain and neural development and extragonadal functions. The specific and exclusive expression of cyp19a1a in M. fluviatilis, combined with the biological attributes of the species renders it as an ideal model to investigate the mechanisms associated with tight ovarian regulation of aromatase, its modulation by external cues and its perturbation by environmental effluents.
CHAPTER 4

Expression of cyp19a isoforms during early embryonic and larval development of the Murray River rainbowfish, *Melanotaenia fluviatilis*

Shanthanagouda A H, Patil J G and Nugegoda D

The manuscript in preparation— Journal of Fish Biology.

Abstract

This study investigated the expression of aromatase gene expression in selected developmental stages of the Murray River rainbowfish, *M. fluviatilis* using qPCR. The ontogenic study revealed the absence of *cyp19a1a* expression at embryonic and larval developmental stages until day 7 post hatch in *M. fluviatilis*. On the contrary, the expression of *cyp19a1b* transcript was strongly evident at all selected developmental stages. The expression of *cyp19a1b* in unfertilized eggs in the present study clearly indicated that it is maternally transferred. The expression of only the *cyp19a1b*, transcript in the developmental stages studied appears to be unique to *M. fluviatilis*, thus far. In addition, a significant increase in the expression of *cyp19a1b* was observed in
just-hatched larvae. This increased transcript level at about hatching could reflect the timing and onset of zygotic cyp19a1b transcription in this species. This zygotic expression of the cyp19a1b form alone during early development further suggested a primary or sole role for cyp19a1b in neural and brain development in this species, with cyp19a1a strictly restricted to ovarian development. Absence of cyp19a1a transcripts during the early developmental stages indicates that sex differentiation has not yet begun in M. fluviatilis.

**Keywords:** M. fluviatilis, Developmental stages, cyp19a, sex differentiation

1. Introduction

Estrogen ratio must be balanced and regulated in all life stages of an organism (Trant *et al.*, 2001) as they participate in development and differentiation of organs including neural and cardiovascular development (Guigen *et al.*, 2010). Estrogens also influence sex differentiation and sexual behaviour in vertebrates (Kishida and Callard, 2001). Biological synthesis of estrogens is catalysed by cytochrome P450 aromatase (Cyp19a)— key enzyme converting androgens to estrogens. Regulation and appropriate expression of this enzyme is critical for many functions including sexual differentiation, maturation, reproduction and also in other physiological functions (Kishida and Callard, 2001).

The Cyp19a enzyme is encoded by two structurally and functionally different genes in teleosts. Among them one is predominantly expressed in ovarian tissues and designated cyp19a1a (*CYP19a/P450AromA/CYP19A1*), and the
other one is preferentially expressed in brain and designated \textit{cyp19a1b} \textit{(CYP19b/P450AromB/CYP19A2)} (Chang \textit{et al.}, 2005). It has been hypothesized that during the developmental stages, if more of the aromatase gene is transcribed, higher the levels of estrogens are synthesized resulting in a higher female population. In contrast, if less of the aromatase gene is transcribed lesser the estrogen levels leading to more of males (Eggen, 2008). Expression of both isoforms have been studied in the developmental stages of several fish species including zebrafish (Trant \textit{et al.}, 2001), Atlantic halibut (Van Nes \textit{et al.}, 2005), common carp (Barney \textit{et al.}, 2008) and medaka (Patil and Gunasekera, 2008).

Several studies suggest that \textit{cyp19a1a} is more involved in the development of the lateral line, sensory organs, sexual differentiation and gonad development, while \textit{cyp19a1b} is involved in neural development and sexual behaviour (Eggen, 2008). However, the precise functions of estrogens and the functions of aromatase isoforms at developmental stages are still unclear and appear to vary from one teleost to another (Patil and Gunasekera, 2008). Moreover the functions of these isoforms can be altered by exposing fish to endocrine-disrupting chemicals (Scholz and Gutzeit, 2000; Langlois \textit{et al.}, 2010; Vosges \textit{et al.}, 2010; Wang \textit{et al.}, 2010) and can be influenced by external factors such as temperature (Nagy \textit{et al.}, 1981; Bull, 1983; Pieau, 1996; Tsai \textit{et al.}, 2003; Barney \textit{et al.}, 2008). If fish were exposed to EDCs at an early life stage, the sex ratio would be skewed to either male or female. The percentage of skewness would depend on the exposure duration and concentrations of EDCs. Many reports have supported these hypotheses (Mouriec \textit{et al.}, 2009; Wang \textit{et al.}, 2010). Consequently, the genes involved in estrogen
biosynthesis, particularly cyp19a has been viewed as a biomarker of exposure and effect of xenoestrogens. Different life stages respond and cope differently to EDC exposure, with early embryonic and larval stages known to be particularly sensitive and vulnerable (Jin et al., 2009; Vosges et al., 2010).

The primary objective of the experiment described in this chapter was to understand and establish expression patterns of cyp19a isoforms during early embryonic and larval development of the Murray River rainbowfish. The results presented should serve as baseline for future investigations on effects of exposure to EDC’s, external factors such as temperature and assist in understanding mechanisms of sex differentiation in this model species.

4.2. Materials and Methods

4.2.1. Fish breeding and developmental staging.

Details of fish rearing are presented in Chapter 2. Briefly, reproductively active female and male (ratio 7:2) M. fluviatilis were held in 82 L of water at about 25 °C. Bundles of cotton strings were provided as substrate to deposit eggs (Fig. 4.1). During this period fish were fed frozen beef heart and blood-worms. Fish typically bred at dawn. The substrate along with the deposited eggs were then collected and treated with malachite green at 4 ppm for 5 min to prevent fungal growth as recommended (Holdway et al., 1994). The treated eggs were washed in clean water and placed in separate aquaria for hatching. Developing embryos were collected and observed under a Leica MZ95.
microscope (Leica Microsystems, Switzerland) at regular intervals to ascertain developmental stage.

The hatchlings were fed four times a day on a premixed diet which included algae (Chlorella), live Paramecium and Vinegar eels until 15 days (Fig. 4.2). Larvae older than 15 days were fed with live brine shrimp larvae and finely powdered Sera micron (Sera micron, D 52518, Heinsberg, Germany) baby fish food. Throughout the nursery stage, water temperature was maintained at 25±1 °C with continuous aeration with a 16:8 light: dark illumination cycle. Three individuals at each of the key developmental stage were sampled and preserved in RNAlater.

Figure 4.1. Fish breeding in normal plastic tubs with substrates
4.2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from three individuals at each selected developmental stages using RNeasy lipid tissue mini kits (Qiagen Pty. Ltd. Australia) according to the manufacturer’s instructions as explained in chapter 2, section 2.11 and 2.12.

4.2.3. Oligonucleotides for the real-time PCR analysis

Gene-specific oligonucleotide primers were designed as explained in section 3.2.2, chapter 3 and are presented in Table 3.2, chapter 3.
4.2.4. Real-time PCR analysis

Real-time PCR assays were carried out on a MJ MiniOpticon system version 3.1 (Bio-Rad Pty. Ltd.) with SYBR green fluorescent label using gapdh as an endogenous control as explained in section 2.15, chapter 2.

4.2.5. Data analysis

Real-time PCR was carried out on a MiniOpticon system for real-time detection version 3.1 (Bio Rad Pty. Ltd. Australia) with SYBR green fluorescent labelling using gapdh as an endogenous control as explained in section 2.15, chapter 2.

4.3. Results

4.3.1. General observations

The key developmental stages of the *M. fluviatilis* are listed in Table 4.1. The Murray River rainbowfish eggs required 6 days (~144 h) to hatch under the stated laboratory conditions (Section 4.2.1) and the developmental stages are presented in Table 4.1.

4.3.2. Expression of *cyp19a1a* and *cyp19a1b* during embryonic and early larval development

To understand the expression pattern of aromatase isoforms in the Murray River rainbowfish, both ovarian (*cyp19a1a*) and brain (*cyp19a1b*) aromatase genes were analysed at select developmental stages.
As presented in Fig. 4.4, *cyp19a1a* expression was not detected in any of the larval stages tested. In contrast *cyp19a1b* was expressed in all the developmental stages tested (Fig. 4.4). Analysis of results by one-way variance (ANOVA) showed significant differences in *cyp19a1b* isoform expression between the developmental stages ($P_{10, 22} = 6.798$, $n=3$) of the Murray River rainbowfish. It is interesting to note that the unfertilized eggs expressed *cyp19a1b*. The mean normalized expressions of *cyp19a1b* transcript were initially higher, and the levels were not significantly different ($P<0.05$) among the stages starting from unfertilized eggs to the neurula. After the neurula stage there was a progressive and significant ($P<0.05$) decrease in the expression of *cyp19a1b*, starting 1 dpf until the day just prior to hatch. However this decrease was not significant between all the intermediate stages as the levels of *cyp19a1b* between 1 and 2 dpf as well as those between 3-6 dpf were not significantly different from one another.

The quantitative expression of *cyp19a1b* in just hatched (JH) larvae was significantly ($P<0.05$) higher than those in the embryo just prior to hatch (6 d). Following hatching, similar levels of *cyp19a1b* expression were observed from 1 to 5 dph. This was followed by a significant ($P<0.05$) increase of *cyp19a1b* transcription at 6 dph but again on 7dpf the levels fell and were comparable those observed between 1-5 dph.
Table 4.1. Embryonic developmental stages of *M. fluviatilis* (Figures in this table are adapted from Reid and Holdway, 1995)

<table>
<thead>
<tr>
<th>Development Stage</th>
<th>Hours of post fertilization (h–hour, d-day)</th>
<th>Sketches of the developmental stages</th>
<th>Observation and description of the developmental stages</th>
</tr>
</thead>
<tbody>
<tr>
<td>JF - Just Fertilized</td>
<td>~1.0 h</td>
<td><img src="image1" alt="Sketch" /></td>
<td>Eggs were clear and uniform sized and spherical. Variable sized numerous oil droplets were observed around the yolk. Y-Yolk, O-Oil droplets, C-Chorion, F-Filaments, PV-Perivetilline</td>
</tr>
<tr>
<td>Mo- Morula</td>
<td>3.5 h</td>
<td><img src="image2" alt="Sketch" /></td>
<td>The peripheral blastomeres were in flattened shape and the cells were arranged in 2-3 layers. Nearly 16 cells were observed at this stage. PV-Perivetilline, Y-Yolk, CE-Cells, O-Oil droplets,</td>
</tr>
</tbody>
</table>
Table 4.1. Continued

<table>
<thead>
<tr>
<th>Developmental Stage</th>
<th>Hours of post fertilization (h–hour, d-day)</th>
<th>Observation and description of the developmental stages</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL-Blastula</td>
<td>9.0 h</td>
<td>Large number of similarly sized (nearly 1000 cells) were observed forming blastodisc. B- Blastodisc, Y- yolk, C-Chorion, O- Oil droplets</td>
</tr>
<tr>
<td>NL-Neurula</td>
<td>15 h</td>
<td>The blastodisc size decreased and spread on the surface of the yolk and a ring like structure was observed. At the posterior end a few oil droplets were also observed. EA-Embryonic Axis, C-Chorion, G–Germ Ring, Y-Yolk, O-Oil droplets</td>
</tr>
</tbody>
</table>
Table 4.1. Continued

<table>
<thead>
<tr>
<th>Developmental Stage</th>
<th>Hours of post fertilization (h–hour, d-day)</th>
<th>Sketches of the developmental stages</th>
<th>Observation and description of the developmental stages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early Somitogenesis</td>
<td>24 h (1 d)</td>
<td><img src="image" alt="Early Somitogenesis Sketch" /></td>
<td>At this stage tiny head (rudimentary brain) like structure at the anterior part of body with eyes development and tiny tail structure appeared, with 1-3 somites. In this stage appearance of heart was observed. Y- Yolk, OV- Otic Vesicle, H- Head, T-Tail, S- Somites</td>
</tr>
<tr>
<td>Late Somitogenesis</td>
<td>56 h (2 d)</td>
<td><img src="image" alt="Late Somitogenesis Sketch" /></td>
<td>Tail like structure appeared with nearly 20 somites on the body and melanophore pigmentation started to appear. O-Oil droplets, OV-Optic Vesicles, PC-Pericardial Cavity, FP-Future Pectoral fin, M–Melanophores, NC-Notochord, S-Somites,</td>
</tr>
</tbody>
</table>
Table 4.1. Continued

<table>
<thead>
<tr>
<th>Developmental Stage</th>
<th>Hours of post fertilization (h–hour, d-day)</th>
<th>Observation and description of the developmental stages</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 day old embryo</td>
<td>72 h (3 d)</td>
<td>On both sides of the head otic vesicles were clearly visible. Melanophore pigmentation was also observed. Just beneath the somites bright kidney structure was clearly observed. OC–Optic Capsule, L-Lens, PC–Pericardial Cavity, O-Oil droplets, YS–Yolk Sac, T-Tail, NC-Notocord, M-Melanophores, OT-Otolith</td>
</tr>
<tr>
<td>4 day old embryo</td>
<td>96 h (4 d)</td>
<td>Throughout the body, increased pigmentation and the body fluid movement was observed. Transparent tiny swim bladder was clearly recognized beneath the notocord. OC-Optic Capsule, L-Lens, DM-Dorsal Melanophores, NC-Notocord, VM-Ventral Melanophores, YS-Yolk Sac</td>
</tr>
<tr>
<td>Developmental Stage</td>
<td>Hours of post fertilization (h–hour, d-day)</td>
<td>Sketches of the developmental stages</td>
</tr>
<tr>
<td>---------------------</td>
<td>-------------------------------------------</td>
<td>-------------------------------------</td>
</tr>
<tr>
<td>5 day old embryo</td>
<td>120 h (5 d)</td>
<td><img src="image" alt="Sketch" /></td>
</tr>
<tr>
<td>6 day old embryo</td>
<td>144 h (6 d)</td>
<td><img src="image" alt="Sketch" /></td>
</tr>
</tbody>
</table>
Figure 4.3. Lateral view of the newly hatched larvae. A- Anus, DM-Dorsal Melanophores, E-Eye, F- Finfold, LM-Lateral Melanophores, MO-Mouth, NC-Notochord, O-Oil Droplets, OT- Otolith, PF-Pectoral fin, YS-Yolk Sac (Figure adapted from Reid and Holdway, 1995).
Figure 4.4. Expression of cyp19a1a, cyp19a1b and gapdh in early developmental stages of Murray River rainbowfish. A 1000 bp molecular marker (M) was used to assess the size of the PCR products. The sizes of the ovarian (cyp19a1a), brain aromatase (cyp19a1b) and gapdh were 229, 161 and 100 bp, respectively. Expression of aromatase was analysed in triplicate. Real-time PCR reactions and amplicons were separated on 1% agarose gel. UF-Unfertilized egg, JF-Just Fertilized, Mo-Morula, BL-Blastula, NL-Neurula and 1-6 d (d-day old embryo). JH-Just Hatched, 1-7dph (dph – days post hatch), respectively.
4.4. Discussion

The results presented in this chapter demonstrated the absence of *cyp19a1a* expression both at embryonic and larval developmental stages of the Murray River rainbowfish. However, the expression of *cyp19a1b* transcript was strongly evident at all the selected developmental stages. The expression of *cyp19a1b* in unfertilized eggs in the present study clearly indicates that it is maternally transferred. Based on the available literature it appears that
maternal inheritance of cyp19a transcripts in teleosts exhibit four possible patterns of maternal inheritance—inheritance of a) none, b) both cyp19a1a and cyp19a1b, c) cyp19a1a but not cyp19a1b and d) cyp19a1b but not cyp19a1a. For example, mRNA of neither isoforms was maternally inherited in the medaka Oryzias latipes (Patil and Gunasekara, 2008). Contrastingly maternal inheritance of both cyp19a1a and cyp19a1b transcripts has been observed in zebrafish (Sawyer et al., 2006). Maternal inheritance of cyp19a1a transcript alone has been reported in common carp (Barney et al. 2008) and that of only cyp19a1b, transcript appears to be unique to M. fluviatilis, thus far. Reasons for such differences in maternal inheritance of the cyp19a transcripts across teleosts is unclear, but are likely to be associated with subtle variations in the reproductive strategies of the respective species. When data on maternal inheritance pattern for more species become available it might be possible to infer an association between the patterns and reproductive strategies adopted by different teleosts.

The observation that cyp9a1a transcript is not zygotically (endogenously) transcribed during embryonic or early larval stages, implies no role for the isoform during non-reproductive life stages in this species. The absence of endogenous transcription of cyp19a1a throughout embryonic or larval stages appears to be unique to this species, thus far. In medaka, despite its non-maternal inheritance, cyp19a1a is transcribed zygotically just before hatching (Patil and Gunasekera, 2008). Zygotic expression of cyp19a1a during early larval development has been reported in zebrafish (Trant et al., 2001) and common carp (Barney et al., 2008). The first endogenous expression of
*cyp19a1a* in this species might occur only when the putative gonad has differentiated into ovary an argument further supported by the observation that *cyp19a1a* is exclusively expressed in ovarian tissue in this species (Chapter 3, Section 3.3.4). Therefore the *cyp19a1a* is unlikely to play a role in early development and gonadal differentiation in the Murray River rainbowfish. However this will require future verification encompassing gene expression studies in later developmental stages and histological observations. In contrast, studies have shown that the *cyp19a1a* transcript plays a pivotal role in the sex differentiation in Nile tilapia (Chang *et al.*, 2005) and common carp (Barney *et al.*, 2008).

The maternally inherited *cyp19a1b* transcripts will likely contribute to the biosynthesis of estrogen required for early larval development and differentiation. The expression of only *cyp19a1b* during the developmental stages of the Murray River rainbowfish indicates that it is of greater importance than the ovarian form of aromatase in non-reproductive life stages of this species. Sustained level of maternal transcripts up to neurulation and their subsequent decline may reflect its active role in biosynthesis of estrogen around the time of neural differentiation. A role for estrogens in the neural differentiation both at embryonic and in brain of adults has been previously suggested in goldfish (Callard *et al.*, 1993), zebrafish (Trant *et al.*, 2001; Sawyer *et al.*, 2006), European Seabass (Blázquez and Piferrer, 2004), Nile tilapia (Chang *et al.*, 2005), common carp (Barney *et al.*, 2008) and Japanese medaka (Patil and Gunasekera, 2008),
Increased transcript levels at about hatching (Fig. 4.5) reflect the timing and onset of zygotic \textit{cyp19a1b} transcription in this species. This zygotic expression of the brain aromatase form alone during early development further suggests a primary or sole role for \textit{cyp19a1b} in neural and brain development in this species, with \textit{cyp19a1a} strictly restricted to ovarian development. Similar inferences have been made in zebrafish (Lassiter and Linney, 2007) although just based on significantly higher \textit{cyp19a1b} expression compared to \textit{cyp19a1a} throughout the embryonic and larval development.

The fluctuating levels of \textit{cyp19a1b} expression through early development, may correspond to, it’s varying role at different stages. A similar pattern of varied expression of \textit{cyp19a1b} was also demonstrated in zebrafish developmental stages (Trant et al., 2001) and common carp (Barney et al., 2008). While the first decline in expression post neurula reflects depletion of the maternal transcripts, the first increase at just hatch, represents timing of zygotic transcription in this species. The level of \textit{cyp19a1b} was significantly increased in just hatched larvae compared to just prior to hatch. This increase is in agreement with previous studies on zebrafish, where the enhanced levels of \textit{cyp19a1b} were well correlated with increased estrogen receptors (ERs) (Sawyer et al., 2006; Mouriec et al., 2009). The observed increase in just hatched larvae of the Murray River rainbowfish could also correspond to increased ERs. However, the expressions of ERs have not been studied in this species.
4.5. Conclusions

The results presented in this chapter provide the expression pattern of both *cyp19a1a* and *cyp19a1b* during early development of *M. fluviatilis*. The *cyp19a1b* was not only maternally inherited; its zygotic transcription was initiated at hatching. In contrast, *cyp19a1a* was neither maternally inherited nor transcribed during the early developmental stages. Together the results suggest a primary role for *cyp19a1b* during early larval development in the species, with *cyp19a1a* primarily restricted to the role of ovarian differentiation and development. More broadly the results provide baseline data for future investigations into, the effect of environmental modulators and aquatic pollutants in this model species.
CHAPTER 5

Exposure to xenoestrogens disrupts the *cyp19a* (aromatase) gene expression in the Murray River rainbowfish, *Melanotaenia fluviatilis*

Shanthanagouda A H, Patil J G and Nugegoda D

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Abstract

This study investigated the influence of two endocrine disrupting chemicals-exogenous estrogen 17β-estradiol (E2) and the estrogen mimic nonylphenol (NP) on the expression of aromatase isoforms in both sexes of adult Murray River rainbowfish. Reproductively active male and female fish were exposed to 1, 3, 5 µg/L of E2 or 100, 500 µg/L of NP for 96 h. The expression analyses of *cyp19a* isoforms in the brain and gonads of both sexes of adult fish were studied using quantitative Real-time PCR (qPCR). The exposure of rainbowfish to E2 and NP resulted in significant reduction in the expression of *cyp19a1a* isoform in ovarian tissues at the
two lowest exposure concentrations of 1 µg/L of E2 and 100 µg/L of NP with complete inhibition at 96 h. At higher concentrations of E2 and NP, \textit{cyp19a1a} was completely inhibited in ovarian tissues. Similarly, \textit{cyp19a1b} was significantly downregulated in the brain of male fish exposed to E2 and NP. However, it’s expression in testicular, ovarian and brain tissues of female fish exposed to E2 was initially upregulated until 72 h of exposure and then showed down regulation trend at 72 and 96 h. Expression of \textit{cyp19a1b} in female fish brain was upregulated at both exposure concentrations of NP. The observations support the hypothesis that E2 regulates expression of \textit{cyp19a1b} via both positive and negative feedback mechanisms, with differential modulation based on the type of estrogen, duration of exposure, fish tissue and sex of the fish. Collectively the results suggest that, the xenoestrogens can have a disruptive effect on the steroidogenic pathways and hence sex differentiation, sexual behaviour and reproductive cycles in this fish. Both isoforms have potential as biomarkers of exposure to xenoestrogens.

\textbf{Keywords:} \textit{Melanotaenia fluviatilis}, Xenoestrogens, \textit{cyp19a} isoforms, qPCR

\textbf{5.1. Introduction}

Estrogens are steroid hormones found in aquatic as well as terrestrial vertebrates including fish, amphibians, reptiles and mammals. They are C-18 compounds (synthesised in the granulosa or sertoli cells), converted from C-19 (synthesised in the thecal or leydig cells) compounds by the
aromatase enzyme in the gonads, placenta, adipose and nervous tissues (Simpson et al., 1997; Cheshenko et al., 2008; Lubzens et al., 2010; Schulz et al., 2010). It is well recognised that estrogens play a vital role in neural development, metabolism, growth, sex differentiation, sexual behaviour, reproduction and regulate other physiological functions (Fukuda et al., 1996; Gen et al., 2001; Diotel et al., 2010). They also play a role in the control of cell cycles and proliferation including uterine growth and control of neuronal growth and differentiation (Cheshenko et al., 2008). They play a prominent role in normal sexual development and are also associated in shaping and sexual behaviour (Sharpe, 1997).

Estrogens act via the estrogen receptor, where they diffuse the target cell and bind to the estrogen receptors (ERs). These ERs will facilitate their activation, dimerization and binding to the specific DNA sequences called estrogen responsive elements (EREs) leading to the transcription mechanism (Cheshenko et al., 2007). This mechanism is often disturbed by natural and synthetic endocrine disrupting chemicals (Gray et al., 1989; Colborn et al., 1993; McKinlay et al., 2008) and the profile of disruption is being studied in various organisms at various levels.

Often aquatic as well as terrestrial organisms are exposed to various chemicals which disrupt their endocrine system and physiology. These chemicals affect the organisms similarly or antagonistically to that of endogenous hormones (Kuhl et al., 2005). Effects of these chemicals include skewed sex ratios of alligators in pesticide ravaged lake Apopka, Florida, skewed sex ratios in birds (Abinawanto et al., 1996) and reptiles
(Wibbels and Crews, 1994), inter-sex fish downstream of sewage treatment plant in the United Kingdom and United States (White et al. 1994; Folmar et al., 2001), feminized testes in common carp and medaka treated with xenoestrogens (Gimeno et al., 1996; Gronen et al., 1999).

Recently the effects of EDCs have been investigated at molecular levels including transcriptomic, proteomic and metabolomic. There are many genes that are affected by EDCs, among which cyp19a is widely studied in different fish species. This gene is a member of the cytochrome super family and it encodes for the aromatase enzyme. In higher vertebrates (with the exception of porcines), there is a single cyp19a gene which is expressed in different tissues. Whereas in many teleosts two different cyp19a isoforms have been isolated, which are products of different cyp19a gene loci—one predominantly expressed in the ovary (cyp19a1a) and the other preferentially in the brain (cyp19a1b) (Tchoudakova and Callard, 1998; Blázquez and Piferrer, 2004; Chang et al., 2005; Guigen et al., 2010). The cyp19a1a is believed to play a role in the sex differentiation and gonad development and the cyp19a1b is presumed to play a role in neuroprotection, neurogenesis, shaping and mating behaviour (Barney et al., 2008; Patil and Gunasekera, 2008).

Presently, over 200,000 varieties of chemicals are being released/entered into the aquatic system (Tyler et al., 2008). These are a wide range of natural and synthetic chemicals mimic endogenous steroid hormones of animals (Colborn et al., 1993). These chemicals include pesticides, herbicides, PCBs, phthalates, alkylphenols and synthetic hormones. They
interfere with the endocrine system of the non-target organisms, mainly fish leading to deleterious effects on the animals as well as on their progeny (Kavlock et al., 1996; Vos et al., 2000). These EDCs induce physiological changes by binding to the ERs and subsequently activate the genes via estrogen responsive elements (Tsai and Malley, 1994).

During the last decade, considerable research has been focused on synthetic chemicals which could alter the endocrine system of the aquatic organisms mainly fish species. Fishes are considered sentinel for aquatic pollution signalling and they are the most suitable test organisms for EDCs research because their endocrine system is well understood and they are often exposed to various sources of EDCs in the aquatic environment (Matthiessen, 2003). Sources include sewage, industrial effluent, urban and agricultural runoff (Kazeto et al., 2004). In order to understand the effect of these EDCs, a number of biomarkers have been identified with aromatase and vtg receiving increased attention. Aromatase and vitellogenin expression have been evaluated to understand the impact of EDCs on the endocrine system in various fish species including fathead minnows (Halm et al., 2002), medaka (Min et al., 2003; Sun et al., 2007), sticklebacks (Hahlbeck et al., 2004), zebrafish (Kazeto et al., 2004; Kallivretaki et al., 2006) and common carp (Letcher et al., 2005). Aromatase P450 encoded by cyp19a gene(s) is considered to be a potential EDCs target, since it catalyses the final step in the biosynthesis of estrogens. Therefore, it is a key gene involved in the control of many physiological processes (Simpson et al., 2002).
In this study, the Australian native Murray River rainbowfish (class Actinopterigii, order artheriniformes, family Melanotaeniidae), was used as a model species. This species has been used as a laboratory test organism for toxicological studies for over 20 years. Hence, its sensitivity to toxicants is well established (Pollino et al., 2007). The effect of exogenous estrogen 17β-estradiol on the ERs and vtg expression of this species was reported recently by Woods et al. (2009). The current study investigated the effect of exogenous E2 and the estrogen mimic NP on the expression of aromatase mRNA in key tissues including the brain and gonads in both sexes of *M. fluviatilis*.

5.2. Materials and Methods

5.2.1. Experimental fish

Reproductively active adult Murray River rainbowfish were purchased from a commercial aquarium fish wholesaler (Aquarium Industries, Epping, Melbourne, Victoria, Australia) and reared at 25±1 °C in 16:8 h light: dark regime in flow-through aquaria with carbon filtered aerated water. Throughout the maintenance, water quality parameters including temperature, dissolved oxygen, pH and conductivity were monitored and the fish were fed commercial fish pellets (Tetra colour™, Tetra Holding (US). Inc. 3001, Commerce street, Blacksburg, VA 24060, Germany) twice daily. Experimental fish were fed with frozen brine shrimp larvae and rotifers twice daily and 25% of the water exposure solution was replaced daily.
5.2.2. Chemicals and experimental design

All chemicals used in this study were of molecular biology grade and were purchased from Sigma-Aldrich Pty. Ltd. Reproductively active male and female fish were exposed to either 1, 3, 5 µg/L of exogenous E2 or 100, 500 µg/L of estrogen mimic NP for 96 h in static partial renewal exposure. Control and carrier controls were also run in parallel. Ethanol was used as a solvent to dissolve both chemicals. These concentrations were selected based on previous research on other fish species (Hemmer et al., 2002: Islinger et al., 2002: Yang et al., 2006: Harries et al., 2000). Each treatment had 20 fish in three replicates, every 24 h three fish were removed from each treatment and anaesthetised in AQUIS-(Isoeugenol (-2-methoxy-4-propenylphenol) and decapitated. Samples of brain, gonad, liver, spleen, eyes and body tissues were collected. Samples were stored in RNA later (Sigma-Aldrich Pty. Ltd.) according to the manufacturer’s instructions for total RNA extractions. Only the brain and the gonad tissues of both sexes were used for total RNA extraction and real-time analysis. All experimental procedures were conducted under the RMIT AEC approved project.

5.2.3. RNA extraction and reverse transcription (cDNA synthesis)

Total RNA was extracted using RNeasy lipid tissue mini kits (Qiagen Pty. Ltd. Australia) according to the manufacturer’s instructions. For adult fish, the whole organs/tissues were used for RNA extraction. The tissues were homogenised in 1 mL QIAzol lysis buffer using a sterile disposable syringe head, extracted with 200 µL chloroform and centrifuged. Final RNA extraction was carried out using RNeasy lipid tissue mini kits and during the procedure RNase-Free DNase was used to eliminate genomic DNA
contamination according to the manufacturer’s (Qiagen Pty. Ltd. Australia) instructions. The total RNA concentration and A260nm/A280nm ratios were measured on a spectrophotometer (Bio Rad Pty. Ltd. Australia). Total RNA quality was verified in all the samples from each tissue by agarose gel (1%) electrophoresis under denaturing conditions with ethidium bromide detection, to confirm the presence of two sharp RNA bands indicating that the RNA samples were not degraded (Sambrook and Russell, 2001). Following which cDNA synthesis for qPCR was carried out using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Pty. Ltd. Australia) according to manufacturer’s instructions. An equal volume of total RNA (~1.0 µg) was added to each 10 µL of master mix. The thermocycler program consisted of 25 °C for 10 mins, 37 °C for 120 mins and 85 °C for 5 mins with a final holding step at 4 °C. Resulting cDNA templates were quantified by spectrophotometer and stored at -20 °C for the real-time assay.

5.2.4. Oligonucleotides for real-time PCR assay

Gene-specific oligonucleotide primers were designed on Primer Express software (Applied Biosystems) and synthesized by Sigma-Proligo (Sigma–Aldrich Pty. Ltd. Australia) and GeneWorks (GeneWorks, Australia). The list of primers used for gene expression studies is given in Chapter 3, Table 3.2.
5.2.5. Real-time expression

Real-time PCR assays were carried out on a MJ MiniOpticon system version 3.1 (Bio-Rad Pty. Ltd.) with SYBR green fluorescent label using \textit{gapdh} as an endogenous control as explained in section 2.15, chapter 2.

5.2.6. Expression of \textit{cyp19a} isoforms in gonads and brain tissues

Expression of \textit{cyp19a1a} and \textit{cyp19a1b} isoforms were measured in gonad and brain tissues of both sexes in triplicate and normalised to \textit{gapdh}. cDNA templates were used as standards to monitor amplification efficiency.

5.2.7. Data Analysis

Real-time data was collected and compiled and the cycle threshold was calculated automatically using CFX software package (Bio Rad. Pty. Ltd. Australia). The data was analysed using the publicly available Q-gene excel script package (Muller \textit{et al.}, 2002). The efficiency values for each aromatase primer and the average efficiency value for \textit{gapdh} were used in subsequent analysis. Throughout the exposure study the efficiency values were consistently higher than 0.97. Data from each sample was averaged and shown as Mean Normalized Expression (MNE±SE). The data was tested for normality and subsequently analysed by two way analysis of variance (ANOVA) followed by Tukey’s post hoc test for multiple
comparisons between the treatments and time of exposure for each genes using SPSS17.0 (SPSS Inc. 2008). The significance was set at P<0.05.

5.3. Results

To understand the effects of E2 and NP on the Murray River rainbowfish, the expression patterns of ovarian (cyp19a1a) and brain (cyp19a1b) aromatase genes were analysed in the gonads and brain tissues of both sexes using qPCR. To normalize the data for the tested genes, gapdh mRNA was used as an endogenous control for all the tissues tested.

5.3.1. Expression of cyp19a isoforms in female fish exposures

5.3.1.1. Expression of cyp19a1a in the brain and ovarian tissues

The exposure of rainbowfish to exogenous E2 and the estrogen mimic NP resulted in a significant reduction in the expression of cyp19a1a isoform in the ovarian tissues with exposure to 1 µg/L of E2 and 100 µg/L of NP and it was completely inhibited at 96 h of exposure (Fig. 5.1 A and B). Further at higher concentrations of these selected EDCs; cyp19a1a was completely inhibited in the ovarian tissues throughout the exposure period (Fig. 5.1 A and B). The expression of cyp19a1a was not detected in brain tissues at any time point, including controls (data not shown). The results showed significant differences between the treatments and exposure time (cyp19a1a, E2: F_{10, 22} =6.496; NP: F_{10, 22} = 6.511, n=3, P<0.05).
Figure 5.1. A: Expression of cyp19a1a in the ovarian tissues of female fish exposed to E2, B: Expression of cyp19a1a in the ovarian tissues of female fish exposed to NP. Values described by the same letter are not significantly different from each other (P<0.05, n=3).
5.3.1.2. Expression of \textit{cyp19a1b} in the brain

Expression of \textit{cyp19a1b} in female brain tissues following exposure to 1 µg/L of E2 was significantly higher than controls throughout the exposure period (Fig. 5.2 A). Whereas exposure to 3 µg/L of E2 significantly increased \textit{cyp19a1b} expression until 72 h and then showed decreasing trend at 96 h but remained significantly above the controls (Fig. 5.2 A). With exposure to 5 µg/L E2 the gene was significantly upregulated at 24 h, then fell to control levels at 48 h, followed by significant downregulation at 72 h and was completely undetectable at 96 h (Fig. 5.2 A). The results showed significant differences between the treatments and exposure time for E2 (\textit{cyp19a1b}, F$_{19, 40}$ = 3.844; P<0.05). The expression of \textit{cyp19a1b} in the brain tissues of fish exposed at both 100 µg/L and 500 µg/L of NP showed significant increase throughout the experimental period, with the exception of 500 µg/L exposure at 72 h, at which it was significantly lower than control (Fig. 5.2 B). The results showed significant differences between the treatments and exposure time for NP (\textit{cyp19a1b}, F$_{15, 32}$ = 4.834, n=3, P<0.05).
Figure 5.2. A: Expression of *cyp19a1b* in the brain of female fish exposed to E2, B: Expression of *cyp19a1b* in brain of female fish exposed to NP. Values described by the same letter are not significantly different from each other (P<0.05, n=3).
5.3.1.3. Expression of cyp19a1b in the ovarian tissues

Immediately following exposure at 24 h, the expression of cyp19a1b significantly decreased with all the concentrations of E2, followed by a slightly different pattern at later time point. The expression of cyp19a1b in the ovarian tissues decreased with exposure to 1 µg/L of E2 with increase in exposure time from 24 to 72 h, but it significantly increased at 96 h (Fig. 5.3 A). Whereas, 3 µg/L of E2 showed similar pattern to 1 µg/L exposure, till 48 h, then increased at 72 h (more than controls) and decreased at 96 h. With exposure to 5 µg/L of E2, it increased (significantly more than controls) at 48 and 72 h, and then decreased significantly at 96 h compared to controls (Fig. 5.3 A). The results showed significant differences between the treatments and exposure time for E2 (F₁₉, ₄₀ = 4.070, P<0.05). Its expression with exposure to both concentrations of NP significantly decreased from 24 h until the termination of the experiment (Fig. 5.3 B). The results showed significant differences between the treatments and time of exposure (cyp19a1b, NP; F₁₅, ₃₂ = 2.608, n=3, P<0.05)
Figure 5.3. A: Expression of cyp19a1b in the ovarian tissues of female fish exposed to E2, B: Expression of cyp19a1b in the ovarian tissues of female fish exposed to NP. Values described by the same letter are not significantly different from each other (P<0.05, n=3).
5.3.2. Expression of *cyp19a* isoforms in male fish exposures

5.3.2.1 Expression of *cyp19a1a* in the brain and testis tissues

Male rainbowfish exposed to exogenous E2 and the estrogen mimic NP throughout the experiment showed no expression of *cyp19a1a* in either brain or testis (data not shown).

5.3.2.2. Expression of *cyp19a1b* in the brain tissues

The expression of *cyp19a1b* was significantly lower in the brain of male fish exposed to both E2 and NP compared to the controls (Fig. 5.4 A and B), with the exception at 24 h, wherein 1 and 5 µg/L showed significantly higher expression compared to controls (Fig 5.4 A). Results of two way ANOVA showed significant differences between the treatments and exposure time (*cyp19a1b*, E2: FF19, 40 =2.693; NP: F15, 32 =2.239, n=3, P<0.05).
Figure 5.4. A: Expression of cyp19a1b in brain of male fish exposed to E2, B: Expression of cyp19a1b in brain of male fish exposed to NP. Values described by the same letter are not significantly different from each other (P<0.05, n=3).
5.3.2.3. Expression of cyp19a1b in the testes tissues

In general the expression of cyp19a1b in the testes tissues increased with exposures to both E2 and NP (Fig. 5.5 A and B). The highest expression of cyp19a1b at exposure to 1 µg/L E2 was observed at 72 h. Whereas, with exposure to 3 µg/L E2 the transcription was maximum at 48 h and started decreasing from 72 h. The expression of cyp19a1b in the testes at 5 µg/L reached a maximum at 72 h and reduced at 96 h. A similar pattern of result was also observed with NP exposure. The expression of cyp19a1b in fish exposed to E2 and NP remained elevated above the controls throughout the experiment. The results of two way ANOVA showed significant differences between the treatments and time of exposure to E2 and NP (cyp19a1a, E2: $F_{19, 40} =3.631$; NP: $F_{15, 32} =5.893$, n=3, P<0.05).
Figure 5.5. A: Expression of *cyp19a1b* in the testes of male fish exposed to E2, B: Expression of *cyp19a1b* in the testes of male fish exposed to NP. Values described by the same letter are not significantly different from each other (P<0.05, n=3).
5.4. Discussion

The two xenoestrogens studied (E2 and NP) had a measurable effect on cyp19a isoform expression in M. fluviatilis exposed for 96 h. It was observed that ovarian aromatase (cyp19a1a) in the ovarian tissues of female was initially downregulated and later completely inhibited. In the brain tissues of male throughout the experiments in both exposures, brain aromatase (cyp19a1b) was downregulated. The possible mechanisms and their impacts are discussed in the following sections.

5.4.1. Expression of ovarian aromatase (cyp19a1a) in both sexes

Exposure to exogenous estrogens resulted in different pattern of aromatase gene expression in many teleosts studied (Kortner et al., 2009). In this study, exposure to exogenous estrogens resulted in downregulation (1 µg/L of E2 and 100 µg/L of NP exposure) and complete inhibition (exposure to 3, 5 µg/L of E2 and with 500 µg/L of NP) of cyp19a1a in the gonads of female M. fluviatilis. Similar results were also observed in zebrafish exposed to ethinylestradiol (EE2) at 100 nmol and E2 at 10 nmol (Kazeto et al., 2004; Hinfray et al., 2006); Rivulus marmoratus exposed to 300 µg/L of 4-nonylphenol for 96 h (Lee et al., 2006) and in Atlantic salmon following exposure to 5 and 50 µg/L of nonylphenol (Kortner et al., 2009). The xenoestrogens (in this study exogenous E2 and the estrogen mimic NP) can conceivably and directly supplement or replace endogenous estrogens trigering a negative feed back loop and shut cyp19a1a
transcription or they may inhibit the processes at the level of testosterone biosynthesis. Mechanistically direct binding of the xenoestrogens to the transcription factors upstream of cyp19a genes could bring about their regulation. Alternatively the xenoestrogens may bind to other molecules in the regulatory pathway, such as the arylhydrocarbon receptor (AhR) resulting in indirect downregulation and/or inhibition of cyp19a1a as has been suggested in the case of dioxin exposures (Kazeto et al., 2004). The inhibitory action of E2 and NP on the expression of cyp19a1a in the ovarian tissues could also be due to a negative feed back mechanism on gonadotropin release hormone (GnRH), a known stimulator of aromatase gene expression in the ovarian follicles (Gen et al., 2001; Kagawa et al., 2003). The xenoestrogens may inhibit GnRH in the hypothalamus or the pituitary (Jobling et al., 1996) thereby inhibiting the ovarian aromatase transcription. There is also evidence that xenoestrogens may downregulate (Kijima et al., 2006) or inhibit cAMP receptor element binding protein (CREB), which in turn downregulate or inhibit aromatase expression and their activity (Whitehead and Rice, 2006). The inhibition of cyp19a1a is thought to be caused by modulation of CREB signals by EDCs, in the ovarian tissues, particularly during the time of vitellogenin production (Cheshenko et al., 2008). It may be possible that one or more of these modulatory pathways are responsible for the repression of cyp19a1a activity observed in this study. Moreover, precise responses of the cyp19a1a to the estrogenic compounds are known to be complex and depend on nature of the chemical, fish species, developmental stages, tissue context, fish exposure patterns, exposure duration and habitat of the fish (Filby et al., 2006: Cheshenko et al., 2008). It has been suggested
that, overall the inhibition of the *cyp19a1a* isoform could lead to the deleterious reproductive consequences in many teleosts (Kazeto *et al*., 2004; Cheshenko *et al*., 2008) and may have similar consequences in *M. fluviatilis* if subjected to chronic environmental concentrations of EDCs.

The downregulation and inhibition of aromatase (*cyp19a1a*) gene expression in female fish exposed to exogenous E2 and the estrogen mimic NP suggests that, it could lead to testicular differentiation in female gonads yet with female phenotypic appearance resulting in compromised reproductive fitness. Similarly exposure of larvae and juveniles of *M. fluviatilis* to either exogenous E2 or NP may inhibit ovarian differentiation. This hypothesis is based on the downregulation and inhibition of ovarian *cyp19a1a* seen in our study since this is believed to play a key role in sex differentiation and gonad development.

Results of this study showed that the *cyp19a1a* transcription in males did not occur with exposure to the estrogenic chemicals. This result is also different to the response of *vtg* in male teleosts where exposure to exogenous estrogens leads to the production of vitellogenin (Leusch *et al*., 2005; Cionna *et al*., 2006; Woods *et al*., 2009, Chapter 6).

### 5.4.2. Expression of brain aromatase (*cyp19a1b*) in both sexes

In the current study, the exposure of Male *M. fluviatilis* to E2 and NP significantly downregulated *cyp19a1b* in the brain tissues. Similar results were also observed in adult fathead minnows exposed to 100 and 320
ng/L of E2 (Halm et al., 2002). The reduced expression of cyp19a1b in the male brain tissue implies activation of a negative feed back mechanism following exposure to E2 and NP. This significant reduction of cyp19a1b expression in males could lead to change in the sexual behaviour, reduced reproduction, impaired courtship behaviour and eventually may affect the reproductive success of males followed by effect on the progeny health and population sex ratio. The exposure of male sand gobies to 17α-ethynilestradiol resulted in reduced male courtship behaviour, delayed nest building and resulted in significant reduction in the aggressiveness in male fish compared to unexposed males (Saaristo et al., 2010). Male Japanese medaka exposed to 17β-estradiol exhibited reduced sexual behaviour, reduced frequency of spawning and reduced fecundity (Oshima et al., 2003).

In contrast to males, upregulation of cyp19a1b was observed in the brain of female M. fluviatilis exposed to both E2 and NP. Similar upregulation of cyp19a1b in both female and male zebrafish has been observed (Kazeto et al., 2004; Hinfray et al., 2006; Kallivretaki et al., 2006). Similarly medaka exposed to E2 (200 ng/L) and and EE2 (1-100 nM) showed increased activity of aromatase in the brain of both sexes (Contractor et al., 2004). Brain aromatase activity was also stimulated in male and female guppies exposed to 0.7 µg/L of androgen, androstenedione (A) and 10 ng/L of 17α-ethynylestradiol (EE2) (Hallgren and Olsen, 2010).

The contrasting and sex specific response in M. fluviatilis compared to other teleosts is intriguing and may arise as consequence of a unique
reproductive and physiological strategy of the species. Vosges et al. (2010) hypothesized that upregulation of cyp19a1b could lead to the local over production of estrogens in the brain and surrounding area and may lead to disruption of GnRH neurogenesis. A detailed study is necessary to understand the key reasons for the differential expression across the species and between genders of the same species. Long term chronic exposure studies to environmental concentrations of xenoestrogens are also necessary to understand the impact on the exposed fish and their progeny.

5.4.3. Expression of brain aromatase (cyp19a1b) in the gonads

This study observed that both E2 and NP downregulated the expression of cyp19a1b in the ovarian tissues. The selected concentrations of NP were more effective than estradiol. In contrast upregulation of cyp19a1b in the ovary was observed in adult fathead minnows exposed to 32-320 ng/L of E2 in a dose dependent manner (Halm et al., 2002). The inhibition of cyp19a1a and the downregulation of cyp19a1b isoforms in the ovarian tissues show that the ovarian tissue is more sensitive to xenoestrogens in M. fluviatilis. The effect of the xenoestrogens was greater in the females in agreement with evidence that there are more estrogen receptors in females than in males (Jobling et al., 1996).

Meanwhile the expression of cyp19a1b in the testes contrasted with that of ovarian tissues. The expression of cyp19a1b was greater in the testes of both exposures throughout the experiment and remained above the
controls. Similar results were also observed in adult fathead minnows exposed to 32-320 ng/L E2 for 14 days (Halm et al., 2002). This indicates activation of a positive feedback mechanism with exposure to xenoestrogens in the testes. This upregulation of cyp19a1b in the testes could be compensation for the downregulation of cyp19a1b in the brain tissues observed. In this study, the cyp19a1b increased in testis and decreased in ovary compared to corresponding controls suggesting different mechanism of regulation in different tissues. The mechanism of action of cyp19a1b in the testes may be very different to the mechanism in the ovarian or the brain tissues (Goto-Kazeto et al., 2004).

The differences in expression of cyp19a1a or cyp19a1b with differences in exposure concentrations and time of exposure could be attributed to the reproductive stage of the fish. At cellular and molecular level (either genomic or non genomic), the differences in the amino acid sequences, the promoter structure and difference in the sequence of the promoters of aromatase genes may account for different levels of activity and different responses to various EDCs (Shilling et al., 1999; Zhao et al., 2001). These responses may vary among the two isoforms depending on the EDCs as was observed in the current study.

5.4. Conclusions

The present study demonstrates that the cyp19a isoforms in M. fluviatilis used has potential as molecular markers of exposure to E2 and NP. The observations in the study support the hypothesis that E2 and NP regulate
expression of cyp19a1b via both positive and negative feedback mechanisms, with differential modulations based on the type of estrogen, duration of exposure, tissues and sex of the fish. cyp19a1b in both sexes of *M. fluviatilis* was expressed differently with exposure to EDCs (E2 and NP). Therefore, the effect of selected EDCs in *M. fluviatilis* depends on sex of the fish, physiology and also the exposure concentrations.

Both estrogenic chemicals negatively regulated cyp19a1a expression in the ovarian tissues. In this study we show for the first time that, cyp19a isoforms show different sensitivity to both E2 and NP. The cyp19a1a and cyp19a1b in ovarian tissues showed higher sensitivity to the exposures. Collectively, the results suggest that, the xenoestrogens can have a disruptive effect on the steroidogenic pathways and hence sex differentiation, sexual behaviour and reproductive cycles in this species. Both aromatase isoforms have potential as biomarkers for detecting and monitoring environmental exposures of xenoestrogens and the ovarian aromatase gene was found to be very sensitive to estrogenic chemicals in this species. Note that, this study examined only mRNA expression; therefore further research is necessary to characterize the post transcriptional changes to further elucidate the effect and mechanisms of EDCs action on development, metabolism, reproduction and sex ratios.

This study demonstrates that cyp19a1b expression in *M. fluviatilis* males and females with exposure to E2 and NP is consistently altered, suggesting its potential as biomarker of exposure to environmentally relevant estrogenic compounds in this species.
CHAPTER 6

Vitellogenin as a biomarker of exposure to estrogenic chemicals in the Murray River rainbowfish, *Melanotaenia fluviatilis*

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Abstract

This study investigated the induction of vitellogenin (Vtg) as a biomarker to evaluate the effect of estrogenic chemicals on *M. fluviatilis*. Vtg is the major egg-yolk protein, which provides energy for embryonic development in oviparous organisms. In mature females, Vtg is synthesised in response to endogenous estrogens. In males and juveniles, *vtg* although present is normally silent. However, it may be activated by xenoestrogens. In the present study adult male *M. fluviatilis* were exposed to either 1, 3, 5 µg/L of 17β-estradiol (E2) or 100, 500 µg/L of nonylphenol (NP) for 96 h. Vtg induction was studied using commercial antibody raised against Vtg of medaka. Gene specific primers were used for *vtg* expression using qPCR.
in liver and testis of *M. fluviatilis*. The detection of vitellogenin protein in plasma was not consistent. However, the expression of *vtg* in the liver and testis of exposed males was strongly evident as analysed by qPCR. Overall, *vtg* was easily detected compared to Vtg protein in the plasma. Therefore, detection of *vtg* using qPCR is more sensitive, quick and reliable. This technique can be readily utilized to study biomarkers of exposure to xenobiotics particularly in smaller fish where it is difficult obtain adequate tissue or blood samples for immunodetection studies.

**Keywords:** *M. fluviatilis*, Vitellogenin, qPCR

### 6.1.1. Introduction

Presently, pollution in aquatic environments is increasing due to tremendous growth in population. Due to a huge demand for food, many pesticides and herbicides are applied to crops, enabling high food production in intensive farming practices to meet the demand of the rapidly expanding population. It has been demonstrated that some of these chemicals mimic endogenous hormones and can interfere with the endocrine system (Arukwe *et al.*, 1997), and in particular, can interfere with the reproductive physiology in fish.

Teleost vitellogenin (Vtg) is a glycolipophosphoprotein that is synthesised in the parenchymal cells of the liver during oogenesis in mature female fish, and it’s production is due to increasing concentrations of estrogens within the ovarian tissues acting on the vitellogenin gene (*vtg*) (Denslow *et
The term vitellogenin was first adopted for female specific egg yolk precursor proteins by Pan et al. (1969) in an insect, the cecropia moth, *Hyalophora cecropia*. In teleosts, this protein is released into the blood stream from the liver and is then taken up by oocytes in the ovary through receptor-mediated endocytosis (Wallace and Selman, 1981; Momsen and Walsh, 1988; Sumpter and Jobling, 1995; Tyler et al., 1998). The molecular weight or size of this protein varies amongst fish species (Watts et al., 2003; Leusch et al., 2005). The production of endogenous estrogens which subsequently leads to the production of Vtg is influenced by environmental cues including water temperature and photoperiod (Arukwe et al., 1997). Whilst vitellogenesis is normally restricted to mature females, the *vtg* gene is also present in males and juveniles, and therefore, upon exposure to xenoestrogens this gene will be activated and *vtg* mRNA will be synthesised followed by protein translation (Baker and Shapiro, 1977; Valotairo et al., 1984; Guellec et al., 1988; Ding et al., 1990). Therefore, the presence of *vtg* mRNA and Vtg protein in the liver as well as blood plasma of male and juvenile fish has been widely utilized as a potential biomarker of exposure to xenoestrogens (Purdom et al., 1994; Folmar et al., 1996; Harries et al., 1997; Arukwe et al., 1998; Denslow et al., 1999; Kime et al., 1999; Porter and Janz, 2003; Nakari, 2004; Barucca et al., 2006; Henry et al., 2009; Woods et al., 2009).
6.1.2. Mechanism of vitellogenin synthesis in teleosts

Environmental parameters including temperature and photoperiod act as cues on the Central Nervous System (CNS) that trigger maturation processes (Fig. 6.1). In response, the hypothalamus secretes gonadotropin-releasing hormone (GnRH). The GnRH stimulates the release of gonadotropins (GtHs) from the pituitary gland (Fig. 6.1). Gonadotropins released from the pituitary gland and steroid hormones produced in ovarian tissue regulate the growth of oocytes and maturation in teleosts and other vertebrates (Nagahama, 2000). Two GtHs have been identified in the brain of teleosts and are involved in vitellogenesis and zonagenesis. Vitellogenesis is the synthesis of vitellogenin protein for the developing eggs and zonagenesis is the synthesis of zona radiata protein during oocyte development which makes an envelope for the developing eggs (Arukwe and Goksoyr, 2003). GtH-I plays a role in vitellogenesis whereas GtH-II is involved in final oocyte maturation and ovulation (Swanson, 1991; Nagahama, 2000). The secretion of these gonadotropin hormones are regulated by a positive feedback mechanism involving 17β-estradiol and testosterone (Peter and Yu, 1997).

The primary function of Vtg is to provide nutritional support to the developing embryo rather than playing a major role as a functional protein (Denslow et al., 1999). According to the literature the Vtg sequence is not conserved amongst fish species (Wahli et al., 1981; Lee et al., 1992; Carnevali and Belvedere, 1991; Denslow et al., 1999). But, because there are some segments of vitellogenin that appear to be highly conserved
among species, it is possible to develop antibodies with wide cross-reactivity among species (Folmar et al., 1996; Heppell et al., 1995).

This project investigated the induction of vtg mRNA in male liver and testis, and Vtg protein induction in plasma of *M. fluviatilis* as a potential biomarker to evaluate the effect of estrogenic chemicals including exogenous E2 and the estrogen mimic NP. This species has been used as a model test organism in Australian ecotoxicological studies (Pollino et al., 2007) for number of excellent reasons presented in chapter 1 (Section 1.11.4).
6.2. Materials and Methods

To understand the effects of EDCs including exogenous E2 and the estrogen mimic NP on the Murray River rainbowfish, the expression patterns of \( vtg \) mRNA in the livers and testes of male fish were studied. Expression was analysed using qPCR and compared with control samples without exposure to EDCs and fish exposed to the carrier control ethanol. Female fish liver was used as a positive control. To normalize the data for \( vtg \) mRNA expression, \( gapdh \) was used as an endogenous control for all tissues tested.

Vitellogenin protein was also tested in plasma samples of male fish exposed to exogenous E2 and the estrogen mimic NP. Female plasma was used as a positive control. The antibody used in the immunodetection was raised against vitellogenin of Japanese medaka. Therefore, purified Japanese medaka vitellogenin protein was used as a reference protein to confirm the methodology and the specificity of the antibody.

6.2.1. Fish exposure

Exposure experiments were conducted as explained in section 5.2.2, chapter 5.

Figure 6.2. Aqueous exposure of adult male \( M. \) fluviatilis to exogenous E2 and NP.
6.2.2. Sample collection

Fish were euthanased as described in Chapter 3, Section 3.2.1. Blood was collected either via, i) by direct injection at the region of caudal peduncle or ii) dorsal incision at anterior to the dorsal fin by direct puncturing the dorsal aorta –to obtain more blood sample (Fig. 6.3). Immediately after collection, blood was spun at 5,000 rpm for 10 mins at 4 °C to separate the plasma from the blood cells (Denslow et al., 1999). Plasma samples were snap frozen in liquid nitrogen and stored at –80 °C for Vtg protein expression. Samples liver, brain, gonads, eyes and spleen of the corresponding individuals were removed and stored in RNAlater for 24 h at 4 °C for RNA stabilization according to the manufacturer’s instructions (Qiagen Pty. Ltd. Australia). After 24 h RNAlater was removed and the stabilised organs were stored at -80 °C for real-time PCR assays.

Fig. 6.3. Fish dissection to collect blood A) via dorsal incision near head region and B) via dorsal near caudal peduncle
6.2.3. Oligonucleotides for real-time PCR assay

Gene-specific oligonucleotide primers were designed on Primer Express software (Applied Biosystems Pty. Ltd. Australia) and synthesized by Sigma-Proligo (Sigma–Aldrich Pty. Ltd. Australia). The primers were based on a published cDNA sequence of *M. fluviatilis* vitellogenin (Woods, 2007). The primer sequences are given in Table 6.1. *gapdh* was used as an internal control to normalize *vtg* cDNA abundance, and *gapdh* primers were based on an *M. fluviatilis* sequence described (Ponza, 2006).

Table 6.1. List of primers used in real-time PCR for *vtg* expression

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5'-3')</th>
<th>Amplicon</th>
</tr>
</thead>
<tbody>
<tr>
<td>VtgF</td>
<td>GAAAGCGGTTGTGCTTGCCCTGACTCTG</td>
<td><em>vtg</em></td>
</tr>
<tr>
<td>VtgR</td>
<td>GGCATTCCTCCTGGGAGGTCTGCC</td>
<td><em>vtg</em></td>
</tr>
<tr>
<td>GAPDHF</td>
<td>ACACCCACTCTCCATCTTT</td>
<td><em>gapdh</em></td>
</tr>
<tr>
<td>GAPDHR</td>
<td>GTTGCTGTAGCCGAACTCAT</td>
<td><em>gapdh</em></td>
</tr>
</tbody>
</table>

6.2.4. Sample preparation from adult fish tissues for real-time PCR

Total RNA extraction and cDNA synthesis was carried out according to the manufacturer’s instructions as explained in Chapter 2, Section 2.11 and 2.12.
6.2.5. Expression analysis (Quantitative real-time PCR)

Real-time assays were carried out on a MJ MiniOpticon system version 3.1 (Bio Rad Pty. Ltd. Australia) with SYBR green fluorescent labelling using gapdh as an endogenous control to normalize the vtg expression. Gene specific primers were designed (Table 6.1) and tested for specificity and efficiency. Primers were designed to amplify 126 bp for vtg and 100 bp for gapdh.

Real-time PCR analysis was carried out with a final reaction volume of 20 µL: using 50 ng/µL of cDNA, 1x SYBR green PCR master mix (Applied Biosystems Pty. Ltd. Australia) and primers at a final concentration of 100nM. Each sample was run in triplicate with vtg and gapdh as an internal control. Cycling parameters for the real-time PCR were as follows: 50 °C for 2 mins, 95 °C for 10 mins, then 40 cycles of 95 °C for 15 s and 62 °C for 1 min. A melting curve analysis was performed at the end of the amplification phase between a minimum of 30 °C and maximum of 95 °C to test the specificity and identity of the real-time PCR products.

6.2.6. Quantification of total protein

The concentration of total protein in the plasma of the fish was quantified using the Bradford method (Bradford, 1976). The plasma was thawed for a few minutes and diluted 2 fold. The quantity of total protein was analysed using a Bovine Serum Albumin (BSA) standard curve with a range of 0.125-2.0 mg/L.
6.2.7. Polyacrylamide gel electrophoresis to separate proteins

The size of the Vtg protein varies amongst fishes, from 70–220 kDa; therefore, 8% acrylamide gels were used for electrophoresis. Composition of the Sodium Dodecyl Sulfate (SDS) polyacrylamide gel is given in Appendix A. 12 µg of the total protein was mixed with 5x sample buffer (refer appendix A) and the samples were heated at ~100 °C to denature the complex structure of the proteins. Next the samples were loaded on each well and electrophoresed at 110V until the dye reached the bottom of the glass plates. Two identical gels with equal quantities of protein in each well were run simultaneously. One of the SDS gels was used for coomassie blue staining and destaining, the remaining gel was used to transfer the proteins to a nitrocellulose membrane for immunodetection (Western blot).

6.2.8. Immunodetection

The nitrocellulose membrane was soaked in absolute methanol for one minute to block the membrane pores before contact with the SDS gel. The membranes were then placed on top of the gel and the whole gel sandwich was placed in transfer buffer inside the western blotting gel tank (Bio Rad laboratories. Australia) (Fig. 6.4 B), following which the proteins were transferred into a membrane at 110V for 70 minutes. The membrane was soaked in blocking solution (5% skimmed milk) for 1 h on a shaker, and then washed two times for 5 mins in Tris Buffer Saline Tween-20 (TBST). The primary antibody was prepared using 2% blotto (2% skimmed milk), then the membranes were immersed in 1:1000 diluted primary antibody
(Japanese medaka monoclonal anti Vtg (CK-4B3), Biosense Laboratories, Norway) for 1 h at RT on a shaker then they were washed with TBST 2 times and each wash was done for 10 mins. The membrane was then soaked in 1:1000 (2% blotto) diluted Anti-Mouse IgG (Fab specific)-Alkaline Phosphatase, secondary antibody (# A1293, Sigma Aldrich) for 1 h at RT on a shaker. The membrane was again washed 2 times with TBST, each wash was done for 10 mins and the membranes were then washed with Tris Buffered Saline (TBS) two times for 5 mins. Immediately after the final wash, 2 mL of alkaline phosphatase (Promega Pty. Ltd. Australia) substrate was added and the membrane incubated in the dark until bands of protein were clearly visible. Immediately after the bands appeared the substrate was drained out and the membrane was washed in MilliQ water to stop the reaction and then air dried and preserved.

Figure 6.4 A. Separation of protein on the SDS gel, B. Transfer of separated protein from SDS gel to PVDF membrane
6.2.9. Data analysis

Real-time data was collected and compiled and the cycle threshold was calculated automatically using a CFX software package (Bio Rad Pty. Ltd. Australia) as explained in section 2.15 of chapter 2. The data was subjected for two way analysis of variance (ANOVA) followed by Tukey’s post hoc test using SPSS17.0 (SPSS Inc. 2008). Data means were considered significantly different from each other at P<0.05.

6.3. Results

6.3.1. Hepatic vtg mRNA induction in male fish exposed to E2 and NP

Exposure to exogenous E2 (Fig. 6.5) and the estrogen mimic NP (Fig.6.6) resulted in significantly increased expression of vtg in the livers of male fish in comparison to controls.

Induction of vtg mRNA in liver of male fish exposed to E2 was observed throughout the experiment and the expression remained above the controls (Fig.6.5). With exposure to 1 µg/L of E2, vtg induction was more than in the controls (significantly higher). Whereas, with 3 µg/L of E2 vtg mRNA levels fluctuated—initially increased at 24 and 48 h, decreased at 72 h and again increased at 96 h. Meanwhile with 5 µg/L of E2, the vtg level was elevated at 24 h of exposure and reached a peak at 48 and 72 h and declined at 96 h. However, throughout the exposure vtg in male fish treated with all three concentrations (1-5 µg/L of E2) remained significantly
higher than in controls. In unexposed female livers (positive control) the level of *vtg* was significantly higher than in the livers of exposed and control males (Fig 6.5). The two-way analysis of variance (ANOVA) showed significant differences in *vtg* expression in livers of fish between treatments and exposure duration (*vtg*, $F_{14, 30} = 6.45$, $n=3$, $P<0.05$).

![Liver E2 expression graph](image)

Figure 6.5. Expression of *vtg* mRNA in livers of male fish exposed to E2. F. Liver- Female Liver, M.C.Liver-Male Control Liver, M.CC.Liver- Male Carrier Control Liver. Values described by the same letter are not significantly different from each other ($P<0.05$, $n=3$).

With exposure to 100 µg/L of NP, *vtg* mRNA in male fish, a succession of increase and decrease in expression at the four sampling time points was observed (Fig. 6.6)—increasing at 24 and 72 h, and decreasing at 48 and 96 h respectively. Nonetheless the induction was significantly greater than controls throughout the experimental period. In slight contrast, with
exposure to 500 µg/L of NP, the expression of vtg mRNA remained elevated but comparable at both 24 and 48 h exposure. Significant reduction was observed at 72 h, followed by significant increase at 96 h. Again the expression in exposed fish remained significantly higher than that of controls (Fig 6.6). Two way analysis of variance (ANOVA) showed a significant differences between the treatments and time points in vtg mRNA expression in fish livers (vtg, $F_{10, 22} = 10.658$, n=3, P<0.05).

Figure 6.6. Expression of vtg mRNA in livers of male fish exposed to NP. F.Liver-Female Liver, M.C.Liver-Male Control Liver, M.CC.Liver- Male Carrier Control Liver. Values described by the same letter are not significantly different from each other (P<0.05, n=3).
6.3.2. Expression of vtg mRNA in testes of fish exposed to E2 and NP

The expression of vtg was also analysed in the testes of fish exposed to both chemicals and compared with that of controls. The vtg expression with exposure to E2 with all concentration was significantly higher throughout the exposure with different pattern as showed in Fig. 6.7. The results of two way analysis of variance showed significant differences between the treatments and time in testes (vtg, $F_{14, 32} = 4.057$, n=3, $P<0.05$).

![Testes E2 exposure](image)

Figure 6.7. Expression of vtg in testes of male fish exposed to E2. C. control testes- Carrier control testes. Values described by the same letter are not significantly different from each other ($P<0.05$, n=3).
The *vtg* expression with exposure to NP with both concentrations was significantly higher throughout the exposure with different pattern as showed in Fig. 6.8. The results of two way analysis of variance (ANOVA) showed significant differences between the treatments and the exposure time in testes of fish exposed to NP (*vtg*, $F_{10, 22} = 3.191$, $n=3$, $P<0.05$).

![Testes NP exposure](image)

Figure 6.8. Expression of *vtg* in testes of male fish exposed to NP. C. Control testes- Carrier control testes. Values described by the same letter are not significantly different from each other ($P<0.05$, $n=3$).
6.3.3. Expression of vitellogenin in the plasma of male fish exposed to E2 and NP

Vitellogenin protein expression was not observed in males exposed to 1 µg/L of E2 throughout the exposure period (Fig 6.9 B). Its expression was observed only at exposure concentrations of 3 and 5 µg/L at 48 h with E2 (Fig. 6.9 B) and no protein expression was observed at 72 and 96 h (data shown). Also with exposure to NP (both concentrations) no Vtg protein expression was detected throughout the experiment (data not shown). Meanwhile, Vtg was detected in female plasma (positive control) and the molecular size of the protein was similar to the expression observed in males with exposure to E2 at 3 and 5 µg/L at 48 h (Fig. 6.9 B).
Figure 6.9. A. Total protein staining of SDS gel with Coomassie blue of fish exposed to 1, 3 and 5 µg/L of E2 for 24 and 48 h. B. Immunodetection of vitellogenin with Japanese Medaka anti Vtg monoclonal Antibody (CK-4B3), Biosense Laboratories, Norway.
6.4. Discussion

The results of this chapter illustrate that induction of the vtg gene and Vtg protein in tissues of male rainbowfish occurs following exposure to E2 and the estrogen mimic NP. The expression of vtg transcripts in the liver and testes of exposed males was strongly evident as analysed by qPCR. However, the detection of vitellogenin protein in plasma was not consistent throughout the experiment in males treated with either E2 or NP.

6.4.1. Expression of vtg mRNA in male fish exposed to E2 and NP

An increase in vtg expression was observed in exposed male livers with both E2 and NP at all concentrations. Similar results were also observed in male sheepshed minnows exposed to both E2 (0.089, 0.71 µg/L) and p-NP (5.6, 59.6 µg/L) in individual exposures (Hemmer et al., 2002), medaka exposed to either E2, nonylphenol (Islinger et al., 2002) or EE2 (Yamaguchi et al., 2005), mosquitofish exposed to E2 at 250 ng/L for 4 days (Leusch et al., 2005), Japanese Sillago exposed to 50 ng/L of E2 and 40 µg/L of 4-tert-octylophenol (Yoon et al., 2008). Male carp injected with E2 (0.033 mg/kg body weight) showed significant expression of vtg measured by RT-PCR (Lattier et al., 2001) and juvenile fathead minnows injected with 5 mg/kg body weight showed a significant increase in vtg within 24 h (Thomas-Jones et al., 2003). As these examples indicate, expression of vtg is consistently induced in liver of male teleosts. Hence
vtg serves as an ideal biomarker of exposure to E2 and NP and potentially other xenoestrogens.

Throughout this study, the vtg expression in treated males was higher than in control testis as well as ovarian tissues. This clearly indicates that the vitellogenin gene is activated in the testis with exposure to xenoestrogens. Such ectopic expression of vtg in testis of exposed M. fluviatilis could potentially impair testis development, via induction of ovo-testis like activity or chronic exposures leading to complete inhibition of testis development. In agreement with this, it has been shown that brown trout exposed to alkylphenolic chemicals (including NP) displayed inhibition of their testes development (Jobling et al., 1996). This study also observed very low levels of vtg expression in unexposed ovaries. The transcripts could have been either transported from liver to the ovaries along with Vtg or a low level of vtg is synthesised in the ovaries itself (Wang et al., 2005).

6.4.2. Expression of Vtg in male plasma of fish exposed to E2 and NP

The minimum concentration of E2 that resulted in the induction of the Vtg protein in male rainbowfish was 3 µg/L (Fig. 6.9 B). In contrast both 100 and 500 µg/L of NP was not sufficient to induce Vtg protein synthesis in male rainbowfish. As expected Vtg protein was detected in female plasma (Fig. 6.9 B), but the response was not consistent. This may reflect individual differences or inconsistency in reproducibility of the technique adopted. The former is likely as the medaka Vtg protein (positive control)
generated consistent banding patterns verifying robustness of the antibody detection technique adopted.

Although the medaka Vtg antibody appears to cross-react with *M. fluviatilis* plasma Vtg, the banding pattern observed between different individual was inconsistent. Unfortunately, the low volume (5-10 µL) of plasma obtained from each fish restricted verification of the banding patterns in different individuals. One reason for the multiple banding pattern observed is that they represent different states of post translation processing of Vtg in different individuals or only a sub-unit of *M. fluviatilis* Vtg cross reacted with the medaka Vtg antibody. Therefore, raising specific antibody against the Murray River rainbowfish Vtg could assist more accurate Immunodetection of Vtg and its induction in male rainbowfish exposed to xenoestrogens.

In contrast to the observations in this study, ability of nonylphenol (NP) to induce Vtg induction in immature fish and in males has been reported in a number of teleosts including male zebrafish exposed to 100 and 500 µg/L of NP for 3 weeks (Yang et al., 2006), immature rainbow trout exposed to 20.3 µg/L of NP for 3 weeks (Jobling et al., 1996), and male fathead minnows exposed to 100 µg/L of NP (Harries et al., 2000). Perhaps the shorter exposure duration in this study (96 h) was insufficient to induce Vtg expression in livers. Therefore, studies incorporating longer exposure periods may be required to detect any Vtg responses following exposure to Vtg in the livers.
6.5. Conclusions

In conclusion, throughout the experiment it was observed that the induction of \textit{vtg} mRNA in male liver, testes and \textit{vtg} mRNA was easily detected compared to Vtg protein in the plasma of both sexes. Since the rainbowfish is small it was difficult to obtain high volume of plasma for Vtg protein detection and it restricted the number of tests that could be done. Therefore, detection of the \textit{vtg} mRNA in liver and testis using real-time PCR is more sensitive, quick and reliable and can be readily utilized as biomarker to detect effects of xenoestrogens such as E2 and NP particularly so in both liver and testes of male \textit{M. fluviatilis}. This unscheduled ectopic hepatic and testicular \textit{vtg} expression in males may lead to reproductive dysfunction in the physiology of male \textit{M. fluviatilis}. 

CHAPTER 7

Responses of the aromatase gene isoforms in adult Murray River rainbowfish exposed to BPA and Fadrozole

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Abstract

The current study investigated the effect of a weak estrogen mimic, BPA and a non steroidal aromatase inhibitor (AI), fadrozole on the expression of \textit{cyp19a} isoforms in \textit{M. fluviatilis}. Reproductively active male and female fish were exposed to 100, 500 µg/L of BPA or 10, 50 µg/L of fadrozole for 96 h. The expression analyses of \textit{cyp19a1a} and \textit{cyp19a1b} was studied using qPCR. BPA did not affect \textit{cyp19a1a} expression significantly, whereas 50 µg/L of fadrozole significantly upregulated it’s expression in the ovary. In contrast BPA exposures increased expression of \textit{cyp19a1b} in brain of both males and females, whilst fadrozole had contrasting effects in brain—increased in male, but decreased in female. Similar contrasting responses of \textit{cyp19a1b} were induced by BPA, in gonads—upregulating in ovary and downregulating in the testis. Fadrozole did
not have significant effect in gonadal expression of *cyp19a1b*. Both aromatase isoforms can be used to detect and or monitor environmental exposures of *M. fluviatilis* to BPA and fadrozole.

**Keywords**: *Melanotaenia fluviatilis*, BPA and AI, *cyp19a* isoforms

### 7.1. Introduction

The aquatic environment receives huge quantities of chemical pollutants from different anthropogenic sources including industrial wastes, sewage treatment plants, intensive agriculture, institutional waste, mining activities, landfill and others. Some of these pollutants mimic endogenous hormones of animal. More than 562 chemicals are known to have the capacity to disrupt the endocrine system of humans and animals and many more chemicals are presumed to act on the endocrine system (Kibria *et al.*, 2010). Among these chemicals Bisphenol A (BPA) is used in huge quantities in the plastic and polycarbonate industries (Minghong *et al.*, 2011) and it acts as a weak estrogenic mimic (Chapin *et al.*, 2008; Wang *et al.*, 2010). BPA is one of the chemicals produced in very high quantities globally; the estimated production in 2008 was 5,108,500 tons (Burridge, 2008). The half-life of BPA is relatively short, it has a low tendency for bioaccumulation (Staples *et al.*, 1998); still it has adverse effects on aquatic organisms and the ecosystem (Minghong *et al.*, 2011) due to its large production and application. The concentration of BPA detected in surface waters, streams of intense urbanization and livestock production sites ranged from 0.04 to 12 µg/L (Kolpin *et al.*, 2002; Peng *et al.*, 2008; Gong *et al.*, 2009; Klecka *et al.*, 2009). Among aquatic organisms, molluscs (snail, *Marisa cornuarietis*) and fish (fathead minnows) are the most sensitive at 0.015 and 1.0 µg/L respectively.
(Oehlmann et al., 2008). Therefore, the effects of BPA on Australian native aquatic organisms needs to be evaluated.

Fadrozole is used as an aromatase inhibitor in breast cancer treatment, in post menopausal patients (Bonnefoi et al., 1996) and also aromatase inhibitors are used to induce sex change in fish to obtain single sex populations in aquaculture for maximum production. For example fadrozole has been used to masculinise the genetic females in many gonochoristic fish species (Piferrer et al., 1994; Nakamura et al., 1999; Kitano et al., 2000; Kwon et al., 2000; Afonso et al., 2001; Kwon et al., 2002; Uchida et al., 2004; Komatsu et al., 2006; Ruksana et al., 2010) and in sex-changing protogynous species (Bhandari et al., 2004; Kroon et al., 2005; Alam et al., 2006). These chemicals act on the steroidogenesis pathway which includes biosynthesis of estrogens from cholesterol involving various steps through the synthesis of different androgens. In fish, estrogens play an important role in reproduction and development, including sex determination, sex differentiation, and sexual behaviour (Wibbels et al., 1998; Bjerselius et al., 2001). The role of estrogens is of major interest in reproductive regulation in animal biology (Cakmak, et al., 2006). There are no studies on the effect of BPA and fadrozole in *M. fluviatilis* or any Australian species to date. Therefore, the current study investigated the effect of the weak estrogen mimic BPA and the non steroidal aromatase inhibitor fadrozole on the expression of *cyp19a* isoforms in key tissues including the brain and gonads in both sexes of *M. fluviatilis*. 
7.2. Materials and Methods

7.2.1. Chemicals and experimental design

All chemicals used in this study were of molecular biology grade and were purchased from Sigma-Aldrich Pty. Ltd. Reproductively active male and female fish were exposed to 100 or 500 µg/L of the weak estrogen mimic BPA or to 10 or 50 µg/L of fadrozole for 96 h in a static partial renewal exposure. These concentrations were selected based on previous research on other fish species (Lee et al., 2006; Villeneuve et al., 2006; Tompsett et al., 2010). Control and carrier controls were also run in parallel. Dimethyl sulfoxide (DMSO) was used as a solvent to dissolve both chemicals. Each treatment had 20 fish in three replicates, every 24 h for 96 three fish were removed from each treatment and anaesthetised in AQUIS-(Isoeugenol (-2-methoxy-4-propenylphenol) and decapitated. Samples of brain, gonad, liver, spleen, eyes and body tissues were collected. Samples were stored in RNAlater (Sigma-Aldrich Pty. Ltd.) according to the manufacturer’s instructions for total RNA extractions. Only the brain and the gonadal tissue of both sexes were used for total RNA extraction and real-time PCR analysis.

7.2.2. Sample preparation from adult fish tissues for real-time PCR

Total RNA extraction and cDNA synthesis was carried out according to the manufacturer’s instructions as explained in 2.11 and 2.13, chapter 2.
7.2.3. Oligonucleotides for real-time PCR assay

Oligonucleotides were synthesized as explained in section 3.2.2, chapter 3 and the list of primers used for gene expression is given in Table 3.2, chapter 3.

7.2.4. Real-time PCR analysis

Real-time PCR was carried out on a MiniOpticon system for real-time detection version 3.1 (Bio Rad Pty. Ltd. Australia) with SYBR green fluorescent labelling using gapdh as an endogenous control as explained in section 2.14, chapter 2.

7.2.5. Expression of cyp19a isoforms in gonads and brain tissues

Expression of cyp19a1a and cyp19a1b isoforms were measured in gonad and brain tissues of both sexes in triplicate and normalised to gapdh. cDNA templates were used as standards to monitor amplification efficiency.

7.2.6. Data Analysis

Real-time data was collected and compiled and the cycle threshold was calculated automatically using CFX software package (Bio Rad. Pty. Ltd. Australia) and analysed as explained in section 2.15, chapter 2.

To understand the effects of EDCs include - BPA and fadrozole on M. fluviatilis, the expression patterns of ovarian (cyp19a1a) and brain (cyp19a1b) aromatase genes were analysed in the gonads and brain tissue of both sexes using qPCR.
To normalize the data for the tested genes, gapdh mRNA was used as an endogenous control for all the tissues tested. The data was tested for normality and subsequently analysed by two way analysis of variance (ANOVA) followed by Tukey’s post hoc test for multiple comparisons between the treatments and time for each genes using SPSS17.0 (SPSS Inc. 2008). The significance was set at \( P<0.05 \).

7.3. Results

7.3.1. Expression of cyp19a isoforms in female fish exposures

7.3.1.1. Expression of cyp19a1a in the ovary and brain tissues

Throughout the experiment, there were no significant differences observed in cyp19a1a expression in ovary with BPA exposure (Fig. 7.1 A). The results of two way analysis of variance (ANOVA) showed no significant differences between the treatments and time for BPA (cyp19a1a, BPA: \( F_{15, 32} =2.663; n=3, P<0.05 \)). In contrast, exposure to 50 µg/L fadrozole first significantly increased cyp19a1a expression at 24 h, followed by significant reduction at 48, 72 and 96 h post exposure, but remained significantly above the controls throughout the exposure period (Fig. 7.1 B). Fadrozole exposure at 10 µg/L did not have any effect (Fig. 7.1 B). The results of two way analysis of variance (ANOVA) showed significant differences between the treatments and time of exposure for fadrozole (cyp19a1a, Fadrozole: \( F_{15, 32} =4.334, n=3, P<0.05 \)).

The expression of cyp19a1a was not detected in brain tissues at any time point, including controls (data not shown).
Figure 7.1. A: Expression of cyp19a1a in the ovarian tissues of female fish exposed to BPA, B: Expression of cyp19a1a in the ovarian tissues of female fish exposed to fadrozole. Values described by the same letter are not significantly different from each other (P<0.05, n=3).
7.3.1.2. Expression of \textit{cyp19a1b} in the brain

Expression of \textit{cyp19a1b} in female brain tissue with exposure to 100 µg/L of BPA increased at 24 and 48 h and was significantly higher than the controls. With exposure to 500 µg/L of BPA, at 24 h the \textit{cyp19a1b} expression was equal to that of controls, and then its expression significantly increased at 48 h compared to controls. At 72 h with both concentrations the expression fell to control levels (Fig. 7.2 A). Meanwhile with exposure to 500 µg/L BPA, expression of \textit{cyp19a1b} at 96 h was significantly less than 48 h, but more than 72 h (Fig. 7.2 A). The two way analysis of variance (ANOVA) showed significant differences between the treatments and time of exposure for BPA (\textit{cyp19a1b}, BPA: $F_{15, 32} = 6.905$, $F_{15, 32} =3.861$, n=3, P<0.05).

The expression of \textit{cyp19a1b} in the brain tissues of fish exposed to 10 and 50 µg/L of fadrozole was significantly lower than that of controls throughout the exposure period (Fig. 7.2 B). However, there was no significant variation observed with exposure to 10 µg/L of fadrozole between different time points throughout the experiment. Expression of \textit{cyp19a1b} with exposure to 50 µg/L fadrozole was significantly higher than in fish exposed to 10 µg/L fadrozole at 24, 48 and 72 h but not at 96 h (Fig. 7.2 B). The two way analysis of variance (ANOVA) showed significant differences between the treatments and time of exposure (\textit{cyp19a1b}, Fadrozole: $F_{15, 32} =3.861$, n=3, P<0.05).
Figure 7.2. A: Expression of cyp19a1b in brain of female fish exposed to BPA, B: Expression of cyp19a1b in the brain of female fish exposed to fadrozole. Values described by the same letter are not significantly different from each other (P<0.05, n=3).
7.3.1.3. Expression of \textit{cyp19a1b} in the ovary

The expression of \textit{cyp19a1b} in the ovarian tissues with exposure to BPA was significantly higher than controls throughout the exposure period (Fig. 7.3 A). However, the pattern was slightly different in different concentrations, wherein the increase in expression for 100 µg/L was 24 h ahead that observed in 500 µg/L. The expression at 72 and 96 h with both concentrations was similar and showed reducing trend (Fig. 7.3 A) but remained significantly above the controls at both exposures. The two way analysis of variance (ANOVA) showed significant differences between the treatments and exposure time (\textit{cyp19a1b}, BPA: $F_{15, 32} = 4.971$, $n=3$, $P<0.05$)

The expression of \textit{cyp19a1b} with exposure to 10 and 50 µg/L of fadrozole did not increase significantly compared to controls ($F_{15, 32}=2.383$, $n=3$, $P<0.05$), although appeared to increase in exposure time.
Figure 7.3. A: Expression of cyp19a1b in the ovarian tissues of female fish exposed to BPA, B: Expression of cyp19a1b in the ovarian tissues of female fish exposed to fadrozole. Values described by the same letter are not significantly different from each other (P<0.05, n=3).
7.3.2. Expression of *cyp19a* isoforms in male fish exposed to BPA and fadrozole

7.3.2.1. Expression of *cyp19a1a* in the brain and testis

Male rainbowfish exposed to BPA and fadrozole throughout the experiment showed no expression of *cyp19a1a* in either brain or testis (data not shown).

7.3.2.2. Expression of *cyp19a1b* in the brain

The expression of *cyp19a1b* was significantly higher in the brain of male fish exposed to BPA at both concentrations throughout the experiment (Fig. 7.4 A). Its expression did not fluctuate at different time points at 100 µg/L, whereas, at 500 µg/L of BPA, a further significant increase at 48 h compared to 24 h exposure was observed (Fig. 7.4 A). The two way analysis of variance (ANOVA) showed significant differences between the treatments and time of exposure to BPA (*cyp19a1b*, BPA: $F_{15, 32} = 2.338$, n=3, $P<0.05$).

With exposure to fadrozole at 10 and 50 µg/L, *cyp19a1b* expression remained significantly higher throughout the 96 h exposure. No change in *cyp19a1b* expression was observed with exposure to 10 µg/L of fadrozole until 72 h, followed by a further significant increase at 96 h, and then it increased significantly compared to previous exposure time points and controls at 96 h (Fig. 7.4 B). Exposure to a high concentration (50 µg/L) of fadrozole resulted in the expression of *cyp19a1b* being significantly higher than controls at 24 h. Then at 48, 72 and 96 h it showed reducing trend but still remained significantly above the controls (Fig. 7.4 B). The two way analysis of variance (ANOVA) showed significant differences between the treatments and time (*cyp19a1b*, Fadrozole: $F_{15, 32} = 3.581$, n=3, $P<0.05$).
Figure 7.4. A: Expression of cyp19a1b in brain of male fish exposed to BPA, B: Expression of cyp19a1b in brain tissues of male fish exposed to fadrozole. Values described by the same letter are not significantly different from each other (P<0.05, n=3).
7.3.2.3. Expression of cyp19a1b in the testis

The expression of cyp19a1b in the testis with exposure to BPA was significantly lower than controls throughout the exposure period at both concentrations (Fig. 7.5 A). With exposure to 100 µg/L of BPA, the cyp19a1b expression reduced significantly at 24 and 48 h, with further significant decrease at 72 h. However the levels at 96 h significantly increased compared to those at 72 h, but remained significantly lower than control (Fig. 7.5 A). Whereas, with exposure to 500 µg/L of BPA, its expression was significantly lower than in controls and in fish exposed to 100 µg/L of BPA at 24 h. Then at 48, 72 and 96 h its expression increased significantly more than at 24 h but remained significantly lower than control (Fig. 7.5 A). The two way analysis of variance (ANOVA) for BPA showed significant differences between the treatments and time (F\textsubscript{15, 32}=2.061, n=3, P<0.05).

In contrast, exposure to fadrozole at 10 and 50 µg/L, cyp19a1b (Fig. 7.5 B) expression did not show any difference between concentration and duration of exposure. During the experiment slight fluctuations in cyp19a1b expression were observed, but were not significantly different (F\textsubscript{15, 32}=0.871, n=3, P>0.05).
Figure 7.5. A: Expression of *cyp19a1b* in the testes of male fish exposed to BPA, B: *cyp19a1b* in the testes of male fish exposed to fadrozole. Values described by the same letter are not significantly different from each other (P<0.05, n=3).
7.4. Discussion

Both BPA and fadrozole had a measurable effect on *cyp19a* isoform expression in both sexes of *M. fluviatilis* exposed for 96 h, except for *cyp19a1a* in testis. It was observed that ovarian aromatase (*cyp19a1a*) in the ovarian tissue was upregulated with fadrozole exposure at high concentration throughout the experiment. In general both chemical exposures tended to enhance the expression of *cyp19a1b* in brain of both males and females, except fadrozole which downregulated *cyp19a1b* in the brain of female fish.

The expression of *cyp19a1b* was significantly increased in male brain throughout the experiment with exposure to BPA and fadrozole. In male gonads BPA significantly downregulated the expression of *cyp19a1b* throughout the experiment.

7.4.1. Expression of ovarian aromatase (*cyp19a1a*) in male and female fish in response to BPA and fadrozole exposure.

With exposure to BPA, *cyp19a1a* in ovary was initially upregulated (until 48 h) and then the levels pleated to those of control fish. Similar results were also observed in juvenile rare minnows, *Gobiocypris rarus* exposed to BPA for 3 days at 0.1 and 1nM, however expression at 10 nm BPA was significantly lower than in controls (Wang *et al.*, 2010). In contrast, upregulation of *cyp19a1a* in the brain was observed in *Rivulus marmoratus* (Lee *et al.*, 2006) exposed to BPA at 600 µg/L for 96 h. The fluctuating trend may suggest that the BPA likely activates and represses the *cyp19a1a* gene, depending on the exposure concentration and duration.
In the current study, the cyp19a1a in the ovarian tissue was upregulated with exposure to 50 µg/L of fadrozole throughout the experiment. Similar results were also observed in gonads of female fathead minnows exposed to 1.85 to 50 µg/L for 7 days (Villeneuve et al., 2006), zebrafish exposed to 100 µg/L for 4 days (Villeneuve et al., 2009) and Japanese medaka exposed to 10 and 100 µg/L for 7 days (Tompsett et al., 2010) to fadrozole. However, in this study (M. fluviatilis) exposure to a low concentration of fadrozole (10 µg/L) did not have any effect on the cyp19a1a expression (Fig. 7.1 B). Similar results were also observed in zebrafish exposed to 25 µg/L of fadrozole for 96 h (Villeneuve et al., 2009). The exposure of M. fluviatilis to 10 µg/L (this study) and zebrafish to 25 µg/L (Villeneuve et al., 2009) of fadrozole may not be sufficient to upregulate the gene or the exposure period was not sufficient. The latter appears likely since the female fathead minnows showed high expression of cyp19a1a at much lower concentration (1.85 µg/L) but only after 7 days of exposure (Villeneuve et al., 2006). Similarly medaka showed high expression at exposure 10 µg/L of fadrozole only after 7 days. The upregulation of cyp19a1a with fadrozole exposure observed in this study (M. fluviatilis) and in previous studies (Villeneuve et al., 2006; Villeneuve et al., 2009; Tompsett et al., 2010) may be indirectly mediated through Steroidogenic Factor-1 (SF-1) a known inducer of aromatase activity (Villeneuve et al., 2006). This is likely as the cyp19a1a promoter contains SF-1 site, whilst lacking the ERE site (Callard et al., 2001; Kazeto et al., 2001). A possibility of fadrozole acting via SF-1 factor in altering the cyp19a1a expression has been suggested in medaka (Tompsett et al., 2010). Moreover SF-1 is known to play a key role in the regulation of many steroidogenic genes (Parker and Schimmer, 1995; Carlone and Richards, 1997; Chau et al., 1997; Zhang and Mellon, 1997) such as GtH that may
influence estrogen synthesis via cAMP-pathways (Parker and Schimmer, 1995; Hearn and Gomme, 2000). In rainbowfish, fadrozole would be expected to cause inhibition of estrogen (endogenous 17β-estradiol) as was observed in fathead minnows exposed to 50 µg/L of fadrozole for 7 and 21 days (Ankley et al., 2002; Villeneuve et al., 2006). Upregulation of cyp19a1a gene following exposure to fadrozole (Ankley et al., 2002; Villeneuve et al., 2006 and this study) may suggest a compensatory response to the reduction in estrogen. Reduction in levels following exposure to fadrozole has been previously reported in medaka (Tompsett et al., 2010). Notably though the estrogen levels and aromatase enzyme activity was not measured during this study. Future studies incorporating these measures and their correlation between gene expressions would be vital to understand the mechanisms underpinning the fadrozole action.

7.4.2. Expression of brain aromatase (cyp19a1b) in the brain of male and female fish in response to BPA and fadrozole (AI) exposure.

Female fish exposed to BPA showed significantly high expression of cyp19a1b in their brains, initially (up to 48 h) but later significantly reduced compared to previous exposure time point. This upregulation followed by decrease in expression may be due to the chemical triggering the gene expression initially but it may have become toxic with increase in exposure time. However, with exposure to 500 µg/L BPA at 96 h (Fig. 7.2 A) the downregulation (72 h) was preceded (24 and 48 h) and followed (96 h) by upregulation. This switch between up and downregualtion may reflect interplay of negative and positive feedback on cyp19a1b in the rainbowfish, but one that is not in sink with the
unexposed fish. In males though exposure to BPA caused significantly elevated cyp19a1b expression throughout the exposure period. No other comparable studies in male fish following BPA exposure are available.

With exposure to fadrozole, expression of cyp19a1b in the brain of both sexes showed contrasting results. The expression of cyp19a1b in female brain was significantly downregulated with exposure to fadrozole throughout the exposure period. Similar results were observed in female fathead minnows exposed to fadrozole (5.5 to 50 µg/L for 7 days) wherein the expression of cyp19a1b decreased with increasing concentration of fadrozole (Villeneuve et al., 2006). Also the aromatase enzyme activity in the brain of female fathead minnows decreased with exposure to 50 µg/L of fadrozole. The response of the aromatase enzyme activity and the gene expression were in contrast to that of cyp19a1a in ovary and aromatase enzyme activity in the ovary of fathead minnows observed by Villeneuve et al. (2006). Similarly female zebrafish exposed to 25 µg/L fadrozole for 96 h, significantly downregulated cyp19a1b expression in the brain (Villeneuve et al., 2009). In direct contrast males in this study showed significantly high expression of cyp19a1b with both concentrations of fadrozole throughout the experiment. Also, male zebrafish exposed to 200 µg/L of fadrozole for 10 days showed significantly higher expression of cyp19a1b in the brain tissues (Paquette, 2008). Collectively, the brain of both sexes is susceptible to aromatase inhibitor, but they appear to respond differently in different sexes. This sex specific response of cyp19a1b to an AI (fadrozole) implies a potential role for the isoform in modulating sexual behaviour in this species and more generally in teleosts.
In this project the studies conducted were limited to gene expression. Parallel studies measuring levels of transcription, translation and or enzyme activity are likely to shed more light on the action of fadrozole on the reproductive pathway of rainbowfish.

7.4.3. Expression of brain aromatase (cyp19a1b) in the gonads

With exposure to BPA, the cyp19a1b expression yielded contrasting results in the gonads of both sexes. cyp19a1b expression in the ovary was significantly upregulated and in testes its expression was significantly decreased compared to controls. This contrast in expression at similar exposure concentrations may be because of the differences in the base level of circulating co-regulators other than estrogens in the respective gonads. The contrast in expression of cyp19a1b between the brain and gonads results further support other reports in the literature indicating that aromatase isoforms are regulated differentially in these tissues (Kazeto et al., 2001; Kishida and Callard, 2001; Fenske and Segner, 2004).

Expression of cyp19a1b in the gonads of female fish exposed to both exposures of fadrozole showed upregulation trend in this study. However fathead minnows exposed to fadrozole (1.85, 5.5, 16 and 50 ug/L) for 7 days showed significant downregulation. In male rainbowfish, this expression was contrasting to that of female rainbowfish. But there are no reports on effect of fadrozole on male fish gonads for comparison studies.
7.5. Conclusions

In conclusion the present study demonstrated that the cyp19a isoforms in *M. fluviatilis* is a useful molecular marker of exposure to BPA and fadrozole, but the responses are sex and tissue dependent. It appears that BPA and Fadrozole regulate cyp19a1a expression in the ovary in a negative and positive manner respectively. In contrast both BPA and fadrozole positively regulated cyp19a1b expression in brain of both sexes and ovary. However, BPA downregulated cyp19a1b in the testis and fadrozole upregulated cyp19a1b in male brain and in ovary. The observations in the study support the hypothesis that BPA and fadrozole regulate expression of cyp19a1b via both positive and negative feedback mechanisms, with differential modulation based on the chemical, tissue and sex of the fish.

Collectively, the results suggest that, the BPA and fadrozole can have a disruptive effect on the steroidogenic pathways and hence sex differentiation, sexual behaviour and reproductive cycles in this species. Both aromatase isoforms have potential as biomarkers for detecting and monitoring environmental exposures to BPA and fadrozole. However, this study examined only mRNA expression; therefore further research is necessary to characterize the post transcriptional effects and confirm the effects of these EDCs on development, metabolism, reproduction and sex ratios in the rainbowfish.
CHAPTER 8

General Discussion

In the current study aromatase gene isoforms and vitellogenin were investigated in the Murray River rainbowfish, \textit{M. fluviatilis} to evaluate their potential as biomarkers of exposure to select EDCs. This type of research on molecular markers of toxicity and more generally ecotoxicogenomics is relatively novel in Australian native species.

In the current study the Murray River rainbowfish, \textit{M. fluviatilis}, native to Australia was chosen as a test organism. The first essential step in this investigation was the cloning of the aromatase gene isoforms in this species. The P450 aromatase genes had been isolated, cloned and characterized from over 20 species of non-native fish prior to the start of this study (see summary of Table 3.1, Chapter 3). This relatively ready availability of sequence information provided information to design degenerate primers for this study. However, no aromatase or \textit{vtg} gene sequences from \textit{M. fluviatilis} were publicly avialable at the time of initiating this research. As presented in chapter 3, aromatase gene isoforms including \textit{cyp19a1a} (ovarian aromatase; Accession No. GU723457) and \textit{cyp19a1b} (brain aromatase; Accession No. GU723458) from \textit{M. fluviatilis} were cloned, characterised and compared with those of other fish and selected higher vertebrates. Like most teleosts it was proven that this species has two aromatase
isoforms—ovarian (cyp19a1a) and brain (cyp19a1b), with each showing high homology with that of the corresponding homologue of other teleost. This result supports the hypothesis that the shared ancestry of the duplicated genes could be due to the duplication of genome during early teleost evolution (Taylor et al., 2003). As expected both aromatase isoforms of the M. fluviatilis showed highest identity with that of pejerrey, a closely related species. The observation reconfirms the close taxonomic relationship of these two artheriniformes. Further, a very high homology observed between the putative Cyp19a functional domains of these two species, compared to other teleosts investigated, suggest a close evolutionary relationship, despite their current geographical separation.

Cloning and characterization of the cyp19a isoforms from M. fluviatilis enabled phylogenic comparisons (Chapter 3), determination of tissue domains of expression in adults (Chapter 3), expression throughout ontogeny (Chapter 4) and more importantly responses of these genes with exposure to select EDCs (Chapters 5 and 7) in laboratory experiments. Unlike aromatase isoforms, partial vtg cDNA sequences of M. fluviatilis were published part way through this investigation (Woods et al., 2007). These sequences were used to develop vtg specific RT-PCR primers and the response of both mRNA and protein following exposure to select EDCs were evaluated (Chapter 6).

8.1. Tissue specific expression (normal) of aromatase isoforms

To characterise normal expression of cyp19a1a and cyp19a1b in the rainbowfish, qPCR was used (Chapter 3). In the current study the cyp19a1a, ovarian aromatase (gonadal aromatase) was exclusively expressed in ovarian tissue.
Similar results were also observed in other species including, *Oreochromis niloticus* (Chang et al., 2005), *Fundulus heteroclitus* (Greytak et al., 2005) and *Odontosthesis bonariensis* (Karube et al., 2007). In contrast the predominant expression of cyp19a1a in ovary and low level expression in other tissues were observed in teleosts including Japanese flounder (Kitano et al., 1999), orange-spotted grouper (Zhang et al., 2004), zebrafish (Sawyer et al., 2006), common carp (Barney et al., 2008) and medaka (Patil and Gunasekera, 2008). Whereas, the brain aromatase (cyp19a1b) in *M. fluviatilis* was predominantly expressed in the brain and also low level expression was observed in other tissues studied including liver, eyes and gonads, with no expression in the spleens of both sexes. Similar expression of cyp19a1b has also been observed in other species including seabass (González and Piferrer, 2003), pejerrey (Strobl-Mazuzulla et al., 2005), Atlantic halibut (Matsuoka et al., 2006) and common carp (Barney et al., 2008).

In the current study it was observed that cyp19a1b expression in male brain was significantly higher (~3 fold) than in female brain. It is possible that in *M. fluviatilis* this higher expression of brain aromatase in the males could be related to the sexual or mating behaviour. However, contrasting results were observed in other teleosts, including zebrafish (Sawyer et al., 2006) and Japanese medaka (Patil and Gunasekera, 2008). This inter-species variation could be due to their reproductive strategies and/or the seasonal variations. Restricted expression of cyp19a1a to the ovary and predominant expression of cyp19a1b in brain suggests that they play a key role in the ovarian development and sexual behaviour respectively in *M. fluviatilis* (Chapter 3).
8.2. Expression through ontogeny

The endogenous aromatase gene expression during early ontogeny was characterised and described in chapter 4. Ovarian aromatase mRNA was not detected during the developmental stages tested, suggesting no role for the isoform during early development, but likely expressed only in differentiated ovary. Ovary specific expression of the isoform was observed in adult fish (Chapter 3). In contrast expression of \textit{cyp19a1b} was detectable in all the developmental stages tested including unfertilized eggs. This implies a significant role for \textit{cyp19a1b} in early larval growth and development including brain. Partly in accordance with these results, the maternal inheritance of both \textit{cyp19a1a} and \textit{cyp19a1b} were also observed in zebrafish (Sawyer et al., 2006). In contrast, Barney et al. (2008) report maternal inheritance of only \textit{cyp19a1a} in common carp. As opposed to these observations, neither of the mRNA isoforms was detected in unfertilized or fertilized eggs or in embryos of medaka before the onset of the mid blastula transition stage (Patil and Gunesekara, 2008). Collectively, these differences in maternal inheritance of transcripts across species indicate their possible association with subtle variations in the reproductive strategies of each of the species.

Although this study did not evaluate the effect of xenobiotics on \textit{cyp19a} expression during ontogeny, the base line expression profiles presented provide a platform for such future studies. This is particularly relevant given that the early larval stages and juveniles are particularly sensitive and vulnerable to xenobiotic exposures (Brion et al., 2004; Hinfray et al., 2006; Vosges et al., 2010).
8.3. Aromatase as a biomarker of exposure

Aromatase has been used as a biomarker of chemical exposures in many fish species (Halm et al., 2002; Kazeto et al., 2004; Kallivretaki et al., 2006). Characterization of cyp19a genes and their expression profile in normal tissues of *M. fluviatilis* (Chapter 3) enabled us to test their suitability as biomarkers of exposure to E2, NP, BPA and an aromatase inhibitor fadrozole.

8.3.1. Effects of E2 and NP on the ovarian aromatase

In the present study it was observed that the E2 and NP significantly reduced and inhibited the ovarian aromatase gene (*cyp19a1a*) transcript (Chapter 5). Similar results were also observed in other species including zebrafish exposed to 100nm of ethinylestradiol (EE2) (Kazeto et al., 2004); *Rivulus marmoratus* exposed to 300 µg/L of 4-nonylphenol for 96 h (Lee et al., 2006) and Atlantic salmon exposed to 5 and 50 µg/L of nonylphenol (Kortner et al., 2009). This downregulation/inhibition may be attributed to negative feedback mechanisms potentially triggered by the presence of high levels of estrogens in the gonads. Excess xenoestrogens may regulate the ovarian aromatase expression mechanistically by either direct binding to the transcription factors (EREs) upstream of the *cyp19a1a* gene. This appears less likely given that promoters of *cyp19a1a* generally lack well-defined EREs (Callard et al., 2001; Kazeto et al., 2001; Guiguen et al., 2010), but this requires validation in this species. Alternatively the xenoestrogens may bind to other molecules in the regulatory pathway such as the arylhydrocarbon receptor (AhR) resulting in indirect downregulation and/or inhibition of *cyp19a1a* as has been suggested in the case of dioxin exposures (Kazeto et al., 2004). The inhibitory action of E2 and NP on
the expression of \textit{cyp19a1a} in the ovarian tissues could also be indirectly brought about by repression of GnRH a known stimulator of aromatase gene expression in the ovarian follicles (Gen \textit{et al.}, 2001; Kagawa \textit{et al.}, 2003).

The response of ovarian aromatase was also evaluated in other tissues (Chapter 3) including testis and the brain of both sexes of \textit{M. fluviatilis} with exposure to selected EDCs, but no expression was observed throughout the exposure experiments in these tissues. This result is not surprising given no basal expression of \textit{cyp19a1a} was observed in tissues other than the ovary in this species and that exposure to EDCs repressed \textit{cyp19a1a} activity in the ovary of this species.

\textbf{8.3.2. Effects of E2 and NP on the brain aromatase}

As presented in Chapter 5, the brain aromatase was upregulated in the brain of females. Similar results were also observed in female zebrafish (Kazeto \textit{et al.}, 2004; Hinfray \textit{et al.}, 2006; Kallivretaki \textit{et al.}, 2006). In contrast to females, its expression was downregulated in the brain of males (Chapter 5) and similar results have been observed in adult fathead minnows exposed to 100 and 320 ng/L of E2 (Halm \textit{et al.}, 2002). The observed repression of \textit{cyp19a1b} in brain of males, but not females, may reflect higher level of basal circulating estrogens in the brain of males compared to females. Conceivably this elevated level of endogenous estrogen in males might explain the observed negative feedback (repression) in male and positive feedback (induction) in female brain. Similarly \textit{cyp19a1b} was upregulated in testis but downregulated in ovaries with exposure to xenoestrogens (Chapter 5). This again suggests that high level of estrogen in ovary triggers an immediate repression of \textit{cyp19a1b} in ovary following exposure
to exogenous E2 and NP. In contrast the absence or low level of endogenous estrogen in testis, appears to induce *cyp19a1b* expression following exposure to E2 and NP, reflective of a positive feedback mechanism. It appears that the *cyp19a1b* isoform is directly regulated via both positive and negative feedback mechanism. This direct regulation of *cyp19a1b* by circulating estrogens is further supported by the observations that only *cyp19a1b* promoter of teleosts contains full EREs (Piferrer and Blázquez, 2005; Diotel et al., 2010).

**8.3.3. Effects of BPA and fadrozole on the ovarian aromatase**

With exposure to 100 and 500 µg/L concentrations of BPA (Chapter 7), the *cyp19a1a* expression in the ovarian tissue did not change. However, with fadrozole exposure, *cyp19a1a* expression was upregulated throughout the 50 µg/L for 96 h. A similar observation was reported previously in other teleosts including fathead minnows exposed to 1.85, 16.7 and 50 µg/L of fadrozole (Villeneuve et al., 2006) and Japanese medaka exposed to fadrozole at 10 and 100 µg/L for 7 days (Tompsett et al., 2010). In *M. fluviatilis* however, there were no significant differences observed with exposure to lower levels (10 µg/L) of fadrozole throughout the study. The observed upregulation of *cyp19a1a* at higher levels of exposure to fadrozole could be because of inhibition of endogenous estrogen as was observed in fathead minnows exposed to fadrozole for 21 and 7 days (Ankley et al., 2002; Villeneuve et al., 2006) respectively leading to upregulation of the *cyp19a1a* gene in the exposed fish to compensate for the reduced estrogen (Tompsett et al., 2010). However, in the current study enzyme activity responses were not measured in *M. fluviatilis* and this remains a potential area of investigation.
cyp19a1a expression in testis and brain of both sexes were also investigated with exposure to E2 and xenoestrogens. However no response was detected throughout the experiment. This result is not surprising given the absence of any cyp9a1a expression in these tissues of control fish (Chapter 3). The results confirm that cyp19a1a is not expressed in tissues other than the ovary even with exposure to xenoestrogens.

8.3.4. Effects of BPA and fadrozole on the brain aromatase

The expression of cyp19a1b in the brain of opposite sexes showed contrasting results with exposure to fadrozole. The expression of cyp19a1b in female brain was significantly downregulated with fadrozole exposure to both selected concentrations throughout the exposure period. Similar results were also observed in female fathead minnows (Villeneuve et al., 2006) exposed to 5.5, 16.7 and 50 µg/L of fadrozole for 7 days and in their experiment the expression of cyp19a1b decreased with increasing concentration of fadrozole. However, the aromatase enzyme activity in the brain of fathead minnows decreased with 50 µg/L of fadrozole. The authors found that the response of the aromatase enzyme was in complete contrast to the expression of cyp19a1a gene. In contrast male rainbowfish showed significantly higher expression of cyp19a1b in brain with exposure to both concentrations of fadrozole throughout the experiment. The gene might have been upregulated to compensate for the reduced estrogen following its inhibition of the enzyme by fadrozole in male M. fluviatilis. This pattern of upregulation in male and downregulation in females is diametrically opposite to what was observed following exposure to E2 and NP (chapter 5 and section 8.3.2 above). This implies that the non steroidogenic inhibitors of
aromatase (e.g. fadrozole) may disrupt as well as activate aromatase activity in a tissue dependent fashion. However, there are no previous studies on the cyp19a1b expression in any other male teleost species exposed to fadrozole for comparison.

In the gonads of both sexes, the expression of cyp19a1b was not significantly affected by fadrozole throughout the exposure studies. However, with exposure to BPA, the cyp19a1b expression in male testis contrasted with the cyp19a1b expression in ovary. The cyp19a1b in the ovary was significantly upregulated and in the testis its expression was significantly downregulated compared to controls. This contrasting response of testis and ovary to BPA exposure may be attributed to the differences in the basal level of circulating estrogens or unknown sex specific co-regulators. These results support other reports in the literature indicating that aromatase isoforms are regulated differentially based on the sex of the individual (Kazeto et al., 2001; Kishida and Callard, 2001; Fenske and Segner, 2004).

At cellular and molecular level (either genomic or non genomic), the differences in the promoter structure and or amino acid sequences of aromatase genes may account for different levels of activity and hence different responses to various EDCs (Shilling et al., 1999; Zhao et al., 2001).

In this project, the experiments conducted were all continuous with static partial renewal exposures. Continuous exposure to EDCs was chosen in this study since there are reports that pulse exposures or injection of xenoestrogens into the fish body may trigger the mRNA expression during exposures and when the
stimulants were removed, the mRNA expression may disappear (Ponza, 2006). Continuous exposure also simulates exposures in field conditions. Hence, with continuous exposures, it was possible to measure the effect of chemicals on mRNA expression of the genes studied, that are likely to reflect the situations in the field.

The aromatase isoforms expression varied between the chemicals and also across species. Throughout the studies it was observed that both aromatase isoforms were very sensitive to the chemicals selected. In addition vitellogenin gene and protein expressions were also investigated in fish exposed to 17β-estradiol and nonylphenol. It was observed that both genes could be used as potential biomarkers of exposures in small fish species like Murray River rainbowfish.

Collectively, the results of this project showed that the effect of the selected xenobiotics will have disruptive effect on the steroidogenesis pathway. Similar results were also observed in zebrafish (Kazeto et al., 2004; Hinfray et al., 2006; Kallivretaki et al., 2006), Japanese medaka (Oshima et al., 2003; Contractor et al., 2004) and fathead minnows (Halm et al., 2002) exposed to various chemicals as discussed in chapters 5 and 7.

However, the observations made in the current project were based just on transcription levels of cyp19a isoforms. It will be of interest to compare the responses of gene transcription, translation and enzyme activity of the aromatase gene isoforms in parallel. Synthesis of an antibody against each of the aromatase isoforms and the examination of their activity in M. fluviatilis would be an obvious follow up in establishing these as robust biomarkers of exposure to EDCs in this Australian native species.
Table 8.1. Effect of EDCs exposure on *cyp19* isofoms in different teleosts

<table>
<thead>
<tr>
<th>Species</th>
<th>EDCs</th>
<th>Concentration</th>
<th>Effect on <em>cyp19a1a</em></th>
<th>Effect on <em>cyp19a1b</em></th>
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<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 µg/L</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Pimephales promelas</td>
<td>E2</td>
<td>100 nm</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>320 nm</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Fadrozole</td>
<td>1.85 – 50 µg/L</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

NE = No expression; - Downregulation; + Upregulation; NA – Not Analysed
8.4. Vitellogenin as a biomarker of exposure

Expression of vtg mRNA in males exposed to EDCs E2 and NP, are presented and discussed in more detail elsewhere in the thesis (Chapter 6). The observations support the estrogenic activity of both E2 and NP as they significantly induced the activation of vtg synthesis in males. Similar results were also observed in sheepshed minnows exposed to either E2 with 0.089 and 0.71 µg/L or p-NP with 5.6 and 59.6 µg/L (Hemmer et al., 2002), medaka exposed to either 25 ng/L of EE2, 0.1 and 10 µg/L of E2, 20 and 500 µg/L of nonylphenol (Islinger et al., 2002; Yamaguchi et al., 2005), mosquitofish exposed to E2 at 250 ng/L for 4 days (Leusch et al., 2005), Japanese Sillago exposed to either 50 ng/L of E2 or 40 µg/L of 4-tert-octylophenol (Yoon et al., 2008), whilst male carp...
injected with E2 (0.033 mg/kg body weight showed significant expression of vtg mRNA measured by RT-PCR (Lattier et al., 2001) and juvenile fathead minnows injected with 5 mg/kg body weight showed significant increase in vtg within 24 h (Thomas-Jones et al., 2003). We also observed the expression of vtg in the testis of males exposed to E2 and the estrogen mimic NP throughout the experiment. These results indicate that the expression of vtg mRNA in liver and gonads of male fish could be used as a biomarker of exposure to xenoestrogens in this species. Further verification of vtg translation in testis and its potential reproductive impacts will be valuable in establishing vtg as a biomarker of estrogenic effluents in male M. fluviatilis. Detection of vitellogenin at transcription will be a useful biomarker for small fish such as M. fluviatilis, which generally yield insufficient plasma and for which specific antibodies are not available.

8.5. Conclusions

In conclusion, this is the first study to isolate and characterize the cyp19a1a and cyp19a1b aromatase isoforms and their expression during ontogeny and in adult tissues of M. fluviatilis. The sequence information was reported on the NCBI database (GU723457 and GU723458). Using the sequence information the responses of these isoforms when exposed to four EDCs was investigated in M. fluviatilis. The results revealed that the selected chemicals had a disruptive effect on the cyp19a and vtg expression patterns, suggesting their utility as biomarkers of exposure to Endocrine Disrupting Chemicals in this native Australian fish species. The clear mechanisms of action of such chemicals on the steroidogenic pathways of M. fluviatilis remain to be elucidated.
The current study provides valuable base line information on expression of *cyp19a* and *vtg* in this native freshwater fish species, including localization and tissues distribution of these genes in female and male tissues. Based on these investigations the Murray River rainbowfish, *M. fluviatilis* can be used as a model species to assess the impact of the EDCs in the Australian freshwater ecosystem.

**8.6. Future directions**

The observations and resources generated form a basis for establishing *cyp19a* and or *vtg* as a useful biomarker to monitor the effects of EDCs in natural environments, using a native fish as a test model. It would be intuitive to develop antibodies for the *M. fluviatilis* aromatase isoforms using the protein sequences available for the aromatase isozymes. Together with qPCR it would be ideal to use other techniques including *In situ* hybridization, Southern blotting and immunodetection to understand and or to detect how these chemicals affect the localization of these proteins in different tissues. More recently it has been shown that, microRNA (miRNAs) play a role in the post transcriptional and or translation regulation of the messenger RNA. In this regard, this study establishes an ideal platform for future investigations to establish the identity and role of potential miRNAs in regulation of Cyp19a and vtg and their responses to EDC exposure.
References


Gelinas, D., Pitoc, G.A., Callard, G.V., 1998. Isolation of a goldfish brain cytochrome P450 aromatase cDNA: mRNA expression during the


Gronen, S., Denslow, N., Manning, S., Barnes, S., Barnes, D., Brouwer, M.,
1999. Serum Vitellogenin levels and reproductive impairment of male
Japanese medaka (Oryzias latipes) exposed to 4-tert-octylphenol,

Vitellogenin gene expression in male rainbow trout (Salmo gairdneri).

Guiguen, J.F., Baroiller, J.F., Ricordel, M.J, Iseki, K., Mcmeel, O.M., Martin,
S.A., Fostier, A., 1999. Involvement of estrogens in the process of sex
differentiation in two fish species: the rainbow trout (Oncorhynchus
mykiss) and a tilapia (Oreochromis niloticus), Mol. Reprod. Dev. 54,
154–162.

Guigen, Y., Fostier, A., Piferrer, F., Chang, C. F., 2010. Ovarian aromatase and
estrogens: A pivotal role for gonadal sex differentiation and sex change

Hahlbeck, E., Katsiadaki, I., Mayer, I., Adolfsson-Erici, M., James, J.,
Bengtsson, B-E., 2004. The juvenile three-spined stickleback
(Gasterosteus aculeatus L.) as a model organism for endocrine
disruption II--kidney hypertrophy, vitellogenin and spiggin induction.
Aquat. Toxicol. 70, 311-326.

in sediments and effluents associated with diverse wastewater outfalls.

Hallgren, S., Olsen, K.H., 2010. Effects on guppy brain aromatase activity
following short-term steroid and 4-nonylphenol exposures. Environ.
Toxicol. 25, 261-271.


Langlois, V.S., Duarte-Guterman, P., Ing, S., Bruce, D., Pauli, B.D., Cooke, G.M., Trudeau, V.L., 2010. Fadrozole and finasteride exposures modulate sex steroid- and thyroid hormone-related gene expression in *Silurana*


encoding human aromatase cytochrome P-450, the enzyme responsible for estrogen biosynthesis. J. Biol. Chem. 264, 19385–19391.


Penman, D.J., Piferrer, F., 2008. Fish gonadogenesis. Part I: Genetic and environmental mechanisms of sex determination. Rev. Fish. Sci. 16 (S1), 16-34.


APPENDIX A - List of common buffers and reagents

1. 5xTAE Buffer

\[
\begin{align*}
\text{H}_2\text{O} & : 1.0 \text{ L} \\
\text{Tris base} & : 24.2 \text{ g} \\
\text{Acetic acid} & : 5.71 \text{ mL} \\
0.2 \text{ M EDTA} & : 10.0 \text{ mL}
\end{align*}
\]

2. 1xTAE Buffer

\[
\begin{align*}
\text{5xTBE Buffer} & : 100 \text{ mL} \\
\text{Milli-Q water} & : 400 \text{ mL}
\end{align*}
\]

3. 0.2M EDTA

\[
\begin{align*}
0.744 \text{ g Na}_2 \text{ EDTA} \\
10 \text{ mL autoclaved Milli-Q water}
\end{align*}
\]

4. Agarose gel (1%)

\[
\begin{align*}
\text{Agarose in 100 mL of 1x TAE buffer} & : 1.0 \text{ g}
\end{align*}
\]

5. LB Media (per litre)

\[
\begin{align*}
\text{Bacto®-tryptone} & : 10 \text{ g} \\
\text{Bacto®-yeast extract} & : 5 \text{ g} \\
\text{NaCl} & : 5 \text{ g}
\end{align*}
\]

Adjusted to pH 7.0 by adding NaOH. To prepare LBA plates, 15 g of agar was added to 1 litre Milli-Q water and autoclaved at standard duration of 121 °C for 20 mins. Then cooled to 50 °C and added 1 mL
of ampicillin (50 mg/mL). Then the medium was poured into petri dishes. When the agar was solidified, the plates were wrapped withPara film to avoid contamination and kept at 4 °C until they were used.

To make LBA/IPTG/X-gal, plates were incubated at 37 °C for 30 mins, then 40 µL of IPTG/X-gal mix (Promega Pty. Ltd. Australia) was spread on to the plates, followed by transformed cells were spread on the plates. Then the plates were wrapped in aluminium foil and incubated at 37 °C for 12 –16 h.

6. Composition of separating gel

<table>
<thead>
<tr>
<th>8 % gel</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution A</td>
<td>2.7 mL</td>
</tr>
<tr>
<td>Solution B</td>
<td>2.5 mL</td>
</tr>
<tr>
<td>Milli-Q water</td>
<td>4.8 mL</td>
</tr>
<tr>
<td>10% Ammonium Persulphate</td>
<td>50.0 µL</td>
</tr>
<tr>
<td>N, N, N', N'-tetramethylene diamine (TEMED)</td>
<td>5.0 µL</td>
</tr>
</tbody>
</table>
7. Composition of stacking gel

<table>
<thead>
<tr>
<th>8 % gel</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution A</td>
<td>0.67 mL</td>
</tr>
<tr>
<td>Solution C</td>
<td>1.0 mL</td>
</tr>
<tr>
<td>Milli-Q water</td>
<td>2.3 mL</td>
</tr>
<tr>
<td>10 % Ammonium Persulphate</td>
<td>30.0 µL</td>
</tr>
<tr>
<td>N, N, N', N'-tetramethylethylenediamine (TEMED)</td>
<td>5.0 µL</td>
</tr>
</tbody>
</table>

8. Solution A

Acrylamide Bis-Solution (Bio Rad, Pty. Ltd. Australia)

9. Solution B

a. 2 M Tris-HCl (pH 8.8) : 75 mL
b. 10 % SDS : 4 mL
c. MQ water : 21 mL

10. Solution C

1 M Tris-HCl (pH 6.8) : 50 mL
10% SDS : 4 mL
MQ water : 46 mL

11. 10% Ammonium Persulphate

Ammonium persulphate : 0.5 g
Milli-Q water : 5 mL
12. Sample buffer

Bromophenol blue : 0.025 g
Xylene cyanol FF : 0.025 g
Glycerol : 3.0 g

Made up to 10 mL using Milli-Q water.

13. Electrophoresis buffer (pH ~ 8.3)

Tris Base : 3.0 g
Glycine : 14.4 g
SDS : 1.0 g
MQ Water : 1.0 Litre
pH was adjusted 7.0 using NaOH/KOH

14. Coomassie blue (SDS gel stain)

Coomassie Blue R-250 : 1.0 g
Methanol : 40.0 mL
APPENDIX B

1. *Melanotaenia fluviatilis* - Brain aromatase cds

GATGAAACGCATGGTAGGGCTGGTCGTCTTCCACCGGGCTGTGCAGA
GGTTGTTGTCGTCCGAGAATCGAAGCTGTTTCTGGAGAAGGTAAG
GAGCAGAATGGAGGAAATGATGTACCTTCTGAGTTCAGAGCTTGGG
GTAAACTCTACGTGGAGCTGGACAAACAAAGTAGATGACTG
GATGCTCACTCCAGACGTGTTGTTTGGGAGGCTGATATGGTAGTA
AATTCTCGTCTTCTGCTGCTGTGTTTTTCCTCTACCTCGAGTAC
CACCGTGCAACTCCAGATGCTCTTGAGCTCTTGAGCTG
TCTATCTACACCAGATTCTCTGGAACACTGCACTGCAACTGCAACT
CTAACAACAAACAATATGGCAGCAGCTTCCGAGGCTGCAGTACTG
AGGACCTTATCTCGAGCAGCTCTCGTCTGCTGAGACTGACTGAC
GCTCAGCCACGTTCCAGGGAACAGGCTGATACGCAGTACTGAC
ATGCTTTTGCTTCTTTGCTTCTGATTTTCAACCTGGAGTCGTTCA
GGAGGAGAGGAAGGAAGTAATTTTTAACTAATCTTGGCATTAAC
AGTTTATTTCTAATGAATCAGCAGATGTTTCGTGTATTCAGACATTGCAAG
TATTAACAGTTAAGGTGCGTATTTTAGCTAGAAAATACATCTCGAGG
AAAGCATGTTTCAATTGTAATTTACTCCCACCATGG
GTCTGCTCAGGTCCAGTCTGTGGAAATGCAGACTATAATAAAGATA
AGTCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAGTACTCTGCGTTGATACCAC
TGCTTGCCCTATAGTGAGTCTGATAG
2. *Melanotaenia fluviatilis* - Ovarian aromatase cds

CGGAGAGGAGACCCCTCCTCAGCCGGCCATCTGCTGTGACCATGTTTCTCAAATGCTACCGCTGGCTCATCACCTGGAATCCCTATGGCGACAAGAAACCTCGTACTGCTGATCTGTTTGCTGCTGGTAGCCTGGAGTCACAGAGACAAAGAAAGCTGTACCAGGTCCTTCTTTCTGTCTGGGTTTGGGGCCACTTCTGTCATATTTTGAGATTTATCTGGACTGGGATTGGCACAGCAAGTAATTACTACAACACCAGATATGGGAGACGCTGCTGACAGCGATTTTGCGTACAGACTCTTCAAGTTTGGCTGGATCCACCAGGACACAAGACAGCAGCTGCAAGATGCCATAGAAAGTCTTGTAGAACAGAAGAGGAGAGATATGGAGCAGGCAGATAAGCTGGACAGCATCAACTTCACAGCAGAGCTCATATTTACGACAGCCATACGGCAGCAGCTTCTTCGTAAGGTACGTCGCTGCTCATTAATGAGAAAGAGCTGCTGACAGCGATTTTGCGTACAGACTCTTCAAGTTTGGCTGGATCCACCAGGACACAAGACAGCAGCTGCAAGATGCCATAGAAAGTCTTGTAGAACAGAAGAGGAGAGATATGG

3. *Melanotaenia fluviatilis* – gapdh partial cds

GTTGCTGTAGCCGAGACTCATCTTCGTCACTAGACAGGAGATATGGAGCAGGCAGATAAGCTGGACAGCATCAACTTCACAGCAGAGCTCATATTTACGACAGCCATACGGCAGCAGCTTCTTCGTAAGGTACGTCGCTGCTCATTAATGAGAAAGAGCTGCTGACAGCGATTTTGCGTACAGACTCTTCAAGTTTGGCTGGATCCACCAGGACACAAGACAGCAGCTGCAAGATGCCATAGAAAGTCTTGTAGAACAGAAGAGGAGAGATATGG

CATTGAGAGCGATTCCACACCAGCATCAAAGATGGAGGAGTGGGTGTT
APPENDIX C

Primer specificity test

Figure 1. Agarose gel analysis for *cyp19a1a* and *cyp19a1b* specificity. Amplified products were obtained from qPCR.

- **M**: Molecular marker
- **1**: Brain primers + Brain plasmid template
- **2**: Brain primers + ovarian plasmid template
- **3**: Ovarian primers + Brain plasmid template
- **4**: Ovarian primers + ovarian plasmid template