# Characterization of candidate genes related to estrogenic activity in *Oreochromis mossambicus*

by

Maria M Esterhuyse

*Dissertation presented for the degree of*

*DOCTOR OF PHILOSOPHY*

*at*

*Stellenbosch University*

Promoter: Prof Johannes H van Wyk

Co-promoter: Prof Caren C Helbing

March 2008

# *Declaration*

*I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.*

*Signature:*

*Date:*

# Abstract

Endocrine disruption is an alteration of the chemical messaging processes in the body. The value of studies- and monitoring of endocrine disruption using techniques included in the field of toxicogenomics is undoubtedly supported by scientific literature over the past four decades, as is demonstrated in Chapter 1 where I review relevant literature on the topic. Clearly, well sustained bio-monitoring will include studies both *in vitro* and *in vivo*, and very well on transcriptional and translational levels. Animals are providing good models for in vivo studies to report or monitor endocrine disruption. It is imperative though to first understand such an animal's biology, especially its endocrine system, and characterize what is considered "normal" for a species before engaging in endocrine disrupting exposures. A multitude of studies report endocrine disruption in relation to reproductive systems, with more recent work illustrating alteration of metabolism related to thyroidogenic disruption within the last decade.

It is therefore essential to consider sex determination and -differentiation when studying sentinel species. Apart from the obvious academic interest in the matter of sex differentiation, altered patterns of sex differentiation in certain appropriate species provide for a very convincing endpoint in monitoring *estrogenic* endocrine disruption. As I approach to study a potential sentinel species for the southern African subcontinent, I set forward to study aspects of endocrine disruption influencing the reproductive system in a piece-meal manner, starting with estrogenic endocrine disruption as this is the best studied facet of the endocrine disruption hypothesis to date. Yet, one learn from vast amounts of literature that in cases where sex is not exclusively determined by the genetic fraction of an individual, a number other characteristics may very well be used to determine estrogenic disruption in ecosystems. Quantitative production of the egg yolk precursor protein (vitellogenin) resides under these characteristics, and in the proposed sentinel, South African tilapiine, *Oreochromis mossambicus* phenotypic sex can be altered by environmental sex determination.

The present study therefore targeted firstly the product most often used in tier I screening processes, vitellogenin (VTG). Specimens of *O. mossambicus* were cultured for this purpose from wild breeding stock, sampled at 5 day

intervals and the transcription levels of vitellogenin gene (*vtg*) studied in those. Hereby, Chapter 2 describes the cloning of partial *vtg* gene and subsequent temporal expression of *vtg* quantitatively in *O. mossambicus*. To shed light on the state of gonadal differentiation sub-samples were subjected to histology, illustrated in Chapter 3. In addition the quantitative *vtg* responses has been described in this study at a transcriptional level, both of adult males and juveniles subjected to low and very high levels of natural estrogens.

In addition, a 3 kb 5' flanking region of *vtg* was cloned and sequenced, and several putative binding sites identified for transcription factors of *vtg*, including several estrogen responsive elements (EREs). These indicate the expected regulational process of *vtg* by estrogens. Subsequently I measured the transcription levels of the only enzyme capable of aromatizing androgens into estrogens, Cytochrome P450 19 (*cyp19*) as has been characterized in Chapter 3.

For stable binding of an estrogen to an ERE, binding of the ligand to its specific nuclear receptor (Estrogen receptor, ESR) is required. Since $E_2$ is known to have different mechanisms of action in vertebrates, the expression levels of the ESRs were evaluated in our sample set after cloning 3 different homologues of ESR in *O. mossambicus*. The results on this matter is discussed in Chapter 4 and provides in addition to data on *vtg* and *cyp19* a platform of "normal" transcription levels of these candidate genes involved in estrogenic endocrine disruption of *O. mossambicus*.

Ultimately, characterization of those candidate genes involved extensively in phenotypic sex, contribute to our understanding of sex determination and differentiation in this species in a small way.

# Opsomming

Endokrien versteuring in mens en dier verander die chemiese boodskappe in die liggaam met merkwaardige gevolge, waarvan wetenskaplike literatuur oor die afgelope vier dekades onteenseglike bewys toon. Huidiglik rapporteer hierdie literatuur hoofsaaklik afwykings in terme van die voorplantingsisteem, hoewel meer onlangse studies ook bemoeid is met metaboliese afwykings wat verband hou met tiroïed versteuring.

Akwatiese diere word tans met groot sukses gebruik om vir endokrien versteurende komponente te toets aangesien sulke middels akkumuleer in waterliggame. Aangesien vis spesies in baie opsigte, veral betreffende die voorplantingsisteem, merkwaardig verskil, is dit dus van uiterse belang om so 'n potensiële spesie se biologie goed te bestudeer.

Een van die Suid-Afrikaanse tilapia spesies, *Oreochromis mossambicus*, word tans bestudeer met die oog op monitering van endokrien versteurders in Suider Afrika. Hierdie varswater spesie wat verwant is aan die Nyl tilapia (*O. niloticus*) en Bloukurper (*O. aureus*) word ook veral in akwakultuur gebruik.

Dus het hierdie studie beoog om uitdrukkingsvlakke van sekere kandidaat-gene wat kwantitatief geslagsspesifiek is, te bestudeer in Mosambiek tilapia. Vitellogeen (VTG, voorloper proteien van dooier in eierlêende diere) word onder andere differensiëel verskillend in mannetjies en wyfies vervaardig – in wyfies baie meer as in mannetjies vanweë hul funksie om eiers te lê. Geneties kan beide mannetjies en wyfies dus vir hierdie geen (*vtg*) kodeer, maar wel kwantitatief reguleer. 17β-Estradiol ($E_2$), 'n steroiëd hormoon wat ook teen verskillende vlakke in mannetjies en wyfies voorkom, is bekend daarvoor om die uitdrukking van *vtg* te beheer. $E_2$ word geproduseer vanaf cholesterol deur 'n reeks ensimatiese stappe, gekataliseer deur verskeie Sitochroom P450 ensieme (CYP). Daar is egter slegs een ensiem (CYP19) wat die vermoë besit om 'n koolstof-19 androgeen te aromatiseer om 'n koolstof-18 estrogeen te vorm. Verder, vir $E_2$ om die uitdrukking van *vtg* te reguleer, vereis die proses dat die ligand ($E_2$) met 'n spesifieke kern-reseptor (Estrogeen reseptor, ESR) bind.

Op grond hiervan het ons die uitdrukking van *vtg* onder normale omstandighede bestudeer in ontwikkelende Mosambiek tilapia. Klonering en volorde-bepaling van gedeeltelike *vtg* en ook die promoter area hiervan werp in

hierdie studie verdere lig op regulering van hierdie veelbesproke geen in verband met endokrienversteuring. Gevolglike blootstellingseksperimente, beide aan hoë en lae konsentrasies van estrogeen aan volwasse en ontwikkelende visse, toon tentatief aan dat hierdie spesie uiters geskik is as bio-monitor vir estrogeen-verwante komponente in water. Hierdie inligting, tesame met die onwikkeling van 'n kragtige metode, verskaf dus 'n soliede platvorm vanwaar omgewingstudies nou standaard uitgevoer kan word.

Voorts illustreer hierdie studie op histologiese vlak gonadale ontwikkeling, met bypassende data om ook die uitdrukkings vlakke van beide *cyp19* isoforme asook drie *ESR* isoforme gedurende geslagsontwikkeling te toon. Uiteindelik beskik ons nou oor die geenvolgordes, tegniek en inligting om genetiese aspekte van geslagsontwikkeling rakende *vtg* uitdrukking te karakteriseer en dus hierdie spesie as bio-monitor vir endokrien versteuring te kan gebruik.

# Acknowledgements

I thank my promoters, **Proff JH van Wyk** and **CC Helbing** for supervision; the **University of Stellenbosch** (South Africa) and **University of Victoria** (BC, Canada) for providing infrastructure to conduct the study, and **National Research Foundation** (SA), **Water Research Counsil** (SA) and **University of Stellenbosch** for their financial support.

Specific laboratories (**Institute for Plant Biotechnology**, **Genetwister SA**, **Retief laboratory** (UStell), **Helbing laboratory** (UVic), **Institute for Wine BioTechnology**) were providing kindly their equipment and/or lab space.

I thank the following people for their involvement in this study: **Ronelle Roth**, **Mauritz Venter**, **Lan Ji**, **Nik Veldhoen**, **Sue Bosch**, **Susana Clusella Trullas, Wolfgang Schäffer, John S Terblanche**, **Johannes P Groenewald**, **Fawzia Gordon**, **Melissa Doyle**, **Piet Grobler, Mauro Introna.**

I thank my parents, **Stollie** and **Chrissie Esterhuyse**, **family** and **friends**, **staff** and **students from Ecophysiology laboratory** (UStell), **Helbing laboratory** (UVic), **Institute for Plant Biotechnology** (UStell) for their support during the course of the study.

Finally, this work was done in obedience to my **Heavenly Father**, Whom I thank for the privilege to be part hereof.

# List of abbreviations

| | |
|---|---|
| AF | Activation function |
| AhR | Aryl hydrocarbon receptor |
| ANOVA | Analysis of Variation |
| AP1 | Activating protein 1 |
| AR | Androgen receptor |
| bactin | Beta actin |
| cAMP | Cyclic adenosine monophosphate |
| cDNA | Complimentary DNA |
| CDS | Coding sequence |
| CNS | Central nervous system |
| CRE | cAMP responsive element |
| Ct | Critical threshold |
| CYP | Cytochrome P450 |
| *cyp19* | Cytochrome P450 19 (gene) |
| DEPC | Diethyl pyrocarbonate |
| DES | Diethylstilbestrol |
| DHT | Dihydrotestosterone |
| DM domain | Doublesex/Mab3 domain |
| DMO | DM domain gene on the ovary |
| DMRT1 | Doublesex- and Mab3-related transcription factor 1 |
| DMY | DM domain gene on the Y chromosome |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxyribonucleotide triphosphate |
| dpf | Days post fertilization |
| E2 | 17β-Estradiol |
| EDC | Endocrine disrupting compound |
| eNOS | Endothelial nitric oxide synthase |
| ERE | Estrogen responsive element |
| ESD | Environmental sex determination |
| ESR | Estrogen receptor |
| FSH | Follicle stimulating hormone |
| GATA-4 | GATA binding protein 4 |
| gDNA | Genomic DNA |
| GPCR | G-protein coupled receptor |
| GSD | Genetic sex determination |

| | |
|---|---|
| HLF | Hepatic leukaemia factor |
| IGF-1 | Insulin-like growth factor 1 |
| IPB | Institute for Plant biotechnology, UStell, South Africa |
| iPCR | inverse PCR |
| IWBT | Institute for Wine biotechnology, UStell, South Africa |
| KTS | Tripeptide: Lys-Thr-Ser |
| LH | Luthenizing hormone |
| mER | Membrane-bound estrogen receptor |
| mRNA | Messenger RNA |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NF-kB | Nuclear factor kappaB |
| NRF | National research foundation, South Africa |
| *Oavtg* | *Oreochromis aureus* vitellogenin gene |
| *Omvtg* | *Oreochromis mossambicus* vitellogenin gene |
| PCR | Polymerase chain reaction |
| PI3K | Phosphatidylnositol 3-kinase |
| PLC | Phosolipase C |
| PPAR | Peroxisome Proliferator Activated Receptor |
| QPCR | Quantitative real-time RT-PCR |
| $R^2$ | Correlation coefficient |
| RAR | Retinoid acrid receptor |
| RNA | Ribonucleic acid |
| rpl8 | Ribosomal protein L8 |
| RT-PCR | Reverse transcription PCR |
| RXR | Retinoid X receptor |
| SF-1 | Steroidogenic factor 1 |
| siRNA | Short inhibitory RNA |
| SRY | Sex-determining region Y |
| TSD | Temperature sex determination |
| VBP | Vitellogenin binding protein |
| VTG | Vitellogenin (protein) |
| *Vtg* | Vitellogenin (gene) |
| WBH | Whole body homogenate |
| WRC | Water Research Council, South Africa |
| WT1 | Wilm's tumor gene |
| WT1-KTS | WT1 lacking the KTS tripeptide |

# Table of contents

# General introduction

In the course of the following pages I attempt to resolve some matters of gene expression against the background of a Tilapiine fish as a bio-monitor for pollution by endocrine disrupting compounds (EDCs) in the sub continent of Southern Africa.

EDCs pose a definite threat to modern society as well as the ecology (Jobling & Sumpter 1993; Colborn *et al.* 1993; Segner *et al.* 2006), and needs to be addressed as it appears. For this reason, monitoring areas of possible contamination is mandatory and asks for good understanding of (a) the biology of each component in the monitoring system, and (b) defining the "normal" state before adverse effects can be reported in any such system.

The vast majority of physiological systems currently known to be affected by EDCs are those that activate the parts of the endocrine system associated with the steroid-, retinoid- and thyroid receptors (Crews & McLachlan 2006), of which most often reported to involve the female hormone $E_2$ in relation to other receptors (Segner *et al.* 2006). Estrogens are known to affect especially juveniles and foetuses of pregnant woman (Carey & Bryant 1995), and in particular their reproductive systems.

Some of the most abundant sources of EDCs are from industries such as farming (pesticides), paper mills and plastic factories. In these cases, the compounds enter an ecological system by water runoffs from farms/factory plants whereby the aquatic environment serves as a sink for chemical substances, particularly endangering aquatic animals by the action of EDCs. Consequently, EDCs can be monitored in aquatic animals where these compounds tend to accumulate due to the nature of the biology of these animals (e.g. water running over gills) (Kime 1998; Guerriero & Ciarcia 2006).

In this regard, a product that stands out as a reaction on this subset of EDCs is Vitellogenin. The translated product of the vitellogenin gene (*vtg*) is synthesized in the liver of aquatic egg-producing animals primarily under the influence of $E_2$. Vitellogenin (VTG) is modified extensively post-translationally in the liver, secreted into the bloodstream, and sequestered by the oocytes via specific VTG receptors (Lim *et al.* 1991). Here VTG is cleaved into subunits of yolk proteins. The process of vitellogenesis (formation of VTG) has been studied in many aquatic species throughout the past two decades which include both *in*

*vivo* and *in vitro* effects of marine and freshwater species (Wahli 1988; Ding *et al.* 1993; Dodson & Shapiro 1994; Sumpter & Jobling 1995; Kime *et al.* 1999; Perazzolo *et al.* 1999; Tong *et al.* 2004; Craft *et al.* 2004; Radice *et al.* 2004; Barucca *et al.* 2006).

Kim et al. (2003) illustrated the enhanced and induced effects of $E_2$ on hepatocyte cultures in male and female tilapia (*O. mossambicus*) respectively, and therefore confirm the strong *vtg* inducing effect in this species.

On account of the available literature (Chapter 1), a need was identified to characterize the expression of *vtg* during development of the South African tilapiine, *Oreochromis mossambicus* (Mozambique tilapia) (Chapter 2). *Vtg* Is known to be under regulation of $E_2$, which in turn is dependant on a catalyzation reaction by Cytochrome P450 19 (aromatase). Therefore, the characterization of the aromatase gene (*cyp19*) has been documented in Chapter 3. Along with a histological view of gonadal development, the substrate specific transcription levels of *cyp19* reveals possible production of $E_2$ in cell types other that classically known to be the $E_2$ producing cells. Moreover, *vtg* transcription is potentially regulated by various transcription factors as was found in Chapter 2. However, regulation via $E_2$ requires a ligand-receptor complex of $E_2$ and its nuclear receptor (estrogen receptor, ESR, alias: Er, Nr3A, ER, *er*, *ER*) to several available estrogen response elements (EREs). Chapter 4 subsequently characterizes three homologues of the ESR genes in *O. mossambicus* which also adds to the knowledge of teleostean ESR evolution.

Finally, *O. mossambicus* proves to be a good sentinel for monitoring estrogenic endocrine disruption (Chapter 2). The present study provides hereby a sound platform from where the journey can continue in developing this species as monitor for other types of endocrine disruption, as well as providing a reference point to be referred to for estrogen-induction studies/estrogen bio-monitoring in the Southern African subcontinent.

## REFERENCE LIST

**Barucca M., Canapa A., Olmo E. & Regoli F.**, 2006. Analysis of vitellogenin gene induction as a valuable biomarker of estrogenic exposure in various Mediterranean fish species. *Environmental Research.* 101, 68-73.

**Carey C. & Bryant C.J.**, 1995. Possible interrelations among environmental toxicants, amphibian development, and decline of amphibian populations. *Environmental Health Perspectives.* 103, 13-17.

**Colborn T., vom Saal F. & Soto A.**, 1993. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environmental Health Perspectives.* 299, 163-172.

**Craft J.A., Brown M., Dempsey K., Francey J., Kirby M.F., Scott A.P., Katsiadaki I., Robinson C.D., Davies I.M., Bradac P. & Moffat C.F.**, 2004. Kinetics of vitellogenin protein and mRNA induction and depuration in fish following laboratory and environmental exposure to oestrogens. *Marine Environmental Research.* 58, 419-423.

**Crews D. & McLachlan J.A.**, 2006. Epigenetics, Evolution, Endocrine Disruption, Health, and Disease. *Endocrinology.* 147, s4-10.

**Ding J., Ng W., Lim E. & Lam T.**, 1993. In situ hybridization shows the tissue distribution of vitellogenin gene expression in *Oreochromis aureus*. *Cytobios.* 73, 294-295.

**Dodson R.E. & Shapiro D.J.**, 1994. An estrogen-inducible protein binds specifically to a sequence in the 3' untranslated region of estrogen-stabilized vitellogenin mRNA. *Molecular and Cellular Biology.* 14, 3130-3138.

**Guerriero G. & Ciarcia G.**, 2006. Stress biomarkers and reproduction in fish. In Reinecke,M., Zaccone,G. & Kapoor,B. *Fish Endocrinology*. Science publishers,Enfield, pp 665-692.

**Jobling S. & Sumpter J.P.**, 1993. Detergent components in sewage effluent are weakly oestrogenic to fish: An in vitro study using rainbow trout (Oncorhynchus mykiss) hepatocytes. *Aquatic Toxicology.* 27, 361-372.

**Kim B.H., Takemura A., Kim S.J. & Lee Y.D.**, 2003. Vitellogenin synthesis via androgens in primary cultures of tilapia hepatocytes. *General and Comparative Endocrinology.* 132, 248-255.

**Kime D.E., Nash J.P. & Scott A.P.**, 1999. Vitellogenesis as a biomarker of reproductive disruption by xenobiotics. *Aquaculture.* 177, 345-352.

**Kime D.** 1998. *Endocrine disruption in fish*. Kluwer Academic Press. Boston .

**Lim E.H., Ding J.L. & Lam T.J.**, 1991. Estradiol-induced vitellogenin gene expression in a teleost fish, *Oreochromis aureus*. *General and Comparative Endocrinology.* 82, 206-214.

**Perazzolo L.M., Coward K., Davail B., Normand E., Tyler C.R., Pakdel F., Schneider W.J. & Le Menn F.**, 1999. Expression and Localization of Messenger Ribonucleic Acid for the Vitellogenin Receptor in Ovarian Follicles Throughout Oogenesis in the Rainbow Trout, Oncorhynchus mykiss. *Biology of Reproduction.* 60, 1057-1068.

**Radice S., Fumagalli R., Enzo C., Ferraris M., Frigerio S. & Marabini L.**, 2004. Estrogenic activity of procymidone in rainbow trout (*Oncorhynchus mykiss*) hepatocytes: a possible mechanism of action. *Chemico-Biological Interactions.* 147, 185-193.

**Segner H., Eppler E. & Reinecke M.**, 2006. The imact of environmental hormonally active substances on the endocrine and immune systems of fish. In Reinecke,M., Zaccone,G. & Kapoor,B. *Fish endocrinology*. Science publishers,Enfield, pp 809-865.

**Sumpter J.P. & Jobling S.**, 1995. Vitellogenesis as a biomarker for estrogenic contamination. *Environmental Health Perspectives Supplements.* 103, 173.

**Tong Y., Shan T., Poh Y.K., Yan T., Wang H., Lam S.H. & Gong Z.**, 2004. Molecular cloning of zebrafish and medaka vitellogenin genes and comparison of their expression in response to 17[beta]-estradiol. *Gene.* 328, 25-36.

**Wahli W.** 1988. Evolution and expression of vitellogenin genes. *Trends in Genetics.* 4, 227-232.

Chapter 1

# Sex determination and differentiation control pathways in fish and the relevance to bio-indicating endocrine disruption in aquatic systems*

**TABLE OF CONTENTS**



## INTRODUCTION

Endocrine disrupting compounds (EDCs) pose a definite threat to modern society as well as the ecology (Colborn *et al.* 1993). For this reason monitoring areas of possible contamination is mandatory, which asks for good understanding of (a) the biology that is monitored, and (b) what the "normal" state is before we can report adverse effects in any monitoring system. The vast majority of physiological systems currently known to be affected by EDCs are those that activate the parts of the endocrine system associated with the steroid-, retinoid- and thyroid receptors (Crews & McLachlan 2006), of which most often involve the female hormone 17β-Estradiol ($E_2$) in relation to other receptors. $E_2$ is known to affect especially juveniles or a fetus of pregnant woman (Carey & Bryant 1995), in particular their reproductive systems. Moreover this steroid hormone is reported to affect some male-associated traits (Gunderson *et al.* 2001; Pawlowski *et al.* 2004; Santos *et al.* 2006). Furthermore, some of the most

* Submitted for publication

abundant sources of EDCs are from industries such as farming (pesticides), paper mills and plastic factories. In these cases the compounds enter ecological systems by runoffs from farms/factory plants. Consequently, EDCs can be monitored in aquatic animals where these compounds may accumulate by the nature of the physiology of these animals (e.g. water running over gills) (Kime 1998).

Processes of sex determination and differentiation are reviewed here, with emphasis on aquatic animals, specifically fish, followed by a discussion on how the "-omics" can be used to assist us in monitoring areas of possible EDC contamination.

Sex determination has been defined by the process that determines whether the bipotential gonad primordium will develop into a testis or ovary (Schartl 2004). For the purpose of this discussion, I refer to *sex determination* specifically where sex has been "pre" determined/determined before or at the time of gonad differentiation and not cases where sex changes during the lifespan of an individual as has been found in some species of fish (Shapiro & Boulon 1982; Godwin & Thomas 1993). In this regard, phenotypic sex can be determined either genetically or by the environment. What is understood under genetic sex determination (GSD) is species that have their gonochoristic sex determined by (i) specific sex chromosomes (or areas on the sex chromosomes), (ii) species that have sex specific areas on autosomes or (iii) species that express epigenetic effects to determine sex. In contrast to GSD, environmental sex determination (ESD) is also briefly discussed although this phenomenon is greatly illustrated by reptiles and some amphibians and is considered additional rather than explanatory.

Secondly, *sex differentiation* involves the epigenetic and genetic determination effects that leads to the differentiation of a particular type of tissue derived from the bipotential primordium. These processes are subject to the biochemical pathways with specific gene products as a result of sex determination.

A number of studies have demonstrated the existence of genes that specifically respond to a particular environmental state by triggering a given pattern of morphogenetic changes (Pigliucci 1996). Some species of fish demonstrate a high level of plasticity in sex-determining mechanisms which make them particularly sensitive to environmental pollutants capable of

mimicking or disrupting sex hormone actions. These EDCs are often present in the environment. Although plasticity of sex reversal in teleost fish has some advantages in commercial fish farming (Beardmore *et al.* 2001), environmentally induced sex reversal in lower vertebrates can pose a direct threat to proper functioning of populations and therefore also the conservation status of ecosystems and its inhabitants, including invertebrates, fish, amphibians, reptiles, birds and mammals (Uguz *et al.* 2003). The use of animal models for *in vivo* or *in vitro* modeling (a) shed some light on mechanism of action which these compounds exert, and (b) monitor the levels of these compounds as they may appear during certain seasons or upon accumulation after years.

Finally, the importance of environmental interaction on the organizational level (Guillette *et al.* 1995) and implications of this interaction in terms of response biology, makes the field of toxicogenomics such an attractive one. Toxicogenomics is a fast evolving science which addresses the global gene expression changes in biological samples exposed to toxic agents (Lee *et al.* 2005). Moreover, combining information on sex related genetic markers with actual phenotypic responses, allows for a historical view on pollution events and the implication on population dynamics in natural populations. The understanding of how animals maintain homeostasis in a changing environment, including subtle interaction between genes and environmental factors, link directly to the recent evolved toxicogenomics approach.

## SEX DETERMINATION

Traditionally sex determining mechanisms in vertebrates include (i) male heterogamety (XX female/XY male), (ii) female heterogamety (ZZ male/ZW female), (iii) polygenic determination (where sex is determined in the zygote by many factors with individually small effects, perhaps also with an arbitrary environmental effect. Thus the cumulative effect of many factors controls sex), (iv) ESD (where sex is determined during embryogenesis in response to the local environment), and (v) arrhentoky (a genetic system in which males arise from unfertilized eggs, females from fertilized eggs – implicating that determination of sex can be environmental (based on fertilization) or genetic (based on ploidy) (Bull 1983; Bull 1985).

## *I Genetic sex determination*

*Sex chromosomes*

Evolution of sex determination mechanisms was considered for the first time by Darwin (1871), and it is generally accepted that sex chromosome heteromorphism may have evolved from homomorphic chromosomes with an autosomal ancestry (Muller 1914; Muller 1918). According to Bull (1983), sex chromosome differentiation is a process limited to certain types of sex determining mechanisms: two-factor systems without environmental influences (male and female heterogamety, maternal monogeny), and the few multiple-factor systems in which YY does not arise. A major influence as to whether sex chromosomes evolved, and specifically a sex determining area on such chromosome(s), is whether it is exposed to recombination (or suppression thereof) in at least all diploid organisms (Charlesworth *et al.* 2005). It is even suggested that, on account of asexual decay, the recombining part of the Y chromosome (in mammals) will become smaller and finally vanish (Vallender & Lahn 2004). The sex determining area, because of recombination suppression, may persist, but finally will be lost and either a new Y chromosome can emerge from an autosome, or the *mode* of sex determination may change (Vallender & Lahn 2004). However with regards to evolution of sex chromosomes, there are processes that may work against the asexual decay of the sex chromosome, such as genes associated with spermatogenesis, which can accumulate on the male chromosome in mammals (Schartl 2004). Detailed discussions on sex chromosome evolution and sex determining mechanisms in vertebrates are addressed in several reviews (Charlesworth 1991; Barton & Charlesworth 1998; Volff & Schartl 2001; Charlesworth 2002; Koopman & Loffler 2003; Schartl 2004; Charlesworth 2004; Sakata & Crews 2004; Page *et al.* 2005) and are considered complementary to this discussion rather than explanatory.

In vertebrates, genotypic sex determination is built into sex chromosomes which individuals inherit. Autosomal chromosomes include all the chromosomes that are shared by males and females while a single sex chromosome pair in mammals include a larger X chromosome and a smaller Y chromosome. The genetic make-up of these differs between sexes and sex determination depends on the combination of these chromosomes. The genotypic sex is therefore determined at the time of conception (Sherwood, Klandorf & Yancey, 2005).

In fish, sex chromosomes have been detected and found in approximately 10% of the species examined to date, to be the major role-player in sex determination (Devlin & Nagahama 2002). The Y chromosome in this group of animals is dominant over the X chromosome in determining maleness, while the W chromosome is dominant over the Z, X and the Y chromosomes in determining femaleness. Males with XY and YY chromosome pairs, as well as WW, WZ, WY and WX combinations in females have been reported in teleosts (Solari 1994; Uguz *et al.* 2003).

To determine the presence of particular sex chromosomes in species in which these are known to exist, the use of genetic markers has been exploited. Sex specific markers were authentically chromosome specific, but markers associated with phenotypic sex (regardless whether it appear on the sex chromosome or not) are being investigated increasingly. The latter has been reviewed for several fish species by Devlin et al. (2001), and are very effective tools for studying phenotypic sex disruption in those species which follow a pure genetic sex determining regime, at least to some stage, during development. For fish, Devlin and Nagahama (2002), in a well documented review, discuss sexual differentiation types, including (a) gonochoristic species which possess ovarian or testicular tissues, and (b) hermaphroditic species that can initially mature either as males or females. An extreme example mentioned elsewhere, is that of the cyprinodont *Rivulus marmoratus*, which is a self-fertilizing, simultaneous hermaphrodite (Schartl 2004). Both these differentiation types are potentially influenced by external factors such as, environment, behaviour and physiological factors, putatively affecting both somatic and germ cells (Devlin & Nagahama 2002; Uguz *et al.* 2003). As for environmental factors influencing gonochoristic species, the most prominent seem to be the influence of incubation temperature of the embryos and larvae, and environmental contaminants mimicking some agents in the signal transduction pathways related to phenotypic gender and secondary responses in the endocrine system of these fish. Research concerning most species studied to date, were conducted in the laboratory, but lately some studies in the field have shown temperature to influence the direction of sex differentiation (Piferrer *et al.* 2005; Black *et al.* 2005).

*Epigenetic*

In 1759 Caspar Wolff proposed an alternative theory to what had been believed until that time for the mechanism of development – that of epigenesis (Wolff 1759). According to this, the adult gradually develops from a rather formless egg as originally proposed by Aristotle, but as Wolff made careful observations during chicken development, the early embryo is entirely different from the adult and development is progressive, with new parts being formed continually.

*Epi*genetics ("epi-"= Greek: upon, in addition, over, besides) in the earlier days referred to the multitude of ways genes give rise to the phenotypes due to different expression and activation (Waddington 1942). As the source of available scientific information increased, it also included relevant forms of epigenetic information such as the histone code or DNA methylation (Tycko 2000). Today epigenetic states can be divided into three broad categories: euchromatin, constitutive heterochromatin and facultative heterochromatin (Arney & Fisher 2004).

Epigenetic effects on living organisms can be extended to include influences of both the internal and external environments of such an organism and environmentally induced changes can occur at all levels of biological organization, from molecular to the organism's behavior and place in society (Figure 1).

Epigenetic silencing of genes refers to nonmutational gene inactivation that can be faithfully propagated from precursor cells to clones of daughter cells. Several studies to date has confirmed that epigenetic change through DNA methylation (a process by which methyl groups are added to the base cytosine residues in CpG dinucleotides in DNA) is generally known to suppress expression of a gene, whereas less DNA methylation is associated with gene activation (Ellegren 2000) as is reviewed by Crews and McLachlan (2006), Esteller (2007) and Tycko (2000).

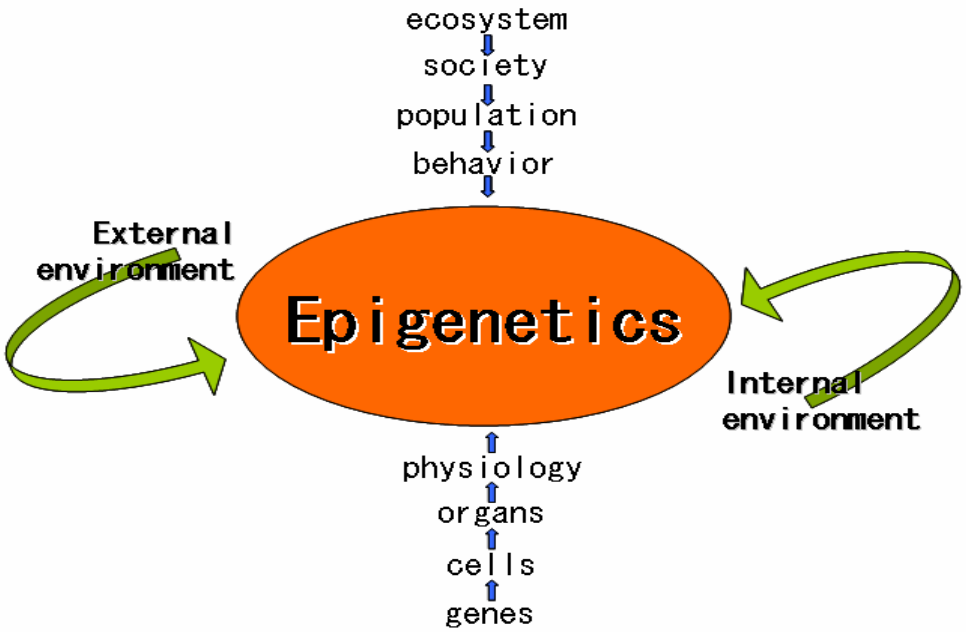


**Figure 1**. Epigenetics being influenced by both the internal (genetics and beyond) and external (individual-independent) environment. Adapted from Crews and McLachlan (2006) and Wong et al. (2005).

If epigenetic distress occurs during specific stages of development, these changes are permanent and can be inherited by offspring (McLachlan 2001; Welshons *et al.* 2003; Arney & Fisher 2004; Anway *et al.* 2005; Duman & Newton 2007). The steroid hormone, 17β-Estradiol ($E_2$), has been prominently implicated in hormonal epigenetic effects and has been reported to cause persistent alterations in gene expression and reprogramming of cell fate – a phenomenon called epigenetic imprinting (Alworth *et al.* 2002; Huang *et al.* 2005) which may provide a potential mechanism for the concept of genetic assimilation (Waddington 1953). Proof of imprinted genes maintaining a defined DNA methylation pattern that is transmitted through the mammalian male or female germline has been reported (Anway & Skinner 2006; Chang *et al.* 2006). However, recently, augmentation of effects of interferon-stimulated genes by reversal of epigenetic silencing has been documented by Borden, (2007) proposing a possible therapeutic application against melanoma.

Important to keep in mind is that, as for transcription, regulation is maintained mainly through DNA binding proteins that affect RNA polymerase recruitment or local chromatin structure, but there is also a functional relationship between gene expression and nuclear organization. The nucleus of eukaryotic cells have different compartments which include the nucleolus, nuclear envelope and nuclear pores, each having distinct functions within the cell. Nuclear pores provide a gateway, enabling the exchange of proteins and mRNA between the nucleus and the cytoplasm, whereas the nucleolus serves as the site for ribosomal component assembly and synthesis. Transcriptionally active genes are most often found at the edge of such territories, and it has been proposed that the localization enables better access to stable transcriptional "factories" between territories (Ahmed & Brickner 2007). Recent work has been reviewed by Ahmed & Brickner (2007) which dictate that certain genes can undergo dynamic recruitment to the periphery upon transcriptional activation. Localization to the periphery for such genes has been suggested to improve mRNA export and favor optimal transcription which is again epigenetic in principle.

Epigenetic modulation via DNA modulation occurs twice during development: first, in the lineage-specific pattern during gastrulation and secondly, during the germ-line-specific pattern in the gonad after sex determination (Reik *et al.* 2001). In mammals, the lineage-specific pattern

establishes the DNA methylation for somatic cell development after fertilization whereas the germ-line DNA methylation pattern is established during gonadal development and is sex specific (Anway & Skinner 2006). It is therefore an obvious consideration when studying endocrine disruption, in particular during the time of sex determination of vertebrates, and provides some understanding for the fact that the embryonic period is the most sensitive for chemical and environmental effects on the epigenetics of the male germ line (Anway *et al.* 2005; Chang *et al.* 2006). A remarkable example of such endocrine disruption is illustrated by Kelce and colleagues (Kelce *et al.* 1994; Kelce *et al.* 1997) where a pregnant rat was transiently exposed to the endocrine disruptor, the fungicide, vinclozolin which caused spermatogenetic cell defect and subfertility in the F1 generation up to the fourth (F4) generation at which time no further examinations were performed.

Hormones are known to imprint epigenetically in non-mammalian vertebrates of which examples include the African clawed frog (*Xenopus laevis*) (Andres *et al.* 1984; Kloas 2002; Urbatzka *et al.* 2007) in which EDCs have been reported to result in epigenetic distress (Anway & Skinner 2006; Chang *et al.* 2006). Epigenetic memory in the vitellogenin (VTG) gene (*vtg*) shows that hormonal treatments early in life alter the response of hormonally regulated genes to the same or different hormones later in life (Andres *et al.* 1984; Edinger *et al.* 1997).

Further aspects of epigenetics complementary to this discussion can be found reviewed by Crews and McLachlan (2006) and Jones and Takai (2001).

### *II Environmental sex determination (ESD)*

In addition to epigenetic effects, exogenous effects overriding the genetic pre-determined sex has been studied, mostly with regards to monitor endocrine disruption, and the mechanism of action of these EDCs in these species or secondly to determine the effects of temperature sex determination (TSD) in species where it occur.

Many discussions have seen the light on the selection pressures underlying the evolution and maintenance of ESD, of which TSD is a specific subset. A hypothesis by Charnov and Bull (1977) claims that ESD will evolve when the environment is patchy (e.g. resource distribution, predation) and the

sexes differ in the relative benefits gained by specific niches – a hypothesis most consistent with available data whereas some additional hypotheses emphasize phylogenetic inertia (i.e. no current advantage to TSD) or inbreeding avoidance (Sakata & Crews 2004). Evolution of TSD illustrates that specific incubation temperature of eggs produce offspring with traits that are differentially advantageous to one sex over the other, with consequent selection pressure for the sex that benefits most from the trait to become more abundant at that specific environment.(Sakata & Crews 2004).

**SEX DIFFERENTIAION**

Sex differentiation has been defined by Sakata and Crews (2004) as the process that sculpts the masculinity and femininity of the individual and is said to be dependent on gonadal sex steroid exposure perinatally and in adulthood.

The bi-potential gonad in gonochoristic species is undifferentiated in males and females until a critical stage when sex determination mechanisms (genetically driven) dictate development into either a testis or ovary, providing an opportunity to delineate the molecular pathways that lead to distinctly different tissues. Even though the components of the machinery that determines sex seem to be conserved between many vertebrates, their interaction and most importantly the initial "switch" is not the same, giving origin to this enormous variety of chromosomal sex determining mechanisms in the animal kingdom, especially with regards to fish (Charlesworth 1991; Uguz *et al.* 2003; Charlesworth 2004).

A paradigm, known as the *organization-activation concept* (Arnold & Breedlove 1985) has been pointed out as the major infrastructure guiding research into the mechanisms underlying the display of social behaviour (Sakata & Crews 2004) and in brief posits that organizational effects which occur early in an individual's lifetime induce permanent effects, whereas activational effects usually are transitory actions occurring during adulthood (Guillette *et al.* 1995). Therefore, sex differences in gonadal hormone secretion perinatally cause the differential development of the neuroendocrine system in males and females, which in turn establishes differences in circulating concentrations of steroid hormones in adulthood. These differences in the levels of hormones in adulthood elicit different behaviour in males and females. Furthermore, sex

differences in early sex steroid exposure organize neural circuits to react differently to sex steroid hormone exposure in adulthood (Sakata & Crews 2004).

*Genes related to direct differentiation of sex*

In general, studies have shown SRY (or SOX, SRY-related HMG box in non-mammalians), GATA-4, WT1-KTS and SF-1 to be sex determining factors in mammals and some other vertebrates, and dose-dependant interactions among these genes are critical to initiation of the cascade of sex differentiation (Parker *et al.* 1999; Knower *et al.* 2003).

With regards to genetic control of sex determination in fish, a hypothesis has been put forward on the basis of the situation in Medaka (*Oryzias latipes*), explaining possible evolutionary mechanisms in the duplication of sex determining genes and their regulatory regions (Schartl 2004). Moreover, the genetic factors involved in sex differentiation are becoming more defined. Genes indicative of some sort of relatedness to determination and differentiation of sex are being identified and are becoming more abundant for some species. In non-mammalian vertebrates, most genes that function downstream of the mammalian *Sry* have been found intact and active, providing the blueprint for a totally functional phenotypic sexual mechanism, regardless the presence or absence of a sex chromosome.

In fish, a factor influencing determination of sex in tilapia (*Oreochromis sp*.), although downstream in the sex determining pathway, is Cytochrome P450 19a (aromatase ovary type, CYP19a). *Cyp19* genes encode Cytochrome P450 aromatase (CYP), a heme-binding protein of the enzyme complex responsible for the conversion of $C_{19}$ androgens into $C_{18}$ estrogens. This enzyme complex consisting of CYP19 and the flavoprotein NADPH-cytochrome P450 reductase, is bound to the membrane of the smooth endoplasmic reticulum of several steroidogenic cells (Conley AJ & Walters 1999). In teleosts studies to date, two homologues of *cyp19* have been identified. Chang et al. (2005) analysed the promoter structure of two *cyp19* homologues and found binding sites within the promoter of the ovary form which are related to sex differentiation (Sry; Wt1-ktS; Sf-1/Ad4 BB). These do not occur in the brain homologue of this gene (discussed below). The argument currently stands that although genes resembling SRY have not been identified in lower vertebrates, other than DMRT1 (denoted DMY in Medaka) (Matsuda *et al.* 2002), the selective existence

of diverse sex determination factors in the tilapiine *cyp19a* (*tCyp19a*) strongly implies that this form of *cyp19* is a down-stream target of the sex determining pathway in tilapia (Chang *et al.* 2005) due to its ability to regulate estrogen synthesis. The second *cyp19* (*cyp19b*), expressed predominantly in brain tissue, has been identified in several teleosts and is indicated to be related to adaptation of the animal to the environment (Sakai *et al.* 1988; Tchoudakova & Callard 1998; Tong & Chung 2003).

In general, it seems that the dimorphic expression of the two known homologues of *cyp19* is regulated by the promoter region and the way it is spliced (Tong & Chung 2003). Among teleosts, *cyp19a* exclusively has Sf-1 binding to its promoter region whereas *cyp19b* exclusively has ERE (Kazeto *et al.* 2001; Tong & Chung 2003; Chang *et al.* 2005). Along with the high levels of expression of *cyp19b* in the brain, these differential binding sites in the promoter area indicate its main involvement in estrogen-mediated neural estrogen synthesis. Moreover, some binding regions were found in the 5'-flanking region of *cyp19a*, which are known male sex-determining factors in mammals, but the expression of this gene is completely absent in male tilapia gonads (Chang *et al.* 2005). Same binding regions were found also for zebrafish and goldfish (Tchoudakova & Callard 1998; Kazeto *et al.* 2001; Kishida & Callard 2001; Tong & Chung 2003). This dichotomous nature of the two transcript-homologues for *cyp19* appears to be similar among the vertebrates in spite of the evolution of two distinct *cyp19* genes in teleost fish, which suggests that the common nature of *cyp19* genes and their tissue-specific transcriptional mechanism have been maintained despite an evolutionary duplication (Kazeto *et al.* 2001).

In addition to Sf1 being a common transcriptional factor in *cyp19a*, and not so in the brain homologue from fish to mammals, Kazeto et al (2001) illustrates in zebrafish that ethinylestradiol and methyltestosterone (an aromatizable androgen) modulates the expression of *cyp19b*, whereas no effect by these hormones was found on *cyp19a* transcription. The mechanism of the transcriptional regulation of *cyp19* in the brain by sex steroids appears to be different between mammals and teleost fishes (Kazeto *et al.* 2001).

In addition, both *cyp19* genes in zebrafish has been found to contain one or more cAMP responsive element (CRE) in its 5'-flanking regions (Kazeto *et al.* 2001; Tong & Chung 2003). In mammals transcription of *cyp19* is stimulated by gonadotropins via the cAMP second messenger (Steinkampf *et al.* 1987) in

ovarian granulosa cells. Therefore, the presence of CRE in *cyp19* promoter area of zebrafish, Medaka and Atlantic stingray indicate possible similar regulation via cAMP such as in mammals. What makes this more interesting is that in Zebrafish, three of these sites were found in the 5'-flanking are of *cyp19a* as apposed to the one in *cyp19b* (Kazeto *et al.* 2001). In Medaka, only the *cyp19a* is known to have a CRE in its 5'-flanking region (Tanaka *et al.* 1995). No CRE sites were reported for tilapia (*Oreochromis niloticus*) by Chang et al (2005).

In flounder (*Platichthys flesus*) the activators of aryl hydrocarbon responsive elements (AhR), polycyclic aromatic hydrocarbons, reduced the activities of steroidogenic enzymes in the ovarian follicles (Rocha Monteiro *et al.* 2000). AhR was found in the 5'-flanking regions of Zebrafish *cyp19a* and b and propose a potential site for endocrine disruption since aryl hydrocarbons may regulate aromatase transcription directly (Kazeto *et al.* 2001; Tong & Chung 2003). As far as *start* of transcription regulation goes for the two homologues found in teleosts – one transcription initiation site has been found in the ovarian form, but multiple sites in the brain-specific transcript (Tanaka *et al.* 1995; Tchoudakova *et al.* 2001; Kazeto *et al.* 2001; Tong & Chung 2003).

Trying to find the mechanism of sex determination in these fish, a variety of hypotheses has been made and tested, one of which the influence by *cyp19* genes, and more potentially the regulatory elements thereof. The 5'-flanking region of *cyp19a* has not been found to contain an estrogen responsive element (ERE), whereas that of *cyp19b* (brain form) does (Chang *et al.* 2005), and therefore, along with the high expression of latter gene in the brain, indicate its main involvement in estrogen-mediated neural estrogen synthesis. Moreover, some binding regions were found in the 5'-flanking region of Cyp19a, which are known male sex-determining factors in mammals, but the expression of this gene is completely absent in male tilapia gonads. Same binding regions were found also for zebrafish and goldfish (Callard & Tchoudakova 1997; Tchoudakova *et al.* 2001). In conclusion, this may indicate a decisive role in sex differentiation in these fish species, but not necessarily a mechanism totally dependent on genetic determination of sex at the time of fertilization.

Another gene family found to be prominent in phenotypic sex of many non-mammalian vertebrates is the DM domain genes, of which one homologue is found on the W chromosome of birds and on autosomes of most fish: *Dmrt1* (*Doublesex* and *Mab-3*-related transcription factor 1) (Raymond *et al.* 2000). DM-

domain genes encode novel zinc finger transcription factors, and in particular, *Dmrt1* is thought to be playing a pivotal role in determining sex for birds and many fish species. It is referred to by some as the "*Sry* gene in non-mammalian vertebrates" (Schartl 2004).

In tilapia (*O. niloticus*) the *Dmrt1* homologue (*tDMRT1*) possess a male-specific binding motif referred to by the authors as *DSX* (Guan *et al.* 2000), which seems to be testis-specific. A homologue of this gene is found only to be expressed in the ovary (denoted *tDMO*), and lacked the *DSX* motif. The absence of *DSX* in *tDMO* suggested a close linkage between *Sox* and *DMRT1* gene products in sex determination pathways, at least for this tilapiine species. Furthermore phylogenetic analysis of these two homologues in Nile tilapia strongly suggested that *tDMRT1* is a homologue of the human *DMRT1*, whereas *tDMO* represents a novel gene (Guan *et al.* 2000). Adding to this, the authors sequenced the 5'-flanking regions for these two gene homologues, and a number of putative motifs are present in both upstream regions. But since a comparison of these regions shows little nucleotide homology, it has been suggested that expression of *tDMRT1* and *tDMO* are probably controlled by different regulatory elements. It is important to note here that when using phenotypic XX males, only *tDMRT1* (and not *tDMO)* have been found to be expressed in the testis, indicating that *tDMRT1* may not be a Y-linked gene. Since the expression thereof restricted to testicular tissue, therefore fails to serve as a genetic sex specific marker.

Devlin & Nagahama (2002) point out that, along with the expression data of *DMRT1* in trout (Marchand *et al.* 2000) and Medaka (Brunner *et al.* 2001), this gene is expressed in these species in response to testis differentiation and is therefore located downstream in the sex determination pathway, and thus the genomic material not being sex linked. There is, however in Medaka (*Oryzias latipes*), a DM-domain gene on the Y chromosome (*DMY*), and found to be expressed in male somatic gonadal cells at the time of initial sex determination (Matsuda *et al.* 2002). It is noted by some (Lutfalla *et al.* 2003; Kondo *et al.* 2003; Volff *et al.* 2003) that a homologue of *dmrt1* in Medaka (*dmrt1bY*), originated during evolution of the genus *Oryzias*, and it is therefore not surprising that this gene has not been found to be the main sex determination gene of other fish species (Kondo *et al.* 2003).

Finally, the genes coding for estrogen and androgen receptors are prominent in the usage of these hormones which are important in the primary differentiation of sex in all gonadotophic animals. These genes are discussed in the following section along with the ligands they bind.

Differential expression of all genes discussed above, is regulated by binding of upstream components to their promoters – often this regulation also involves binding of some gene products to receptors on or within cells. Recognition by promoters or receptors is often mistaken for their upstream components because of the mimicking ability of certain chemicals or unnatural occurring hormones display – those are called EDCs.

*Hormonal basis of sex differentiation*

Endocrine control of sex differentiation is a topic well studied in mammals and to a lesser degree in non-mammalian vertebrates, including fish. It involves a complex interplay of gonadotropins and steroids produced by the pituitary or gonads and brain respectively (Camerino *et al.* 2006). Steroid hormones directly affects the germ-cell development by acting locally in the cells, but may also influence the organs and other cell types involved in secondary sex differentiation. This multitude of biochemical, neurological, and physiological pathways provide necessary plasticity for gonadal development to proceed in context with intrinsic and environmental factors. The complexity of this system provides for many levels at which reproduction can be disrupted.

On a histological level, male or female germ lines form together with the somatic organization of the gonads. Yamamoto (1969) explained that steroid hormones are the natural inducers of gonadal sex differentiation and sexual dimorphic traits in fish. Indeed, the genetically prescribed sex can be overridden with exogenous steroids if applied at the appropriate time (window) and dose during early development (see discussion on organization-activation above). The common theory of steroid action predicts that steroids modulate gene transcription by interaction with nuclear receptors, acting as ligand dependent transcription factors (Evans 1988).

Primary role players amongst the steroid hormones include androgens and estrogens, the latter of which is an aromatized product of the former in a cytochrome-catalyzed reaction. Numerous studies have confirmed that steroid hormones are required for induction and maintenance of gonad differentiation

(Nakamura & Nagahama 1989; Kwon *et al.* 2000; Kobayashi *et al.* 2003), however a multitude of effects are described to be totally or partially dependant on $E_2$. Therefore, I briefly mention androgens here and continue to discuss estrogens in more detail thereafter.

***Androgens:*** Testosterone, the major endogenous androgen, is transformed in the central nervous system (CNS) by 5$\alpha$-reductase to the pure AR-agonist dihydrotestosterone (DHT) or by CYP19 to the major estrogen, $E_2$ (Tsai & O'Malley 1994; Patchev *et al.* 2004). Androgens are involved in homeostasis and maintenance of male reproductive functions, including sexually dimorphic characteristics in non-genital tissues, and is recognized for numerous aspects of the central nervous system function (Mooradian *et al.* 1987; Patchev *et al.* 2004).

***Androgen receptor:*** The action of androgens in target cells is mediated by high affinity intracellular receptors which belong to the steroid-thyroid hormone superfamily of ligand-modulated DNA binding protein that act directly in altering cellular gene expression. Following ligand induction, androgen receptor (AR) regulates transcription by binding as a homodimer to specific upstream DNA sequences in the target genes (Tsai & O'Malley 1994). Three major functional domains have been identified: (a) transcription regulating amino-terminal domain, (b) central DNA binding domain and (c) carboxyl-terminal hormone binding domain (Rana *et al.* 1999). Nuclear receptors interact with distinct DNA sequences in the promoter region of target genes – an action which is a pre-requisite for their influence on gene transcription. There are however some genes specific to the central nervous system whose transcriptional regulation depends on the exclusive presence of androgen responsive elements. Patchev *et al.* (2004) review these and other factors influencing the functioning of ARs in mammals. In some teleost fish, the AR gene has been sequenced and found to be coded for by either of two homologues (Park *et al.* 2007; Harbott *et al.* 2007). To date no evidence was found to support any departure of mechanism of action of androgen receptors in fish from those in mammals.

***Estrogen:*** $E_2$ is a steroid hormone classified as both a true endocrine hormone and neurotransmitter (Falkenstein *et al.* 2000), from here onwards referred to as the "dual nature" of estrogen (Figure 2). Apart from the

reproductive functions of the hormone, $E_2$ is also a factor in maintenance of bone mass and exhibits cardio-protective effects (Grumbach 2000; Vasudevan *et al.* 2002) in mammals. Furthermore, it exhibits effects known to be permanent organizing effects on the CNS development and general neurotrophic factors in many different brain regions and life stages (Maclusky & Naftolin 1981; Tchoudakova *et al.* 2001).

Production of estrogens is regulated by the hypothalamic-pituitary axis, where the hypothalamus secretes gonadotropin-releasing hormones that further increase or decrease FSH and LH which in turn regulates estrogen production in granulosa cells (Murray *et al.* 1993). More specifically, in fish and mammals, cholesterol is transformed by a CYP11a catalyzed reaction into pregnenolone to be catalysed by CYP17 into androgens (Tsuchiya *et al.* 2005). Classically, testosterone produced by the thecal cells then serves as an essential substrate for *cyp19a* in granulosa cells, to synthesize $E_2$. $E_2$ can thus be biosynthesized only by *cyp19* and 17β-hydroxysteroid dehydrogenase (17β-HSD) from androstenedione via testosterone or estrone respectively (Figure 2). Metabolites of $E_2$ include catachol metabolites via hydroxyestradiols produced by either CYP1A1/2, CYP3A4 or CYP1B1 which can finally be metabolized by catechol *O*-methyltransferases to result in methoxyestradiols which in turn can either be non-carcinogenic and inhibits the proliferation of cancer cells (2-Methoxyestradiol), or alternatively cause cell damage (Estradiol-3,4-quinone, Figure 2) (MacLusky *et al.* 1987; Tsuchiya *et al.* 2005).

Under the dual nature of estrogen (Figure 2), its mechanisms of actions can be classified into two sets: neuro-endocrine functions which include its nature of a neuro transmitter or secondly its endocrine hormone functions which include a variety of actions, mainly by acting as ligand to bind to receptors on or within cells. As either a neurotransmitter or endocrine hormone, $E_2$ has illustrated modes of action via a genomic and non-genomic mechanism.

Firstly, $E_2$ in brain tissue binds to intracellular receptors, which then act as transcription factors to influence behavior, where changes in aromatase activity result from slow steroid-induced modifications of enzyme transcription (Balthazart *et al.* 1996; Falkenstein *et al.* 2000). More recently, rapid (minutes to hour) non-genomic mechanisms has been described. Aromatase activity in the hypothalamus is rapidly down-regulated in conditions that enhance protein phosphorylation, and in particular, increases the intracellular calcium

concentration, such as those triggered by neurotransmitter (e.g., glutamate) activity (Balthazart *et al.* 2004; Balthazart *et al.* 2006). These data support an emerging concept in neuroendocrinology, namely that estrogen, locally produced in the brain, regulates male sexual behavior via a combination of genomic and non-genomic mechanisms (Haynes *et al.* 2000; Falkenstein *et al.* 2000; Cornil *et al.* 2006; Balthazart & Ball 2006). Rapid and slower changes of brain aromatase activity match well with these two modes of estrogen action and provide temporal variations in the estrogen's bioavailability that can support the entire range of established effects for this steroid (Balthazart *et al.* 2006).

Secondly, $E_2$ is traditionally known and described extensively as an endocrine hormone by which it can affect target cells again in a slow genomic or rapid non-genomic manner for which resulting evidence has been reviewed (Falkenstein *et al.* 2000; Cornil *et al.* 2006; Balthazart & Ball 2006; Janosek *et al.* 2006). In brief, $E_2$ modulates gene transcription by interaction with intracellular estrogen receptors (ESRs), which act as ligand-dependent transcription factors after conformational change which allows the dimerized complex to enter the nucleus (Mangelsdorf *et al.* 1995). Here it binds to a 13 basepair palindromic target DNA sequence known as an ERE within estrogen responsive genes. This mechanism referred to as "genomic" is slow (hours to days) to produce results which are indeed more stable and often includes long term effects. Alternatively, $E_2$ acts via non-genomic pathways (Figure 2) to produce rapid (seconds to minutes) responses in non-neural cell types (Pietras & Szego 1977).

Current research supports several such pathways by which $E_2$ effects cell metabolism (Cornil *et al.* 2006). These mechanisms include either that $E_2$ allosterically modulates the activity of ionotropic receptors or G-protein coupled receptors (GPCR) or that $E_2$ can bind to membrane-bound ER (mER) that is coupled to a G-protein. Thereby, $E_2$ can modulate the activity of ionic conductance through phosphorylation of ionotropic receptors or uncoupling of GPCR from their ionic channels to intracellular effectors. $E_2$ can further mobilize intracellular $Ca^{2+}$ through activation of phospholipase C (PLC) (Haynes *et al.* 2000; Hisamoto & Bender 2005).

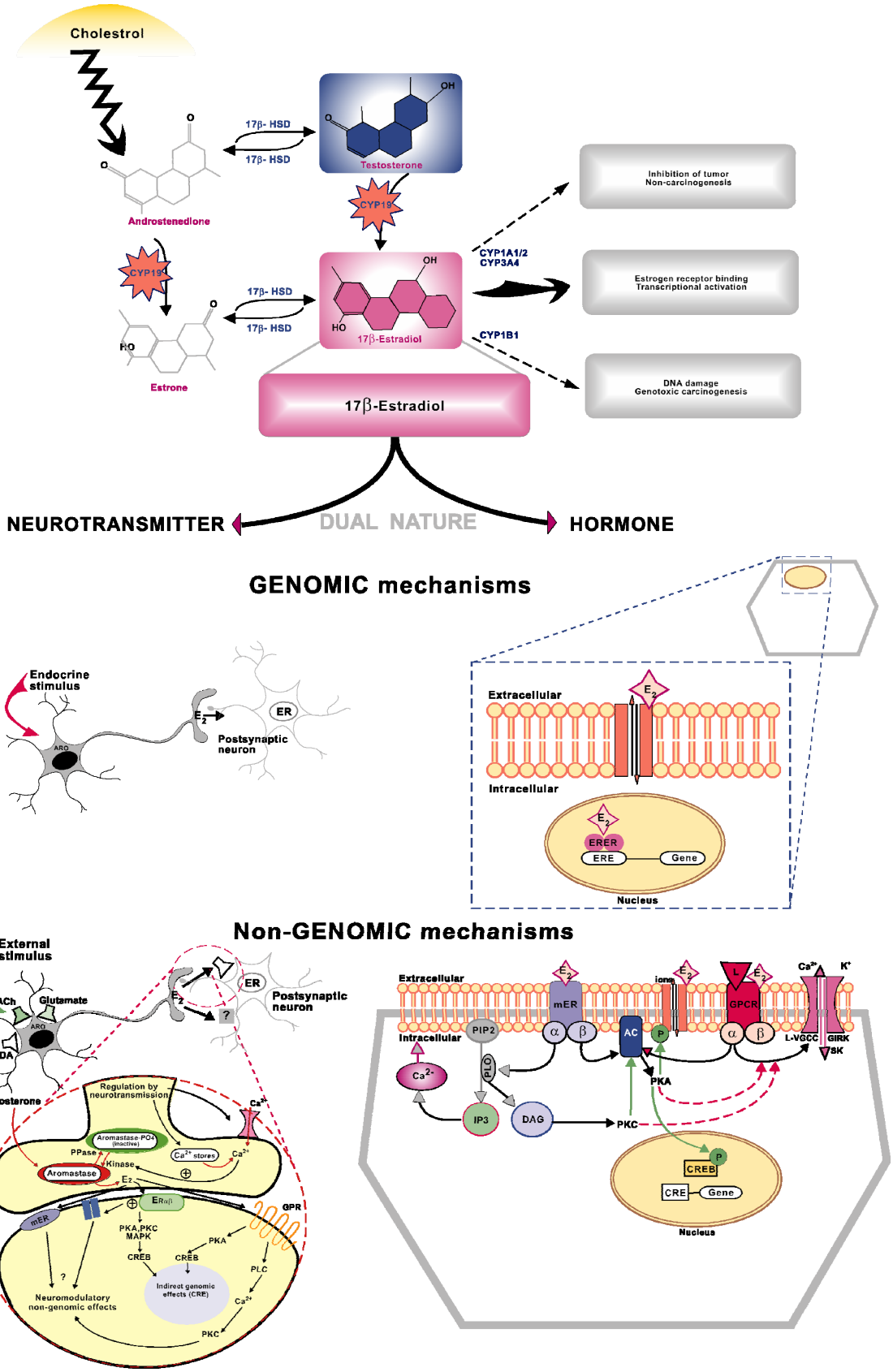


**Figure 2** Diagram illustrating production, function and mode of action for 17β-Esradiol ($E_2$). The metabolic pathway of $E_2$ includes several cytochrome P450 (CYP)-

mediated reactions starting with the primary steroid cholesterol. Estradiol is biosynthesized by both *cyp19* and 17β-hydroxysteroid dehydrogenase (17β-HSD) from androstenedione via testosterone or estrone respectively. Metabolites of $E_2$ include catachol metabolites via hydroxyestradiols produced by either CYP1A1/2, CYP3A4 or CYP1B1 which can finally be metabolyzed by catechol *O*-methyltransferases to result in methoxyestradiols which can either be non-carcinogenic and inhibits the proliferation of caner cells (2-Methoxyestradiol), or alternatively cause cell damage (Estradiol-3,4-quinone) $E_2$ act as either the classical endocrine hormone or as neurotransmitter (Balthazart & Ball, 2006). As a neurotransmitter, $E_2$ can function either along a genomic pathway where its action results in the transcription of various other proteins that modify neural activity in the long term and have consequently long-lasting effects. Secondly, $E_2$ can function as a neurotransmitter along rapid non-genomic pathways as been illustrated in the left lower corner of this illustration. See text for details. In its role as a true endocrine hormone, $E_2$ again employs either a genomic or non-genomic mechanism with the former allowing the ligand to enter the cell, bind to estrogen receptors in the cytoplasm where conformational change allows the ligand-receptor complex to enter the nucleus and binds to ERE sites of DNA resulting in the activation of transcription of specific genes. (Adapted from Cronil et al., 2006; Tsuchiya et al., 2005 and Balthazart & Ball, 2006)

Specifically in the endothelial cells, assembly of a membrane-associated molecular complex is rapid and comprises of an ESR, c-Src and the regulatory unit of phosphatidylinositol 3-kinase (PI3K), p85 in response to $E_2$ (Caulin-Glaser *et al.* 1997; Kim & Bender 2005). This complex triggers some sequential enzyme activation, involving endothelial nitric oxide synthase (eNOS) (Hisamoto & Bender 2005).

Finally, Klinge (2001) describes another mode of activation as "tethering" where ER interacts with yet another type of DNA-bound transcription factors such as AP1, SP1, or NF-κB in a way that stabilizes the DNA binding of that transcription factor and/or recruits co-activators to the complex. This mechanism does not bind DNA, though. The implication hereof is demonstrated in a study where tamoxifen (well-known anti-estrogen) could function as an agonist on promoters where ESR interacted indirectly by pairing to AP1, whereas it functioned as a pure antagonist when analyzed on ERE-containing promoters (McDonnell 2004).

***Estrogen receptor:*** ESRs (Evans 1988) are traditionally known as ligand-activated transcription activators or repressors, located in the cytosol and/or nucleus of cells (Pham *et al.* 1992; Kim & Bender 2005). In response to ligand

binding, ESR undergoes conformational changes, termed "activation", accompanied by dissociation of some proteins, forming a ligand-occupied ESR dimer. ESR is known to be involved in the regulation of expression of a multitude of specific target genes or gene networks since these gene activation events require the recruitment of specific co-activators by ligand-bound nuclear receptors, all which are implicated in growth and differentiation of female reproductive tissues. Transcriptional regulation is therefore ligand dependent and occurs via binding of the ligand-receptor complex to specific sequences of target genes (Beato 1989). This leads to chromatin being remodeled and "opened up", Since many coactivators possess histone acetyltransferase activity (Chen *et al.* 1997; Vasudevan *et al.* 2002).

In oviparous species, the hepatic ESR concentration is markedly increased by $E_2$ as can be expected because the liver is one of the main target organs for estrogens during the adult life of these egg-laying females. The question with regards to target organs for $E_2$ during the developmental stages in these animals largely still remains unanswered and hybridization techniques could shed some light on this issue. Flouriot et al. (1996) discuss implications of the steroids which regulate the receptor concentration, and points out that this autoregulation is generally mediated by a combination of regulatory mechanisms involving gene transactivation and the turnover of mRNA and protein.

For most vertebrates in which the coding sequence for ESR has been sequenced, at least two homologues of are known, denoted ESR1 (Greene *et al.* 1986) and ESR2 (Kuiper *et al.* 1996), with ESR1 the subtype that appears to be required for most of the known estrogenic responses (Couse & Korach 1999), and ESR2 being specifically involved at differentiation of granulosa cells (surrounding the ovarian follicle), and the ovulatory response to gonadotropins (Couse *et al.* 2005). In addition, several teleosts have been studied in this respect for which a some were found to have two homologues for ESR2, denoted ESR2a or ESR2b including alternatively spliced and truncated forms (Haynes *et al.* 2000; Hawkins *et al.* 2000; Menuet *et al.* 2002; Wang *et al.* 2005; Greytak & Callard 2007; Caviola *et al.* 2007).

Following translocation of the $E_2$–ESR complex to the nucleus, it binds to a specific 13-basepair palindromic target DNA sequence within the estrogen responsive genes (Brown & Sharp 1990; Haynes *et al.* 2000; Hisamoto & Bender 2005) which is located on the 5'-flanking region of such genes, and allows three

ambiguities in the middle of the sequence. In humans, an activated ESR can bind to half-palindromic sequences and increase gene transcription, however, unliganded ESRs can also bind to ERE consensus sequences and activate transcription, but interaction of the receptor with $E_2$ stabilizes dimer-initiation and enhances its interaction with target sequences within estrogen responsive genes (Brown & Sharp 1990; Kato *et al.* 1992). The specific domains within ESR that are required for each of these functions have been described by McDonnell (1999), and reviewed by McDonnell (2004) and McDonnell and Norris (2002). A short review by McDonnell and Norris (2002) points out why different cells can respond to the same hormone in a different manner, which include that (a) two (in primates, three in teleosts) genetically and functionally distinct estrogen receptors that have distinct expression patterns *in vivo* exist, (b) positive and negative transcriptional activities of these receptors require them to engage transcription cofactors in target cells, and (c) not all cofactors are functionally equivalent, nor are they expressed in the same manner in all cells.

ESR1 has been shown to be expressed in a variety of tissues, but subsequent work on ESR2 has indicated the presence of ESR2 in vascular cells of mammals, and additionally some more variants of the ESR2 (Greytak & Callard 2007; Takase & Iguchi 2007; Traupe *et al.* 2007; Caviola *et al.* 2007; Leanos-Castaneda & Van Der Kraak 2007). ESR1 and ESR2 comprises at least six domains from the N- to C terminus, encoded by 8-9 exons, of which three are the major functional domains: (a) a variable N terminus that modulates transcription in a gene- and cell-specific manner through its N-terminal activation function-1 (AF1); (b) a central DNA-binding domain consisting the C domain, comprised of two functionally distinct zinc fingers through which the receptor interacts directly with the DNA helix; and (c) the ligand-binding domain that contains activation function-2 (AF2) (Montano *et al.* 1995).

Klinge (2001) points out that ESR1 binding affinity does not relate linearly with $E_2$-induced transcriptional activation, and suggest some reasons for this phenomenon: cellular amounts of co-activators and adaptor proteins that play roles both in ESR binding and transcriptional activation; phosphorylation of ESR and other proteins involved in transcriptional activation, and thirdly sequence-specific and protein-induced alteration in chromatin architecture.

In conclusion, genes responsive to $E_2$, whether direct via the genomic pathway or indirect via second messangers are thus potential "reporter" genes

for increased levels of $E_2$ in aquatic ecosystems. One such gene (*vtg*) and its translational product (VTG, protein) has enjoyed much attention over the past 3 decades where estrogenic endocrine disruption is considered as was demonstrated by Leanos-Castaneda & Van der Kaak (2007).

## SEX DETERMINATION AND DIFFERENTIATION AFFECTED BY EDCs

An EDC has been defined by the International Program for Chemical Safety as "exogenous substances that alter function(s) of the endocrine system and consequently cause adverse health effects in an intact organism, its progeny, or (sub)populations" (Ropero *et al.* 2006). The endocrine system of both human and animals is hereby disrupted by EDCs to either (i) mimic endogenous hormones, (ii) antagonize normal hormones, (iii) alter the natural pattern of hormone synthesis or metabolism, or (iv) modify hormone receptor levels (Sonnenschein & Soto 1998). Important to keep in mind is that although *in vivo* exposure assays are highly integrated assessments of whole animal responses, they often lack information about mechanisms of action.

As for modification during gonadal development, many factors are known to act at the transcriptional level. Park and Jameson (2005) explains that, based on homology to other transcription factors, some affect DNA conformation or modulate chromatin remodeling, whereas others form interactive complexes that activate transcription or have a role in specifying progenitor cell types. Little is known about the repression of transcription factors, which potentially have an equally drastic effect on gene expression.

Studying signaling pathways, the question often arises as to assessing the components of such pathways, especially working upstream from a reference process. Andersen et al. (2005) shed some light on this matter, by discussing "reverse genetic screens", which involves cell-based assays constructed with various cellular endpoints or reporter genes. These indicate activation of a specific pathway, introducing full-length cDNA or short inhibitory RNAs (siRNAs) into cells to provide a gain-of-expression or loss-of-expression form of the genetic manipulation, respectively.

Three potential targets of EDCs are discussed by Segner et al. (2006) which includes sexual differentiation of gonads, the IGF-I hormone system, and the immune system. Today it is known that exposure of adult human males to

$E_2$ during adulthood results in gynecomastia and interferes with the normal function of the hypothalamus-hypophyseal-gonadal axis, resulting in decreased libido, impotence, decreased blood androgen levels, and lowered sperm counts. In women, exposure to natural estrogens holds the principal risk factor in the development of breast and endometrial cancer (Sonnenschein & Soto 1998).

Amongst potential endocrine disrupted systems studied to date, that of estrogenic, androgenic and thyrodogenic disruption is most prominent. Hereby the sex differentiating pathways in several species received attention and in appropriate gonochoristic species serves as a reliable endpoint in screening for EDCs in aquatic systems. However, some aquatic species are not totally dependant on genetic sex determination, providing a challenge in this regard. Therefore, another well studied endpoint in estrogenic endocrine disruption provides a suitable solution: vitellogenesis (discussed below), and is still being used to date as a reliable tier I screen (Hiramatsu *et al.* 2005; Peters *et al.* 2007; Leanos-Castaneda & Van Der Kraak 2007). Other endpoints include zona radiata (Zr) proteins (Arukwe *et al.* 1997; Boon *et al.* 2002; Meucci & Arukwe 2005) and ESRs (Greytak & Callard 2007; Leanos-Castaneda & Van Der Kraak 2007).

Estrogens (Segner *et al.* 2006), as has been described above, follow the same mechanisms of action as the gonadal hormone $E_2$, but is now known to also act as a neuro-endocrine as well as functioning to activate additional transcription factors outside the nucleus (Figure 2). In spite of this, estrogenic bioassays have not been developed to monitor these rapid effects of xenoestrogens (Ropero *et al.* 2006). Estrogen-like compounds bind to estrogen receptors at a cellular level and promote cell proliferation and hypertrophy of female secondary sex organs and induce the synthesis and secretion of cell type-specific proteins (Hertz 1985).

**Sex differentiation as estrogenic endpoint:** differentiation of primary phenotypic sex and subsequent reproduction in fish are complex processes of which much can be genetically pre-determined but ultimately regulated by endogenous hormones (Arukwe & Goksoyr 1998) and accompanying proteins serving, when bound to appropriate ligands, as transcription regulators in these complex signal transduction pathways. During sex differentiation of the bipotential gonad, gonadal development is regulated through the

hypothalamus-pituitary-gonadal-hepatic axis as these organs altogether produces substances influencing one another (Arukwe & Goksoyr 1998).

Alligators (Crain & Guillette 1998) and teleost fish (Afonso *et al.* 2000; Anway & Skinner 2006; Chang *et al.* 2006; Vega-Lopez *et al.* 2007) has been used in several studies to report sex differentiation alteration due to of estrogenicity in aquatic environments. This endpoint of monitoring EDCs has especially relevance to whole ecosystems, changing population structures detrimentally, as has been reviewed by Hahlbeck *et al.* (2004) and Kime (1998).

**Vitellogenin as estrogenic endocrine disruption endpoint:** VTG is a term first used to describe a female-specific protein in a moth (*Cecropia*) (Pan *et al.* 1969; Hiramatsu *et al.* 2005). The formation of yolk precursor protein, termed vitellogenesis, has been studied extensively in many egg-laying animals. It became such an important factor in ovarian development of oviparous species, that ovarian follicle growth if fishes are broadly classified into pre-vitellogenic and vitellogenic stages (Hiramatsu *et al.* 2005) However, the term vitellogenesis includes also other processes related to oocytes growth. VTG is a large glucolipophosphoprotein (350-600 kDa) circulating in blood, usually as homodimer (Vlaming *et al.* 1980; Sundararaj & Nath 1981; Sawaguchi *et al.* 2005).

VTG is primarily synthesized in the liver of fish under the influence of estrogenic compounds, as the promoter region of animals for which this has been sequenced, contain several ERE sites. This and other regulatory enhancers have been even found to both the 5′- and 3′-flanking regions in some species (Seiler-Tuyns *et al.* 1986; Dodson & Shapiro 1994; Teo *et al.* 1998). VTG is modified extensively post-translationally in the liver, secreted into the bloodstream, and sequestered by the oocytes via specific VTG receptors (Lim *et al.* 1991). Here VTG is cleaved into subunits of yolk proteins, lipovitellin I, phosvitin, and lipovitellin II.

Within the past two decades multiple cDNAs encoding VTG has been described for species including chicken, *Xenopus laevis*, with evidence that multiple proteins may exist in various taxa (Wahli *et al.* 1979; Felber *et al.* 1980; Stifani *et al.* 1990; Hiramatsu *et al.* 2002; Sawaguchi *et al.* 2005). In teleosts, full-length cDNA encoding a variety of two to twenty distinct VTGs has been reported (Mouchel *et al.* 1997; Reith *et al.* 2001; Hiramatsu *et al.* 2002; Sawaguchi *et al.* 2005; Davis *et al.* 2007) and discussed in more detail by Hiramatsu et al

(2005). Basically three classes of VTG exist, denoted VgA, VgB and VgC. The true relations of these various *vtg* genes and proteins are investigated, and information about splicing of these genes are unavailable.

Kim et al. (2003) illustrated the enhanced and induced effect of $E_2$ on hepatocyte cultures in male and female tilapia respectively, and thereby confirmed the strong *vtg* inducing effect in this species. Administered tamoxifen, known as a nonsteroidal anti-estrogen which competitively binds ER (Tsai & O'Malley 1994), can reduce hepatic *vtg*, despite increased circulating estradiol levels arising from blockage of feedback inhibition of steroid synthesis (Peyon *et al.* 1997; Devlin & Nagahama 2002). Interestingly, *vtg* expression is stimulated over a wide range of $E_2$ concentrations, much above that needed to induce maximal levels of ESR gene expression found in two species of Tilapia (Lazier *et al.* 1996). This makes sense in the light of *vtg* expression which occurs in response to binding of estrogen receptors with estrogen-responsive elements associated with regulatory regions of the VTG gene promoter.

Further work by Kim et al. (2003) on *O. mossambicus*, illustrated an increased or at least a maintained VTG concentration after females were treated with testosterone, 17$\alpha$-methyltestosterone or 5$\alpha$-dihydrotestosterone. The latter androgen also increased the VTG level on the medium of male hepatocytes cultures. Co-treatment of $E_2$ and androgens to the male hepatocytes enhanced VTG concentration in the medium, suggesting that the androgens have some roles in VTG synthesis in the hepatocytes. As one can expect, co-treatment by Testosterone and an aromatase inhibitor failed to inhibit the effect on VTG synthesis by testosterone alone, since on a transcriptional level, RT-PCR resulted in none of either of the two types *cyp19*s being expressed. $E_2$ in *O. mossambicus* produced a significant and concentration-related stimulation of VTG release in both male and female hepatocytes *in vitro* primary cultures (Riley *et al.* 2004) or *in vivo* by adult males (Davis *et al.* 2007). In several studies, *vtg* expression is observed within the first 8 hours after exposure to estrogenic compounds, making this one of the most rapid reported impacts to date as an estrogenic biomarkers in vertebrate species (Lim *et al.* 1991; Scholz *et al.* 2004; Gordon *et al.* 2006).

In a well referenced chapter, Hiramatsu et al. (2005) reviews the various VTG assay systems developed to date, depending on various types of immunological methods.

**Estrogenic EDCs:** It is noted that exposure to natural estrogens is the principal risk factor in the development of breast and endometrial cancer (Sonnenschein & Soto 1998). Several estrogenic bioassays has been described (Hertz 1985; Sonnenschein & Soto 1998; Larkin *et al.* 2002; Terasaka *et al.* 2004) where $E_2$ (or estrogen-like compounds) binds to ESRs at a cellular level and promotes cell proliferation and hypertrophy of female secondary sex organs and induces the synthesis and secretion of type-specific cell proteins.

Estrogen-related abnormalities include: expression of cancer genes and *Hox* genes which are induced by perinatal $E_2$ exposure (Iguchi & Watanebe 2003); nongenital abnormalities induced by perinatal exposure to $E_2$ – evidence from mice and alligators having polyovular follicles and polynuclear oocytes when exposed to DES/high dose of Bisphenol A (150 μg/pup) or dicofol/DDT respectively, as well as humans which show higher rates of infertility and anatomical structural defects after being exposed to the DES (Iguchi & Watanebe 2003). In fish treated with $E_2$, many abnormalities has been found ranging from decreased rates of hatching and survival to malformations including eye extrusion, crooked vertebral column, reduced body weight and head-body length (Urushitani *et al.* 2002). Ultimately some fish respond by a complete change phenotypic sex, but this may depend greatly on the kind of mechanism the particular species use for determining/differentiating sex, and is not a rule throughout all taxa.

In estrogen related cancers, much attention is therefore directed at aromatase inhibitors (Janicke 2004). Aromatase inhibitors can be separated into two classes based on their mechanisms of action, denoted (a) steroidal substrate analogs, which inhibit the enzyme activity by competing for the active site of the enzyme, or (b) non-steroidal inhibitors which interact with the heme prosthetic group of aromatase (Brodie *et al.* 1977; Salhanick 1982). Studies on the activity of aromatase, whichever form thereof, is therefore to be dealt with carefully in terms of negative controls when investigating up-regulation of these genes, as steroidal analogs and non-steroidal inhibitors may have different effects upon induction by steroids.

According to Lazier *et al*. (1996) the action of methyl-testosterone at the level of hypothalamic-pituitary, it mimics $E_2$ negative feedback or has direct negative feedback effects through AR binding. Kim *et al.* (2003) confirmed the

binding of androgens to ESR and, consequently, to exert an estrogenic action such as the synthesis of VTG, the precursor protein of egg yolk in oviparous animals, which is partly under the control of $E_2$. They found the stimulatory effect of androgens on VTG production levels to be more effective in female and $E_2$ treated male hepatocytes than in control male hepatocytes, and attribute this difference to high ESR expression levels which were found in livers of $E_2$ treated males rather that that of untreated male fish, finally suggesting that probably a certain threshold level of ESR in the liver is necessary for multiplying androgen action. The effects of *cyp19* expression is mentioned by some (Trant *et al.* 1997; Di Fiore *et al.* 1998; Kim *et al.* 2003; Greytak *et al.* 2005) as to have an effect resulting in this behaviour of *vtg* expression in response to androgens in the liver of male and female teleosts. Some matters regarding the post transcriptional regulation and modulation of *vtg* is however unresolved for several teleosts, including *O. mossambicus*.

As androgens are reported to also induce VTG production in cultured hepatocytes of some fish (Peyon *et al.* 1997; Mori *et al.* 1998), this effect is blocked with tamoxifen, indicating that stimulation is occurring through the ESR. Peyon *et al.* (1997) demonstrates that in eels *in vivo* effects of androgens included that VTG synthesis was not affected by these steroids. In contrast, in a tilapia, treatments with 17$\alpha$-methyltestosterone dramatically reduced VTG protein and hepatic VTG mRNA levels. In females androgen treatment also lowered serum $E_2$ levels, suggesting these effects to be occurring by feedback of steroids at the hypothalamic or hypophyseal levels (Lazier *et al.* 1996).

**Matters on Monitoring EDCs:** Modeling EDCs in a dose-response manner is of integral importance when a system is developed for toxicological testing. It is pointed out by Andersen et al. (2005) that genome-wide functional screens, bioinformatics tools, and network mapping technologies together can provide directed graph representations of the cellular signaling networks. Furthermore, during development of such a system, the graphical representations can be converted into mathematical models that permit predicting the shapes of dose-response curves for altered cell signaling by test compounds. However, predicting the shapes of these dose-response curves provide another challenge, that of organizing the qualitative information quantitatively in order to provide a full risk assessment process. Qualitative

studies provide information about the hazard, mode-of-action, progression and finally, susceptibility between the test compound and the biological system used whereas quantitative measurements are often necessary for management of water bodies, especially where threshold considerations are implicated (Funkenstein *et al.* 2004; Bustin *et al.* 2005; Kubista *et al.* 2006).

It is imperative to study these pathways/processes at both transcription and translational level. The first level of expression (mRNA) shows that genes have been turned on (or off) and to what degree induction has occurred. The second level (proteins) indicates a functional response in the organism (Shrader *et al.* 2003). In eukaryotes, mRNA expression has a relatively low correlation with protein abundance. In addition, Park and Jameson (2005) point out that the functional interaction between extra cellular ligand molecules and nuclear transcription factors also merits emphasis. For example, extracellular signals can induce transcription factor release and translocation of the nucleus. Increasing lines of evidence suggest that cross talk among intracellular signaling pathways mediate downstream transcriptional response by which they argue the importance to identify the extracellular ligands, membrane receptors, and signal transduction pathways associated with gonadal development, as well as the transcription factors.

An integral concept to apply in all molecular studies today, is the relationship between DNA sequence information and nonlinear cellular responses. However, quantitative transcriptional analyses are not necessarily coinciding relative translational levels of product, and expression data may not provide sufficient information to fully predict dynamic biological response (Hatzimanikatis & Lee 1999). It is therefore imperative to study multiple levels of control including transcript and translated product because each have unique, non-overlapping attributions to add to a study which might be overlooked in assessing the mechanisms of actions in EDC studies.

It is a challenging approach to apply expression profiling to ecotoxicological studies where it is applied to organisms without sequenced genomes or poorly defined genetically. These organisms are not as established as classical models are, but they may still have high ecotoxicological relevance and are worthy of investigation, as is the subject of this study – *Oreochromis mossambicus*. The degree to which such investigation should occur is arguable however. The scarcity of evidence linking impacts of environmental

contaminants to changes in reproductive success of indigenous fish populations may reflect a critical need for a dependable method or indicator to assess reproduction of fish *in situ* (Mills & Chichester 2005). Iguchi and Watanabe (2003) underlined the importance of understanding the molecular basis of hormonal actions, especially the effects of hormonally active chemicals on developing organisms. More so should one understand the interaction of exposure levels and gene responses to xenobiotic chemicals.

Finally, from the abundant reports on possible biomonitoring species across the world, and the variety of lessons I learn from them, I argue strongly a range of model species should be included for monitoring endocrine disruption which should incorporate both invertebrates and vertebrates. Even more important is to understand their biology before engaging any such species in a bio-monitoring programme.

**Toxicogenomics:** Toxicogenomics is a term recently developed to describe a field of study in toxicology and pharmacology (Iguchi *et al.* 2007). Since the 1970s toxicological studies have increased exponentially primarily including behaviour and protein investigations. As molecular knowledge and techniques evolved, more attention was drawn to applying genomic basis to the study of toxins (especially EDCs) and pharmaceuticals. Therefore the term "toxicogenomics" attempts to define how the regulation and expression of genes are mediated by the toxicological effects associated with exposure to a particular chemical or chemical mixture (Neumann & Galvez 2002; Schmidt 2003; Sarrif *et al.* 2005). Gene expression changes may provide more sensitive, immediate and comprehensive markers for toxicity compared to typical toxicological endpoint studies, including morphological changes, carcinogenicity, and reproductive toxicity (Marchant 2002; Lee *et al.* 2005). Global gene expression changes in bio-indicator species exposed to toxicants can include genomic scale mRNA expression (transcriptomics), cell and tissue-wide protein expression (proteomics), metabolite profiling (metabolomics) and bioinformatics as well as epidemiological population studies linking genetic risk factors with environmental factors (Lee *et al.* 2005). Defining the process of transcription and its regulation by proteins (i.e. transcription factors) has however became a challenge and therefore transcriptomic studies can be confusing if clear distinctions are not determined at the onset of such a study.

One of the first factors which affected the development of this branch of toxicology was the possibility of sequencing the genome or parts thereof. A further major technical success was the invention of the microarray without which it took days to analyze a single gene. The chip technology now allows for analysis of expression data of thousands of genes at one time. Quantitative real-time PCR (QPCR) is taking this analysis even a step further, enabling the researcher to measure the level of specific gene expression at a high resolution.

DNA microarrays are typically used to analyze patterns of gene expression, presence of markers, or nucleotide sequence. The major advantage of microarrays is the extent to which the process of genotyping can be automated, thereby enabling large numbers of individuals to be simultaneously genotyped at many loci. DNA chips can be categorized into two general types: oligonucleotide arrays and PCR-amplicon arrays, with the former having the ability to discriminate between closely related gene sequences because of the greater specificity for target sequences (Inoue & Pennie 2003). Whereas amplicon arrays are relatively cost effective to generate (Gant & Zhang 2005; Iguchi *et al.* 2006; Iguchi *et al.* 2007), they require that PCR-amplified DNA products be obtained from tissues or cloned libraries and subsequently spotted onto an array.

As for microarrays, the ultimate approach would be for a particular species, to have a chip of the complete set of genes in order to identify which genes are up- or down-regulated by a particular response to toxicant stress. Unfortunately, there are very few species of which the whole genome is completely sequenced and the genes of that species have been characterized. According to the NCBI database (http://www.ncbi.nlm.nih.gov/), there are only 132 whole genome sequences available to date, of which 104 belong to prokaryotes (79 %).

Gene expression profiling implementing micro chips therefore has the potential to provide a fast, cheap initial screen of potential toxicants, provided the development for the specific set of genes is known for a particular species. Otherwise procedures incorporating individual proteins may serve as a cheaper option, but will provide answers at a post translation level as apposed to the transcription level of DNA chips and QPCR. Oberemm et al. (2005) suggested that molecular fingerprinting could be used, *in vivo* and *in vitro*, to categorize chemicals and mixtures of chemicals into different mode of action groups. A

question also asked by critics and needed to be kept in mind, is at what stage can one apply gene expression data to translate adverse effects on population level in the ecological framework. A better understanding of genomic expression for particular gene products will certainly enable a greater insight into the factors behind the observed variability in susceptibility to chemical exposures seen in populations (Oberemm *et al.* 2005).

According to Sariff et al. (2005), toxicogenomics has entered a period of reflection and validating whether the particular techniques are applicable or not, and whether they reflect the real picture of what happens in the ecosystems. As has been discussed before, the first level of expression (mRNA) can indicate that genes have been turned on, whereas the second level (proteins) may indicate a functional response in the organism (Shrader *et al.* 2003). In a mini-review by Park and Jameson (2005), the issue of including transcripts and protein analyses is discussed explaining the various roles of genes and ligands influencing gonadal development. According to them, many of the gonadal development factors described to date are known to act at the transcriptional level, and for the most part, their functions are incompletely understood. Transcription factors may either form interactive complexes that activate transcription or have a role in specifying progenitor cell types, or can affect DNA conformation or modulate chromatin re-modeling. Moreover, inhibition of gene expression is equally likely to be important as a means to dictate cell fate, but less is known about potential transcriptional repressors (Park & Jameson 2005).

The first customized DNA microarray for EDC related work has been developed and applied to study the expression profiling of the estrogen-responsive genes to evaluate estrogenic activity among natural estrogens (e.g. $E_2$) and industrial chemicals (Inoue *et al.* 2002; Terasaka *et al.* 2004). Terasaka et al. (2004) performed a comprehensive analysis of estrogen-responsive genes among approximately 20 000 human genes, selected 172 of these genes for a microarray chip, and examined estrogen activity among a variety of natural estrogens, industrial chemicals and dioxin. Vezina et al. (2004) assessed the effect of sub-chronic exposure of toxic levels of some halogenated aromatic hydrocarbons (HAHs) on hepatic gene expression in mice. A set of 8799 gene probes were compared on the microchip, with many genes being co-expressed during the exposure period, some of which were known AhR-regulated genes, and some not previously characterized as being AhR regulated. Hereafter, the

authors applied QPCR to confirm the increased expression of the "new" genes in exposed rats, as well as the up- and down- regulation of several novel dioxin-responsive genes, demonstrating the value of toxicogenomic work.

It is anticipated that toxicogenomics will greatly improve the sensitivity, accuracy, and speed of toxicological investigations and it is argued by some (Hamadeh *et al.* 2002; Steiner *et al.* 2004) that a transcriptome-wide overview of altered expression patterns can assist the mechanistic understanding of underlying changes induced by chemicals, but requires a comprehensive knowledge of the biological system under investigation and only known genes should be considered for such an analysis. Different classes of toxicants have been discriminated using transcript profiling (Steiner *et al.* 2004). Steiner et al. (2004) aimed to determine if biological samples from rats treated with various compounds can be classified based on gene expression profiles, providing a solid study to demonstrate gene expression analysis using microarrays, a complete serum chemistry profile and liver and kidney histopathology. Steiner et al. (2004) derived predictive models which were able to discriminate between hepatotoxic and non-hepatotoxic compounds, and also predicted the correct class of hepatotoxicant in most cases, illustrating the use of this newly evolved branch discipline in toxicological studies. What is still important to be kept in mind, is that same as with quantitative structure-activity relationship methods (Tong *et al*. 2004), it is impossible to predict the activity of an unknown chemical. Therefore, once obtaining the data from microarrays, further biological validation and additional analyses through proteomics and QPCR are warranted, provided appropriate housekeeping standards are applied. Typical housekeeping genes are involved in basic cellular functions and assumed to express invariably under all conditions tested for during any toxicogenomic study (Arukwe 2006). As toxicological application of QPCR can deem this a misconception, care should be taken when choosing a housekeeping gene for a species and specific experiment.

An application of toxicogenomics in combination with bioinformatics is a technique called Quantitative structure-activity relationship (QSAR), yet not much exploited, but having real potential, setting the stage for initial screening of EDCs. See Tong *et al*. (2004) for a demonstration where QSAR is applied, testing models for prediction of estrogen receptor binding activity. They assessed objectively and quantitatively the applicability domains of the models

by computing prediction confidence and domain extrapolation for predicting unknown chemicals with an extensive cross-validation. The authors note that QSAR models could be important in prioritizing chemicals for testing based on likelihood of activity, and finally propose a particular model most suitable for aiding in prioritizing chemicals for testing as possible EDCs.

When studying toxicogenomics, one unravels most often signaling pathways which provide clues for various other pathways (Terasaka *et al.* 2004), sometimes even pointing to a whole new pathway not yet known. However one of the greatest challenges in the wildlife environment linking expression profiling to eco-toxicological studies, is working with organisms without sequenced genomes and that are generally poorly defined genetically.

To conclude, toxicogenomics is a fairly new, fast evolving field of research (Inoue & Pennie 2003; Adler 2003; Sarrif *et al.* 2005) by which trusted, but relatively new techniques, in molecular biology are applied to give either information of mechanisms of action or endpoint result. Ultimately the success of a toxicogenomics approach will depend on our ability to interpret the data in relation to existing information (screening of a pollutant induced gene expression fingerprint against a database containing chemical-related gene expression toxicity profiles) (Frueh *et al.* 2004; Sarrif *et al.* 2005; Iguchi *et al.* 2007). Still one of the greatest challenges in the wildlife environment linking expression profiling to eco-toxicological studies, is working with organisms without sequenced genomes and that are generally poorly defined genetically.

## CONCLUSION

"Historically, much of our understanding of transcription has been derived from piece-meal biochemical and genetic approaches, usually focusing on the mechanism of action of a singular transcription factor or regulation of a singular locus. However, the advent of the so-called "post genomic" era together with the attendant rise in array technology has changed this perspective. Genome sequence derivation and annotation provide the potential to identify regulatory sites *in silico*, a task aided by the rise of comparative genomics whilst DNA microarrays offer a platform to interrogate thousands, if not millions of interactions in parallel. This has changed the landscape in which we view transcriptional control" (Buckley 2007).

The paragraph above is of course written on account of research done in specifically human biology – much of which has provided a platform in understanding the total biology which can now be applied to perform future studies as well as understand and treat diseases. However, this is not the state that research in all organisms has been progressed to. It very well provides for faster or more pin pointed tests in other groups of animals and plants. But if we need to make inferences on environmental effects for human or any other organisms in the ecosystem under influence of such environment, we need to be sure of processes in such a modeling species.

Although it has been argued that aquatic species provide excellent biomonitors for endocrine disruption, the matter is further complicated by the vast array of mechanisms these species adopted throughout the years of evolution – including amongst others, mechanisms of sex differentiation as has been described.

## REFERENCE LIST


**Adler T.** 2003. The new biology. *Environmental Health Perspectives Supplements.* 111, A14-A15.

**Afonso L.O.B., Iwama G.K., Smith J. & Donaldson E.M.**, 2000. Effects of the aromatase inhibitor Fadrozole on reproductive steroids and spermiation in male coho salmon (*Oncorhynchus kisutch*) during sexual maturation. *Aquaculture.* 188, 175-187.

**Ahmed S. & Brickner J.H.**, 2007. Regulation and epigenetic control of transcription at the nuclear periphery. *Trends in Genetics.* 23, 396-402.

**Alworth L.C., Howdeshell K.L., Ruhlen R.L., Day J.K., Lubahn D.B., Huang T.H.M., Besch-Williford C.L. & vom Saal F.S.**, 2002. Uterine Responsiveness to Estradiol and DNA Methylation Are Altered by Fetal Exposure to Diethylstilbestrol and Methoxychlor in CD-1 Mice: Effects of Low versus High Doses. *Toxicology and Applied Pharmacology*. 183, 10-22.

**Andersen M.E., Thomas R.S., Gaido K.W. & Conolly R.B.**, 2005. Dose-response modeling in reproductive toxicology in the systems biology era. *Reproductive Toxicology.* 19, 327-337.

**Andres A., Muellener D. & Ryffel G.**, 1984. Persistece, methylation and expression of bitellogenin gene derivatives after injection into fertilized eggs of *Xenopus laevis*. *Nucleic Acids Research.* 12, 2283-2302.

**Anway M.D. & Skinner M.K.**, 2006. Epigenetic Transgenerational Actions of Endocrine Disruptors. *Endocrinology.* 147, s43-s49.

**Anway M., Cupp A., Uzumcu M. & Skinner M.**, 2005. Epigenetic transgenerational actions of endocrine disruptors and male fertility. *Science.* 308, 1466-1469.

**Arney K.L. & Fisher A.G.**, 2004. Epigenetic aspects of differentiation. *Journal of Cell Science.* 117, 4355-4363.

**Arnold A.P. & Breedlove S.M.**, 1985. Organizational and activational effects of sex steroids on brain and behavior: A reanalysis. *Hormones and Behavior.* 19, 469-498.

**Arukwe A.** 2006. Toxicological housekeeping genes: Do they really keep the house? *Environmental Science & Technology.* 40, 7944-7949.

**Arukwe A. & Goksoyr A.**, 1998. Xenobiotics, xenoestrogens and reproduction disturbances in fish. *Sarsia.* 83, 225-241.

**Arukwe A., Knudsen F.R. & Goksoyr A.**, 1997. Fish zona radiata (eggshell) protein: A sensitive biomarker for environmental estrogens. *Environmental Health Perspectives.* 105, 418-422.

**Balthazart J., Cornil C.A., Taziaux M., Charlier T.D., Baillien M. & Ball G.F.**, 2006. Rapid changes in production and behavioral action of estrogens. *Neuroscience.* 138, 783-791.

**Balthazart J., Foidart A., Absil P. & Harada N.**, 1996. Effects of testosterone and its metabolites on aromatase-immunoreactive cells in the quail brain: Relationship with the activation of male reproductive behavior. *The Journal of Steroid Biochemistry and Molecular Biology.* 56, 185-200.

**Balthazart J., Baillien M., Cornil C.A. & Ball G.F.**, 2004. Preoptic aromatase modulates male sexual behavior: slow and fast mechanisms of action. *Physiology & Behavior.* 83, 247-270.

**Balthazart J. & Ball G.F.**, 2006. Is brain estradiol a hormone or a neurotransmitter? *Trends in Neurosciences.* 29, 241-249.

**Barton N.H. & Charlesworth B.**, 1998. Why Sex and Recombination? *Science.* 281, 1986-1990.

**Beardmore J.A., Mair G.C. & Lewis R.I.**, 2001. Monosex male production in finfish as exemplified by tilapia: applications, problems, and prospects. *Aquaculture.* 197, 283-301.

**Beato M.** 1989. Gene regulation by steroid hormones. *Cell.* 56, 335-344.

**Black M.P., Moore B., Canario A.V.M., Ford D., Reavis R.H. & Grober M.S.**, 2005. Reproduction in context: Field testing a laboratory model of socially controlled sex change in *Lythrypnus dalli* (Gilbert). *Journal of Experimental Marine Biology and Ecology.* 318, 127-143.

**Boon J.P., van Zanden J.J., Lewis W.E., Zegers B.N., Goksoyr A. & Arukwe A.**, 2002. The expression of CYP1A, vitellogenin and zona radiata proteins in Atlantic salmon (*Salmo salar*) after oral dosing with two commercial PBDE flame retardant mixtures: absence of short-term responses. *Marine Environmental Research.* 54, 719-724.

**Borden E.C.** 2007. Augmentation of effects of interferon-stimulated genes by reversal of epigenetic silencing: Potential application to melanoma. *Cytokine & Growth Factor Reviews.* 18, 491-501.

**Brodie A.M., Schwarzel W.C., Shaikh A.A. & Brodie H.J.**, 1977. The effect of an aromatase inhibitor, 4-hydroxy-4-androstene-3,17- dione, on estrogen-dependent processes in reproduction and breast cancer. *Endocrinology.* 100, 1684-1695.

**Brown M. & Sharp P.A.**, 1990. Human estrogen receptor forms multiple protein-DNA complexes. *Journal of Biological Chemistry.* 265, 11238-11243.

**Brunner B., Hornung U., Shan Z., Nanda I., Kondo M., Zend-Ajusch E., Haaf T., Ropers H., Shima A., Schmid M., Kalscheuer V. & Schartl M.**, 2001. Genomic organization and exression of the doublesex-related gene cluster in vertebrates and detection of putative regulatory regions for DMRT1. *Genomics.* 77, 8-17.

**Buckley N.J.** 2007. Analysis of Transcription, Chromatin Dynamics and Epigenetic Changes in Neural Genes. *Progress in Neurobiology.* 83, 195-210.

**Bull J.** 1985. Sex determining mechanisms: an evolutionary perspective. *Experientia.* 41, 1285-1296.

**Bull J.** 1983. *Evolution of sex determining mechanisms*. Benjamin/Cummings. Menlo Park, CA .

**Bustin S., Benes V., Nolan T. & Pfaffl M.**, 2005. Quantitative real-time RT-PCR - a perspective. *Journal of Molecular Endocrinology.* 34, 597-601.

**Callard G.V. & Tchoudakova A.**, 1997. Evolutionary and functional significance of two CYP19 genes differentially expressed in brain and ovary of goldfish. *The Journal of Steroid Biochemistry and Molecular Biology.* 61, 387-392.

**Camerino G., Parma P., Radi O. & Valentini S.**, 2006. Sex determination and sex reversal. *Current Opinion in Genetics & Development.* 16, 289-292.

**Carey C. & Bryant C.J.**, 1995. Possible interrelations among environmental toxicants, amphibian development, and decline of amphibian populations. *Environmental Health Perspectives.* 103, 13-17.

**Caulin-Glaser T., Garcia-Cardena G., Sarrel P., Sessa W.C. & Bender J.R.**, 1997. 17beta-Estradiol Regulation of Human Endothelial Cell Basal Nitric Oxide Release, Independent of Cytosolic Ca2+ Mobilization. *Circulation Research.* 81, 885-892.

**Caviola E., la Valle L., Belvedere P. & Colombo L.**, 2007. Characterisation of three variants of estrogen receptor [beta] mRNA in the common sole, *Solea solea* L. (Teleostei). *General and Comparative Endocrinology.* 153, 31-39.

**Chang H.S., Anway M.D., Rekow S.S. & Skinner M.K.**, 2006. Transgenerational Epigenetic Imprinting of the Male Germline by Endocrine Disruptor Exposure during Gonadal Sex Determination. *Endocrinology.* 147, 5524-5541.

**Chang X., Kobayashi T., Senthilkumaran B., Kobayashi-Kajura H., Sudhakumari C.C. & Nagahama Y.**, 2005. Two types of aromatase with different encoding genes, tissue distribution and developmental expression in Nile tilapia (*Oreochromis niloticus*). *General and Comparative Endocrinology.* 141, 101-115.

**Charlesworth B.** 2002. The evolution of chromosomal sex determination. *Novartis Found Symp.* 244, 207-219.

**Charlesworth B.** 1991. The evolution of sex chromosomes. *Science.* 251, 1030-1033.

**Charlesworth B.** 2004. Sex Determination: Primitive Y Chromosomes in Fish. *Current Biology.* 14, R745-R747.

**Charlesworth D., Charlesworth B. & Marais G.**, 2005. Steps in the evolution of heteromorphic sex chromosomes. *Heredity.* 95, 118-128.

**Charnov E. & Bull J.**, 1977. When is sex determined environmentally? *Nature.* 266, 828-830.

**Chen H., Lin R.J., Schiltz R.L., Chakravarti D., Nash A., Nagy L., Privalsky M.L., Nakatani Y. & Evans R.M.**, 1997. Nuclear Receptor Coactivator ACTR Is a Novel Histone Acetyltransferase and Forms a Multimeric Activation Complex with P/CAF and CBP/p300. *Cell.* 90, 569-580.

**Colborn T., vom Saal F. & Soto A.**, 1993. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environmental Health Perspectives.* 299, 163-172.

**Conley AJ & Walters K.**, 1999. Aromatization. In Knobel,E. & Neill,J. *Encyclopedia of reproduction*. Academic Press,London, pp 280-291.

**Cornil C.A., Ball G.F. & Balthazart J.**, 2006. Functional significance of the rapid regulation of brain estrogen action: Where do the estrogens come from? *Brain Research.* 1126, 2-26.

**Couse J.F. & Korach K.S.**, 1999. Estrogen Receptor Null Mice: What Have We Learned and Where Will They Lead Us? *Endocrine Reviews.* 20, 358-417.

**Couse J.F., Yates M.M., Deroo B.J. & Korach K.S.**, 2005. Estrogen Receptor-{beta} Is Critical to Granulosa Cell Differentiation and the Ovulatory Response to Gonadotropins. *Endocrinology.* 146, 3247-3262.

**Crain D.A. & Guillette L.J.**, 1998. Reptiles as models of contaminant-induced endocrine disruption. *Animal Reproduction Science.* 53, 77-86.

**Crews D. & McLachlan J.A.**, 2006. Epigenetics, Evolution, Endocrine Disruption, Health, and Disease. *Endocrinology.* 147, s4-10.

**Darwin C.** 1871. *The descent of man, and selection in relation to sex*. Princeton University Press. Princeton .

**Davis L.K., Hiramatsu N., Hiramatsu K., Reading B.J., Matsubara T., Hara A., Sullivan C.V., Pierce A.L., Hirano T. & Grau E.G.**, 2007. Induction of Three Vitellogenins by 17beta-Estradiol with Concurrent Inhibition of the Growth Hormone-Insulin-Like Growth Factor 1 Axis in a Euryhaline Teleost, the Tilapia (*Oreochromis mossambicus*). *Biology of Reproduction.* 77, 614-625.

**Devlin R.H., Biagi C.A. & Smailus D.E.**, 2001. Genetic mapping of Y-chromosomal DNA markers in Pacific salmon. *Genetica.* 111, 43-58.

**Devlin R.H. & Nagahama Y.**, 2002. Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. *Aquaculture.* 208, 191-364.

**Di Fiore M.M., Assisi L. & Botte V.**, 1998. Aromatase and testosterone receptor in the liver of the female green frog, *Rana esculenta*. *Life Science.* 62, 1949-1958.

**Dodson R.E. & Shapiro D.J.**, 1994. An estrogen-inducible protein binds specifically to a sequence in the 3' untranslated region of estrogen-stabilized vitellogenin mRNA. *Molecular and Cellular Biology.* 14, 3130-3138.

**Duman R.S. & Newton S.S.**, 2007. Epigenetic Marking and Neuronal Plasticity. *Biological Psychiatry.* 62, 1-3.

**Edinger R.S., Mambo E. & Evans M.A.**, 1997. Estrogen-dependent transcriptional activation and vitellogenin gene memory. *Molecular Endocrinology.* 11, 1985-1993.

**Ellegren H.** 2000. Microsatellite mutations in the germline:: implications for evolutionary inference. *Trends in Genetics.* 16, 551-558.

**Esteller M.** 2007. Epigenetic gene silencing in cancer: the DNA hypermethylome. *Human Molecular Genetics.* 16, R50-R59.

**Evans R.** 1988. The steroid and thyroid hormone receptor superfamily. *Science.* 240, 889-895.

**Falkenstein E., Tillmann H.C., Christ M., Feuring M. & Wehling M.**, 2000. Multiple Actions of Steroid Hormones - A Focus on Rapid, Nongenomic Effects. *Pharmacological Reviews.* 52, 513-556.

**Felber B.K., Maurhofer S., Jaggi R.B., Wyler T., Wahli W., Ryffel G.U. & Weber R.**, 1980. Isolation and translation in vitro of four related vitellogenin mRNAs of estrogen-stimulated *Xenopus laevis*. *European Journal of Biochemistry.* 105, 17-24.

**Flouriot G., Pakdel F. & Valotaire Y.**, 1996. Transcriptional and post-transcriptional regulation of rainbow trout estrogen receptor and vitellogenin gene expression. *Molecular and Cellular Endocrinology.* 124, 173-183.

**Frueh F., Huang S. & Lesko L.**, 2004. Regulatory acceptance of toxicogenomics data. *Environmental Health Perspectives.* 112, A663.

**Funkenstein B., Dyman A., Levavi-Sivan B. & Tom M.**, 2004. Application of real-time PCR for quantitative determination of hepatic vitellogenin transcript levels in the striped sea bream, *Lithognathus mormyrus*. *Marine Environmental Research.* 58, 659-663.

**Gant T.W. & Zhang S.D.**, 2005. In pursuit of effective toxicogenomics. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis.* 575, 4-16.

**Godwin J.R. & Thomas P.**, 1993. Sex Change and Steroid Profiles in the Protandrous Anemonefish *Amphiprion melanopus* (Pomacentridae, Teleostei). *General and Comparative Endocrinology.* 91, 144-157.

**Gordon D.A., Toth G.P., Graham D.W., Lazorchak J.M., Reddy T.V., Knapp C.W., deNoyelles J., Campbell S. & Lattier D.L.**, 2006. Effects of eutrophication on vitellogenin gene expression in male fathead minnows (*Pimephales promelas*) exposed to 17[alpha]-ethynylestradiol in field mesocosms. *Environmental Pollution.* 142, 559-566.

**Greene G., Gilna P., Waterfield M., Baker A., Hort Y. & Shine J.**, 1986. Sequence and expression of human estrogen receptor complementary DNA. *Science.* 231, 1150-1154.

**Greytak S.R. & Callard G.V.**, 2007. Cloning of three estrogen receptors (ER) from killifish (*Fundulus heteroclitus*): Differences in populations from polluted and reference environments. *General and Comparative Endocrinology.* 150, 174-188.

**Greytak S.R., Champlin D. & Callard G.V.**, 2005. Isolation and characterization of two cytochrome P450 aromatase forms in killifish (*Fundulus heteroclitus*): Differential expression in fish from polluted and unpolluted environments. *Aquatic Toxicology.* 71, 371-389.

**Grumbach M.** 2000. Estrogen, bone, growth and sex: a sea change in conventional wisdom. *Journal of Pediatric Endocrinology and Metabolism.* 13, 1439-1455.

**Guan G., Kobayashi T. & Nagahama Y.**, 2000. Sexually Dimorphic Expression of Two Types of DM (Doublesex/Mab-3)-Domain Genes in a Teleost Fish, the Tilapia (*Oreochromis niloticus*). *Biochemical and Biophysical Research Communications.* 272, 662-666.

**Guillette L., Crain D., Rooney A. & Pickford D.**, 1995. Organization versus Activation: The role of Endocrine-disrupting Contaminants (EDC's) durig Embryonic Development in Wildlife. *Environmental Health Perspectives.* 103, 157-164.

**Gunderson M.P., LeBlanc G.A. & Guillette J.**, 2001. Alterations in Sexually Dimophic Biotransformation of Testosterone in Juvenile American Alligators (*Alligator mississippiensis*) from Contaminated Lakes. *Environmental Health Perspectives.* 109, 1257.

**Hahlbeck E., Griffiths R. & Bengtsson B.E.**, 2004. The juvenile three-spined stickleback (*Gasterosteus aculeatus*) as a model organism for endocrine disruption: I. Sexual differentiation. *Aquatic Toxicology.* 70, 287-310.

**Hamadeh H.K., Bushel P.R., Jayadev S., Martin K., DiSorbo O., Sieber S., Bennett L., Tennant R., Stoll R., Barrett J.C., Blanchard K., Paules R.S. & Afshari C.A.**, 2002. Gene Expression Analysis Reveals Chemical-Specific Profiles. *Toxicological Sciences.* 67, 219-231.

**Harbott L., Burmeister S., White R., Vagell M. & Fernald R.**, 2007. Androgen receptors in a cichlid fish, *Astatotilapia burtoni*: Structure, localization, and expression levels. *The journal of comparative neurology.* 504, 57-73.

**Hatzimanikatis V. & Lee K.**, 1999. Dynamical analysis of gene networks requires both mRNA and protein expression information. *Metabolic Engineering.* 1, 275-281.

**Hawkins M.B., Thornton J.W., Crews D., Skipper J.K., Dotte A. & Thomas P.**, 2000. Identification of a third distinct estrogen receptor and reclassification of estrogen receptors in teleosts. *Proceedings of the National Academy of Sciences U.S.A.* 97, 10751-10756.

**Haynes M.P., Russell K.S. & Bender J.R.**, 2000. Molecular mechanisms of estrogen actions on the vasculature. *Journal of Nuclear Cardiology.* 7, 500-508.

**Hertz R.** 1985. The estrogen problem: retrospect and prospect. In McLachlan,J.A. *Estrogens in the Environment. II. Influences on Development*. Elsevier Science B.V.,Amsterdam, pp 1-11.

**Hiramatsu N., Cheek A.O., Sullivan C.V., Matsubara T. & Hara A.**, 2005. Vitellogenesis and endocrine disruption. In Mommsen,T.P. & Moon,T.W. *Biochemical and Molecular biology of fishes*. Elsevier Science BV,Amsterdam, pp 431-471.

**Hiramatsu N., Hara A., Hiramatsu K., Fukada H., Weber G.M., Denslow N.D. & Sullivan C.V.**, 2002. Vitellogenin-Derived Yolk Proteins of White Perch, *Morone americana*: Purification, Characterization, and Vitellogenin-Receptor Binding1. *Biology of Reproduction.* 67, 655-667.

**Hisamoto K. & Bender J.R.**, 2005. Vascular cell signaling by membrane estrogen receptors. *Steroids.* 70, 382-387.

**Huang W.W., Yin Y., Bi Q., Chiang T.C., Garner N., Vuoristo J., McLachlan J.A. & Ma L.**, 2005. Developmental Diethylstilbestrol Exposure Alters Genetic Pathways of Uterine Cytodifferentiation. *Molecular Endocrinology.* 19, 669-682.

**Iguchi T., Watanabe H. & Katsu Y.**, 2006. Application of Ecotoxicogenomics for Studying Endocrine Disruption in Vertebrates and Invertebrates. *Environmental Health Perspectives.* 114, 101-105.

**Iguchi T. & Watanebe H.**, 2003. Developmental effects of hormonally active agents on animals: from *Daphnia* to humans. *Environmental Sciences.* 10, 43-60.

**Iguchi T., Watanebe H. & Katsu Y.**, 2007. Toxicogenomics and ecotoxicogenomics for studying endocrine disruption and basic biology. *General and Comparative Endocrinology.* 153, 25-29.

**Inoue A., Yoshida N., Omoto Y., Oguchi S., Yamori T. & Kiyama R.**, 2002. Development of cDNA microarray for expression profiling of estrogen-responsive genes. *Journal of moecular endocrinodogy.* 29, 175-192.

**Inoue T. & Pennie W.**, 2003. *Toxicogenomics*. Springer-Verlag. Tokyo, Japan .

**Janicke F.** 2004. Are all aromatase inhibitors the same? A review of the current evidence. *The Breast.* 13, 10-18.

**Janosek J., Hilscherova K., Blaha L. & Holoubek I.**, 2006. Environmental xenobiotics and nuclear receptors--Interactions, effects and in vitro assessment. *Toxicology in Vitro.* 20, 18-37.

**Jones P.A. & Takai D.**, 2001. The Role of DNA Methylation in Mammalian Epigenetics. *Science.* 293, 1068.

**Kato S., Tora L., Yamauchi J., Masushige S., Bellard M. & Chambon P.**, 1992. A far upstream estrogen response element of the ovalbumin gene contains several half-palindromic 5'-TGACC-3' motifs acting synergistically. *Cell.* 68, 731-742.

**Kazeto Y., Ijiri S., Place A.R., Zohar Y. & Trant J.M.**, 2001. The 5'-Flanking Regions of CYP19A1 and CYP19A2 in Zebrafish. *Biochemical and Biophysical Research Communications.* 288, 503-508.

**Kelce W.R., Monosson E., Gamcsik M.P., Laws S.C. & Gray L.E.**, 1994. Environmental Hormone Disruptors: Evidence That Vinclozolin Developmental Toxicity Is Mediated by Antiandrogenic Metabolites. *Toxicology and Applied Pharmacology.* 126, 276-285.

**Kelce W.R., Lambright C.R., Gray L.E. & Roberts K.P.**, 1997. Vinclozolin andp,p'-DDE Alter Androgen-Dependent Gene Expression:In VivoConfirmation of an Androgen Receptor-Mediated Mechanism. *Toxicology and Applied Pharmacology.* 142, 192-200.

**Kim B.H., Takemura A., Kim S.J. & Lee Y.D.**, 2003. Vitellogenin synthesis via androgens in primary cultures of tilapia hepatocytes. *General and Comparative Endocrinology.* 132, 248-255.

**Kim K.H. & Bender J.R.**, 2005. Rapid, Estrogen Receptor-Mediated Signaling: Why Is the Endothelium So Special? *Science's STKE.* 2005, e28.

**Kime D.** 1998. *Endocrine disruption in fish*. Kluwer Academic Press. Boston .

**Kishida M. & Callard G.V.**, 2001. Distinct Cytochrome P450 Aromatase Isoforms in Zebrafish (*Danio rerio*) Brain and Ovary Are Differentially Programmed and Estrogen Regulated during Early Development. *Endocrinology.* 142, 740-750.

**Klinge C.M.** 2001. Estrogen receptor interaction with estrogen response elements. *Nucleic Acids Research.* 29, 2905-2919.

**Kloas W.** 2002. Amphibians as a model for the study of endocrine disruptors. In*International Review of Cytology*. Academic Press,pp 1-57.

**Knower K., Kelly S. & Harley V.**, 2003. Turning on the male - SRY, SOX9 and sex determination in mammals. *Cytogenetic Genome Research.* 101, 185-198.

**Kobayashi T., Kajiura-Kobayashi H. & Nagahama Y.**, 2003. Induction of XY sex reversal by estrogen involves altered gene expression in a teleost, tilapia. *Cytogenetic Genome Research.* 101, 289-294.

**Kondo M., Nanda I., Hornung U., Asakawa S., Shimizu N., Mitani H., Schmid M., Shima A. & Schartl M.**, 2003. Absence of the candidate male sex-determining gene dmrt1b(Y) of medaka from other fish species. *Current Biology.* 13, 416-420.

**Koopman P. & Loffler K.A.**, 2003. Sex determination: the fishy tale of Dmrt1. *Current Biology.* 13, R177-R179.

**Kubista M., Andrade J.M., Bengtsson M., Forootan A., Jonak J., Lind K., Sindelka R., Sjoback R., Sjogreen B. & Strombom L.**, 2006. The real-time polymerase chain reaction. *Molecular Aspects of Medicine.* 27, 95-125.

**Kuiper G.G., Enmark E., Pelto-Huikko M., Nilsson S. & Gustafsson J.A.**, 1996. Cloning of a novel estrogen receptor expressed in rat prostate and ovary. *Proceedings of the national academy of sciences.* 93, 5925-5930.

**Kwon J., Haghpanah V., Kogson-Hurtado L., McAndrew B. & Penman D.**, 2000. Masculinization of genetic female nile tilapia (*Oreochromis niloticus*) by dietry administration of an aromatase inhibitor during sexual differentiation. *Journal of Experimental Zoology.* 46-53.

**Larkin P., Sabo-Attwood T., Kelso J. & Denslow N.D.**, 2002. Gene expression analysis of largemouth bass exposed to estradiol, nonylphenol, and p,p'-DDE. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology.* 133, 543-557.

**Lazier C.B., Langley S., Ramsey N.B. & Wright J.M.**, 1996. Androgen Inhibition of Vitellogenin Gene Expression in Tilapia (*Oreochromis niloticus*). *General and Comparative Endocrinology.* 104, 321-329.

**Leanos-Castaneda O. & Van Der Kraak G.**, 2007. Functional characterization of estrogen receptor subtypes, ER[alpha] and ER[beta], mediating vitellogenin production in the liver of rainbow trout. *Toxicology and Applied Pharmacology.* 224, 116-125.

**Lee K.M., Kim J.H. & Kang D.**, 2005. Design issues in toxicogenomics using DNA microarray experiment. *Toxicology and Applied Pharmacology.* 207, 200-208.

**Lim E.H., Ding J.L. & Lam T.J.**, 1991. Estradiol-induced vitellogenin gene expression in a teleost fish, *Oreochromis aureus*. *General and Comparative Endocrinology.* 82, 206-214.

**Lutfalla G., Roest Crollius H., Brunet F.G., Laudet V. & Robinson-Rechavi M.**, 2003. Inventing a sex-specific gene: a conserved role of DMRT1 in teleost fishes plus a recent duplication in the medaka Oryzias latipes resulted in DMY. *Journal of Molecular Evolution.* 57 Suppl 1, S148-S153.

**MacLusky N.J., Clark A.S., Naftolin F. & Goldman-Rakic P.S.**, 1987. Estrogen formation in the mammalian brain: possible role of aromatase in sexual differentiation of the hippocampus and neocortex. *Steroids.* 50, 459-474.

**Maclusky N. & Naftolin F.**, 1981. Sexual Differentiation of the Central Nervous System. *Science.* 4488, 1294-1302.

**Mangelsdorf D.J., Thummel C., Beato M., Herrlich P., Schutz G., Umesono K., Blumberg B., Kastner P., Mark M., Chambon P. & Evans R.M.**, 1995. The nuclear receptor superfamily: The second decade. *Cell.* 83, 835-839.

**Marchand O., Govoroun M., D'Cotta H., McMeel O., Lareyre J.J., Bernot A., Laudet V. & Guiguen Y.**, 2000. DMRT1 expression during gonadal differentiation and spermatogenesis in the rainbow trout, *Oncorhynchus mykiss*. *Biochimica et Biophysica Acta - Gene Structure and Expression.* 1493, 180-187.

**Marchant G.E.** 2002. Toxicogenomics and toxic torts. *Trends in Biotechnology.* 20, 329-332.

**Matsuda M., Nagahama Y., Shinomiya A., Sato T., Matsuda C., Kobayashi T., Morrey C.E., Shibata N., Asakawa S., Shimizu N., Hori H., Hamaguchi S. & Sakaizumi M.**, 2002. DMY is a Y-specific DM-domain gene required for male development in the medaka fish. *Nature.* 417, 559-563.

**McDonnell D.P.** 1999. The Molecular Pharmacology of SERMs. *Trends in Endocrinology and Metabolism.* 10, 301-311.

**McDonnell D.P.** 2004. The molecular determinants of estrogen receptor pharmacology. *Maturitas.* 48, 7-12.

**McDonnell D.P. & Norris J.D.**, 2002. Connections and Regulation of the Human Estrogen Receptor. *Science.* 296, 1642-1644.

**McLachlan J.A.** 2001. Environmental Signaling: What Embryos and Evolution Teach Us About Endocrine Disrupting Chemicals. *Endocrine Reviews.* 22, 319-341.

**Menuet A., Pellegrini E., Anglade I., Blaise O., Laudet V., Kah O. & Pakdel F.**, 2002. Molecular Characterization of Three Estrogen Receptor Forms in Zebrafish: Binding Characteristics, Transactivation Properties, and Tissue Distributions. *Biology of Reproduction.* 66, 1881.

**Meucci V. & Arukwe A.**, 2005. Detection of vitellogenin and zona radiata protein expressions in surface mucus of immature juvenile Atlantic salmon (*Salmo salar*) exposed to waterborne nonylphenol. *Aquatic Toxicology.* 73, 1-10.

**Mills L.J. & Chichester C.**, 2005. Review of evidence: Are endocrine-disrupting chemicals in the aquatic environment impacting fish populations? *Science of The Total Environment.* 343, 1-34.

**Montano M.M., Muller V., Trobaugh A. & Katzenellenbogen B.S.**, 1995. The carboxy-terminal F domain of the human estrogen receptor: role in the transcriptional activity of the receptor and the effectiveness of antiestrogens as estrogen antagonists. *Molecular Endocrinology.* 9, 814-825.

**Mooradian A.D., Morley J.E. & Korenman S.G.**, 1987. Biological actions of androgens. *Endocrine Reviews.* 8, 1-28.

**Mori T., Matsumoto H. & Yokota H.**, 1998. Androgen-induced vitellogenin gene expression in primary cultures of rainbow trout hepatocytes. *The Journal of Steroid Biochemistry and Molecular Biology.* 67, 133-141.

**Mouchel N., Trichet V., Youssef Naimi B., Le Pennec J.P. & Wolff J.**, 1997. Structure of a fish (*Oncorhynchus mykiss*) vitellogenin gene and its evolutionary implication. *Gene.* 197, 147-152.

**Muller H.** 1914. Mutation rate and dominance of genes affecting viability in *Drosophila*. *Journal of Experimental Zoology.* 17, 325-336.

**Muller H.** 1918. Genetic variability, twin hybrids and constant hybrids, in a case of balanced lethal factors. *Genetics.* 3, 422-499.

**Murray R., Granner D., Mayes P. & Rodwell V.**, 1993. *Harper's biochemistry*. Apleton & Lange. Connecticut .

**Nakamura M. & Nagahama Y.**, 1989. Differentiation of Leydig cells and changes of testosterone levels dufing testicular differentiation in tilapia, *Oreochromis niloticus*. *Fish physology and biochemistry.* 7, 211-219.

**Neumann N.F. & Galvez F.**, 2002. DNA microarrays and toxicogenomics: applications for ecotoxicology? *Biotechnology Advances.* 20, 391-419.

**Oberemm A., Onyon L. & Gundert-Remy U.**, 2005. How can toxicogenomics inform risk assessment? *Toxicology and Applied Pharmacology.* 207, 592-598.

**Page J., Berrios S., Parra M.T., Viera A., Suja J.A., Prieto I., Barbero J.L., Rufas J.S. & Fernandez-Donoso R.**, 2005. The Program of Sex Chromosome Pairing in Meiosis Is Highly Conserved Across Marsupial Species: Implications for Sex Chromosome Evolution. *Genetics.* 170, 793-799.

**Pan M., Bell W. & Telfer W.**, 1969. Vitellogenic blood protein synthesis by insect fat body. *Science.* 165, 393-394.

**Park C.B., Takemura A., Aluru N., Park Y.J., Kim B.H., Lee C.H., Lee Y.D., Moon T.W. & Vijayan M.M.**, 2007. Tissue-specific suppression of estrogen, androgen and glucocorticoid receptor gene expression in feral vitellogenic male Mozambique tilapia. *Chemosphere.* 69, 32-40.

**Park S.Y. & Jameson J.L.**, 2005. Minireview: Transcriptional regulation of gonadal development and differentiation. *Endocrinology.* 146, 1035-1042.

**Parker K., Schedl A. & Schimmer B.**, 1999. Gene interactions in gonadal development. *Annual Reviews of Physiology.* 61, 417-433.

**Patchev V.K., Schroeder J., Goetz F., Rohde W. & Patchev A.V.**, 2004. Neurotropic action of androgens: principles, mechanisms and novel targets. *Experimental Gerontology.* 39, 1651-1660.

**Pawlowski S., Sauer A., Shears J.A., Tyler C.R. & Braunbeck T.**, 2004. Androgenic and estrogenic effects of the synthetic androgen 17[alpha]-methyltestosterone on sexual development and reproductive performance in the fathead minnow (*Pimephales promelas*) determined using the gonadal recrudescence assay. *Aquatic Toxicology.* 68, 277-291.

**Peters R.E.M., Courtenay S.C., Cagampan S., Hewitt M.L. & MacLatchy D.L.**, 2007. Effects on reproductive potential and endocrine status in the mummichog (Fundulus heteroclitus) after exposure to 17[alpha]-ethynylestradiol in a short-term reproductive bioassay. *Aquatic Toxicology.* 85, 154-166.

**Peyon P., Baloche S. & Burzawa-Gerard E.**, 1997. Investigation into the possibe role of androgens in the induction of hepatic vitellogeniesis in the European eel: in vivo and in vitro studies. *Fish physology and biochemistry.* 16, 107-118.

**Pham T.A., Hwung Y.P., Santiso-Mere D., McDonnell D.P. & O'Malley B.W.**, 1992. Ligand-dependent and -independent function of the transactivation regions of the human estrogen receptor in yeast. *Molecular Endocrinology.* 6, 1043-1050.

**Pietras RJ. & Szego C.**, 1977. Specific binding sites for oestrogen at the outer surfaces of isolated endometrial cells. *Nature.* 265, 69-72.

**Piferrer F., Blazquez M., Navarro L. & Gonzalez A.**, 2005. Genetic, endocrine, and environmental components of sex determination and differentiation in the European sea bass (*Dicentrarchus labrax* L.). *General and Comparative Endocrinology.* 142, 102-110.

**Pigliucci M.** 1996. How organisms respond to environmental changes: from phenotype to molecules (and vice versa). *Trends in Ecology & Evolution.* 11, 168-173.

**Rana S., Bisht D. & Chakraborti P.K.**, 1999. Synergistic activation of yeast-expressed rat androgen receptor by modulators of protein kinase-A. *Journal of Molecular Biology.* 286, 669-681.

**Raymond C.S., Murphy M.W., O'Sullivan M.G., Bardwell V.J. & Zarkower D.**, 2000. Dmrt1, a gene related to worm and fly sexual regulators, is required for mammalian testis differentiation. *Genes & Development.* 14, 2587-2595.

**Reik W., Dean W. & Walter J.**, 2001. Epigenetic Reprogramming in Mammalian Development. *Science.* 293, 1089.

**Reith M., Munholland J., Kelly J., Finn R. & Fyhn H.**, 2001. Lipovitellins derived from two forms of vitellogenin are differentially processed during oocyte maturation in haddock (*Melanogrammus aeglefinus*). *Journal of Experimental Zoology*. 291, 58-67.

**Riley L.G., Hirano T. & Grau E.G.**, 2004. Estradiol-17[beta] and dihydrotestosterone differentially regulate vitellogenin and insulin-like growth factor-I production in primary hepatocytes of the tilapia *Oreochromis mossambicus*. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology.* 138, 177-186.

**Rocha Monteiro P.R., Reis-Henriques M.A. & Coimbra J.**, 2000. Polycyclic aromatic hydrocarbons inhibit in vitro ovarian steroidogenesis in the flounder (*Platichthys flesus* L.). *Aquatic Toxicology.* 48, 549-559.

**Ropero A., Alonso-Magdalena P., Ripoll C., Fuentes E. & Nadal A.**, 2006. Rapid endocrine disruption: Environmental estrogen actions triggered outside the nucleus. *Journal of steroid biochemistry and molecular biology.* 102, 163-169.

**Sakai N., Iwamatsu T., Yamauchi K., Suzuki N. & Nagahama Y.**, 1988. Influence of follicular development on steroid production in the medaka (*Oryzias latipes*) ovarian follicle in response to exogenous substrates. *General and Comparative Endocrinology.* 71, 516-523.

**Sakata J.T. & Crews D.**, 2004. Developmental sculpting of social phenotype and plasticity. *Neuroscience & Biobehavioral Reviews.* 28, 95-112.

**Salhanick H.A.** 1982. Basic studies on aminoglutethimide. *Cancer research.* 42, 3315s-33321.

**Santos M.M., Micael J., Carvalho A.P., Morabito R., Booy P., Massanisso P., Lamoree M. & Reis-Henriques M.A.**, 2006. Estrogens counteract the masculinizing effect of tributyltin in zebrafish. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology.* 142, 151-155.

**Sarrif A., van Delft J.H.M., Gant T.W., Elliott B.M., van Ravenzwaay B., van Schooten F.J., van Steeg H. & Vrijhof H.**, 2005. Toxicogenomics in genetic toxicology and hazard determination: Introduction and overview. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis.* 575, 1-3.

**Sawaguchi S., Koya Y., Yoshizaki N., Ohkubo N., Andoh T., Hiramatsu N., Sullivan C.V., Hara A. & Matsubara T.**, 2005. Multiple Vitellogenins (Vgs) in Mosquitofish (*Gambusia affinis*): Identification and Characterization of Three Functional Vg Genes and Their Circulating and Yolk Protein Products. *Biology of Reproduction.* 72, 1045-1060.

**Schartl M.** 2004. Sex chromosome evolution in non-mammalian vertebrates. *Current Opinion in Genetics & Development.* 14, 634-641.

**Schmidt C.W.** 2003. Toxicogenomics. *Environmental Health Perspectives Supplements.* 111, A20-A25.

**Scholz S., Kordes C., Hamann J. & Gutzeit H.O.**, 2004. Induction of vitellogenin in vivo and in vitro in the model teleost medaka (*Oryzias latipes*): comparison of gene expression and protein levels. *Marine Environmental Research.* 57, 235-244.

**Segner H., Eppler E. & Reinecke M.**, 2006. The imact of environmental hormonally active substances on the endocrine and immune systems of fish. In Reinecke,M., Zaccone,G. & Kapoor,B. *Fish endocrinology*. Science publishers,Enfield, pp 809-865.

**Seiler-Tuyns A., Walker P., Martinez E., Merillat A.M., Givel F. & Wahli W.**, 1986. Identification of estrogen-responsive DNA sequences by transient expression experiments in a human breast cancer cell line. *Nucleic Acids Research.* 14, 8755-8770.

**Shapiro D.Y. & Boulon J.**, 1982. The influence of females on the initiation of female-to-male sex change in a coral reef fish. *Hormones and Behavior.* 16, 66-75.

**Shrader E., Henry T., Greeley M.Jr. & Bradley P.**, 2003. Proteomics in Zebrafish exposed to endorine disrupting compounds. *Ecotoxiclogy.* 12, 485-488.

**Solari A.** 1994. *Sex chromosomes and sex determination in vertebrates*. CRC Press. Florida .

**Sonnenschein C. & Soto A.M.**, 1998. An updated review of environmental estrogen and androgen mimics and antagonists. *The Journal of Steroid Biochemistry and Molecular Biology.* 65, 143-150.

**Steiner G., Suter L., Boess F., Gasser R., de Vera M.C., Albertini S. & Ruepp S.**, 2004. Discriminating different classes of toxicants by transcript profiling. *Environmental Health Perspectives.* 112, 1236-1248.

**Steinkampf M., Mendelson C. & Simpson E.**, 1987. Regulation by follicle-stimulating hormone of the synthesis of aromatase cytrochrome P-450 in human granulosa cells. *Molecular Endocrinology.* 1, 465-471.

**Stifani S., Nimpf J. & Schneider W.J.**, 1990. Vitellogenesis in *Xenopus laevis* and chicken: cognate ligands and oocyte receptors. The binding site for vitellogenin is located on lipovitellin I. *Journal of Biological Chemistry.* 265, 882-888.

**Sundararaj B.I. & Nath P.**, 1981. Steroid-induced synthesis of vitellogenin in the catfish, Heteropneustes fossilis (Bloch). *General and Comparative Endocrinology.* 43, 201-210.

**Takase M. & Iguchi T.**, 2007. Molecular cloning of two isoforms of *Xenopus (Silurana) tropicalis* estrogen receptor mRNA and their expression during development. *Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression.* 1769, 172-181.

**Tanaka M., Fukada S., Matsuyama M. & Nagahama Y.**, 1995. Structure and promoter analysis of the cytochrome P-450 aromatase gene of the teleost fish, medaka (*Oryzias latipes*). *Journal of Biochemistry.* 117, 719-725.

**Tchoudakova A. & Callard G.V.**, 1998. Identification of Multiple CYP19 Genes Encoding Different Cytochrome P450 Aromatase Isozymes in Brain and Ovary. *Endocrinology.* 139, 2179-2189.

**Tchoudakova A., Kishida M., Wood E. & Callard G.V.**, 2001. Promoter characteristics of two cyp19 genes differentially expressed in the brain and ovary of teleost fish. *The Journal of Steroid Biochemistry and Molecular Biology.* 78, 427-439.

**Teo B.Y., Tan N.S., Lim E.H., Lam T.J. & Ding J.L.**, 1998. A novel piscine vitellogenin gene: structural and functional analyses of estrogen-inducible promoter. *Molecular and Cellular Biology.* 146, 103-120.

**Terasaka S., Aita Y., Inoue A., Hayashi S., Nishigaki M., Aoyagi K., Sasaki H., Wada-Kiyama Y., Sakuma Y., Akaba S., Tanaka J., Sone H., Yonemoto J., Tanji M. & Kiyama R.**, 2004. Using a customized DNA microarray for expression profiling of the estrogen-responsive genes to evaluate estrogen activity among natural estrogens and industrial chemicals. *Environmental Health Perspectives.* 112, 773-781.

**Tong S.K. & Chung B.c.**, 2003. Analysis of zebrafish cyp19 promoters. *The Journal of Steroid Biochemistry and Molecular Biology.* 86, 381-386.

**Tong W., Xie Q., Hong H., Shi L., Fang H. & Perkins R.**, 2004. Assessment of prediction confidence and domain extrapolation of two structure-activity relationship models for predicting estrogen receptor binding activity. *Environmental Health Perspectives.* 112, 1249-1254.

**Trant J.M., Lehrter J., Gregory T., Nunez S. & Wunder J.**, 1997. Expression of cytochrome P450 aromatase in the channel catfish, *Ictalurus punctatus*. *The Journal of Steroid Biochemistry and Molecular Biology.* 61, 393-397.

**Traupe T., Stettler C.D., Li H., Haas E., Bhattacharya I., Minotti R. & Barton M.**, 2007. Distinct Roles of Estrogen Receptors {alpha} and {beta} Mediating Acute Vasodilation of Epicardial Coronary Arteries. *Hypertension.* 49, 1364-1370.

**Tsai M.J. & O'Malley B.W.**, 1994. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annual Reviews of Biochemistry.* 63, 451-486.

**Tsuchiya Y., Nakajima M. & Yokoi T.**, 2005. Cytochrome P450-mediated metabolism of estrogens and its regulation in human. *Cancer Letters.* 227, 115-124.

**Tycko B.** 2000. Epigenetic gene silencing in cancer. *Journal of Clinical Investigation.* 105, 401-407.

**Uguz C., Iscan M. & Togan I.**, 2003. Developmental genetics and physiology of sex differentiation in vertabrates. *Environmental Toxicology and Pharmacology.* 14, 9-16.

**Urbatzka R., Lutz I. & Kloas W.**, 2007. Aromatase, steroid-5-alpha-reductase type 1 and type 2 mRNA expression in gonads and in brain of *Xenopus laevis* during ontogeny. *General and Comparative Endocrinology.* 153, 280-288.

**Urushitani H., Shimizu A., Katsu Y. & Iguchi T.**, 2002. Early estrogen exposure induces ambormal development of *Fundulus heteroclitus*. *Journal of Experimental Zoology.* 293, 693-702.

**Vallender E.J. & Lahn B.T.**, 2004. How mammalian sex chromosomes acquired their peculiar gene content. *Bioessays.* 26, 159-169.

**Vasudevan N., Ogawa S. & Pfaff D.**, 2002. Estrogen and Thyroid Hormone Receptor Interactions: Physiological Flexibility by Molecular Specificity. *Physiological Reviews.* 82, 923-944.

**Vega-Lopez A., Martinez-Tabche L. & Galar Martinez M.**, 2007. Toxic effects of waterborne polychlorinated biphenyls and sex differences in an endangered goodeid fish (*Girardinichthys viviparus*). *Environment International.* 33, 540-545.

**Vezina C.M., Walker N.J. & Olson J.R.**, 2004. Subchronic exposure to TCDD, PeCDF, PCB126, and PCB153: effect on hepatic gene expression. *Environmental Health Perspectives.* 112, 1636-1644.

**Vlaming L., Wiley H.S., Delahunty G. & Wallace R.A.**, 1980. Goldfish (*Carassius auratus*) vitellogenin: Induction, isolation, properties and relationship to yolk proteins. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology.* 67, 613-623.

**Volff J.N., Kondo M. & Schartl M.**, 2003. Medaka dmY/dmrt1Y is not the universal primary sex-determining gene in fish. *Trends in Genetics.* 19, 196-199.

**Volff J.N. & Schartl M.**, 2001. Variability of genetic sex determination in poeciliid fishes. *Genetica.* 111, 101-110.

**Waddington C.** 1942. Canalization of development and the inheritance of acquired charcters. *Nature.* 150, 563-565.

**Waddington C.** 1953. Genetic assimilation of an acquired character. *Evolution.* 7, 126.

**Wahli W., Dawid I.B., Wyler T., Jaggi R.B., Weber R. & Ryffel G.U.**, 1979. Vitellogenin in *Xenopus laevis* is encoded in a small family of genes. *Cell.* 16, 535-549.

**Wang D., Senthilkumaran B., Sudhakumari C.C., Sakai F., Matsuda M., Kobayashi Tokimasa, Yoshikuni M. & Nagahama Y.**, 2005. Molecular cloning, gene expression and charaterization of the third estrogen receptor of the Nile tilapia, *Oreochromis niloticus*. *Fish physology and biochemistry.* 31, 255-266.

**Welshons W., Thayer K., Judy B., Taylor J., Curran E. & vom Saal F.**, 2003. Large effects from small exposures I. Mechanisms for endocrine-disrupting chemicals with estrogenic activity. *Environmental Health Perspectives.* 111, 994-1006.

**Wolff C.** 1759. *Theoretica generationis*. Halle.

**Wong A.H.C., Gottesman I.I. & Petronis A.**, 2005. Phenotypic differences in genetically identical organisms: the epigenetic perspective. *Human Molecular Genetics.* 14, R11-R18.

**Yamamoto T.** 1969. Sex differentiation in fishes. In Hoar,W. & Randall,D. *Fish Physiology*. Academic Press,New York, pp 117-175.

Chapter 2

# Characterization of vitellogenin gene expression in *Oreochromis mossambicus* during development and the establishment of an estrogen-dependent gene expression assay[#]

**ABSTRACT**

*Oreochromis mossambicus* is a tilapiine species indigenous to Southern Africa and represents a potential animal model for the development of an endocrine disruptor screening assay based upon the widely used estrogen-responsive vitellogenin (*vtg*) biomarker gene. However, little is known about *vtg* mRNA expression during normal development in *O. mossambicus.* A 3kb upstream promoter region of the *vtg* gene was isolated and putative binding sites identified for several regulatory factors including estrogen receptor (ESR), the sex-determining protein, SRY, and vitellogenin binding protein. Evidence for the expression of several splice-site *vtg* mRNA variants was found in a number of tissue types. A quantitative real time polymerase chain reaction (QPCR) assay was developed based upon a specific primer pair (OMV6/9) that selectively amplified the liver-enriched transcript. The level of this transcript in liver tissue was high in females and lower, but detectable, in males and was significantly increased in male fish exposed to 17β-estradiol ($E_2$). Juvenile whole-body homogenates (WBH) were found to contained extremely low levels of liver-specific *vtg* mRNA between 5 and 110 days post fertilization (dpf) compared to adult male liver and that exposure of 20 dpf juveniles to $E_2$ and diethylstilbestrol showed a substantial increase in this transcript within hours. These data reiterate the importance of selecting appropriate *vtg*-specific primers for determining exposure to estrogenic chemicals and establish that the combination of QPCR using the OMV6/9 primer pair with juvenile fish WBH is a feasible alternative to the standard use of adult male livers as indicators of endocrine disruption.

[#] Submitted for publication

Keywords: *Oreochromis mossambicus*, vitellogenin, development, quantitative real-time polymerase chain reaction

## INTRODUCTION

Evidence presented over the last century has led to the formulation of the endocrine disruption hypothesis, stating that anthropogenic and natural chemical substances in the aquatic environment have the potential to disrupt the normal functions of the endocrine system and its hormones in wildlife and humans (Guillette, Jr. *et al.* 1995; Krimsky 2000). These chemical compounds mimic, antagonize or modulate the normal actions of hormones or affect hormonal synthesis or degradation pathways and are generally referred to as endocrine disrupting chemicals (EDCs). However, because environmental EDCs are mostly found as part of complex mixtures, understanding the context in which EDCs operate remains problematic and sensitive biological screening platforms are required that are amenable to high throughput methodologies. Tier I screening applications favor aquatic systems, due to the water borne nature of many chemical pollutants and their accumulation in sediment and groundwater (Jobling & Tyler 2003). Various aquatic species have been studied and currently serve as sentinels for low concentration chemical exposure with endocrine modulation activity (Bruton & Boltt 1975; Nagahama 1990; Helbing *et al.* 1992; Nagahama 1994; Kloas 2002; Noaksson *et al.* 2003; Hahlbeck *et al.* 2004; Sharpe *et al.* 2004; Zhong *et al.* 2005; Piferrer *et al.* 2005; Kuhl *et al.* 2005; Tao *et al.* 2006; Zhang *et al.* 2006; Filby *et al.* 2007; Cheshenko *et al.* 2008) .

Initially, most research on endocrine disruption in fish was related to the actions of environmental estrogens: EDCs that elicit an estrogenic response similar to the actions of the natural female hormone 17β-estradiol ($E_2$) (Segner *et al.* 2006). Assay development included measurement of changes in expression of estrogen-regulated gene products. Synthesis of one such product, vitellogenin (the egg yolk precursor phospholipoglycoprotein; VTG), is generally associated with oviparous reproduction and forms the basis of many estrogenic screening programs in aquatic systems (Sumpter & Jobling 1995; Hiramatsu *et al.* 2005; Porte *et al.* 2006). The *vtg* is expressed in the liver where it is extensively modified post-translationally, secreted into the bloodstream, and sequestered by the oocytes *via* specific vitellogenin receptors in coated pits (endocytosis) (Lim *et al.* 1991). Kim et al (2003) illustrated the enhanced and induced effect of $E_2$ on

hepatocyte cultures in male and female tilapia, *Oreochromis mossambicus*, respectively, and thereby confirm the strong VTG inducing effect by this hormone in this species *ex vivo* (Kim *et al.* 2003) and *in vivo* (Takemura & Kim 2001). While egg-producing females normally produce high levels of VTG, males do not produce significant amounts. However, *vtg* expression in males is inducible following exposure to estrogenic substances and represents a useful biological screening platform for estrogen-related EDCs (Vaillant *et al.* 1988; Miracle *et al.* 2006). Although immunological methods to quantify circulating VTG levels in either juvenile WBH or adult plasma samples are currently used, the detection and quantification of *vtg* mRNA transcripts using a quantitative real-time RT-PCR (QPCR) assay is an additional highly sensitive tool to assess temporal variation in expression of *vtg* genes as well as show early signs of estrogenic EDC activity.

The tilapiine species, *Oreochromis mossambicus*, (Mozambique Tilapia), is native to Southern Africa and extensively used in aquaculture (Trewavas 1983). Many aspects of the life history and ecology of this mouthbrooding species have been described (Neil 1966; Popma & Masser 1999), but what remains unclear, are many aspects related to the development and reproductive biology of this species; particularly with respect to molecular mechanisms (Majumdar & McAndrew 1986; Wright J.M. 1989; Wohlfarth & Wedekind 1991; Nussey *et al.* 1995; Tsai & Wang 1997; Wang & Tsai 2000; Vijayan *et al.* 2001; Schmid *et al.* 2003). Because of the potential for a sex non-differentiated period and the contribution of environmental sex determination of *O. mossambicus*, juvenile fish may serve as a powerful alternative to adult males in the context of an EDC assay. In addition, reduced assay duration may benefit from increased ease of husbandry and no separation of test populations by sex.

In order for juvenile tilapia to serve as a good animal model in an EDC assay, it is important to establish the expression pattern of *vtg* mRNA during normal development and determine early developmental stages at which *vtg* expression is suitably low. Then an investigation of $E_2$-responsive nature of *vtg* can be performed. The aim of this study was to clone the *vtg* gene promoter region and related partial cDNA from *O. mossambicus* in order to develop a quantitative PCR (QPCR) protocol that effectively measures the expression levels of *vtg* transcript (a) in different adult tissue types, (b) during early development and (c) following estrogen-dependent induction in both adult

males and juveniles. Our results confirmed tissue-restricted expression of the normally spliced *vtg* transcript in the liver of both males and females; whereas alternatively spliced transcripts were observed in all other tissue types examined. Adult female liver expressed significantly more *vtg* transcript than males. *In silico* analysis of the *vtg* promoter region indicated the presence of several potential estrogen responsive elements (ERE) under stringent multilevel consensus sequence testing. Induction studies confirmed that *vtg* mRNA abundance increased in *O. mossambicus* adult male hepatic tissue following exposure to $E_2$. Juvenile WBH displayed much lower levels of liver-specific *vtg* transcript which increased significantly upon $E_2$ exposure confirming the great potential of juvenile tilapia as an effective bio-indicator of aquatic estrogen-based screening in Southern Africa.

## MATERIALS AND METHODS

*Experimental Animals*

Adult breeding stock of *Oreochromis mossambicus* were obtained from Aquastel (South Africa) and maintained in aquaria with water under constant aeration and filtered through activated charcoal. Water temperature was maintained at 27 °C (±1 °C). Fish were fed once daily with Tilapia pellets (AquaNutro, South Africa). The light regime followed a 14:10 light:dark cycle. Offspring production was monitored daily in this mouthbrooding species. Females carrying eggs in their mouths were removed from the breeding aquaria to culturing tanks. Each brooding female was kept alone until the offspring reached the swim-up fry stage, at which time the adult females were removed and re-introduced into the breeding tank. Each batch of offspring was reared separately in the same water conditions as for breeding stock. Animals at the appropriate developmental stage (determined by age in 5 day intervals after fertilization) were collected, euthanized using 0.01 % Benzocaine (Heynes Mathew, Ltd., South Africa) and preserved in RNA*later* (Ambion Inc., USA) at 4°C. At least three different breeding pairs were used to generate offspring that were sampled at each developmental stage. For the adult tissue scan for determination of *vtg* mRNA expression, at least five males and five females were dissected and the total RNA prepared as outlined below.

*Genomic DNA isolation*

Tilapia finclips of about 25 $mm^2$ were homogenized in 700 µl extraction buffer (50 mM Tris, pH 8; 0.7 M NaCl; 10 mM EDTA; 1 % CTAB; 0.1 % β-mercaptoethanol) and genomic DNA was isolated using a CTAB-method according to D'Amato *et al.* (2007).

*Total RNA isolation and cDNA preparation*

Anesthized fish were decapitated, dissected and appropriate tissues/whole bodies were homogenized in TRIzol reagent (Invitrogen, USA) for 5 seconds, working on ice. Total RNA was prepared from specific adult tissues or from WBH of juveniles using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. Following resuspension of total RNA in diethyl pyrocarbonate (DEPC)-treated water (50 µl), samples were treated with 1 unit of DNase I (Promega, USA) for 30 min at 37°C and precipitated at -20°C following the addition of 0.1 volumes of 3 M sodium acetate pH 5.6 and 2.5 volumes of 95% ethanol. The RNA pellets were washed with 70% ethanol and redissolved in 30 to 60 µl of DEPC-treated water. RNA yields were quantified spectrophotometrically at $Absorbance_{260nm}$ and stored at -70°C. First strand cDNA was prepared from 2 µg of total RNA using oligo $d(T)_{15}$ primers and SuperScript III RNase $H^-$ MMLV reverse transcriptase (Invitrogen, USA) as described by the manufacturer. Each cDNA sample was diluted 40-fold and stored at -20°C prior to gene expression analysis or used as template for *vtg* cDNA isolation as described below.

*Isolation of O. mossambicus vitellogenin cDNA and partial gene sequences*

To obtain partial *O. mossambicus vtg* sequence, PCR was performed using template cDNA derived from *O. mossambicus* liver tissue dissected from three adult females and primers designed against the related *O. aureus vtg* sequence (GenBank accession no. AF072686). Each 25 µl reaction included 1.5 mM $MgCl_2$, 0.05 mM of each dNTP, 1 µM of each primer (VTGforw/VTGrev, Table 1) and 2.5 Units of in-house Taq polymerase. In each reaction, 50 ng nucleic acid was added. The PCR thermocycle included an initial denaturation for 2 min at 94°C, after which followed 30 cycles of 1 min at 94°C, 30 sec at 65°C and 30 sec at 72°C, with a final elongation step of 5 min at 72°C. The same conditions were repeated when using primer pairs OMV1/2 OMV4/10 and OMV6/9 with the adaptation of

annealing temperatures which to 54 °C, 54 °C and 56 °C for the respective primer pairs. PCR products were visualized by agarose gel electrophoresis and ethidium bromide staining. Amplified DNA fragments of interest were cloned into pGEM-T Easy vector (Promega, USA) and transformed into *E. coli* DH5*α*. Plasmid DNA was isolated from positive clones detected by colony PCR (Gussow & Clackson 1989) and insert DNA was sequenced using SP6 and T7 primers. The resulting *O. mossambicus vtg* sequence information was deposited in GenBank (accession no. AJ889835). I performed sequence analysis of the DNA and derived amino acid sequences using ClustalW software according to Chenna et al. (2003). Graphic illustrations were prepared with software available in Bioedit Sequence Alignment Editor v7 (Hall 1999).

The resulting *O. mossambicus vtg* cDNA sequence information was used to design a new species-specific primer pair (iVTG1a and iVTG2a, Table 1) for use in inverse PCR (iPCR) to isolate an upstream *vtg* promoter region from genomic DNA (Ochman *et al.* 1988). Three micrograms of genomic DNA were digested in individual reactions with EcoRI, EcoRV, BamHI, XbaI restriction enzymes (Promega Corporation, Madison, USA) overnight. Digested DNA (1 μg) was resuspended in a ligation mix containing 9U T4 DNA ligase, 40 μl ligase buffer (Promega Corporation, Madison, USA) to a final volume of 400 μl at 4 °C for 12 hours. Circularized DNA was phenol/chloroform (1:1) extracted, precipitated and resuspended in 40 μl distilled water. Digestion and re-ligation of genomic DNA were visualized in ethidium bromide-stained 0.8 % (w/v) agarose gels. Inverse PCR reactions were performed with 150 ng re-ligated genomic DNA in a final volume of 50 μl containing 1 μl Elongase enzyme mix (Invitrogen, USA), 10 μl total volume of Buffer A and B, 0.2 mM of each dNTP and 0.2 mM of each primer. PCR samples were denatured at 94 ˚C for 30 sec followed by 35 cycles of amplification (94 °C denaturation, 1 min; 50 °C annealing, 1 min; 68 °C polymerization, 5 min).

All iPCR reactions were carried out using a Perkin-Elmer GeneAmp Thermocycler 9700. The 5′-flanking region of the VTG gene was cloned into pGEM-T Easy vector (Promega Corporation, Madison, USA) and sequenced in both directions (ABI PRISM 3100 genetic analyser) using the ready reaction kit with AmpliTaq DNA polymerase (The Perkin Elmer Corporation, Norwalk, USA). Two clones were isolated which displayed 100 % overlap within the 5′ flanking area of the *O. mossambicus vtg* gene. These initial sequence results

provided information for the design of additional primers, iVTG1d/2d (Table 1) at the 5′ and 3′ ends of the putative promoter region which were used in the amplification and generation of a third *vtg* promoter-associated clone.

**Table 1**. Primers used in the isolation QPCR of *O. mossambicus vtg* cDNA

| Application | Gene | Primer Name | Primer sequence |
|---|---|---|---|
| Cloning | Vitellogenin | VTG fwd | TCGAGCTGGGGTTAAAATC |
| Cloning | | VTG rev | TGGCAGTGGTTCAGGTC |
| Cloning | | iVTG1a | CATGGAAGGCACTGCCAAGC |
| Cloning | | iVTG2a | GACCTGAACCACTGCCA |
| Cloning | | iVTG1d | AAAAGTCAATAAGCCAACAC |
| Cloning | | iVTG2d | AGGTGCTCTTGGTCATGG |
| Cloning/ QPCR | | OMV4 | TAACTACATCATGAAGCCAGCACCC |
| Cloning/ QPCR | | OMV6 | GTTGGAGTGAGGACTGAGGG |
| Cloning/ QPCR | | OMV9 | GGTCCACTGGCAAACTGGATAAGC |
| Cloning/ QPCR | | OMV10 | AGTGCTGACAATCTGAGCCTCGGC |
| QPCR | *β*-actin | OMBA1 | TGTGATGGTGGGTATGGG |
| QPCR | | OMBA2 | CTGTGGTGGTGAAGGAGTAG |
| QPCR | Ribosomal protein L8 | PAC L8 up | AGAGCCCATGTAAAGCAC |
| QPCR | | PAC L8 dn | CCTGTAAGGGTCACGGAA |
| QPCR | Vitellogenin | OMV1 | TTGTAGACCCTCAGTTGCT |
| QPCR | | OMV2 | CCTCCCTAATGACATAGTTG |

*Bioinformatic analysis of the vtg promoter region*

The *O. mossambicus vtg* promoter sequence (3019 bp including ATG start codon) was analyzed using promoter prediction database software (Neural Network Promoter Prediction, http://www.fruitfly.org/seqtools/promoter.html) to identify putative proximal promoter regulatory elements (Reese & Eeckman 1995). The *O. mossambicus vtg* promoter sequence (designated as *Omvtg*; GenBank accession no. AJ889574) was examined in comparison to *vtg* promoter sequences of *Gallus gallus* (GenBank accession nos. X00345, M28125), *Oreochromis aureus* (GenBank accession no. AF072686) and *Xenopus laevis* (GenBank accession nos. X01173, X01175, X01176), using a probabilistic algorithm MEME (Bailey & Elkan 1995). A series of motif lengths (minimum: 15 bp and maximum: 100 bp) as well as the number of motifs to be detected (2, 5 and 10 respectively) were the primary parameter settings. Distribution of possible occurrences of a single motif was set as "Zero OR One" per sequence. Motif sequences were submitted to the TRANSFAC database for putative transcription factor-binding site identification (Heinemeyer *et al.* 1999). Confirmation of the position of identified estrogen responsive elements (ERE) was further examined by submitting detected motif sequences to the Dragon ERE Finder version 2.0 (Bajic *et al.* 2003) with a 0.83 default sensitivity. Multilevel consensus sequences of the putative ERE motifs are represented in WebLogo format (Crooks *et al.* 2004). All sequence similarity search and alignment analyses were conducted using BLASTn (Altschul *et al.* 1990), ClustalW (Higgins *et al.* 1994) and DNASIS MAX software (Hitachi Software Engineering Co., Ltd., Japan).

*Quantitative gene expression analysis*

Primers for analysis of gene expression by quantitative real time PCR (QPCR) were designed using Primer Premier Version 5.00 software (Premier Biosoft International, USA). These included *vtg* based upon *O. mossambicus* and *O. aureus* sequences (GenBank accession nos. AJ889835, AF072686, respectively, Table1) and the potential normalizer control gene targets *β*-actin (*O. mossambicus* *β*-actin, GenBank accession no. AB037865) and ribosomal protein L8 (rpl8) primers for which cross-species primers were found to provide specific amplification. Gene expression was quantified using either Stratagene MX3000P or MX4000 real-time quantitative PCR systems (Stratagene, USA) as well as an Applied Biosystems 7500 (Applied Biosystems, CA, USA). Each 15 μl QPCR

reaction contained 2 μl of diluted first-strand cDNA, 7.5 μl 2x SYBRgreen mix (Sigma, Germany), 0.08 μl ROX reference dye (Sigma, Germany) and 0.33 μM of each primer. The thermocycle program included 95°C (9 min), followed by 40 cycles of 95°C (15 sec), 56°C for (30 sec) and 72°C (45 sec). Each sample was evaluated in at least triplicate amplification reactions and each QPCR run included control reactions containing no cDNA template and a standard concentration of each target DNA amplicon ($4 \times 10^4$ copies/reaction). Amplification DNA products were quality checked using melting curve analysis. $C_t$ values obtained across independent amplification runs for a given gene target were used to determine relative mRNA abundance by the $\Delta\Delta C_t$ method (Pfaffl 2001; Bustin 2002; Rutledge & Côte 2003; Kubista *et al.* 2006). Two gene transcripts, bactin and rpl8, were used in this study and normalization using either gene transcript yielded highly similar results. I therefore present only the data normalized with rpl8. The standard curve for quantification using all primer sets was linear over 3 orders of magnitude with linear correlation ($R^2$) between $C_t$ and the number of target copies ≥ 0.98 in each case with reactions done in quadruplicate (data not shown). Expression data generated from juvenile fish samples indicating poor RNA quality or suboptimal amplification were not included in further analyses.

*Chemical Exposures*

Animal husbandry, treatment and handling were done according to the South African Standard: the care and use of animals for scientific purposes (SANS 10386:200X) and guidelines for the use of fishes in research (2004) (American Fisheries Society, http://www.fisheries.org/afs/publicpolicy/guidelines2004.pdf). Adult males (n = 8) were exposed to 60 μg/L 17$\beta$-estradiol ($E_2$, Sigma, USA) or an equal volume of vehicle (ethanol) alone (n = 10) for 12 h in order to confirm qualitative induction of *vtg* by $E_2$. Vehicle concentration constituted $6x10^{-6}$:1 of the volume in which animal are exposed. Livers were dissected and weighed to calculate the hepatosomatic index (HSI: liver mass/total body mass) in order to compare data from adults to that of juveniles. Juvenile fish were selected at 20 dpf where sex is not morphologically distinguishable but is believed to be already determined (Chang *et al.* 2005). These juveniles were exposed to 0.5, 1.0 and 60 μg/L $E_2$ (n = 10 for each condition) in a temporal experiment with 0, 12 and 24 hours time points. Additional juvenile exposures included 100 μg/L

diethylstilbestrol (DES) (Schwarz Mann, New York) or an equal volume of vehicle (ethanol) for 3 days. Fish were euthanized and total RNA was prepared from adult livers or juvenile WBH as described below.

*Statistical analyses*

Statistical analyses were performed using the STATISTICA software package v8 (StatSoft Inc., USA). Data was tested for normality and equal variance among groups. For parametric data-sets, I used Analysis of Variation (ANOVA) to test for significant variation ($P<0.05$) together with Holm-Sidak's Multiple Comparison test to determine significance among groups. In cases of non-parametric data-sets, I used Kruskal-Wallis Analysis of Variance (ANOVA) followed by a Mann Whitney U test to establish significance between treatment groups.

**RESULTS**

*Isolation and in silico analysis of the O. mossambicus vtg promoter*

We isolated a 3061 bp gene fragment from *O. mossambicus* (*Omvtg*; Genbank accession no. AJ889574) that showed 84% identity to the available 1695 bp 5'-end of the *O. aureus vtg* (*Oavtg*; GenBank accession no. AF072686). This fragment included 45 bp of exon I which was 93% identical to the *O. aureus* cDNA sequence. Comparative promoter sequence analysis between *Omvtg* and *Oavtg* genes of a 100 bp region upstream of the first codon showed 100 % homology. Compared to previous experimental characterization of the *Oavtg* promoter (Teo et al. 1998), computational prediction using NNPP, confirmed the identical position of a TATA-box in the *Omvtg* sequence with a core sequence of TTAAAAA, and a putative transcriptional start site (indicated by arrow; Figure 1a) which is 2 bp downstream of the experimentally identified site in *Oavtg* (Teo *et al.* 1998).

Initial analyses, using the TRANSFAC database, revealed the presence of several different putative motifs within the *vtg* promoter sequence of *O. mossambicus*. However, for this study, I focused specifically on the available cis-acting elements involved in estrogen-dependent transcriptional regulation of *vtg*. *In silico* analysis assessed the presence of putative estrogen responsive elements (EREs) residing within the 3019 bp (including start codon) promoter region. Six putative EREs were identified using TRANSFAC at a 70% cut-off

score (Figure 1A). The MEME algorithm detected two over-represented motifs within multiple *vtg* promoters to be putative EREs derived from multiple consensus sequences (indicated with dark grey triangles, Figure 1). These EREs were confirmed by the Dragon ERE finder (Bajic *et al.* 2003) at a default sensitivity of 0.83. MEME analyses of multiple *vtg* promoter sequences showed that ERE motif 1 (position -2821 bp to -2837 bp in *Omvtg*) was identified in the (+)-strand and has a core consensus sequence of CAGGTCAAGACAACCTG (Figure 1B). ERE motif 2 (position -2491 bp to -2477 bp in *Omvtg*) was identified in the (-)-strand with a core-consensus sequence of CATGTCAGTGTGCCCTG (Figure 1C).

Further analysis of MEME-identified motifs, using the MATCH-platform (http://www.gene-regulation.com/cgi-bin/pub/programs/match/bin/match.cgi), revealed high probability hits to SRY-related motifs, Sox 5 and Sox 9, as well as to vitellogenin binding protein (VBP), hepatic leukemia factor (HLF) and GATA-1 motifs (data not shown). Using MATCH-parameters at a high stringency "to minimize the sum of both error rates" I identified putative SRY-related (Sox-5/9) binding sites matching the core-sequence 5'-ACAAT-3' (on both strands) at positions -2074 bp, -1726 bp, -1153 bp, -1032 bp and -457 bp relative to the putative transcriptional start site.

*Evidence for differentially spliced products*

Using sequence information garnered from the isolated *O. mossambicus vtg* partial cDNA and the known *O. aureus vtg* gene organization, several DNA primer sets were generated that could amplify different regions of the *O. mossambicus vtg* mRNA transcript. Collectively, these regions represented approximately 17 % of the *vtg* gene (Figure 2A). Primer positions spanned putative intronic regions, based upon the *O. aureus vtg* gene structure, and could be used to assess the presence of genomic DNA contamination during RT-QPCR analysis. Indeed, the lack of amplification of an intron-containing fragment using OMV6/9 primers confirmed the absence of genomic DNA template in the assessed samples (Figures 2A and 2B).

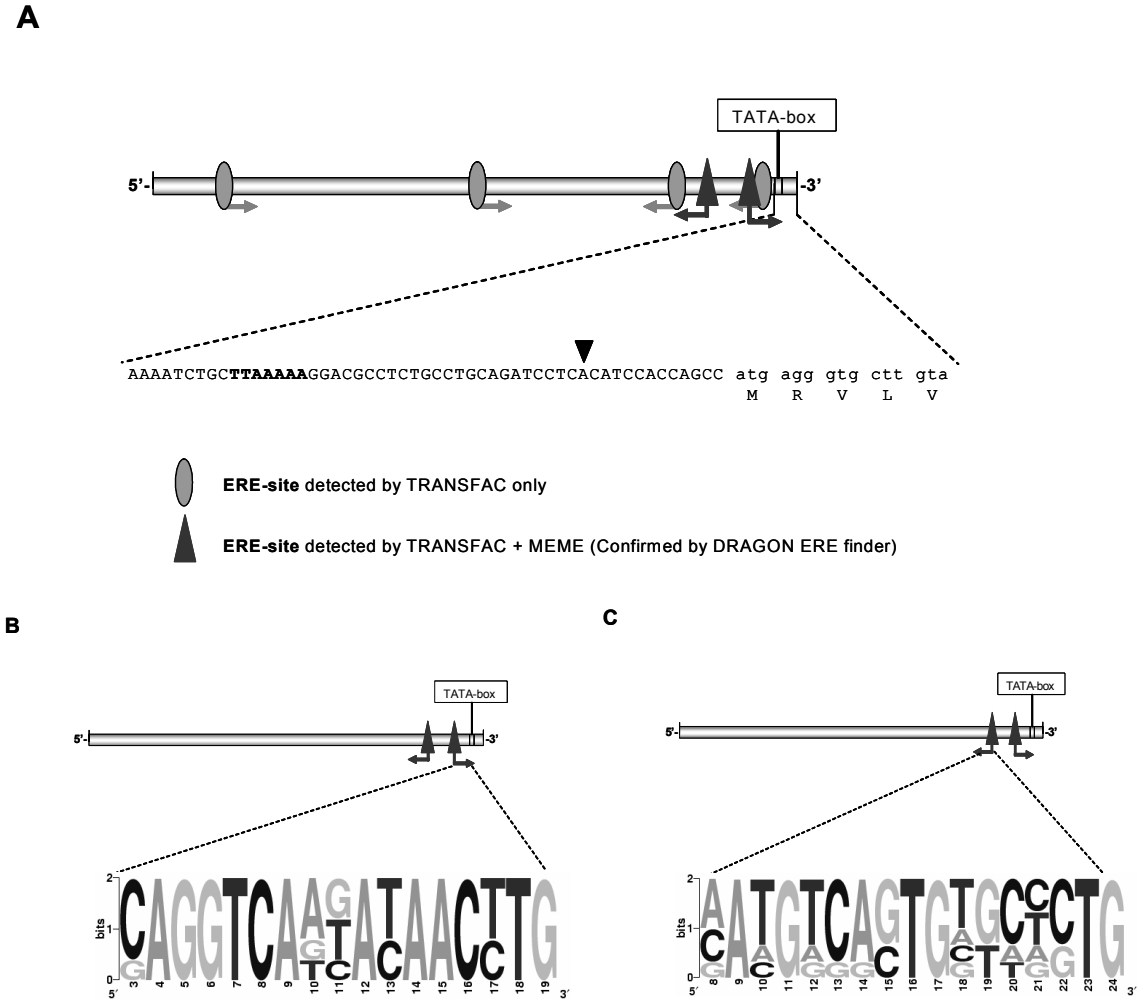


**Figure 1.** Organization of the *O. mossambicus vtg* promoter region. (A) Schematic representation of the *vtg* promoter and partial sequence of the first exon (GenBank accession no. AJ889574). *In silico* analysis revealed the putative TATA-box and transcription start site as indicated in bold and with an arrowhead, respectively. Putative ERE motifs identified by TRANSFAC are denoted by the grey ovals while those identified using multiple consensus sequences, detected by MEME and confirmed by ERE Dragon finder are shown by black triangles. Arrows indicate the direction of each putative ERE binding site within the promoter region. (B) ERE motif 1 detected in the (+) strand located 180-196 bp upstream of the putative transcription start site. (C) ERE motif 2 in the (-) strand and 526-540 bp upstream of the putative transcription start site.

Tissue specific amplification revealed alternatively spliced *vtg* transcripts (Figures 2A and B). OMV1/2 primer-based amplification from adult female and male resulted in a larger than predicted 397 bp product expressed in all tissues examined (brain, gonad, spleen, gill, heart and muscle) with the exception of the liver, where a 297 bp product of the predicted size was obtained (Figures 2A and B).

Characterization of each amplified cDNA revealed that the larger fragment contains exon IV, exon V and the intervening intronic sequence. This intron is absent in the DNA amplicon obtained from the liver (Figure 2C). Putative translation of this transcript homologous region results in the presence of stop codons in all 3 frames (Figure 2A) suggesting that it may not encode a functional VTG protein. Expression of a second alternatively spliced mRNA product was highlighted by the use of the downstream primer pair, OMV4/10, which identified a 365 bp fragment displaying inclusion of the intronic sequence in *vtg* mRNA. This intronic region was absent in *vtg* transcript expressed within the liver, as represented by a 279 bp amplified DNA product (Figure 2B). The liver-specific mRNA containing this region of the *vtg* transcript expressed at higher levels in females compared to males. In contrast to OMV1/2 and OMV4/10 amplified cDNA sequences, primer pair OMV6/9 did not detect *vtg* mRNA transcripts that retained intronic sequence information and demonstrates a tissue-restricted pattern of expression in the livers of both male and female *O. mossambicus*. In the case of all three *vtg*-specific primer sets, the female liver showed higher levels of mRNA expression compared to the male livers. These data suggest that alternate *vtg* transcripts are expressed in tissues other than the liver, while a liver-specific *vtg* transcript is present in *O. mossambicus*. Thus, development of biomarker tools for real-time QPCR must incorporate this novel information in the design of appropriately positioned gene-specific primers.

One possible reason why OMV1/2 and OMV4/10 identify mRNA variants that retain intronic information in tissues other than the liver may be due to suboptimal splice donor and/or acceptor sites at these positions in the *vtg* gene. To investigate this possibility, the mRNA splice donor and acceptor sites were analyzed within these two regions with SpliceView using the Fugu setting (http://l25.itba.mi.cnr.it/~webgene/wwwspliceview.html) and determined that a relatively weak splice acceptor site exists for exon V and no identified donor site

for exon VII, which may contribute to the retention of introns observed for *vtg* transcript homologues in many tissues.

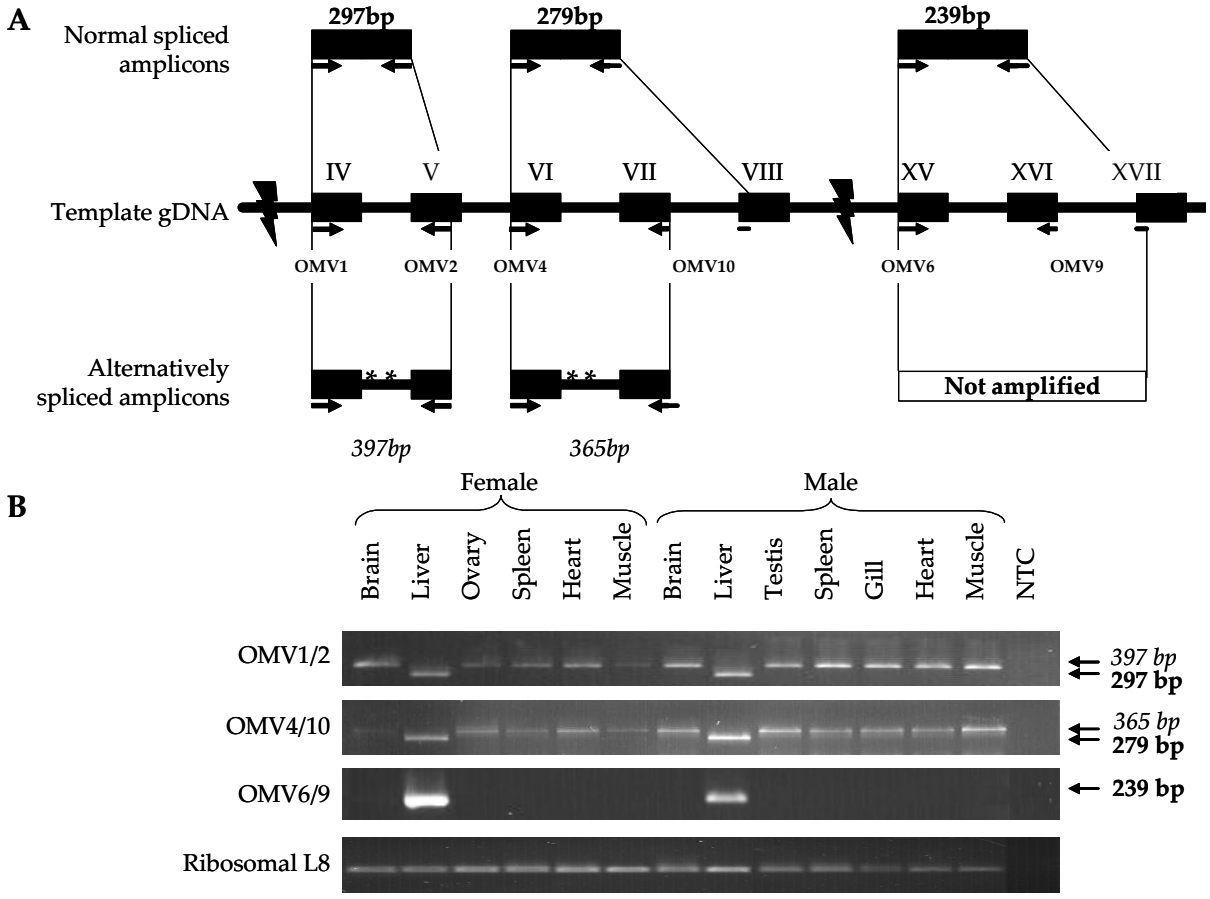


**Figure 2.** Identification of *O. mossambicus vtg* mRNA in various tissues and QPCR primer design. (A) Organization of the related *Oreochromis aureus vtg* gene (gDNA; GenBank accession no. AF072686) displaying the relative locations of exons and intronic sequences. The QPCR amplicons generated from *O. mossambicus* are shown with products from normally spliced mRNA indicated above while those generated from alternate transcripts are indicated below. Asterisks indicate the presence of putative in-frame stop codons. (B) Agarose gel analysis showing the various DNA amplicons generated with RNA isolated from the indicated adult male and female tissues is shown. Amplification of rpl8 was included to assess sample integrity and as a loading control. The relative band sizes are indicated in base pairs (bp). NTC, no template control.

Comparison of sex-associated transcript abundance in the adult tissue scan are consistent with *O. mossambicus vtg* being estrogen-inducible in the liver (Baker & Shapiro 1977; Denslow *et al.* 2001; Craft *et al.* 2004). To lend support for this hormone-dependent regulation, adult males were exposed to a high concentration of 17β-Estradiol ($E_2$) (60 μg/L). Using OMV6/9 primers that detect liver-specific *vtg* mRNA and not alternative transcript forms found in other tissues, a significant 4-fold increase was observed in *vtg* transcript levels upon $E_2$ exposure within 2 h (Figure 3; p=0.003; Mann-Whitney U test).

*Normal and induced expression of vtg mRNA in juvenile O. mossambicus*

Expression of the liver-specific form of *vtg* mRNA was assessed using the OMV6/9 primer set. Overall, the steady-state levels of *vtg* transcript in juvenile liver were found to be extremely low over an early developmental period (5 dpf to 110 dpf) compared with an approximately 60-fold higher abundance measured in adult male liver after correction for WBH (Figure 4). The level of *vtg* mRNA remained relatively steady until 65 dpf where a 2-fold increase compared to 5 dpf was observed. However, a significant increase in *vtg* transcript levels was only attained at 80 dpf (p=0.048, Mann Whitney U test), while abundance levels remained non significant at successive time points (Figure 4).

Juveniles at 20 dpf developmental stage were chosen for $E_2$ induction analysis of *vtg* mRNA expression. The exposure concentrations of $E_2$ employed were at levels similar to or lower than those used with adult males (see Figure 3) in order to get an indication of the relative induction profile at environmentally-relevant concentrations of $E_2$. Following 12h of exposure to 1.0 μg/L and 60 μg/L $E_2$, juveniles displayed a significant increase in liver-specific *vtg* transcript abundance relative to the time-matched controls (3- and 13-fold, respectively).

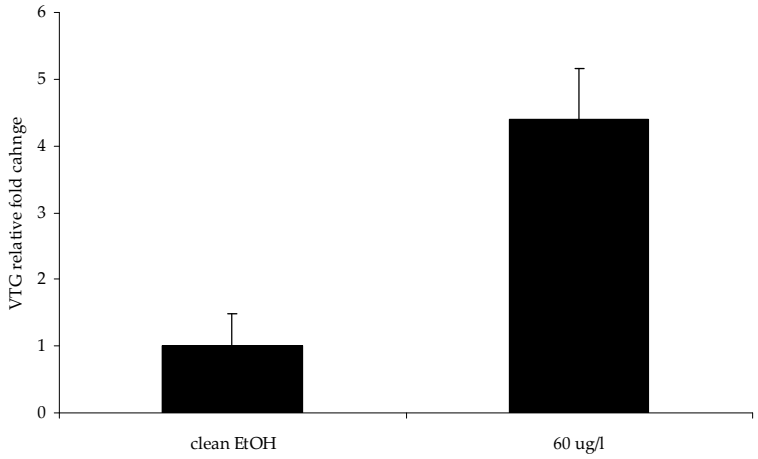


**Figure 3**. Induction of *vtg* mRNA expression in *O. mossambicus* adult male liver. Relative *vtg* transcript levels were examined in livers from adult males exposed to ethanol vehicle or 60 µg/L $E_2$ for 12h (n=10 and 8, respectively) using the OMV6/9 primer set. Expression data were normalized to the invariant endogenous rpl8 transcript. The observed increase in liver-specific *vtg* mRNA abundance was significant at p=0.003, Mann Whitney U test. The error bars represent standard error of the mean.

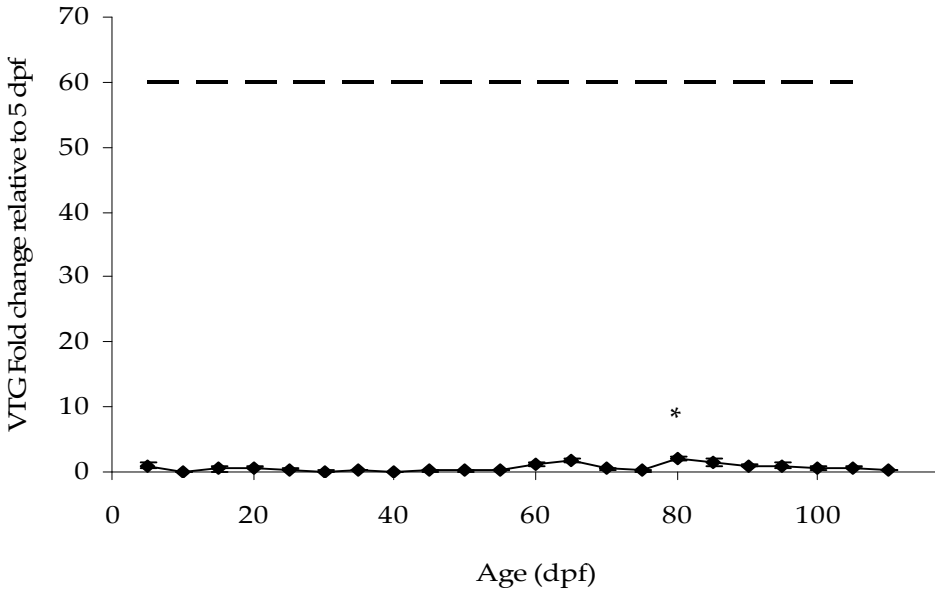


**Figure 4**. Expression of liver-specific *vtg* mRNA during early development of *O. mossambicus*. Liver-specific *vtg* transcript levels were investigated in WBH of juveniles at the indicated days post fertilization (dpf). Each time point was generated from a pool of 3 to 22 individuals except for at days 10 and 40 where n=1. The asterisk indicates a significant increase relative to 5 dpf (p=0.048, Mann Whitney U). The error bars represent standard error of the mean. The dotted line depicts the relative level of *vtg* transcript in adult male liver corrected for HSI which was 0.15. All data were normalized to the invariant endogenous rpl8 transcript.

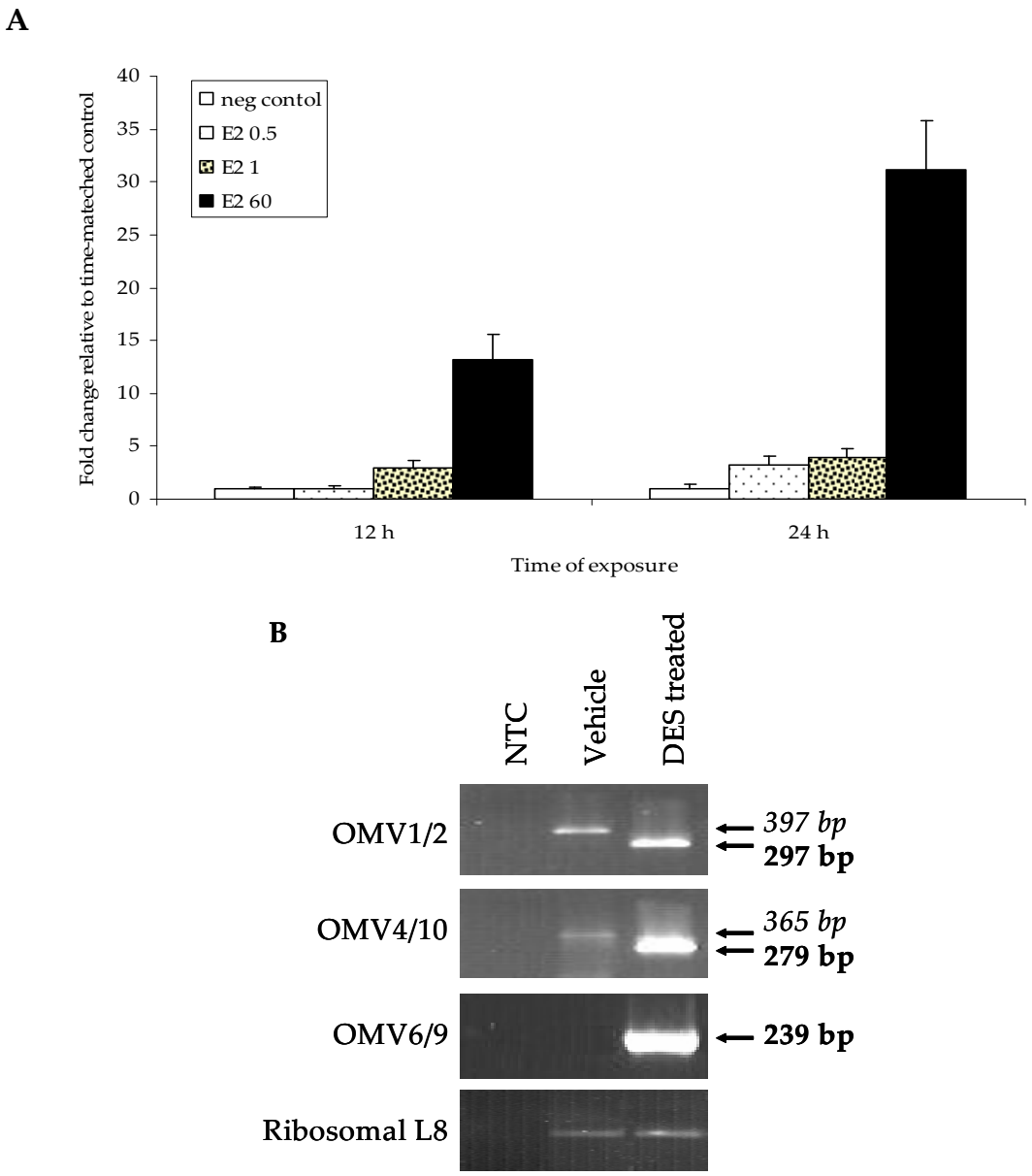


**Figure 5**. Estrogen-dependent induction of liver-specific *vtg* mRNA in juvenile *O. mossambicus*. (A) Relative levels of *vtg* transcripts were determined from WBH of 20 dpf animals exposed to ethanol vehicle (white bars), 0.5 μg/L $E_2$ (grey bars), 1.0 μg/L $E_2$ (hatched bars) or 60 μg/L $E_2$ (black bars) for the indicated times. Error bars represent standard error of the mean. Significance is indicated relative to the time-matched control by a single ($p<0.05$; Mann Whitney U) or triple asterisk ($p\leq0.001$; Mann Whitney U). (B) Exposure to 100 μg/L DES for 3 days also is shown to increase liver-specific *vtg* transcript abundance. Use of the non tissue-specific OMV1/2 or OMV4/10 QPCR primer sets produces an additional band in the vehicle exposed animals representative of alternate vtg transcripts. All data were normalized to the invariant endogenous rpl8 transcript.

**DISCUSSION**

An *in silico* analysis of the isolated 5′ promoter region in *O. mossambicus vtg* identified binding sites for ESR as well as VBP. To have induction of mRNA transcription mediated through the VTG responsive elements, a basal level of VTG is required. Since juveniles at 5-20 dpf are still feeding on their yolk sac, detection of low levels of *vtg* mRNA as early as 5 dpf was not unexpected. However, the translation of these transcripts into functional VTG has not been confirmed. There is some evidence that *O. mossambicus* utilizes a heterozygous male sex determination mechanism (XX female /XY male) (Clemens & Inslee 1968). The presence of putative ESR and SRY-related (Sox-5, Sox-9) binding sites in the *vtg* promoter region indicates that sexually dimorphic modulation of gene expression may also occur involving these transcription factors, although such a regulatory mechanism remains undetermined at present. The data indicate higher expression of *vtg* mRNA in adult females when compared to males and it is interesting to speculate that this sex-dependent difference may arise from a combination of regulatory mechanisms that include ESR and SRY action.

Examination of *vtg* mRNA present within the transcriptome of a variety of tissue types identified splice variants expressed from the *O. mossambicus vtg* gene. The reason for the presence of these alternate transcripts is currently not known and some are not predicted to express functional protein. Interestingly, a tissue-specific transcript that displayed the potential to encode VTG protein was observed in the liver. The complexity in the presence of transcript homologues requires the design and validation of assay tools that successfully evaluate the liver-specific form of *vtg* mRNA. This is especially true in assays that employ WBH where a potential underestimation of gene expression induction may occur due to the higher abundance of the different forms of *vtg* mRNA across tissues other than the liver. Such work highlights the importance of biological characterization of gene expression systems within sentinel species prior to assay development.

Two biological platforms were assessed for the detection of estrogen-dependent induction of gene expression. The first instance represented a widely used system in which the low background expression of *vtg* in adult male liver forms the basis of the screening assay. Examples of this can be found for adult tilapia (*O. aureus*) and other teleost males (Ding *et al.* 1989; Lim *et al.* 1991; Ding *et al.* 1993; Scholz *et al.* 2004; Mikawa *et al.* 2006; Miracle *et al.* 2006; Gordon *et al.*

2006). In accordance with the present work with *O. mossambicus*, these studies demonstrated relatively low levels of *vtg* expressed in the liver prior to estrogen-associated induction. Upon $E_2$ exposure for 12h, the steady-state levels of *O. mossambicus vtg* mRNA show a significant increase (4-fold) (60 μg/L $E_2$). Lim et al. (1991) reported a similar 20-fold increase in *vtg* mRNA in *O. aureus* at 72 hrs post-treatment with $E_2$.

Our present study argues strongly for ensuring the use of correct primer sets when using transcripts as indicators of $E_2$ exposure. A recent publication by Davis et al (2007) describes the cloning of 3 *vtg* fragments in *O. mossambicus*. The partial cDNA fragments that were cloned in this manuscript do not overlap with the ones identified in the present work and data is only presented for adult male livers. Our work looks beyond this tissue type and developmental stage and specifically shows that that various splice alternatives may exist in different tissues. The two partial clones sequenced in the present study which retained their introns had stop codons when translated in any frame. This means that a protein product is not expected from these transcripts and, thus, would not be detected by immunoblotting protein homogenates, as was done by Davis et al. (2007).

While this adult-based assay clearly demonstrates an estrogen-dependent induction of *vtg* expression, it requires husbandry of large numbers of adult males and the isolation of liver tissue for analysis. The second assay platform investigated involves a simpler method that employs WBH preparation from 20 dpf juvenile *O. mossambicus* exposed to estrogenic substances. The use of a QPCR primer set that recognizes only the liver-specific *vtg* transcript along with the application of juvenile stage animals increases assay sensitivity by allowing for the establishment of a low baseline expression when compared with adult male liver. Animals at this early developmental stage were particularly responsive to low concentrations of $E_2$ within a relatively short time frame. Significant induced levels of *vtg* transcript were found following 12h of exposure to 1.0 μg/L $E_2$ whereas lower exposure of $E_2$ (0.5 μg/L) increased *vtg* transcript levels after 24 hours. These results confirm that *O. mossambicus* juveniles are responsive to a range of $E_2$ concentrations. The fact that no mortality was observed for exposed animals along the $E_2$ concentration gradient illustrates this species' robustness to assay conditions.

## CONCLUSION

Real-time quantitative PCR has been shown to be a viable method in the evaluation of estrogen-induced hepatic *vtg* transcript levels (Funkenstein *et al.* 2004). The present study identifies assay criteria and highly sensitive QPCR tools required to establish the indigenous *O. mossambicus* as a sentinel in tier I screening for estrogenic activity in Southern African aquatic systems. The substantially lower pre-induction abundance levels of liver-specific *vtg* found in juveniles compared to adult males increases the dynamic range of the assay and greatly enhances the ability to detect estrogenic EDC effects at environmentally relevant concentrations.

## REFERENCE LIST


**Altschul S., Gish W., Miller W., Myers E. & Lipman D.**, 1990. Basic local alignment search tool. *Nucleic Acids Research.* 215, 403-410.

**Bailey T. & Elkan C.**, 1995. Unsupervised learning for multiple motifs in biopolymers using expectation maximization. *Mach Learn.* 21, 51-80.

**Bajic V., Tan S., Chong A., Tang S., Ström A., Gustafsson J.A., Lin C.-Y. & Liu E.**, 2003. Dragon ERE Finder ver.2: A tool for accurate detection and analysis of estrogen response elements in vertebrate genomes. *Nucleic Acids Research.* 31, 3605-3607.

**Baker H.J. & Shapiro D.J.**, 1977. Kinetics of estrogen induction of *Xenopus laevis* vitellogenin messenger RNA as measured by hybridization to complementary DNA. *Journal of Biological Chemistry.* 252, 8428-8434.

**Bruton M. & Boltt R.**, 1975. Aspects of the biology of *Tilapia mossmabica* Peters (Pisces: Cichlidae) in a natural freshwater lake (Lake Sibaya, South Africa). *Journal of Fish Biology.* 7, 423-445.

**Bustin S.** 2002. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *Journal of Molecular Endocrinology.* 29, 23-39.

**Chang X., Kobayashi T., Senthilkumaran B., Kobayashi-Kajura H., Sudhakumari C.C. & Nagahama Y.**, 2005. Two types of aromatase with different encoding genes, tissue distribution and developmental expression in Nile tilapia (*Oreochromis niloticus*). *General and Comparative Endocrinology.* 141, 101-115.

**Chenna R., Sugawara H., Koike T., Lopez R., Gibson T.J., Higgins D.G. & Thompson J.D.**, 2003. Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Research.* 31, 3497-3500.

**Cheshenko K., Pakdel F., Segner H., Kah O. & Eggen R.I.L.**, 2008. Interference of endocrine disrupting chemicals with aromatase CYP19 expression or activity, and consequences for reproduction of teleost fish. *General and Comparative Endocrinology.* 155, 31-62.

**Clemens H. & Inslee T.**, 1968. The production of unisex broods by *Tilapia mossambica* sex-reversed with methyltestosterone. *Transactions of the Amercian Fisheries Society.* 97, 18-21.

**Craft J.A., Brown M., Dempsey K., Francey J., Kirby M.F., Scott A.P., Katsiadaki I., Robinson C.D., Davies I.M., Bradac P. & Moffat C.F.**, 2004. Kinetics of vitellogenin protein and mRNA induction and depuration in fish following laboratory and environmental exposure to oestrogens. *Marine Environmental Research.* 58, 419-423.

**Crooks G., Hon G., Chandonia J. & Brenner S.**, 2004. A sequence logo generator. *Genome Research.* 14, 1188-1190.

**D'Amato M., Esterhuyse M., Van der Waal B., Brink D. & Volckaert F.**, 2007. Hybridization and phylogeography of the Mozambique tilapia *Oreochromis mossambicus* in southern Africa evidenced by mitochondrial and microsatellite DNA genotyping. *Conservation Genetics.* 8, 475-488.

**Davis L.K., Hiramatsu N., Hiramatsu K., Reading B.J., Matsubara T., Hara A., Sullivan C.V., Pierce A.L., Hirano T. & Grau E.G.**, 2007. Induction of Three Vitellogenins by 17beta-Estradiol with Concurrent Inhibition of the Growth Hormone-Insulin-Like Growth Factor 1 Axis in a Euryhaline Teleost, the Tilapia (*Oreochromis mossambicus*). *Biology of Reproduction.* 77, 614-625.

**Denslow N.D., Bowman C.J., Ferguson R.J., Lee H.S., Hemmer M.J. & Folmar L.C.**, 2001. Induction of Gene Expression in Sheepshead Minnows (*Cyprinodon variegatus*) Treated with 17[beta]-Estradiol, Diethylstilbestrol, or Ethinylestradiol: The Use of mRNA Fingerprints as an Indicator of Gene Regulation. *General and Comparative Endocrinology.* 121, 250-260.

**Ding J.L., Hee P.L. & Lam T.J.**, 1989. Two forms of vitellogenin in the plasma and gonads of male *Oreochromis aureus*. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology.* 93, 363-370.

**Ding J., Ng W., Lim E. & Lam T.**, 1993. In situ hybridization shows the tissue distribution of vitellogenin gene expression in *Oreochromis aureus*. *Cytobios.* 73, 294-295.

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

**Funkenstein B., Dyman A., Levavi-Sivan B. & Tom M.**, 2004. Application of real-time PCR for quantitative determination of hepatic vitellogenin transcript levels in the striped sea bream, *Lithognathus mormyrus*. *Marine Environmental Research.* 58, 659-663.

**Gordon D.A., Toth G.P., Graham D.W., Lazorchak J.M., Reddy T.V., Knapp C.W., deNoyelles J., Campbell S. & Lattier D.L.**, 2006. Effects of eutrophication on vitellogenin gene expression in male fathead minnows (*Pimephales promelas*) exposed to 17[alpha]-ethynylestradiol in field mesocosms. *Environmental Pollution.* 142, 559-566.

**Guillette L.J., Jr., Crain D., Rooney A. & Pickford D.**, 1995. Organization versus Activation: The role of Endocrine-disrupting Contaminants (EDC's) during Embryonic Development in Wildlife. *Environmental Health Perspectives.* 103, 157-164.

**Gussow D. & Clackson T.**, 1989. Direct clone characterization from plaques and colonies by the polymerase chain reaction. *Nucleic Acids Research.* 17, 4000.

**Hahlbeck E., Griffiths R. & Bengtsson B.E.**, 2004. The juvenile three-spined stickleback (*Gasterosteus aculeatus* L.) as a model organism for endocrine disruption: I. Sexual differentiation. *Aquatic Toxicology.* 70, 287-310.

**Hall T.** 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids, Symposium Series.* 41, 95-98.

**Heinemeyer T., Chen X., Karas H., Kel A., Leibich I., Meinhardt T., Reuter I., Schacherer F. & Wingender E.**, 1999. Expanding the TRANSFAC database towards an expert system of regulatory molecular mechanisms. *Nucleic Acids Research.* 27, 318-322.

**Helbing C., Gergely G. & Atkinson B.G.**, 1992. Sequential up-regulation of thyroid hormone beta receptor, ornithine transcarbamylase, and carbamyl phosphate synthetase mRNAs in the liver of *Rana catesbeiana* tadpoles during spontaneous and thyroid hormone-induced metamorphosis. *Developmental Genetics.* 13, 289-301.

**Higgins D., Thompson J., Gibson T., Thompson J., Higgins D. & Gibson T.**, 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research.* 22, 4673-4680.

**Hiramatsu N., Cheek A.O., Sullivan C.V., Matsubara T. & Hara A.**, 2005. Vitellogenesis and endocrine disruption. In Mommsen,T.P. & Moon,T.W. *Biochemical and Molecular biology of fishes*. Elsevier Science BV,Amsterdam, pp 431-471.

**Jobling S. & Tyler C.R.**, 2003. Endocrine disruption in wild freshwater fish. *Pure and applied chemistry.* 75, 2219-2234.

**Kim B.H., Takemura A., Kim S.J. & Lee Y.D.**, 2003. Vitellogenin synthesis via androgens in primary cultures of tilapia hepatocytes. *General and Comparative Endocrinology.* 132, 248-255.

**Kloas W.** 2002. Amphibians as a model for the study of endocrine disruptors. In*International Review of Cytology*. Academic Press,pp 1-57.

**Krimsky S.** 2000. *Hormonal Chaos: The scientific and social origins of the environmental endocrine hypothesis*. The John Hopkins Univeristy Press. Baltimore .

**Kubista M., Andrade J.M., Bengtsson M., Forootan A., Jonak J., Lind K., Sindelka R., Sjoback R., Sjogreen B. & Strombom L.**, 2006. The real-time polymerase chain reaction. *Molecular Aspects of Medicine.* 27, 95-125.

**Kuhl A.J., Manning S. & Brouwer M.**, 2005. Brain aromatase in Japanese medaka (*Oryzias latipes*): Molecular characterization and role in xenoestrogen-induced sex reversal. *The Journal of Steroid Biochemistry and Molecular Biology.* 96, 67-77.

**Lim E.H., Ding J.L. & Lam T.J.**, 1991. Estradiol-induced vitellogenin gene expression in a teleost fish, *Oreochromis aureus*. *General and Comparative Endocrinology.* 82, 206-214.

**Majumdar K. & McAndrew B.**, 1986. Relative DNA content of somatic nuclei and chromosomal studies in three genera, *Tilapia, Sarotherodon*, and *Oreochromis* of the tribe Tilapiini (Pisces, Cichlidae). *Genetica.* 68, 175-188.

**Mikawa N., Utoh T., Horie N., Okamura A., Yamada Y., Akazawa A., Tanaka S., Tsukamoto K., Hirono I. & Aoki T.**, 2006. Cloning and characterization of vitellogenin cDNA from the common Japanese conger (*Conger myriaster*) and vitellogenin gene expression during ovarian development. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology.* 143, 404-414.

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

**Nagahama Y.** 1990. Endocrine control of oocyte maturation in teleosts. *Progress in Clinical Biology Research.* 342, 385-392.

**Nagahama Y.** 1994. Endocrine regulation of gametogenesis in fish. *International journal of Developmental Biology.* 38, 217-229.

**Neil E.** 1966. Observations on the behaviour of *Tilapia mossambica* (Pisces, Cichlidae) in Hawaiian ponds. *Copeia.* 1, 50-56.

**Noaksson E., Linderoth M., Bosveld A.T.C. & Balk L.**, 2003. Altered steroid metabolism in several teleost species exposed to endocrine disrupting substances in refuse dump leachate. *General and Comparative Endocrinology.* 134, 273-284.

**Nussey G., Van Vuren J.H.J. & du Preez H.H.**, 1995. Effect of copper on the haematology and osmoregulation of the Mozambique tilapia, *Oreochromis mossambicus* (Cichlidae). *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology and Toxicology.* 111, 369-380.

**Ochman H., Gerber A. & Hartl D.**, 1988. Genetic applications of an inverse polymerase chain reaction. *Genetics.* 120, 621-623.

**Pfaffl M.W.** 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research.* 29, e45.

**Piferrer F., Blazquez M., Navarro L. & Gonzalez A.**, 2005. Genetic, endocrine, and environmental components of sex determination and differentiation in the European sea bass (*Dicentrarchus labrax* L.). *General and Comparative Endocrinology.* 142, 102-110.

**Popma T. & Masser M.**, 1999. Tilapia: Life history and biology. *SRAC Publication.* 283,

**Porte C., Janer G., Lorusso L.C., Ortiz-Zarragoitia M., Cajaraville M.P., Fossi M.C. & Canesi L.**, 2006. Endocrine disruptors in marine organisms: Approaches and perspectives. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology.* 143, 303-315.

**Reese, MG and Eeckman, FH**. Novel Neural Network Algorithms for Improved Eukaryotic Promoter Site Recognition. 1995. Hilton Head Island, South Carolina. The seventh international Genome sequencing and analysis conference.
Ref Type: Conference Proceeding

**Rutledge R. & Côte C.**, 2003. Mathematics of quantitative kinetic PCR and the application of standard curves. *Nucleic Acids Research.* 31, E93.

**Schmid A.C., Lutz I., Kloas W. & Reinecke M.**, 2003. Thyroid hormone stimulates hepatic IGF-I mRNA expression in a bony fish, the tilapia *Oreochromis mossambicus*, in vitro and in vivo. *General and Comparative Endocrinology.* 130, 129-134.

**Scholz S., Kordes C., Hamann J. & Gutzeit H.O.**, 2004. Induction of vitellogenin in vivo and in vitro in the model teleost medaka (*Oryzias latipes*): comparison of gene expression and protein levels. *Marine Environmental Research.* 57, 235-244.

**Segner H., Eppler E. & Reinecke M.**, 2006. The imact of environmental hormonally active substances on the endocrine and immune systems of fish. In Reinecke,M., Zaccone,G. & Kapoor,B. *Fish endocrinology*. Science publishers,Enfield, pp 809-865.

**Sharpe R.L., MacLatchy D.L., Courtenay S.C. & Van Der Kraak G.J.**, 2004. Effects of a model androgen (methyl testosterone) and a model anti-androgen (cyproterone acetate) on reproductive endocrine endpoints in a short-term adult mummichog (*Fundulus heteroclitus*) bioassay. *Aquatic Toxicology.* 67, 203-215.

**Sumpter J.P. & Jobling S.**, 1995. Vitellogenesis as a biomarker for estrogenic contamination. *Environmental Health Perspectives Supplements.* 103, 173.

**Takemura A. & Kim B.H.**, 2001. Effects of estradiol-17[beta] treatment on in vitro and in vivo synthesis of two distinct vitellogenins in tilapia. *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology.* 129, 641-651.

**Tao L., Shiwei J., Yang F.X., Yang H. & Ying X.**, 2006. An enzyme-linked immunosorbent assay for rare minnow (*Gobiocypris rarus*) vitellogenin and comparison of vitellogenin responses in rare minnow and zebrafish (*Danio rerio*). *Science of The Total Environment.* 364, 284-294.

**Teo B.Y., Tan N.S., Lim E.H., Lam T.J. & Ding J.L.**, 1998. A novel piscine vitellogenin gene: structural and functional analyses of estrogen-inducible promoter. *Molecular and Cellular Biology.* 146, 103-120.

**Trewavas E.** 1983. *Tilapiine Fishes*. British Museum. London .

**Tsai C.L. & Wang L.H.**, 1997. Effects of thermal acclimation on the neurotransmitters, serotonin and norepinephrine in the discrete brain of male and female tilapia, *Oreochromis mossambicus*. *Neuroscience Letters.* 233, 77-80.

**Vaillant C., Le Guellec C., Pakdel F. & Valotaire Y.**, 1988. Vitellogenin gene expression in primary culture of male rainbow trout hepatocytes. *General and Comparative Endocrinology.* 70, 284-290.

**Vijayan M.M., Takemura A. & Mommsen T.P.**, 2001. Estradiol impairs hyposmoregulatory capacity in the euryhaline tilapia, *Oreochromis mossambicus*. *AJP - Regulatory, Integrative and Comparative Physiology.* 281, R1161-R1168.

**Wang L.H. & Tsai C.L.**, 2000. Temperature affects the development of central neurotransmitter systems of tilapia, *Oreochromis mossambicus*. *Neuroscience Letters.* 285, 95-98.

**Wohlfarth G.W. & Wedekind H.**, 1991. The heredity of sex determination in tilapias. *Aquaculture.* 92, 143-156.

**Wright J.M.** 1989. Nucleotide sequence, genomic organization and evolution of a major repetitive DNA family in tilapia (*Oreochromis mossambicus/honorum*). *Nucleic Acids Research.* 17, 5071-5079.

**Zhang F., Degitz S.J., Holcombe G.W., Kosian P.A., Tietge J., Veldhoen N. & Helbing C.C.**, 2006. Evaluation of gene expression endpoints in the context of a *Xenopus laevis* metamorphosis-based bioassay to detect thyroid hormone disruptors. *Aquatic Toxicology.* 76, 24-36.

**Zhong X., Xu Y., Liang Y., Liao T. & Wang J.**, 2005. The Chinese rare minnow (*Gobiocypris rarus*) as an in vivo model for endocrine disruption in freshwater teleosts: a full life-cycle test with diethylstilbestrol. *Aquatic Toxicology.* 71, 85-95.

Chapter 3

# Temporal expression of two Cytochrome P450 Aromatase isoforms during development in *O. mossambicus,* in association with histological development

**ABSTRACT**

Aromatase – an enzyme coded for by two homologues of Cytochrome P450 19 (*cyp19*) in *Oreochromis mossambicus* is the only enzyme able to catalyze the reaction whereby estrogens are aromatized from androgens. As the gene product *vtg* (vitellogenin) is used as a bio marker to report estrogenic activity in oviparous species, and is induced by the presence of estrogens in the aquatic environment, resulting tissue specificity and temporal expression of *cyp19* homologues (*cyp19a* and *cyp19b*) were studied in unexposed populations of this species. To this end a quantitative real-time PCR (QPCR) protocol was developed and quantitative temporal expression profiles for *cyp19a* (ovarian homologue of *cyp19*) and *cyp19b* (brain homologue of *cyp19*) been determined. Maturely spliced *cyp19a* was transcribed only in the ovaries of adult specimens, and temporal expression reflected this result as induction of *cyp19a* coincides with histological development of putative ovaries. Using primers that only identify the ovarian transcript, *cyp19a* transcript levels demonstrates a typical reflection of puberty as after the initial surge in *cyp19a* mRNA, juveniles expressed very low levels of *cyp19a*, which increased again at the time histologically discernable vitellogenic ovaries was witnessed. *Cyp19b* was expressed in brain and muscle tissue of both male and female adults, with the addition of ovarian expression in females. During development, *cyp19b* transcript levels were increased at the same time as *cyp19a* (20 dpf) whereafter basal levels of expression of this isoform were measured in whole body homogenates of juvenile samples.

Keywords: Cytochrome P450 Aromatase, *Oreochromis mossambicus*, Quantitative real-time polymerase chain reaction

## INTRODUCTION

17β-Estradiol ($E_2$) is responsible for a multitude of functions in the vertebrate physiology including primary and secondary sexual characteristics, maintenance of bone bass and cardio-protective effects (Grumbach 2000; Vasudevan *et al.* 2002). In addition, $E_2$ exhibits effects known to be permanent organizing effects on the CNS development and general neurotrophic factors in several brain regions and life stages (Maclusky & Naftolin 1981; Tchoudakova *et al.* 2001).

$E_2$ biosynthesis in mammals involves a series of enzymatic steps, converting cholesterol into the steroid hormone, $E_2$. This process requires various forms of cytochrome P450 (Cyp) gene products, one of which *cyp19* ("aromatase"). In combination with NADPH-cytochrome P450 reductase, *cyp19* catalyze the formation of $E_2$ from testosterone, where the enzyme is bound to the membrane of the smooth endoplasmic reticulum of steroidogenic cells (Conley & Walters 1999; Tsuchiya *et al.* 2005). *Cyp19* is the only Cytochrome P450 that is capable of creating an aromatic ring which is characteristic of estrogenic compounds (Simpson *et al.* 1994).

The cytochrome genes consist of a superfamily encoding heme-containing monoxygenases and are responsible for the oxidative metabolism of many drugs and environmental chemicals as well as endogenous substances including steroids (Nelson *et al.* 1996). *cyp19* is present as a single copy in the genome of most mammals (Simpson *et al.* 1994; Choi *et al.* 1997) whereas in several other vertebrates, including fish, two distinctive genes denoted *cyp19a* and *cyp19b* code for this enzyme and are expressed predominantly in ovarian and brain tissues respectively (Callard *et al.* 2001; Greytak *et al.* 2005; Chang *et al.* 2005). The Japanese eel is an exception, reported to only express a single isoform (Jeng *et al.* 2005). Although both these forms of *cyp19* genes are able to aromatize [$^3$H]androgen to [$^3$H]estrogen, it remains the rate-limiting step in estrogen biosynthesis (Tchoudakova & Callard 1998). Moreover, the two different genes encode functionally different enzymes, with the heme- and steroid binding sites highly conserved between these isoforms and among different species (Cheshenko *et al.* 2008) Whether one or two forms of *cyp19* is carried in a species, all vertebrates to our knowledge illustrate a tissue specific expression of this gene(s) which is known to occur in a differential manner (Schulz *et al.* 2001; Janicke 2004; Piferrer *et al.* 2005; Tsuchiya *et al.* 2005; Pellegrini *et al.* 2005; Cheshenko *et al.* 2008).

*Cyp19a* expression in adult females varies in relation with the vitellogenin cycle (Goto-Kazeto *et al.* 2004). Androgens which are produced predominantly in thecal cells are converted into estrogens in the granulosa cells by ovarian aromatase mainly (Cheshenko *et al.* 2008).

*Cyp19b* expression occurs mostly in the brain but is found in other tissue types too for several species of fish (Callard *et al.* 1993; Balthazart 1997). In teleosts, evidence suggests that brain aromatase may be expressed exclusively in glial cells (Forlano *et al.* 2006). Within the brain, *cyp19b* expression was found mostly in the cells bordering the ventricles in the telencephalon, preoptic area, hypothalamus and mesencephalon (la Valle *et al.* 2005; Wang & Tsai 2006; Forlano *et al.* 2006; Cornil *et al.* 2006; Balthazart & Ball 2006). Levels of *cyp19b* is higher in the hypothalamus of the brain which correspond to the presence of gonadotropins-releasing hormone I neurons that project into the pituitary of teleosts (Blazquez & Piferrer 2004). In males however, neural aromatase is suggested to be involved in the production of neurosteroids that may affect neural development and brain sex differentiation. In addition, the brain of teleosts is traditionally characterized by exceptionally high levels of aromatase, which is continuous throughout life (Blazquez & Piferrer 2004). In Atlantic halibut, the ratio of *cyp19a* to *cyp19b* expression was found to be much higher in ovaries than in testes in the adult fish and was used to some degree of accuracy to infer sex of a specimen (Matsuoka *et al.* 2006).

Vertebrates with only one *cyp19* gene achieve tissue-specific expression by alternative splicing and/or different promoter usage in these species, whereas in species which has two different isoforms of the gene, differential transcript levels are obtained on account of the difference in the promoter area (Tsuchiya *et al.* 2005; Cornil *et al.* 2006). The two different *cyp19* genes in teleosts has sub-divided expression domains and accompanying different promoter regions. When expressed in non-steroidogenic cell lines, both ovarian- and brain aromatase are able to aromatize androgen (Tchoudakova & Callard 1998), but differences in substrate preference and inhibitory constants are consistent with differences in functionally important residues (Zhao *et al.* 2001).

Cheshenko et al (2007) and Forlano et al (2006) reviewed the potential regulatory factors for aromatases in teleosts which include responsive elements for cAMP responsive element binding protein (CREB), Steroidogenic factor 1/adrenal 4 binding protein (SF1/Ad4BP), estrogen receptor (ESR), aryl

hydrocarbon receptor (AhR), and nuclear hormone receptors (including PPAR, RXR and RAR). Some of these elements are exclusively found flanking either the ovary or brain isoforms of the gene. In tilapia, Chang *et al*. (2005) identified SRY, WT1-KTS, GATA-4, CRE and SF-1/Ad4 as binding sites for transcription factors in Cy19a. From these only GATA-4 and CRE was found in *cyp19b*. In addition ERE and RAR was also found in the 5' flanking region of *cyp19b* (Chang *et al.* 2005).

Therefore, in the brain of tilapia and some other teleosts (Callard *et al.* 2001), aromatase expression can potentially be regulated directly by estrogens and its receptor via its conventional genomic pathway (Falkenstein *et al.* 2000). Since this way of regulation is a lengthy process (days/weeks), it makes sense that it can affect sex determination/differentiation. Estrogens induce the transcription of *cyp19b* (Kishida *et al.* 2001; Kazeto *et al.* 2004). This phenomenon underlines the potential effect of estrogenic EDCs on the expression of *cyp19b* and consequently aromatase activity. In zebrafish, estrogenic exposure resulted in increased aromatase expression in the brain of female fish, whereas no change was reported for the male brain (Andersen *et al.* 2003). However, the opposite effect, was reported for fathead minnows (Halm *et al.* 2002). In tilapia, the enzyme activity upon $E_2$ induction decreased in the post-natal fish, whereas it increased significantly at the time of gonadal differentiation (Tsai *et al.* 2000). This and other studies clearly illustrate the importance of stage of development, tissue type and sex when measuring *cyp19* expression and activity (reviewed by Cheshenko et al 2007). Alteration in the aromatase expression and/or activity following exposure to estrogenic EDCs, may drastically affect sex determination/-differentiation pathways adversely.

In oviparous vertebrates, aromatase plays a critical role in vitellogenesis (Nagahama 1994). As a steady increase in the expression of *cyp19a* occurs during vitellogenesis (Yoshiura *et al.* 2003) this phenomenon might be a contributing factor to the reported high levels of aromatase in the brains of teleosts (Callard *et al.* 1990; Blazquez & Piferrer 2004). On the other hand, it impacts greatly on regulation of sex differentiation in many fish species since primary sexual characteristics during development depend on the availability of estrogens (Callard *et al.* 2001). This may however be a secondary effect of sex determination.

Towards understanding the mechanism of sex determination in cichlid fish, several hypotheses have been put forward and tested which implicates the *cyp19* genes, in particular the regulatory elements thereof in several of these studies (Matsuoka *et al.* 2006), since the 5'-flanking region of *cyp19a* has been found not to contain an estrogen responsive element (ERE) (Forlano *et al.* 2006; Cheshenko *et al.* 2008), whereas that of *cyp19b* does (Chang *et al.* 2005). A SRY binding site found in the 5'-flanking region of *cyp19a* in tilapia (Chang *et al.* 2005), which are known male sex-determining factors in mammals, but the expression of SRY itself is completely absent in male Nile tilapia gonads. Same binding regions were found also for zebrafish and goldfish. This may indicate a decisive role in sex *differentiation* in these fish species, but not necessarily a mechanism totally dependent on genetic *determination* of sex at the time of fertilization. A study on Fathead minnows (Villeneuve *et al.* 2006) may indicate that regulation of reproductive activity occur at a transcriptional level with regards to *cyp19a* in that the enzyme activity of aromatase was significantly lower in non reproductive adults' ovaries. Yet, when the two homologues of *cyp19* are differentially measured, not *cyp19b*, but very well *cyp19a* was down regulated in the ovaries of non reproductive adults. Considering the hypothetical role in sex determination and differentiation of these genes specifically in the brain of sexually undifferentiated juveniles, a comparison of gene expression data along with enzyme activity would shed light on the mechanism of sex determination and possibly the signal transduction pathway employed by the species in question.

*Oreochromis mossambicus*, a gonochoristic teleost fish targeted as sentinel species for endocrine disruption in Southern Africa is known to follow a sex determination mechanism largely genetic dependent, but can be altered environmentally by temperature (Wang & Tsai 2000) and hormonal imbalance (Eckstein & Spira 1965; Nakamura *et al.* 1998). As part of background studies relating to EDC monitoring using *O. mossambicus*, I set forward to clone and sequence partial fragments of both isoforms of *cyp19* in order to develop quantitative PCR (QPCR) protocols. I subsequently quantify tissue specific expression in adult specimens, and determine expression levels of *cyp19a* and *cyp19b* during the juvenile development stages of this species in comparison with gonadal development histologically.

## MATERIALS AND METHODS

*Animals*

Adult breeding stock of *Oreochromis mossambicus* was obtained from Aquastel (South Africa) and kept in aquaria with water, constantly aerated and recycled through an activated charcoal filter. Water temperature was kept at 27 °C (±1 °C). Fish were fed once daily with tilapia pellets (AquaNutro, South Africa). Light regime followed 14:10 L:D cycle. On a daily basis aquaria were checked for females carrying eggs in their mouths. These females were removed from the breeding aquaria to culturing tanks. In the culturing tanks each brooding female was kept alone until the offspring reached swim-up fry stage, at which time the adult female was removed and re-introduced into the breeding tank. Each batch of offspring was reared separately in the same water conditions as for breeding stock. Animals at the appropriate developmental stage were collected, euthanized using 0.01 % Benzocaine (Heynes Mathew, Ltd., South Africa) and preserved in RNA*later* (Ambion Inc., USA) at 4 °C or fixed in buffered formaldehyde (Bancroft & Stevens 1977) for genetic and histological purposes respectively. At least three groups from different breeding pairs ($n \geq 5$ per group) were sampled at 5 day intervals starting at 5 days post fertilization (dpf).

*Histological procedures*

Standard histological procedures (Bancroft & Stevens 1977) were used to determine the relationship between age and the formation of gonadal structures. Five (5) specimens per age class were processed. Whole bodies were fixed in buffered formaldehyde (Bancroft & Stevens 1977) and embedded in paraffin wax (Merck, South Africa). Histological sections (5 μm) were stained with Harris hematoxylin and eosin (Humason 1967) within each age group.

*RNA isolation and cDNA preparation*

For the initial cloning of either of the expressed aromatase sequences, RNA was prepared from brain, ovary and liver tissue of an adult female using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. Following resuspension of total RNA in DEPC-treated water, samples were treated with DNase I (Promega, USA) for 30 min at 37 °C and precipitated with 2.5 volume (vol) 95% ethanol and 1/10 vol 3 M sodium acetate pH 5.6 at -20 °C.

The RNA pellets were washed with 70 % ethanol and redissolved in 30 to 60 µl of DEPC-treated water.

For analysis of aromatase gene expression during development, total RNA was isolated from whole body homogenates (WBH), three groups per age class (n = 5 to 10 per age group). RNA yields were quantified spectrophotometrically at $Absorbance_{260nm}$ and stored at -70 °C.

First strand cDNA was prepared from 2 µg of total RNA using oligo $d(T)_{15}$ primers and SuperScript III RNase $H^-$ MMLV reverse transcriptase as described by the manufacturer (Invitrogen, USA). Samples were diluted 40-fold prior to gene expression determination and stored at -20 °C.

*Primer development*

To obtain partial *cyp19* gene sequences, PCR was performed using cDNA derived from *O. mossambicus* brain and ovary tissue as template and primers designed against the related *O. niloticus cyp19a* and b sequences (GenBank accession no. AF472620 and AF472621 respectively). PCR conditions included 1.5 mM $MgCl_2$, 0.05 mM of each dNTP, 1 µM of each primer (Table 1) and 2.5 units of in-house Taq polymerase in a 25 µl reaction. In each reaction, 50 ng cDNA from liver, ovary or brain was added. PCR reaction volumes were denatured for 2 min at 94 °C, after which followed 30 cycles constituting of 1 min at 94 °C, 30 seconds at 55 °C and 30 seconds at 72 °C, with a final elongation step of 5 min at 72 °C. PCR products were checked for size on a 2 % Agarose gel.

Amplified DNA fragments of interest were cloned into pGEM-T Easy vector (Promega, USA) and transformed into *E. coli* DH5α. Plasmid DNA was isolated from positive clones detected by colony PCR and insert DNA sequenced using SP6 and T7 primers. The resulting *O. mossambicus* aromatase sequence information was compared to all sequences in the NCBI database and best similarity was found with aromatase (*cyp19*a and *cyp19b* respectively with the appropriate primer sets) sequences of *O. niloticus*.

**Table 1** Primer sequences used for cloning or QPCR. Asterisk (*) indicate anti sense primers

| Target Gene | Application | Primer | Sequence |
|---|---|---|---|
| *cyp19a* | Cloning | OMAO6 | AATTAAACCCCAGAAAGCCAGG |
| | | OMAO4* | CTGTGAACTAAATATGTATGACATGC |
| | | OMAO1 | CACAAAACCACGGTGAGCTGTCTGCT |
| | | AromaOfwd | CAATCGCATGGGATATCAATGG |
| | | AromaOrev* | GAAGATCTGCTTAGTATGAGCGTC |
| | QPCR | OMAO3 | CACAAGACAGCAACCCAGGAGTTA |
| | | OMAO2* | CTGTCTCTCACCCACAACAGCG |
| *cyp19b* | Cloning and | AromaBfwd | GAGCGTCAGAAGTCACTGC |
| | QPCR | AromaBrev* | GCTCAAAATCAGGGTCTCCTC |
| *β*-actin | QPCR | OMBA1 | TGTGATGGTGGGTATGGG |
| | | OMBA2* | CTGTGGTGGTGAAGGAGTAG |

*QPCR assay development and -verification*

Primers and clones for analysis of gene expression by quantitative PCR (QPCR) are listed in Table 1. For housekeeping gene (internal invariant standard) purposes, β-actin was used which has been developed and described in Chapter 2. Gene expression was quantified using a Applied Biosystems 7500 real-time PCR system (Applied Biosystems, CA, USA). Each 15 µl QPCR reaction contained 2 µl of first-strand cDNA (40-fold dilution), 7.5 µl SYBRgreen mix (Sigma, Germany), 0.08 µl reference dye (Sigma, Germany) and 0.33 µM of each primer. The thermocycle program included 95 °C (9 min), followed by 40 cycles of 95 °C (15 sec), 60 °C (OMAO3/6) or 56 °C (AromaBfwd/rev) for (30 sec) and 72 °C (45 sec). Each amplification run included control reactions containing no cDNA template.

Quadruplicate determinations were performed on samples containing plasmids which consist of pGEM-T Easy (Promega, USA) vector containing either OMAO3 fragment (amplified with primers OMAO2/3) or aromaB fragment (amplified with primers aromaBforw/rev). For each a 1/5 dilution range is prepared and the $C_t$ values obtained were used in combination with the dilution series to obtain a standard curve that describes the relationship between copies of gene target to derived $C_T$ value. The resulting gene expression data was averaged for each sample. Variation between individual samples in RNA input quantity was normalized using the expression of *β*-actin gene as a housekeeping gene.

Primers (Table 1) designed from the available *O. mossambicus* sequences proved to amplify the respective spliced fragments, which resulted in 100% identity with the available sequences in NCBI database. These primers (OMAO3/2 and AromaBforw/rev for *cyp19a* and *cyp19b* respectively) were then used to obtain standard curves from a dilution range of *cyp19a* and *cyp19b* template on a QPCR analyzer. Average $C_T$ values of *cyp19a* (OMAO3/2) and *cyp19b* (AromaBforw/rev), and Beta actin (OMBA1/2) showed a linear relationship with varying concentrations of purified RNA from samples, with correlation coefficients of ≥ 96.0. The assumptions for the $\Delta\Delta C_t$ method (Bustin 2002) is therefore met and the ratio of DNA expression of data to BA is calculated by $2^{-\Delta Ct}$.

*Statistical analysis*

Statistical analyses were performed using the software package STATISTICA© V8 (StatSoft Inc., USA). Normal distributed data was analyzed with parametric analysis of variation (ANOVA) to test for significant variation ($P<0.05$). Non-normally distributed data was analyzed with the non-parametric Kruskal-Wallis analysis of variance (ANOVA) on ranks followed by Dunn's Multiple Comparison Procedures to test for significance among groups. Where appropriate, quantitative gene expression values were tested using both normalized (with housekeeping gene) data or $Log_{10}$ transformed data (Zar 1996) according to methods that agree with the assumptions for the type of data to see correlation of first derivative data. Correlation between variables was analyzed with linear regression on data according to Pearson (Pearson 1896) for normally distributed data or Spearman's rank correlations (Gibbons 1985) for non normal data.

## RESULTS

*Histological development of gonads*

Histological procedures used in this study were insufficient to determine the presence of any gonadal structures for specimens as young as 3 dpf. In *O. mossambicus*, at 20 dpf, differentiation of somatic cells could be observed in the gonadal ridge extending from the peritoneal wall (Figure 1A) with germ cells and somatic cells distinguishable (Figure 1B-D). However, no clear distinction between sexes in this gonochoristic species was possible at this stage of development. At 20 dpf gonads were still primordial. One specimen in this group of 20 dpf juveniles presented with a stromal elongation that may represent the possible formation of the ovarian cavity in Figure 1D.

At 40 dpf, gonadal structures appeared larger and gonadal cells more abundant. Gonadal ridges were clearly discernable as bulbed structures at the end of the gonadal mesenteries extending from the peritoneal wall ventral to the well-developed swim bladder. Male gonads could not be positively recognized at this stage, however, in Figure 2A-D the possible formation of the efferent duct was observed (arrowhead in Figure 2A). These presumptive males exhibited less distinctive germ cells relative to the somatic cells when compared to presumptive female specimens (Figure 2E-H). Contrary to the early male gonadal analogues, several specimens exhibited gonadal structures with well

developed germ cells with clear evidence of meiotic activity (Figure 2E-H). These distinguishable germ cells were supported by somatic cells at the periphery of the developing gonad, showing active germ cell mitosis and meiosis. Germ cells were found to be relatively small (4-8 μm, Figure 2E). In one specimen, appendix-like outgrowths (Figure 2F), what seems to be the early development of the ovarian cavity was observed. At 40 dpf, at the light-microscopic level, I could not identify any other organizational features indicating differential gonadal development.

After 55 dpf, structures were not notably larger than at 40 dpf, and similar to the 40 dpf specimens, the extended gonadal mesenterium (Figure 3C and E) containing clusters of somatic cells and germ cells (Figure 3D). However, similar to the 40 dpf individuals, it was impossible to confidently determine a differentiating testis or ovary with light microscopy.

Juveniles at the 75 dpf stage exhibited well developed ovaries mostly in the primary growth stage, showing clear cellular differentiation with either oocytes in early peri-nucleolus stage or advanced peri-nucleolus stage. This primary growth stage of the oocytes (Figure 4) typically involved a substantial increase in cells size as well as the formation of Balbiani bodies (which corresponds to various cellular organelles) as basophilic cytoplasmatic masses (Figure 4A). As for organizational activities of somatic cells, ovaries in all individuals examined exhibited an ovarian lumen (cavity) (Figure 4B) with primary oocytes enveloped by squamous-type stromal (follicular) cells. Oocyte cytoplasm stained strongly basophilic and showing evidence of lipid droplets (vitellogenic activity) in the peripheral regions of the ooplasma (arrowheads in Figure 4B).

Males at 75 dpf developmental stage exhibited smaller gonadal ridges characterized by a proliferation of somatic-type cells and the absence of larger oocytes (Figure 4C). At the light-microscopic level, no distinction between mitotic and meiotic activity associated with developing spermatogonia could be made. However, discernable pachytene spermatocytes can be observed at this stage, suggesting the onset of spermatogenic development.

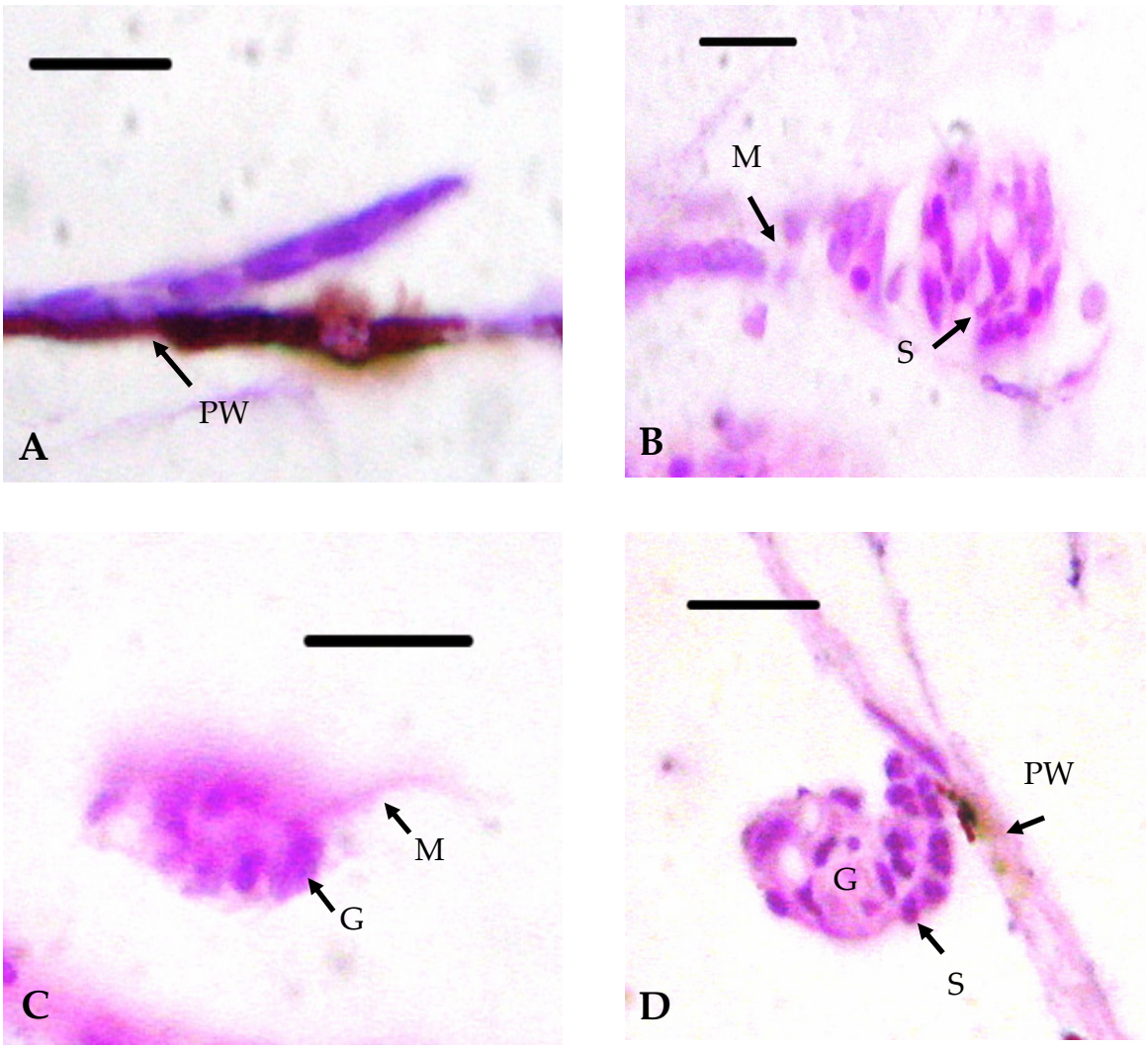


**Figure 1** Light microscopy of gonadal development of *Oreochromis mossambicus* at 20 days post fertilization (dpf). Scale bar = 10 μm. **A**: cross section of primordial gonads suspended from the dorsal peritoneal wall (PW) in an early differentiating specimen. **B-D**: cross sections of differentiating gonads illustrating the presence of germ cells (G) and somatic cells (S).

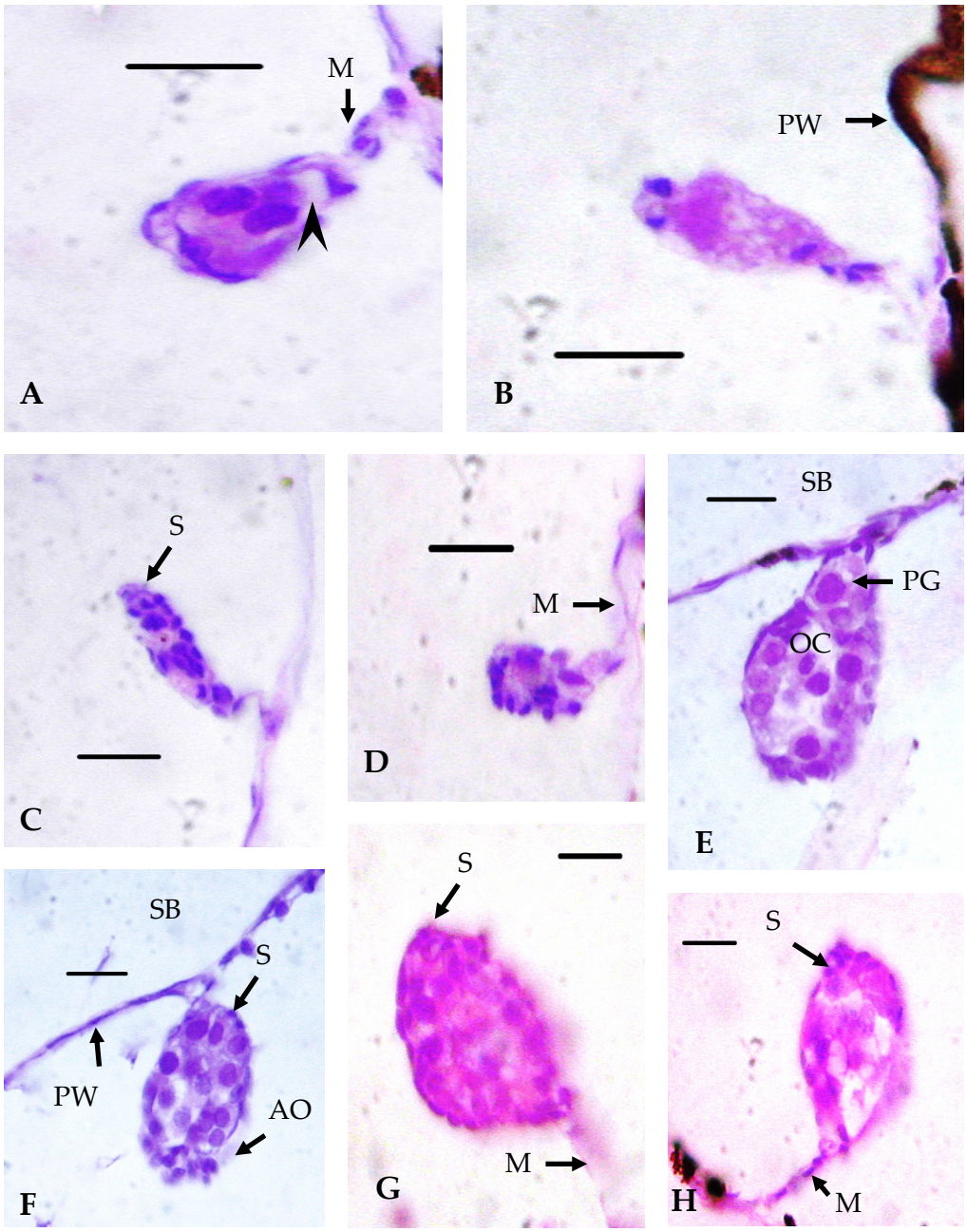


**Figure 2** Light microscopy of gonadal development of *Oreochromis mossambicus* at 40 days post fertilization (dpf). Scale bar = 10 μm. **A-D**: cross section of differentiating gonads suspending from the dorsal peritoneal wall (PW) by short mesenteries (M) with the absence of any germ cells present. Possible formation of an efferent duct is indicated by an arrowhead in A. **E-F** cross sections of differentiating gonads presenting somatic (S) and germ cells (G). In E a putative developing ovary below the caudal tip of the swim bladder (SB) illustrates the formation of the ovarian cavity (OC) and one pre-mitotic germ cell (PG). An appendix-like outgrowth in F predicts the initiation of the formation of an ovarian cavity. A typical aggregation of somatic cells (S) is seen, possibly the origin of an appendix-like outgrowth in specimen photographed in H.

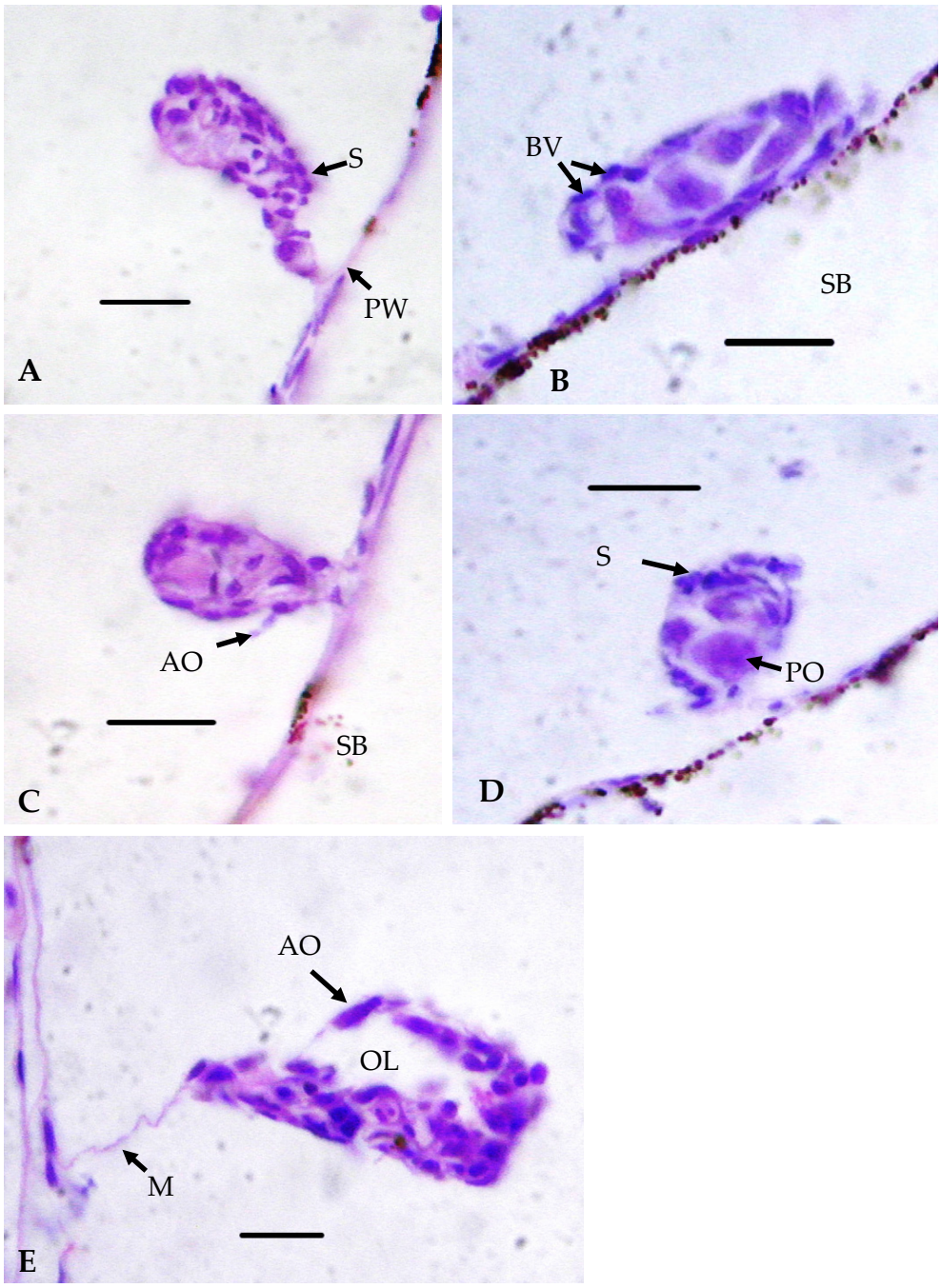


**Figure 3** Light microscopy of gonadal development of *Oreochromis mossambicus* around 55 days post fertilization (dpf). Scale bar = 10 µm. **A** illustrates typical proliferation of somatic cells (S) with no evident germ cells present growing from the peritoneal wall (PW). In addition, blood vessels (BV) in **B** can be seen in the periphery of the differentiating gonad. **C** illustrates again appendix-like outgrowth (AO) in a developing ovary. Note the low number of somatic cells relative to specimen in A. A pachytene oocytes in **D** further indicate the formation of an ovary, whereas **E** illustrates a further developed ovary with advanced formation of the ovarian lumen (OL). (SB, swim bladder; M, mesentary)

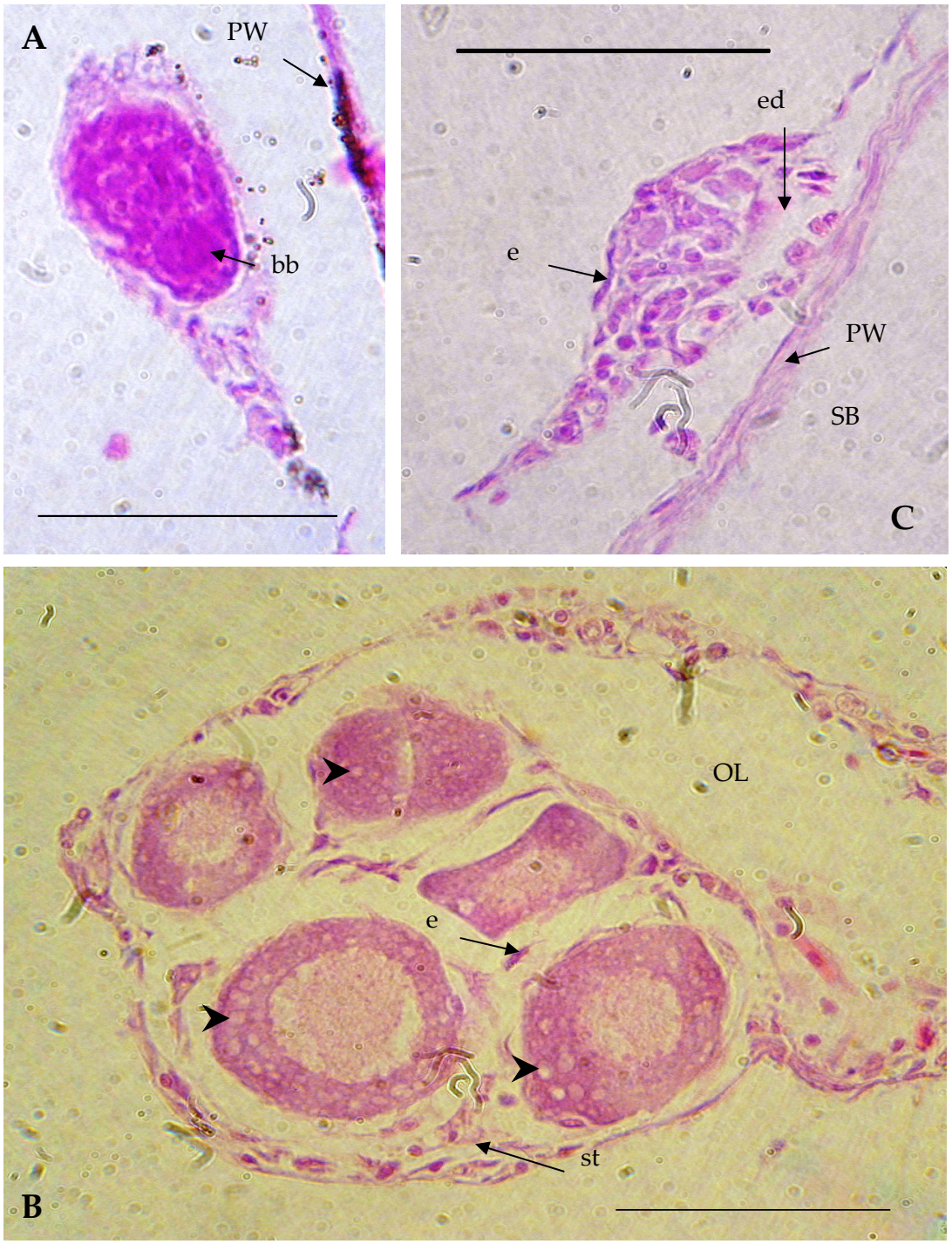


**Figure 4** Light microscopy of gonadal development of *Oreochromis mossambicus* at 70-75 days post fertilization (dpf). Scale bar = 50 µm. **A:** Balbiani bodies (bb) in an early perinucleolus oocytes (75 dpf). **B:** vitellogenic ovary illustrating start of organizational effect by flat epithelium (e) cells and supportive tissue (st). The ovarian lumen (OL) is clearly recognizable at this stage (75 dpf). **C:** pachytene spermatocytes in testis of 70 dpf presenting with surrounding epithelium cells (e) and efferent duct (ed). PW = peritoneal wall; SB = swim bladder

*Isolation and sequencing of partial cyp19 sequences*

Sequencing results of amplified product using primers for *cyp19a* and *cyp19b* was 100% identical to sequences for the two genes in *Oreochromis mossambicus* (GenBank accession nu. AF135851 and AF135850 respectively).

For *cyp19a* alternatively spliced variants was found, which were cloned and sequenced (Figure 5) and subsequently found to be 100 % identical to the genomic (gDNA) sequence mentioned above, which demonstrate the inclusion of introns even though samples were DNase I treated before reverse transcription.

*Tissue specific expression of aromatase*

*Cyp19a* was amplified in ovarian tissue only (Figure 5) and not in males at all when the finally spliced product (primers OMAO3/2) was amplified, however alternatively spliced product (primers AromaO f/r and OMAO1/2) was found in all tissues of male and female specimens for *cyp19a*.

*Cyp19b* mRNA was amplified in brain, ovary and muscle tissue of female and in the brain and muscle of the male specimens. Only the expected spliced product was amplified in the target region of *cyp19b*.

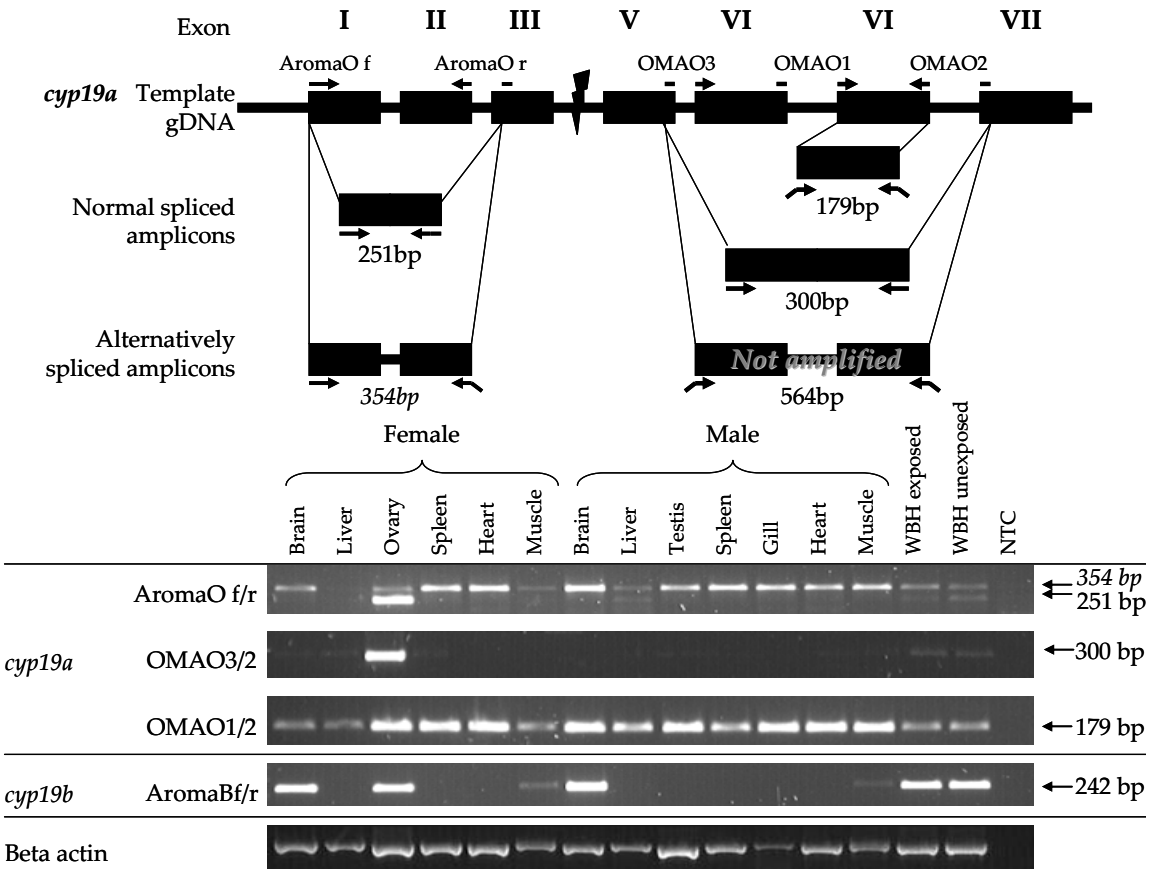


**Figure 5** Schematic representation of cloned loci in *cyp19a* illustrating the intron splice sites and areas to be cloned with primers as listed in table 1. The second frame shows tissue specific amplification of *cyp19a* and *cyp19b* with an internal standard, Beta actin on a 2 % agarose gel.

*Temporal quantitative cyp19a and cyp19b expression*

*Cyp19a* transcript levels (primers OMAO3/2) measured in WBH samples of developing juveniles increased statistically significant for the first time at 20 dpf ($p<0.01$, MWU, Figure 6A). After this initial (primary, 1°, Figure 6A) increase in *cyp19a* mRNA, it dropped again at 30 dpf and continued to show hardly any *cyp19a* expression until a second increase at 65 dpf ($p<0.01$). At 85 dpf all samples had an elevated level (secondary, 2° increase) of *cyp19a* which begs the question of where in its sexual development these animals appear since spliced *cyp19a* was completely absent in gonads of males tested for tissue specific expression of *cyp19a*. During this period of 2° elevated expression, a clear dimorphic pattern was determined of 50 % (n = 13) of samples expressing *cyp19a* at zero or low (<6 fold) transcription levels, whereas the other 50 % (n = 13) of data indicated clear induced expression (>9 fold) of *cyp19a*.

Quantitative expression of *cyp19b* revealed statistically significant increase in expression at 15 dpf for the first time during our range of data. Hereafter, I measured basal levels of *cyp19b* for the remainder of the period of development, with much variation, pointing to no particular phase of high or low transcription rates in *O. mossambicus*'s development. *cyp19b* therefore results in a expression pattern not mirrored by that of *cyp19a*, reiterating the probable distinction in function of the two isoforms in teleosts.

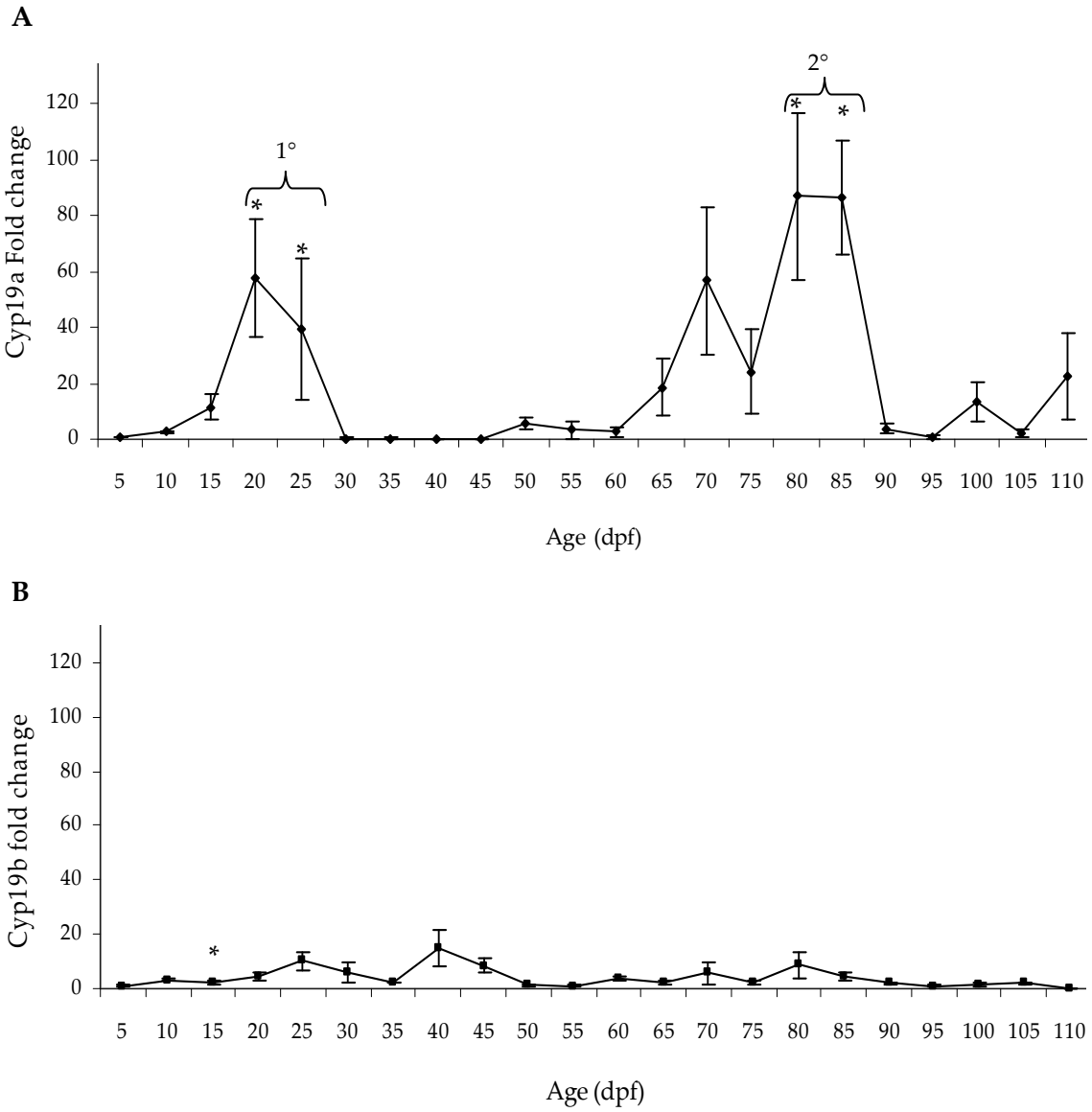


**Figure 6** Temporal expression of *cyp19a* (frame A) and *cyp19b* (frame B) in *O. mossambicus* whole body homogenates during development. In frame A a primary (1°) and secondary (2°) induction (asterisk, statistically significant at $p<0.05$) of *cyp19a* is indicated at 20-25 dpf and 80-85 dpf respectively. Apart from an increase at 15 dpf (asterisk, $p<0.05$), no significant induction relative to 5 dpf fish occurred in *cyp19b* during temporal development.

## DISCUSSION

Aromatase is encoded for by either of two isoforms *cyp19a* or *cyp19b* in teleosts, and is the only enzyme that is capable of aromatization of $C_{19}$ androgens to $C_{18}$ estrogens (Tchoudakova & Callard 1998) in combination with NADPH reductase. Aromatase is therefore an enzyme playing a pivotal role in regulation of the multitude of estrogen responsive genes (Cornil *et al.* 2006; Vasudevan & Pfaff 2007) – one of which is vitellogenin.

Vitellogenin is the precursor protein for egg yolk, the source of nutrients for the developing embryo in teleost fish, and is known to be under control of $E_2$ via its genomic pathway where $E_2$ binds to its nuclear receptor (ESR), permeates the nucleus and binds to estrogen responsive elements (EREs) in the 5'flanking area of vitellogenin gene (*vtg*). This process, known as vitellogenesis, occurs in the liver of both male and female fish, however at differential levels between the sexes.

The differential level of *cyp19* transcripts therefore can affect assays used to measure estrogenic EDCs *in vivo*. The behavior of *cyp19* isoforms in the Southern Africa tilapia, *Oreochromis mossambicus* was therefore studied during development under conditions free of xeno-estrogenic influence, providing sound background to determine departure from normal expression levels, and indicate substrate specificity of the two isoforms (*cyp19a* and *cyp19b*) found.

### *Histology*

Embryological origin of somatic constituents of the gonad in fish (and other vertebrates) is closely related to morphological sex differentiation of the gonads (Nakamura *et al.* 1998), and the undifferentiated gonad consist of two somatic cell components, the cortex and medulla. In addition, the cortex develops and the medulla degenerates during ovarian development (Nakamura *et al.* 1998). In tilapia (*O. mossambicus*) it has been documented that genital ridges already form three (3) dpf with the germ cells of these ridges being enclosed by somatic cells derived from the peritoneal wall (Nakamura *et al.* 1998). Furthermore, it has been documented that the cortex is classically known to originate in the peritoneal wall illustrated in Figure 1A, whereas the medulla derives from the mesonephric blastema (Yamamoto 1969). Nakamura (1998), employ the relative abundance of primordial germ cells to the number of surrounding somatic cells to discriminate presumptive testes and ovaries in tilapia, as early as 20dpf.

Although in the present study, at the light microscopic level I could make such a distinction (see Figs 1A & 1B-D), the detailed gonadal differentiation was unclear. Nakamura (1998) suggested that mitosis and meiosis of germ cells takes place earlier in ovaries of than in testes, at approximately 22dpf. Ovarian differentiation has been described in *O. niloticus* as the proliferation of both stromal and germ cells prior to ovarian meiosis (D'Cotta *et al.* 2001). However, in the current study, this proliferation was not observed before the 20 dpf stage in *O. mossambicus*.

In the present study, at the 40 dpf developmental age, gonadal structures in *O. mossambicus* were enlarged exhibiting a proliferation of cells. Our observations corroborate similar results reported in several studies confirming the presence of germ cells in cord-like, discontinuous clusters intercalated with germ cell-free spaces during the early gonadal development (Parmentier & Timmermans 1985; Strussmann *et al.* 1996). Rocha & Rocha (2007) refer to this stage of oocyte development as the oogonium stage, characterized by proliferating oogonia. Another study on *O. mossambicus* and *Carassius auratus*, describes stromal elongations of the gonad for the formation of ovarian cavity (Nakamura *et al.* 1998) whereas others describes the formation of the ovarian cavity before the appearance of oocytes in two species of tilapia *O. niloticus* and *O. aureus* (Eckstein & Spira 1965; Nakamura & Nagahama 1985). The present study confirmed the initial development of an ovarian cavity by the appendix-like stromal outgrowths (Figure 2F). In the gonochoristic fish *Cichlasoma dimerus* the development of an ovarian cavity started at 58 dpf (Meijide *et al.* 2005). This appendix-like outgrowth is typically a cluster of somatic cells usually indicating the origin of such outgrowths seen in other teleosts (Nakamura *et al.* 1998; D'Cotta *et al.* 2001; Meijide *et al.* 2005). Apart from this initial dimorphic differentiation at 40 dpf, I could not confidently recognize any clear indications of organational changes suggesting putative ovary or testis development.

In a study reviewing sex differentiation in several teleosts (Nakamura *et al.* 1998), spermatogenesis is described to start at 50 - 70 days after hatching for *O. mossambicus,* confirming the absence of accurately distinguishable male gonads at 55 dpf. However, amongst the samples studied at ~55 dpf (52-60 dpf), the behavior of the somatic cells in the gonad clearly appeared sexual dimorphic (Figure 3A) exhibiting a clear absence of meiotic presumptive oocytes amongst abundant somatic cells indicate the possible differentiation of a testis. Although

the basic timing of developmental events correspond to a report for *O. niloticus* at 55 dpf by D'Cotta *et al.* (2001), the presence of germ cells in meiotic prophase, could not be confirmed in the present study.

Specimens at 75 dpf exhibited morphological differentiation with oocytes either in early perinucleolus stage or even advanced perinucleolus stage as has been described by Rocha & Rocha (2006). At this ovarian development stage, female *O mossambicus* juveniles contained oocytes with peripheral ooplasmic lipid droplets pointing to early vitellogenic activity (biosynthesis of lipovitellin). In Nile tilapia, from 60 dpf onwards typical steroid producing cells could be clearly characterized by large mitochondria with tubular cristae (D'Cotta *et al.* 2001). The question of identification of true steroid producing cells in sexually developing fish may however need to be re-visited as evidence prove already elevated levels of ESRs at 50, 20 and 20 dpf (ESR1, ESR2a and ESR2b respectively, Chapter 4). The current study supports the general lack of indisputable morphological characters that suggest early dimorphic differentiation (D'Cotta et al. 2001). Several reports indicated that the first histological signs of differentiation are the occurrence of meiotic activity following the proliferation of germ cells relative to somatic cells (West G 1990; Hourigan *et al.* 1991; Hines *et al.* 1999; Leino *et al.* 2005; Park *et al.* 2007) but, I support the concern of D'Cotta *et al.* (2001) that the molecular differentiation in the presumptive ovary may in fact occur much earlier on account of increase of *cyp19a* levels at an earlier stage as indicated by the present QPCR data (Figure 6A) and histological evidence, (Figure 1) to be ~20 dpf.

*Tissue specificity*

The liver receives circulating $E_2$ from where the ligand traditionally known to be produced in the brain and gonads (Cheshenko *et al.* 2008). In the gonads the dominant isoform of *cyp19* is the ovarian form (*cyp19a*) (Callard & Tchoudakova 1997; Callard *et al.* 2001; Toffolo *et al.* 2007), which is what our results confirmed for *O. mossambicus* (Figure 5). Expression of *cyp19a* the present study was not detectable in the testis tissue in contrast with some reports of occasional expression in other fish species, which also reports expression of *cyp19a* in the pituitary, eye, liver, kidney, heart, muscle, spleen, thyroid, intestine, gill and blood (Kishida & Callard 2001; Goto-Kazeto *et al.* 2004; Chang *et al.* 2005). In trout, however, Guiguen *et al.* (1999) reported high levels of *cyp19* mRNA in

developing ovarian tissue, whereas none in testes. Considering amplification of *cyp19a* with additional primer sets (Table 1), I found amplification of regions of the gene transcript in all tissue types examined in the present study however with different coding potential that the expected transcript (Figure 1). Translating these unspliced products, I identified 2 or more stop codons in each of the clones and therefore suggest that such transcript forms are not functional for coding for the aromatase enzyme. The current study is therefore in agreement with earlier studies indicating expression of *cyp19a* only to be in the ovary of teleosts as been reported by Chiang *et al.* (2001) and Kishida and Callard (2001). Finally, these results underline the importance of using the correct primers in a PCR reaction to avoid measuring additional transcript variants.

As for *cyp19b*, in this study, transcript was amplified in the brain and muscle of both male and female and in addition also in female ovarian tissue. Several studies reported *cyp19b* in testis too, based again on PCR techniques. However, immunohistochemistry fails to find *cyp19* in Sertoli and germ cells in testis (Kobayashi *et al.* 1988; Sunobe *et al.* 2005) so did the present study. Within the brain, vertebrates express *cyp19* most often in the forebrain, whereby it is implicated in the control of reproduction and sexual behavior (Balthazart & Ball 1998). The duplication of the *cyp19* gene on account of two different genes present and expressed in different tissues can be explained by possible partitioning of initial function as has been the theory for similar cases (Lynch & Force 2000).

On a quantitative basis, sexual dimorphism in both mammals (De Vries & Simerly 2002) and teleosts (Goto-Kazeto *et al.* 2004; Forlano *et al.* 2006) is well known for aromatase expression in the brain – specifically males tend to have higher activity in the areas of the brain related to reproduction (Gonzalez & Piferrer 2003). Moreover, aromatase activity and mRNA transcripts in exclusively glial cells seems to be consistent only for teleosts over a range of species (Forlano *et al.* 2006) in contrast with mammals and birds where *cyp19* expression is restricted to neuronal cell bodies and nerve terminals under normal conditions, but appears in glial cells only after brain injury (reviewed by Cheshenko *et al*. 2007). The fact that *cyp19b* is markedly expressed and spliced in the female ovary begs the question as to what the function of this isoform is.

*Temporal expression*

*Cyp19* is being expressed in the early developing tilapia brain (Wang & Tsai 2006), and sexual differentiation in gonochoristic fish appears to be at least partly regulated by the differential expression of *cyp19* genes leading to changes in sex steroid production (Cheshenko *et al.* 2008). This provides an entry point to study sex differentiation if not determination in a South African Tilapia (*Oreochromis mossambicus*). Studies on a few other gonochoristic species (Trant *et al.* 2001; Sawyer *et al.* 2006; Greytak & Callard 2007; Hecker *et al.* 2007) pointed out the significance of using differential expression of aromatase in monitoring endocrine disruption of aquatic ecology.

*Cyp19*a transcript levels were high at 20 dpf when gonad differentiation was observed. These levels dropped significantly such that by 60 dpf, they were very low coinciding with a general dimorphic differentiation of gonadal tissue in histology samples. Compared to expression of *cyp19b* during the same time period (25 dpf – 60 dpf, Figure 2b), I measured elevated levels of the latter gene, albeit much lower. The tissue specific analysis (Figure 5) revealed marked expression in the ovarian tissue of *cyp19b*, which provides a possible explanation for where aromatase may originate. Again the question of how either of these isoforms is regulated during this time may provide remarkable insight into the mechanisms directing gonadal differentiation during development of *O. mossambicus*.

There is evidence shown in zebrafish (*Danio rerio*) that unfertilized eggs had levels of *cyp19a* that compared similarly to that in ovaries and *cyp19b* was higher, suggesting preferential synthesis or accumulation in mature oocytes (Sawyer *et al.* 2006) although the study found that maternally derived *cyp19* homologues were rapidly degraded post-fertilization. Therefore, I hypothesize further that either maternally received $E_2$ or estrogen synthesized *de novo* upon transcription of *cyp19a* in the undifferentiated gonads prior to gonadal differentiation may result in upregulation of *cyp19b* via the presence of ERE in the promoter area during this time period (25 – 60 dpf) whereas *cyp19a* lacks ERE but has amongst others SF1 and SRY binding sites (Chang *et al.* 2005) – both binding sites for gene products known to inhibit the expression of *cyp19a* (Crews *et al.* 2001; Chang *et al.* 2005). In addition, female fathead minnows showed a dose dependant $E_2$ induction of *cyp19b* in agreement of the ERE found in some gonochoristic species (Tchoudakova *et al.* 2001; Kazeto *et al.* 2001; Halm *et al.*

2002; Chang *et al.* 2005). This is in agreement with male teleosts that demonstrate an increased expression of the *cyp19* genes found predominantly in the brain (Goto-Kazeto *et al.* 2004) during some stages of development (Blazquez & Piferrer 2004). A study on fathead minnows by Villeneuve *et al.* (2006) may indicate that regulation of reproductive activity occurs at a transcriptional level with regards to *cyp19a* in that the enzyme activity of aromatase was significantly lower in non reproductive adults' ovaries. Yet, when the two *cyp19s* are differentially measured, not *cyp19b*, but very well *cyp19a* was down regulated in the ovaries of non reproductive adults' ovaries (Villeneuve *et al.* 2006).

Finally, the elevated expression of *cyp19b* as apposed to the virtual absence of *cyp19a* during this time of development further suggests the neuroendocrine role of *cyp19b* in teleosts. In zebrafish, males were grouped according to their levels of *cyp19b* expression in to "high" and "low" expression groups (Trant *et al.* 2001). Hoewever no bipolar distribution of data was found in this study (Figure 2B).

Positive and negative regulation of FSH and LH by steroids illustrates a seasonal variation when dependent on aromatase in these fish (reviewed by Cheshenko et al 2007), possibly explaining some of the variation found in the expression levels for both *cyp19a* and *cyp19b*.

*Aromatase and vtg*

Vitellogenin gene activation in teleosts is known to be receptor mediated as has been pointed out by the molecular basis for vitellogenin gene (*vtg*) expression in juvenile Atlantic salmon (Meucci & Arukwe 2006) – specifically responses that are ligand structure-dependent interactions with ESR in addition with other activators. Moreover, *cyp19b* activity has been found in a gonochoristic fish (bream) to be positively and significant correlated with plasma estradiol in females whereas in males it was found to be correlated to testosterone (11-ketotestosterone) (Hecker *et al.* 2007). In some studies it has been shown that transcriptional level and enzyme activity of aromatase are correlated in tilapia (Chang *et al.* 1999) and other species (Fukada *et al.* 1996; Gelinas *et al.* 1998) and is therefore applied as an indication of $E_2$ production and even sex differentiation in various teleosts (Baroiller *et al.* 1999; Trant *et al.* 2001; Kishida & Callard 2001; Blazquez & Piferrer 2004; Luckenbach *et al.* 2005) including tilapia (D'Cotta *et al.* 2001; Kwon *et al.* 2001; Chang *et al.* 2005). $E_2$ can be produced only by *cyp19*

aromatase (Simpson *et al.* 1994) in combination with NADPH reductase (Conley & Walters 1999) from either testosterone or androstenedione via estrone (Tsuchiya *et al.* 2005). Therefore, if *vtg* of an individual is induced by $E_2$, that sample had to have either high levels of $E_2$ already or had to synthesize it *de novo* and therefore had to have upregulated *cyp19* genes in its body. In this study I correlated the expression *of vtg* with either isoform of *cyp19* for each sample.

In this regard, no correlation was found between *cyp19a* or b with *vtg* when comparing mRNA levels as shown in Chapter 2. This is not surprising as I suspect a time lag phase between expressing *cyp19* and *vtg* induction by $E_2$. The *O. mossambicus vtg* promoter area indicates that regulation of the gene can be orchestrated via EREs or other biding motives including *vtg*-related motifs, Sox 5 and Sox 9, as well VBP, hepatic leukaemia factor (HLF) and GATA-1 (Chapter 2) which might provide a second answer to the question stated above. These results indicate that regulation of *vtg* in fish may not be exclusively receptor mediated as has been suggested by (Meucci & Arukwe 2006) for salmon and I propose possible other mechanisms of *vtg* induction for samples with no transcript of the aromatase genes present. Since regulation of *vtg* via $E_2$ on the ERE of *vtg*'s promoter is receptor mediated, ER levels for each of these samples may shed more light on this matter as will it strengthen the proposed hypothesis if found at low levels in these particular samples.

Ovarian $E_2$ production is known to change depending on the stage of the vitellogenic cycle it engages in various teleosts including tilapia (Chang *et al.* 1997), with an increase from the start of the cycle, and a decrease at the end of vitellogenesis with the beginning of final oocyte maturation which coincides with a steroidogenic shift involving a decrease in *cyp19* ovary expression and activity in the ovary (Chang *et al.* 1997; Goto-Kazeto *et al.* 2004). On the contrary, *cyp19b* change in a different manner with regards to the vitellogenic cycle: in channel catfish (Kazeto *et al.* 2003) found this isoforms of the gene to remain at low levels at recrudescent and vitellogenic stages, but to increase around the time of spawning when ovaries reach the maturing stage, pointing to a regulatory effect of the female reproductive cycle by both these isoforms of the gene, albeit in opposite fluctuations.

When one consider that ovarian *cyp19a* expression is hosted by granulosa cells, it can be that the expression is cell specific and since no cells similar to granulosa cells are found in any of the testicular tissues, the expression of this

gene is regulated in that manner. As for *cyp19b* that is expressed in the ovaries (Figure 1), the question still remains as to in which cell type it is expressed, more so since the testis is not found to have either of these genes expressed and spliced within.

*Sexual dimorphism*

A recent gene expression study on zebrafish aromatase illustrated one tissue – the eye – to exhibit sex differences in terms of quantities of *cyp19b* being expressed in the eyes (Sawyer *et al.* 2006). In a semi quantitative study on juvenile sea bass (*D. labrax*), the brain type aromatase has been shown to exhibit higher in females than males at 200 dpf, the time of gonadal sex differentiation (Blazquez & Piferrer 2004). Hereafter a switch occurred between 200 and 250 dpf with males illustrating higher *cyp19b* levels than females which were maintained up to 300 dpf. These changes in expression profiles along with the sex-related differences found in European sea bass, indicates differences in the functionality of the enzyme between males and females, suggesting and important role for brain aromatase during sex differentiation (Blazquez & Piferrer 2004). These authors suggest also a role in neurogenesis on account of the continuous growth of the teleost brain throughout life.

Without known differentiated sex in *O. mossambicus* throughout the developmental stages, quantitative sexual dimorphism in the expression of *cyp19a* and *cyp19b* is not discernible using our current QPCR technique. However, sexual dimorphism of adults where tissue can be dissected is appropriate, and revealed higher expression of *cyp19b* in the brain of adult female compared to an adult male. The samples used here were mature fish after gonadal sex differentiation – agreeing with the 200 – 250 dpf stage in sea bass.

*Cyp19a* in the ovaries is known to be upregulated at the vitellogenic stage of oocytes (Goto-Kazeto *et al.* 2004) and therefore can be expected to be changing in a mirroring manner along with the vitellogenic cycle. It is therefore not strange to find animals with high *cyp19a* expression to have high *vtg* expression too. But this is expected to hold true for only *cyp19a*, which I indeed found to be the case for *O. mossambicus*. However, one need more information about the availability of $E_2$ (and other estrogens) after being metabolized by aromatase to fully understand what is happening in the gonadal tissues of both male and

female tilapia. Since *cyp19a* might be active but availability of ER could very much be responsible for prevention of the metabolized $E_2$. Again, its also important to keep in mind that $E_2$ might signal in a non-genomic manner involving diverse second messenger systems (Falkenstein *et al.* 2000) albeit for short periods of time (seconds/minutes).

## CONCLUSION

Teleosts carry a duplicated *cyp19* gene which highlights the evolutionary significance of maintaining two active genes for specific functions in specific tissues, and offers an opportunity to characterize the role of this gene product in its particular sites in more detail. In the arena of endocrine disruption, the natural interplay of these two genes can provide classic information of one of the best studied groups of EDCs yet – that of estrogenic substances. However, before one understands the natural biology of $E_2$ responsive genes, making inferences on account of these genes may provide a dubious picture and inaccurate predictions of endocrine disruption by estrogenic compounds.

*Cyp19b* is noted elsewhere to serve as a neural marker of estrogenic effect in teleosts (Sawyer *et al.* 2006) but is also stated to serve as an entry point for studying the role of hormonal and environmental estrogens on neurodevelopment and neuroplasticity. I add hereby to a platform (Chapter 2) for what is normal expression of both *cyp19a* and *cyp19b* with supported histological gonadal development during the various life stages of *O. mossambicus*, and variation especially within the juveniles which might prove to be a valuable asset in this regard.

## REFERENCE LIST

**Andersen L., Bjerregaard P. & Korsgaard B.**, 2003. Vitellogenin induction and brain aromatase activity in adult male and female zebrafish exposed to endocrine disrupters. *Fish Physiology and Biochemistry.* 28, 319-321.

**Balthazart J.** 1997. Steroid control and sexual differentiation of brain aromatase. *The Journal of Steroid Biochemistry and Molecular Biology.* 61, 323-339.

**Balthazart J. & Ball G.F.**, 1998. New insights into the regulation and function of brain estrogen synthase (aromatase). *Trends in Neurosciences.* 21, 243-249.

**Balthazart J. & Ball G.F.**, 2006. Is brain estradiol a hormone or a neurotransmitter? *Trends in Neurosciences.* 29, 241-249.

**Bancroft J. & Stevens A.**, 1977. *Theory and practice of histological techniques*. Churchill Livingstone. New York .

**Baroiller J.F., Guiguen Y. & Fostier A.**, 1999. Endocrine and environmental aspects of sex differentiation in gonochoristic fish. *Cellular and Molecular Life Sciences.* 55, 910-931.

**Blazquez M. & Piferrer F.**, 2004. Cloning, sequence analysis, tissue distribution, and sex-specific expression of the neural form of P450 aromatase in juvenile sea bass (*Dicentrarchus labrax*). *Molecular and Cellular Endocrinology.* 219, 83-94.

**Bustin S.** 2002. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *Journal of Molecular Endocrinology.* 29, 23-39.

**Callard G.V., Drygas M. & Gelinas D.**, 1993. Molecular and cellular physiology of aromatase in the brain and retina. *The Journal of Steroid Biochemistry and Molecular Biology.* 44, 541-547.

**Callard G.V., Schlinger B.A. & Pasmanik M.**, 1990. Nonmamalian vertebrate models in studies of brain-steroid interactions. *Journal of Experimental Zoology (Suppl).* 4, 6-16.

**Callard G.V. & Tchoudakova A.**, 1997. Evolutionary and functional significance of two CYP19 genes differentially expressed in brain and ovary of goldfish*1. *The Journal of Steroid Biochemistry and Molecular Biology.* 61, 387-392.

**Callard G.V., Tchoudakova A.V., Kishida M. & Wood E.**, 2001. Differential tissue distribution, developmental programming, estrogen regulation and promoter characteristics of cyp19 genes in teleost fish. *The Journal of Steroid Biochemistry and Molecular Biology.* 79, 305-314.

**Chang C.F., Hung C.Y., Chiang M.C. & Lan S.C.**, 1999. The concentrations of plasma sex steroids and gonadal aromatase during controlled sex differentiation in grey mullet, *Mugil cephalus*. *Aquaculture.* 177, 37-45.

**Chang X.T., Kobayashi T., Kajiura H., Nakamura M. & Nagahama Y.**, 1997. Isolation and characterization of the cDNA encoding the tilapia (*Oreochromis niloticus*) cytochrome P450 aromatase (P450arom): changes in P450arom mRNA, protein and enzyme activity in ovarian follicles during oogenesis. *Journal of Molecular Endocrinology.* 18, 57-66.

**Chang X., Kobayashi T., Senthilkumaran B., Kobayashi-Kajura H., Sudhakumari C.C. & Nagahama Y.**, 2005. Two types of aromatase with different encoding genes, tissue distribution and developmental expression in Nile tilapia (*Oreochromis niloticus*). *General and Comparative Endocrinology.* 141, 101-115.

**Cheshenko K., Pakdel F., Segner H., Kah O. & Eggen R.I.L.**, 2008. Interference of endocrine disrupting chemicals with aromatase CYP19 expression or activity, and consequences for reproduction of teleost fish. *General and Comparative Endocrinology.* 155, 31-62.

**Chiang E.F., Yan Y.L., Guiguen Y., Postlethwait J. & Chung B.c.**, 2001. Two Cyp19 (P450 Aromatase) Genes on Duplicated Zebrafish Chromosomes Are Expressed in Ovary or Brain. *Molecular Biology and Evolution.* 18, 542-550.

**Choi I., Troyer D., Cornwell D., Kirby-Dobbels K., Collante W. & Simmen F.**, 1997. Closely related genes encode developmental and tissue isoforms of porcine cytochrome P450 aromatase. *DNA and Cell Biology.* 16, 769-777.

**Conley A. & Walters K.**, 1999. Aromatization. In Knobel,E. & Neill,J. *Encyclopedia of reproduction*. Academic Press,London, pp 280-291.

**Cornil C.A., Ball G.F. & Balthazart J.**, 2006. Functional significance of the rapid regulation of brain estrogen action: Where do the estrogens come from? *Brain Research.* 1126, 2-26.

**Crews D., Fleming A., Willingham E., Baldwin R. & Skipper J.K.**, 2001. Role of steroidogenic factor 1 and aromatase in temperature-dependant sex determination in the red-eared slider turtle. *Journal of Experimental Zoology.* 290, 597-606.

**D'Cotta H., Fostier A., Guiguen Y., Govoroun M. & Baroiller J.F.**, 2001. Aromatase plays a key role during normal and temperature-induced sex differentiation of tilapia *Oreochromis niloticus*. *molecular reproduction and development.* 59, 265-276.

**De Vries G. & Simerly R.**, 2002. Anatomy, development, and function of sexually dimorphic neural circuits in the mammalian brain. In Pfaff,D., Arnold,A., Etgen,A., Fahrbach,S. & Rubin,R. *Hormones, Brain and Behaviour*. Elsevier,Amsterdam,

**Eckstein B. & Spira M.**, 1965. Effect of sex hormones on gonadal differentiation in a cichlid, *Tilapia aurea*. *Biological Bulletin.* 129, 482-489.

**Falkenstein E., Tillmann H.C., Christ M., Feuring M. & Wehling M.**, 2000. Multiple Actions of Steroid Hormones - A Focus on Rapid, Nongenomic Effects. *Pharmacological Reviews.* 52, 513-556.

**Forlano P.M., Schlinger B.A. & Bass A.H.**, 2006. Brain aromatase: New lessons from non-mammalian model systems. *Frontiers in Neuroendocrinology.* 27, 247-274.

**Fukada S., Tanaka M., Matsuyama M., Kobayashi D. & Nagahama Y.**, 1996. Isolation, characterization, and expression of cDNAs encoding the medaka (*Oryzias latipes*) ovarian follicle cytochrome P-450 aromatase. *molecular reproduction and development.* 45, 285-290.

**Gelinas D., Pitoc A. & Callard V.**, 1998. Isolation of a goldfish brain cytochrome P450 aromatase cDNA:: mRNA expression during the seasonal cycle and after steroid treatment1. *Molecular and Cellular Endocrinology.* 138, 81-93.

**Gibbons J.** 1985. *Nonparametric statistical inference*.New York .

**Gonzalez A. & Piferrer F.**, 2003. Aromatase activity in the European sea bass (*Dicentrarchus labrax* L.) brain. Distribution and changes in relation to age, sex, and the annual reproductive cycle. *General and Comparative Endocrinology.* 132, 223-230.

**Goto-Kazeto R., Kight K.E., Zohar Y., Place A.R. & Trant J.M.**, 2004. Localization and expression of aromatase mRNA in adult zebrafish. *General and Comparative Endocrinology.* 139, 72-84.

**Greytak S.R. & Callard G.V.**, 2007. Cloning of three estrogen receptors (ER) from killifish (*Fundulus heteroclitus*): Differences in populations from polluted and reference environments. *General and Comparative Endocrinology.* 150, 174-188.

**Greytak S.R., Champlin D. & Callard G.V.**, 2005. Isolation and characterization of two cytochrome P450 aromatase forms in killifish (*Fundulus heteroclitus*): Differential

expression in fish from polluted and unpolluted environments. *Aquatic Toxicology.* 71, 371-389.

**Grumbach M.** 2000. Estrogen, bone, growth and sex: a sea change in conventional wisdom. *Journal of Pediatric Endocrinology and Metabolism.* 13, 1439-1455.

**Guiguen Y., Baroiller J.F., Ricordel M.J., Iseki K., McMeel O., Martin S. & Fostier A.**, 1999. Involvement of estrogens in the process of sex differentiation in two fish species: the rainbow trout (*Onchorhynchus mykiss*) and a tilapia (*Oreochromis niloticus*). *molecular reproduction and development.* 54, 154-162.

**Halm S., Pounds N., Maddix S., Rand-Weaver M., Sumpter J.P., Hutchinson T.H. & Tyler C.R.**, 2002. Exposure to exogenous 17[beta]-oestradiol disrupts P450aromB mRNA expression in the brain and gonad of adult fathead minnows (*Pimephales promelas*). *Aquatic Toxicology.* 60, 285-299.

**Hecker M., Thomas Sanderson J. & Karbe L.**, 2007. Suppression of aromatase activity in populations of bream (*Abramis brama*) from the river Elbe, Germany. *Chemosphere.* 66, 542-552.

**Hines G.A., Boots L.R., Wibbels T. & Watts S.A.**, 1999. Steroid Levels and Steroid Metabolism in Relation to Early Gonadal Development in the Tilapia *Oreochromis niloticus* (Teleostei: Cyprinoidei). *General and Comparative Endocrinology.* 114, 235-248.

**Hourigan T.F., Nakamura M., Nagahama Y., Yamauchi K. & Grau E.G.**, 1991. Histology, ultrastructure, and in vitro steroidogenesis of the testes of two male phenotypes of the protogynous fish, *Thalassoma duperrey* (Labridae). *General and Comparative Endocrinology.* 83, 193-217.

**Humason G.** 1967. *Animal tissue techniques*. WH Freeman and Co. San Francisco .

**Janicke F.** 2004. Are all aromatase inhibitors the same? A review of the current evidence. *The Breast.* 13, 10-18.

**Jeng S.R., Dufour S. & Chang C.-F.**, 2005. Differential expression of neural and gonadal aromatase enzymatic activities in relation to gonadal development in Japanese eel, *Anguilla japonica*. *Journal of Experimental Zoology Part A: Comparative Experimental Biology.* 303A, 802-812.

**Kazeto Y., Goto-Kazeto R., Place A. & Trant J.**, 2003. Aromatase expression in zebrafish and channel catfish brains: changes in transcript abundance associated with the reproductive cycle and exposure to endocrine disrupting chemicals. *Fish Physiology and Biochemistry.* 28, 29-32.

**Kazeto Y., Ijiri S., Place A.R., Zohar Y. & Trant J.M.**, 2001. The 5'-Flanking Regions of CYP19A1 and CYP19A2 in Zebrafish. *Biochemical and Biophysical Research Communications.* 288, 503-508.

**Kazeto Y., Place A.R. & Trant J.M.**, 2004. Effects of endocrine disrupting chemicals on the expression of CYP19 genes in zebrafish (*Danio rerio*) juveniles. *Aquatic Toxicology.* 69, 25-34.

**Kishida M. & Callard G.V.**, 2001. Distinct Cytochrome P450 Aromatase Isoforms in Zebrafish (*Danio rerio*) Brain and Ovary Are Differentially Programmed and Estrogen Regulated during Early Development. *Endocrinology.* 142, 740-750.

**Kishida M., McLellan M., Miranda J.A. & Callard G.V.**, 2001. Estrogen and xenoestrogens upregulate the brain aromatase isoform (P450aromB) and perturb markers of early development in zebrafish (*Danio rerio*). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology.* 129, 261-268.

**Kobayashi M., Aida K. & Hanyu I.**, 1988. Hormone changes during the ovulatory cycle in goldfish. *General and Comparative Endocrinology.* 69, 301-307.

**Kwon J., McAndrew B.J. & Penman D.**, 2001. Cloning of brain aromatase gene and expression of brain and ovarian aromatase genes during sexual differentiation in genetic male and female Nile tilapia *Oreochromis niloticus*. *Molecular Reproduction Development.* 59, 359-370.

**la Valle L., Toffolo V., Vianello S., Ikuo H., Takashi A., Belvedere P. & Colombo L.**, 2005. Genomic organization of the CYP19b genes in the rainbow trout (*Oncorhynchus mykiss* Walbaum). *The Journal of Steroid Biochemistry and Molecular Biology.* 94, 49-55.

**Leino R.L., Jensen K.M. & Ankley G.T.**, 2005. Gonadal histology and characteristic histopathology associated with endocrine disruption in the adult fathead minnow (*Pimephales promelas*). *Environmental Toxicology and Pharmacology.* 19, 85-98.

**Luckenbach J.A., Early L., Rowe A., Borski R.J., Daniels H.V. & Godwin J.**, 2005. Aromatase cytochrome P450: cloning, intron variation, and ontogeny of gene expression in southern flounder (*Paralichthys lethostigma*). *Journal of Experimental Zoology.* 303A, 643-656.

**Lynch M. & Force A.**, 2000. The probability of duplicate gene presevation by subfunctionalization. *Genetics.* 154, 459-473.

**Maclusky N. & Naftolin F.**, 1981. Sexual Differentiation of the Central Nervous System. *Science.* 4488, 1294-1302.

**Matsuoka M.P., van Nes S., Andersen O., Benfey T.J. & Reith M.**, 2006. Real-time PCR analysis of ovary- and brain-type aromatase gene expression during Atlantic halibut (*Hippoglossus hippoglossus*) development. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology.* 144, 128-135.

**Meijide F., Lo Nostro F. & Guerrero G.**, 2005. Gonadal development and sex differentiation in the cichlid fish *Cichlasoma dimerus* (Teleostei, perciformes): A light- and electron-microscopic study. *Journal of morphology.* 264, 191-210.

**Meucci V. & Arukwe A.**, 2006. Transcriptional modulation of brain and hepatic estrogen receptor and P450arom isotypes in juvenile Atlantic salmon (*Salmo salar*) after waterborne exposure to the xenoestrogen, 4-nonylphenol. *Aquatic Toxicology.* 77, 167-177.

**Nagahama Y.** 1994. Endocrine regulation of gametogenesis in fish. *International journal of Developmental Biology.* 38, 217-229.

**Nakamura M., Kobayashi T., Chang X.T. & Nagahama Y.**, 1998. Gonadal sex differentiation in teleost fish. *Journal of Experimental Zoology.* 281, 362-372.

**Nakamura M. & Nagahama Y.**, 1985. Steroid producing cells during ovarian differentiation of the tilapia *Sarotherodon niloticus*. *Development, Growth and Differentiation.* 27, 701-708.

**Nelson D., Koymans L., Kamataki T., Stegeman J., Feyereisen R., Waxman D., Waterman M., Gotoh O., Coon M., Estabrook R., Gunsalus I. & Nebert D.**, 1996. P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics.* 6, 1-42.

**Park C.B., Takemura A., Aluru N., Park Y.J., Kim B.H., Lee C.H., Lee Y.D., Moon T.W. & Vijayan M.M.**, 2007. Tissue-specific suppression of estrogen, androgen and glucocorticoid receptor gene expression in feral vitellogenic male Mozambique tilapia. *Chemosphere.* 69, 32-40.

**Parmentier H. & Timmermans L.**, 1985. The differentiation of germ cells and gonads during development of carp (*Cyprinus carpio* L.). A study with anti-carp sperm monoclonal antibodies. *Journal of Embryonology and Experimental Morphology.* 90, 13-32.

**Pearson K.** 1896. Regression, heredity, and panmixia. *Philosophical Transactions of the Royal Society of London.* 187, 253-318.

**Pellegrini E., Menuet A., Lethimonier C., Adrio F., Gueguen M.M., Tascon C., Anglade I., Pakdel F. & Kah O.**, 2005. Relationships between aromatase and estrogen receptors in the brain of teleost fish. *General and Comparative Endocrinology.* 142, 60-66.

**Piferrer F., Blazquez M., Navarro L. & Gonzalez A.**, 2005. Genetic, endocrine, and environmental components of sex determination and differentiation in the European sea bass (*Dicentrarchus labrax* L.). *General and Comparative Endocrinology.* 142, 102-110.

**Rocha A., Gomez A., Galay-Burgos M., Zanuy S., Sweeney G.E. & Carrillo M.**, 2007. Molecular characterization and seasonal changes in gonadal expression of a thyrotropin receptor in the European sea bass. *General and Comparative Endocrinology.* 152, 89-101.

**Rocha M. & Rocha E.**, 2006. Morphofunctional aspects of reproduction from synchronous to asynchronous fishes - and overview. In Reinecke,M., Zaccone,G. & Kapoor,B. *Fish endocrinolgy*. Science Publishers,Enfield, pp 571-624.

**Sawyer S.J., Gerstner K.A. & Callard G.V.**, 2006. Real-time PCR analysis of cytochrome P450 aromatase expression in zebrafish: Gene specific tissue distribution, sex differences, developmental programming, and estrogen regulation. *General and Comparative Endocrinology.* 147, 108-117.

**Schulz R.W., Vischer H.F., Cavaco J.E.B., Santos E.M., Tyler C.R., Goos H.J.T. & Bogerd J.**, 2001. Gonadotropins, their receptors, and the regulation of testicular functions in fish. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology.* 129, 407-417.

**Simpson E.R., Mahendroo M.S., Means G.D., Kilgore M.W., Hinshelwood M.M., Lorence S., Amarneh B., Ito Y., Fisher C.R. & Michael M.D.**, 1994. Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. *Endocrine Reviews.* 15, 342-355.

**Strussmann C.A., Takashima F. & Toda K.**, 1996. Sex differentiation and hormonal feminization in pejerrey *Odontesthes bonariensis*. *Aquaculture.* 139, 31-45.

**Sunobe T., Nakamura M., Kobayashi Y., Kobayashi T. & Nagahama Y.**, 2005. Aromatase immunoreactivity and the role of enzymes in steroid pathways for inducing sex change in the hermaphrodite gobiid fish *Trimma okinawae*. *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology.* 141, 54-59.

**Tchoudakova A. & Callard G.V.**, 1998. Identification of Multiple CYP19 Genes Encoding Different Cytochrome P450 Aromatase Isozymes in Brain and Ovary. *Endocrinology.* 139, 2179-2189.

**Tchoudakova A., Kishida M., Wood E. & Callard G.V.**, 2001. Promoter characteristics of two cyp19 genes differentially expressed in the brain and ovary of teleost fish. *The Journal of Steroid Biochemistry and Molecular Biology.* 78, 427-439.

**Toffolo V., Belvedere P., Colombo L. & Valle L.D.**, 2007. Tissue-specific transcriptional initiation of the CYP19 genes in rainbow trout, with analysis of splicing patterns and promoter sequences. *General and Comparative Endocrinology.* 153, 311-319.

**Trant J.M., Gavasso S., Ackers J., Chung B. & Place A.**, 2001. Developmental expression of cytochrome P450 aromatase genes (CYP19a and CYP19b) in zebrafish fry (*Danio rerio*). *Journal of Experimental Zoology.* 290, 475-483.

**Tsai C.Γ., Wang L.Γ., Chang C.Γ. & Kao C.Γ.**, 2000. Effects of Gonadal Steroids on Brain Serotonergic and Aromatase Activity During the Critical Period of Sexual Differentiation in Tilapia, *Oreochromis mossambicus*. *Journal of Neuroendocrinology.* 12, 894-898.

**Tsuchiya Y., Nakajima M. & Yokoi T.**, 2005. Cytochrome P450-mediated metabolism of estrogens and its regulation in human. *Cancer Letters.* 227, 115-124.

**Vasudevan N., Ogawa S. & Pfaff D.**, 2002. Estrogen and Thyroid Hormone Receptor Interactions: Physiological Flexibility by Molecular Specificity. *Physiological Reviews.* 82, 923-944.

**Vasudevan N. & Pfaff D.W.**, 2007. Membrane-Initiated Actions of Estrogens in Neuroendocrinology: Emerging Principles. *Endocrine Reviews.* 28, 1-19.

**Villeneuve D., Knoebl I., Kahl M., Jensen K., Hammermeister D., Greene K., Blake L. & Ankley G.**, 2006. Relationship between brain and ovary aromatase activity and isoform-specific aromatase mRNA expression in the fathead minnow (*Pimephales promelas*). *Aquatic Toxicology.* 76, 353-368.

**Wang L.H. & Tsai C.L.**, 2006. Photoperiod influences the ontogenic expression of aromatase and estrogen receptor alpha in the developing tilapia brain. *General and Comparative Endocrinology.* 145, 62-66.

**Wang L.H. & Tsai C.L.**, 2000. Temperature affects the development of central neurotransmitter systems of tilapia, *Oreochromis mossambicus*. *Neuroscience Letters.* 285, 95-98.

**West G** ,1990. Methods of assessing ovarian development in fishes: a review. *Australian Journal of Marine and Freshwater Research.* 41, 199-222.

**Yamamoto T.** 1969. Sex differentiation in fishes. In Hoar,W. & Randall,D. *Fish Physiology*. Academic Press,New York, pp 117-175.

**Yoshiura Y., Senthilkumaran B., Watanabe M., Oba Y., Kobayashi T. & Nagahama Y.**, 2003. Synergistic Expression of Ad4BP/SF-1 and Cytochrome P-450 Aromatase (Ovarian Type) in the Ovary of Nile Tilapia, *Oreochromis niloticus*, During Vitellogenesis Suggests Transcriptional Interaction. *Biology of Reproduction.* 68, 1545-1553.

**Zar J.** 1996. *Biostatistical analysis*. Prentice Hall. New Jersey, USA .

**Zhao J., Mak P., Tchoudakova A., Callard G.V. & Chen S.**, 2001. Different catalytic properties and inhibitor responses of the goldfish brain and ovary aromatase isoforms. *General and Comparative Endocrinology.* 123, 180-191.

Chapter 4

# Isolation and characterization of three estrogen receptors in *Oreochromis mossambicus*

**ABSTRACT**

Exposure of aquatic organisms to 17β-Estradiol ($E_2$) induces a variety of estrogen responsive genes, including vitellogenin (*vtg*), the precursor protein of egg yolk in oviparous species. Transcription regulation of *vtg* by $E_2$ is dependant on binding of the ligand ($E_2$) to a specific nuclear receptor (estrogen receptor, ESR) which in turn binds to the estrogen responsive element (ERE) in the promoter of *vtg*. As *Oreochromis mossambicus* is targeted as a model for estrogenic endocrine disruption in Southern Africa, a platform of knowledge is necessary for the ontogenic and tissue specific behavior of ESR in this species before vitellogenin levels can be interpreted in relation to such endocrine disruption. Answering in part to this demand, I isolated and sequenced three homologues of ESR in *O. mossambicus* and developed subsequent protocols to ascertain quantitative transcription levels of these three isoforms in adult brain, gonadal and hepatic tissues. Finally I used these protocols to describe quantitative gene levels during development in this species.

Keywords: Estrogen receptor (ESR), Mozambique tilapia, quantitative real-time PCR (QPCR), Endocrine disruption, temporal transcription

**INTRODUCTION**

The endocrine steroid hormone 17β-Estradiol ($E_2$,) performs a multitude of functions in both the male and female physiological systems. Apart from its primary reproductive effects which include controlling reproductive processes in male and female vertebrates, gonadal function and many secondary reproductive functions, $E_2$ also plays an important role in liver and cardiovascular physiology, neural growth and differentiation, neuroprotection, cognition, and regulation of mood (Vasudevan & Pfaff 2007; Brann *et al.* 2007). In addition, oviparous species are known to be dependant on the presence of $E_2$

in order to produce large quantities of the egg yolk precursor protein, vitellogenin (VTG). This, along with the vast majority of endocrine disruption studies revealing estrogenic interference, provides a rationale for detailed studies on matters where estrogens are concerned.

$E_2$ is traditionally known to affect target genes through binding to specific nuclear receptors and influencing response genes through transcriptional regulation (Wang et al. 2005). However, $E_2$ has recently been shown to have at least two different mechanisms of action in vertebrates: (i) the classical "genomic mechanism" via its regulation of transcription of target genes, and (ii) its "non-genomic mechanism" where it binds to membrane receptors or recognition sites mediating its action through regulation of transcription but not via its own ligand-receptor complex (Falkenstein *et al.* 2000). The latter mode of action is suggested to represent a more rapid effect and include activation of kinases and release of calcium which in turn activate other signaling cascades in the cytoplasm and finally in the nucleolus where transcriptional regulation is potentiated (Nilsson *et al.* 2001). In turn, the classical genomic mechanism of $E_2$, which incorporates specific nuclear receptors, provides an entry point to study some aspects of the function of $E_2$.

Binding of estrogens to nuclear receptors has been well documented for mammals, in which temporal and tissue-specific actions of estrogens are mediated by either of two isoforms of ESR, denoted ESR1 and 2 (Pearce & Jordan 2004). ESR1 was the first ESR cloned and isolated from MCF-7 human breast cancer cells (Walter *et al.* 1985; Greene *et al.* 1986) with ESR2 cloned about a decade later from rat prostate (Kuiper *et al.* 1996). In fish, three isoforms of ESR has been identified. These are transcribed for by distinctive genes (ESR1, ESR2a and ESR2b, also known as ER$\gamma$) to result in functionally different receptors that have distinct expression patterns *in vivo*, and for which all co-factors are not functionally equivalent (Hawkins *et al.* 2000). Substrate preference has been reported for the different ESRs in various species (McDonnell & Norris 2002; Greytak & Callard 2007) and several different splice variants have been described for ERs in both mammals and teleosts (Pearce & Jordan 2004; Meucci & Arukwe 2006; Martyniuk *et al.* 2007; Traupe *et al.* 2007).
In mammals, both ESR1 and $\beta$ are known to localize in the breast, brain, cardiovascular system, urogenital tract and bone (Pearce & Jordan 2004). In the liver however ESR1 is known to be the predominant isoform, whereas ESR2 is

the main ESR in colon tissue (Pearce & Jordan 2004). Teleost fish has been shown in a few studies to express ESR1 in liver tissue, whereas in ovarian tissue, both ESR2a and ESR2b are the most transcribed isoforms (Menuet *et al.* 2004; Sabo-Attwood *et al.* 2004). This information for Mozambique tilapia under investigation is not available to date, and can provide significant information with regards to the mechanism by which $E_2$ function both during developmental programme, and possibly in reaction on vitellogenic transcription inducers.

The aim of this study was therefore to isolate and sequence ESR cDNA in *O. mossambicus* with the objective to describe the expression of ESR quantitatively in different tissues (spatial variation) and during the normal developmental programme (temporal changes) using quantitative real-time reverse transcription PCR (QPCR). To this end, three ESR transcript isoforms in *O. mossambicus* were cloned and sequenced, and subsequent information was used to develop a QPCR protocol to describe the expression of all isoforms during developmental stages in *O. mossambicus*.

## MATERIALS AND METHODS

*Animals and sampling procedure*

Adult breeding stock of *Oreochromis mossambicus* were obtained from Aquastel (South Africa) and maintained in aquaria with water which is constantly aerated and filtered through activated charcoal. Water temperature was kept at 27 °C (±1 °C). Fish were fed once daily with Tilapia pellets (AquaNutro, South Africa). The light regime followed a 14:10 light:dark cycle. Offspring production was monitored daily in this mouthbrooding species. Females carrying eggs in their mouths were removed from the breeding aquaria to culturing tanks. In culturing tanks each brooding female was kept alone until the offspring reached the swim-up fry stage, at which time the adult female was removed and re-introduced into the breeding tank. Each batch of offspring was reared separately in the same water conditions as for breeding stock. Animals at the appropriate developmental stage (determined by age in 5 day intervals after fertilization) were collected, euthanized using 0.01 % benzocaine (Heynes Mathew, Ltd., South Africa) and preserved in RNA*later* (Ambion Inc., USA) at 4 °C. At least three different breeding pairs were used to generate offspring that were sampled at each developmental stage. For the adult tissue

scan for determination of the various ESR amplicons presence, at least five males and five females were dissected and the RNA prepared as outlined below.

*Total RNA isolation and cDNA preparation*

Total RNA was prepared from specific tissues of adults or from whole body homogenates of juveniles using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. Following resuspension of total RNA in diethyl pyrocarbonate (DEPC)-treated water, samples were treated with DNase I (Promega, USA) for 30 min at 37 °C and precipitated with 0.1 volumes 3 M sodium acetate pH 5.6 and 2.5 volumes of 95 % ethanol at -20 °C. The RNA pellets were washed with 70 % ethanol and redissolved in 30 to 60 µl of DEPC-treated water. RNA yields were quantified spectrophotometrically at $Absorbance_{260nm}$ and stored at -70°C. First strand cDNA was prepared from 2 µg of total RNA using oligo $d(T)_{15}$ primers and SuperScript III RNase $H^-$ MMLV reverse transcriptase (Invitrogen, USA) as described by the manufacturer. Samples were diluted 40-fold prior to gene expression determination and stored at -20 °C or used as template for ESR cloning as described below.

*Isolation of O. mossambicus ESR cDNA*

PCR primer sequences for ESR1, ESR21 and ESR2B cDNA in *O. mossambicus* were initially designed from the closely related *Oreochromis niloticus* (GenBank acc. nu. U75604, U75605 and DQ462608 respectively). cDNA from ovarian tissue of an adult female was used as template to perform long-range PCR for which a reaction consists of 1.5 mM $MgCl_2$, 0.05 mM of each dNTP, 1 µM of each primer (Table 1) and 2.5 Units of SuperTherm Gold (JMR Holdings, UK) Taq polymerase in a 25 µl reaction. To each reaction, 50 ng cDNA was added. PCR reaction volumes were denatured for 9 min at 95 °C, after which followed 30 cycles constituting of 30 seconds at 95 °C, 30 seconds at 64 °C and 3 minutes at 70 °C, with a final elongation step of 5 min at 70 °C. PCR products were checked for size on a 0.8 % agarose gel. Amplified DNA fragments were cloned into pGEM-T Easy vectors (Promega, USA) and transformed into *E. coli* DH5α.

*DNA sequencing and sequence comparisons*

Plasmid DNA was isolated from positive clones detected by colony PCR, and insert DNA sequenced using SP6 and T7 primers on an ABI PRISM® 3100 Genetic Analyser (Applied Biosystems, USA). The resulting *O. mossambicus* ESR sequence information was deposited in GenBank (accession no. AM284390, AM284391 and EU140820 for ESR1, ESR2a and ESR2b respectively). I performed sequence analysis of the DNA and derived amino acid sequences using ClustalW software according to Chenna et al. (2003) and graphic illustrations were prepared with software available in Bioedit Sequence Alignment Editor v7 (Hall 1999). Sequence alignments were done for various vertebrates by using the BLAST program (Altschul *et al.* 1997).

*Phylogenetic tree analysis*

A phylogenetic tree of ESR1, ESR2a and ESR2b was constructed for the derived amino acid sequences of the genes sequenced in this study as well as reported sequences for ESR1, ESR2a and ESR2b in other teleost species. The deduced sequences were aligned using CLUSTALW (Higgins *et al.* 1994) implementing a Gonnet scoring matrix, and the phylogenetic tree was constructed with application of the Neighbor Joining (Saitou & Nei 1987) method where bootstrap analysis calculated the probability of the presented branching of 1000 possible tree values recorded are presented as percentage of times out of 1000 that a node was recovered (Felsenstein 1985). Phylogenetic and molecular evolutionary analyses were conducted using MEGA v4.0 (Tamura, Dudley, Nei, and Kumar 2007).

*Gene expression analysis by QPCR*

Primers for analysis of gene expression by quantitative real-time PCR (QPCR) were designed for ESR1, ESR2a and ESR2b from the distinctive genes sequenced (Table 1). For house keeping gene purposes, a primer pair/locus described in Chapter 2 was used for $\beta$-actin (GenBank accession no. AB037865).

**Table 1** Oligonucleotide primer sequences in a 5′ to 3′ direction. Ta = annealing temperature

| Target gene | Application | Primer | Sequence | Ta used | NCBI reference |
|---|---|---|---|---|---|
| ESR1 | cloning | onERI 3 | ATGTACCCCGAAGAGAGCC | 65 °C | U75604 |
| | | onERI 5 | TCATGGGATGCGGGTGCAGTCG | | U75604 |
| | | omERI 8 | TCCAATCCTGTGCTCTCGTC | | AM284390 |
| | | omERI 9 | CACAGCGTCCCGCTTCC | | AM284390 |
| | | omERI 12 | GCACATGAGCAACAAAGGC | | AM284390 |
| | | omERI 13 | GCCTTTGTTGCTCATGTGC | | AM284390 |
| | | omERI 14 | AGGCACCAGAGTTTAGCA | | AM284390 |
| | QPCR | omERI 11 | TGCTAAACTCTGGTGCCT | 64 °C | AM284390 |
| | | onERI 5 | TCATGGGATGCGGGTGCAGTCG | | AM284390 |
| ESR2a | cloning | omERII 1 | CAACATGTGCCTCAGTTC | | U75605 |
| | | omERII 2 | CTACTGGGATTCACCTCCG | | U75605 |
| | | omERII 3 | GTCATGTCAGTAACAAAGGC | | AM284391 |
| | | omERII 4 | GCCTTTGTTACTGACATGAC | | AM284391 |
| | | omERII 5 | GAAGCTGCGTCCAGGGC | | AM284391 |
| | | omERII 6 | CTGTTGGAGTGCTGCTGGC | | AM284391 |
| | QPCR | omERIIa7* | TAACTGGACCAGCTGAGGGT | 66 °C | AM284391 |
| | | omERIIa8* | AGTTCCTCAGACGGCAGCGA | | AM284391 |
| ESR2b | cloning | omERIIb3 | ATGACCTCCTCCCCTGCCCTGG | 65 °C | DQ462608 |
| | | omERIIb5 | TCAAGCTGTTTCCGTGACAACTCTG | | DQ462608 |
| | QPCR | omERIIb1* | CAGTGCACTATTGACAAGAACCGAC | 66.5 °C | EU140820 |
| | | omERIIb2* | CCAGCATGAGGATCTCCAACCAGC | | EU140820 |

*derived from (Wang *et al.* 2005)

Gene expression was quantified using a using an Applied Biosystems 7500 real-time PCR system (Applied Biosystems, CA, USA). Each 15 µl QPCR reaction contained 2 µl of first-strand cDNA (40-fold dilution), 7.5 µl SYBRgreen mix (Sigma, Germany), 0.08 µl reference dye (Sigma, Germany) and 0.27 µM of each primer. The thermocycle program included 95 °C (9 min), followed by 40 cycles of 95 °C (15 sec), 31 sec at the appropriate annealing temperature (Ta) for each amplicon (Table 1) and 72 °C (45 sec). At the end of each programme a dissociation step was included as confirmation of amplicon size. Each DNA amplification run included control reactions containing no cDNA template and a standard concentration of each target DNA. Triplicate determinations were performed for each sample and the $C_T$ values obtained across independent amplification runs for a given gene target which were used to transform the data into gene levels expressed as fold change compared to a standard (a 20 dpf sample without exposure to additional chemicals) according to the $\Delta\Delta C_t$ method (Pfaffl 2001; Bustin 2002; Rutledge & Côte 2003; Kubista *et al.* 2006). A dilution range was generated for each gene target in 5-fold dilution increments using the appropriate gene contained in a plasmid used for sequencing. QPCR analysis of these were used to determine PCR efficiency (PCR efficiency = $10^{(-1/slope)} - 1$).

In each PCR run, standard curves generated using plasmids containing amplicons of interest showed a linear relationship between Ct-values and plasmid concentration with the correlation coefficient ($R^2$) of 0.992, 0.998 and 0.995 for ESR1, ESR2a and ESR2b respectively. PCR efficiencies were calculated as 92.4, 94.1 and 91.8 % for the respective genes, whereby the assumptions for the $\Delta\Delta C_t$ method (Pfaffl 2001; Bustin 2002; Rutledge & Côte 2003; Kubista *et al.* 2006) are met. QPCR primer sets as described in Table 1 were validated for specificity using the authentic gene-containing plasmid. No product was amplified in plasmids with genes other than the specific gene that has been targeted.

*Statistical analysis*

Statistical analyses were performed using the software package STATISTICA (data analysis software system), version 8 (StatSoft, Inc. 2007). Data sets were first tested for normality. Quantitative data were log transformed before statistical analysis to achieve statistical homogeneity (Zar 1996) when testing for correlations between gene effects. Correlations between genes (variables) were

analyzed using linear regression on scatterplots and Pearson method (Pearson 1896) or in case of data not normally distributed, the Spearman Rank Order Correlations (Spearman 1904). The significance level for each test was set at $p<0.05$ throughout the study. Comparison between groups was calculated using the non-parametric Mann-Whitney U test when all data in groups were not normally distributed.

## RESULTS

*Amplification and cloning of ESR genes*

Following the alignment of nucleotide sequences for full length ESR alpha (ESR1), beta one (ESR2a) and beta two (ESR2b) in *O. mossambicus* as well as several partial fragments, the coding sequence (CDS) for each gene was cloned and sequenced with additional two introns at the 3' region of ESR1 and one intron at the 3' region of ESR2a (Figure 1). This result was significant in the light of splice sites which offered a solution to develop primer sequences which allows to effectively amplifying only spliced (mature) transcript levels of each of these genes. I did not have any splice-site information on *O. mossambicus* for ESR2b and consequently used established primers from an earlier publication (Wang *et al.* 2005) which proved to be sufficient.

Nucleotide (GenBank accession numbers: ESR1: AM284390, ESR2a: AM284391, ESR2b: EU140820) and deduced amino acid (Figure 2) sequences of isolated cDNA are 99, 99 and 98 % identical to the respective genes in *O. niloticus* (GenBank accession numbers U75604, U75605 and DQ462608 respectively). The receptors cloned and analysed in this study showed highly conserved areas for the DNA-binding domain and ligand binding domain.

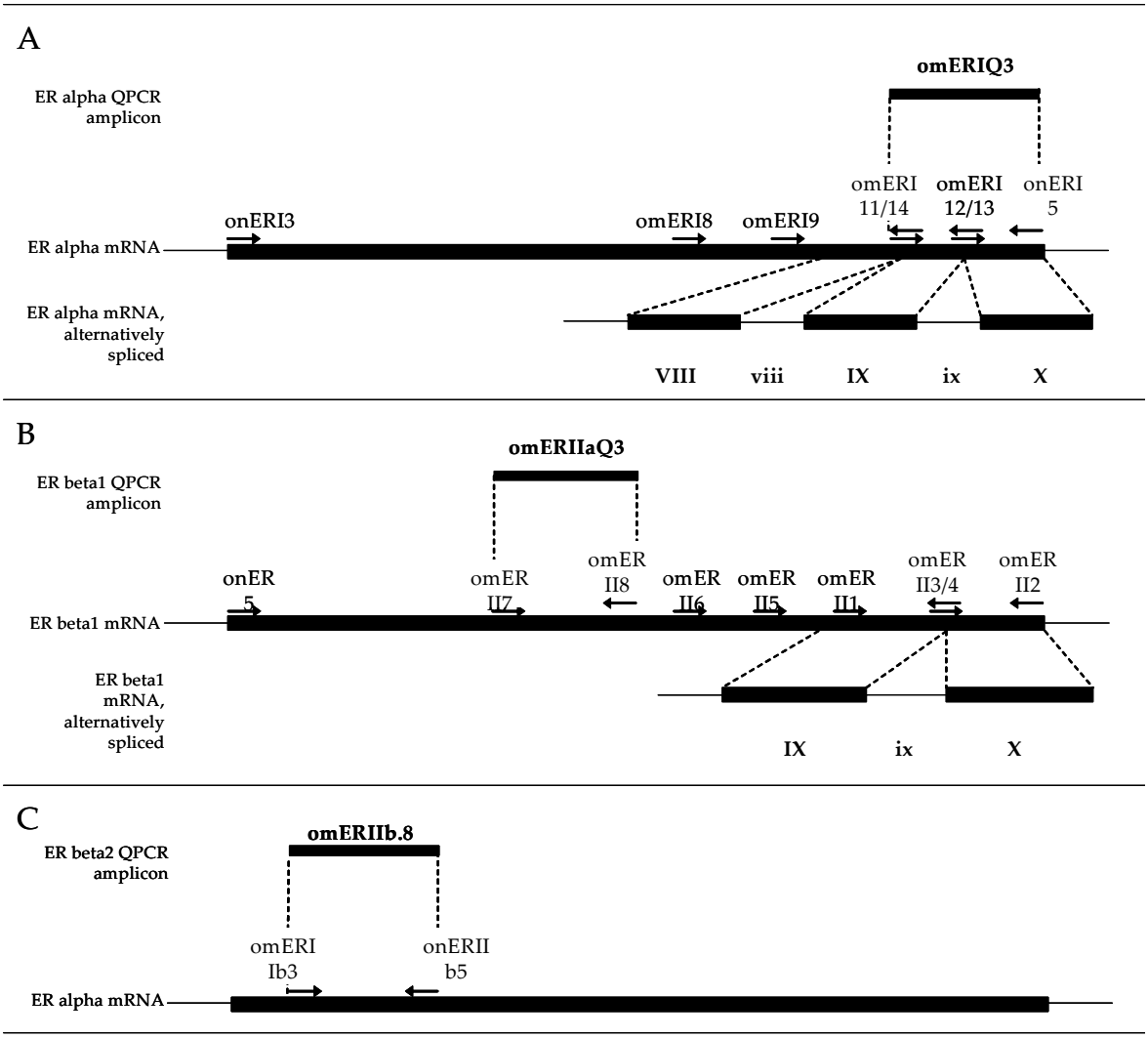


**Figure 1**. Schematic overview of designed primers in ESR1 (A), ESR2a (B) and ESR2b (C) which produced splice variant templates. No splice variants were amplified for ESR2b when using the primer pair OMER2b3/5 which are tested by Wang et al. (2005). Arrows indicate loci of primers (Table 1) tested in this study.

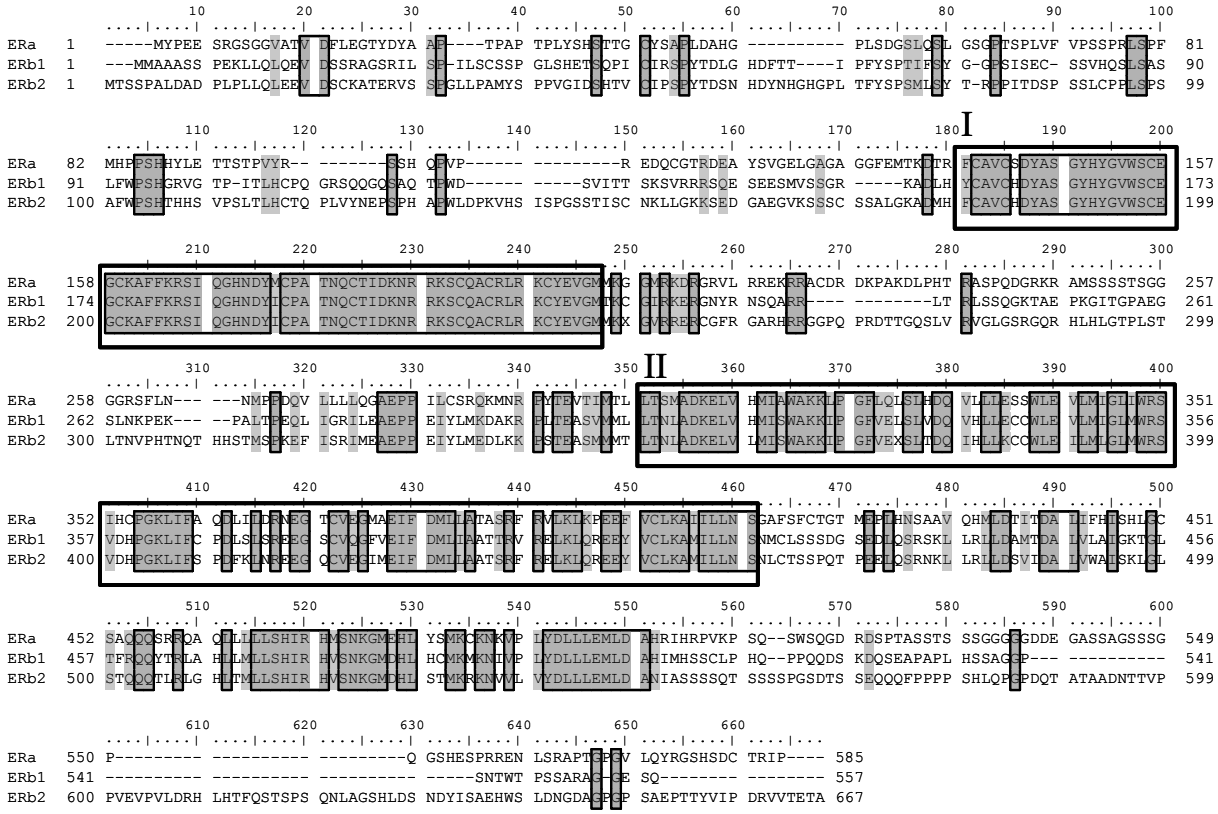


**Figure 2** Alignment of the amino acid sequences of ESR1, ESR2a and ESR2b of Mozambique tilapia. Darker shaded and outlined boxes points out identical amino acids, whereas lighter shaded fragments without oulines illustrates similar amino acids. Box I denotes the putative DNA binding domain, and Box II the putative ligand binding domain.

*Phylogenetic analysis*

To further characterize ESR in *O. mossambicus*, deduced amino acid sequences of 33 entries found in GenBank for teleost ESR1, ESR2a and ESR2b in addition to *O. mossambicus* ESR isoforms were subjected to phylogenetic analysis (Figure 3). The resulting tree is well resolved between the ESR1 and ESR2 groups. However, a further node with 100 % bootstrapping was found between ESR2a and ESR2b. The tree revealed that *O. mossambicus* ESR1, ESR2a and ESR2b are related closest to the respective genes in *O. niloticus*. Within the teleost ESR1 group 100 % bootstrap support distinction between a zebrafish lineage from the tilapia group. Within the ESR2 group, ESR2a and ESR2b are clearly grouped separately with again 100 % bootstrap support. ESR2a group resolve again in the same manner as ESR2, but ESR2b is less convincing with 79 % support (GenBank accession numbers are given in legend of Figure 3).

*Tissue specific gene analysis*

Expression of ESR1, ESR2a and ESR2b was further characterized by quantifying substrate specificity in adult males and females. ESR1 transcript level in females is significantly higher in liver tissue compared to brain ($p<0.05$, Mann-Whitney U) but not ovaries (Figure 4). This isoform was not expressed significantly higher in either male tissue type examined (liver, brain, testes). ESR2a was expressed in all tissue samples except testes at detectable levels, however no polymorphism with statistical significance between any of the tissues. ESR2b was again mostly expressed in livers of the adult females ($p<0.05$, Mann-Whiney U), however males showed no statistical significant tissue specificity for this form of ESR. I found ESR2b expressed in all tissues at very low levels, whereas both male and female liver housed elevated levels ($p<0.05$, Mann-Whitney U) of this isoform but no dimorphism between the sexes.

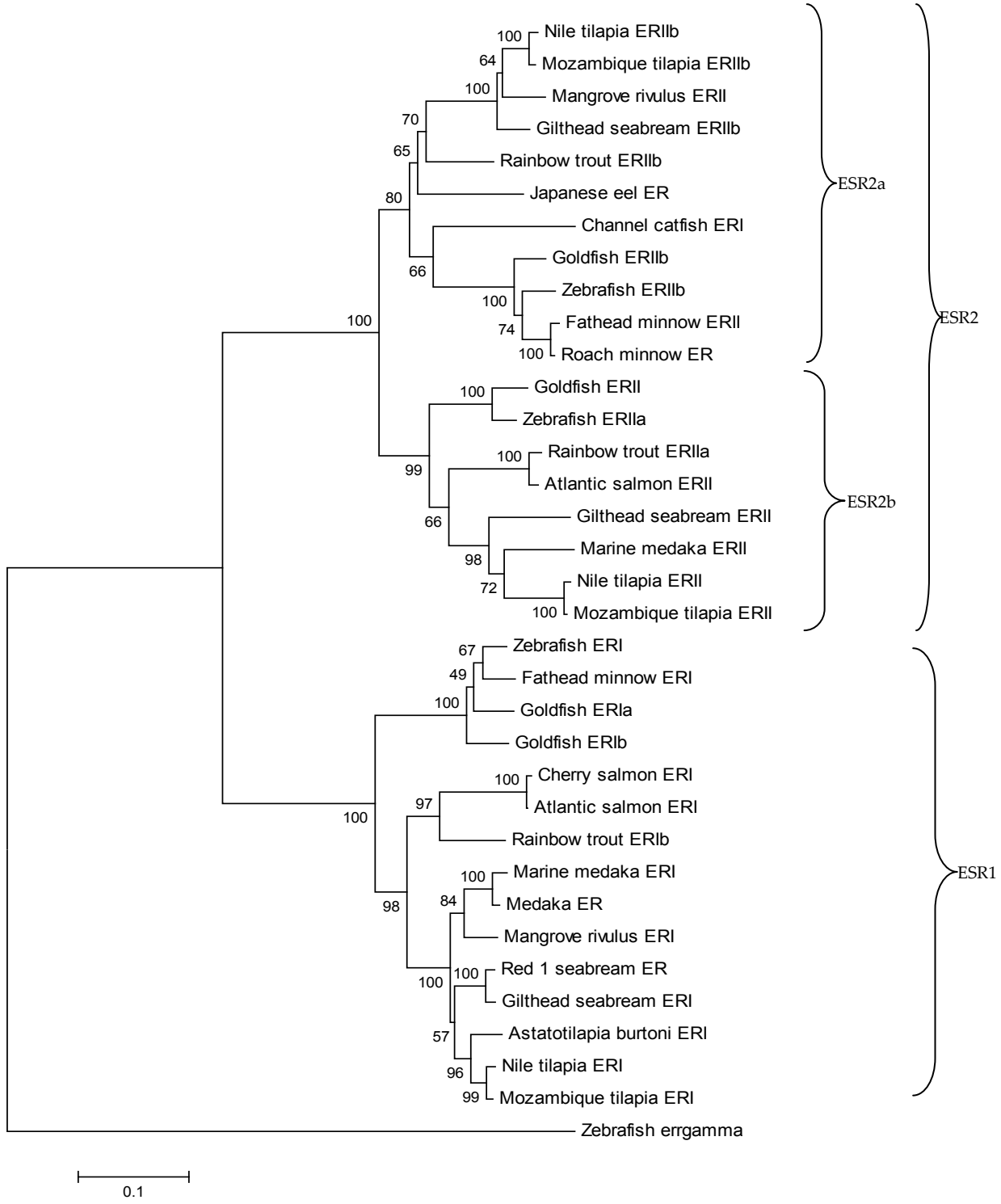


**Figure 3** Phylogenetic tree based upon the alignment of deduced amino acid sequences of Mozambique tilapia ESR sequences and reported ESR sequences in other teleostean species. Phylogenetic analysis was carried out by MEGAv4.1 using p-distance based on Neighbor-Joining method with 1000 boostrap replicates. The tree was rooted by using Zebrafish estrogen related receptor as the outgroup. The number shown at each branch indicated the boostrap values (%). GenBank accession numbers are as follows: Japanese eel (*Anguilla japonica*) ESR, 2073112; Astatotilapia burtoni (*Astatotilapia burtoni)* ESR1,

41056569; Goldfish (*Carassius auratus)* ESR1b, 38327071; Goldfish (*Carassius auratus)* ESR1a, 16118450; Goldfish (*Carassius auratus)* ESR2, 4666317; Goldfish (*Carassius auratus)* ESR2b, 7012682; Red seabream (*Chrysophrys major)* ESR, 2447037; Zebrafish (*Danio rerio)* ESR1, 23308674; Zebrafish (*Danio rerio)* ESR2a, 31340676; Zebrafish (*Danio rerio)* ESR2b, 56711295; Zebrafish (*Danio rerio)* ESR2a, 24421226; Zebrafish (*Danio rerio)* ESR2b, 23466358; Channel catfish (*Ictalurus punctatus)* ESR1, 7527467; Mangrove rivulus (*Kryptolebias marmoratus)* ESR2, 85013466; Mangrove rivulus (*Kryptolebias marmoratus)* ESR1, 85013464; Rainbow trout (*Oncorhynchus mykiss)* ESR2b, 82409101; Rainbow trout (*Oncorhynchus mykiss)* ESR2a, 77020882; Rainbow trout (*Oncorhynchus mykiss)* ESR2b, 77020880; Cherry salmon (*Oncorhynchus masou)* ESR1, 82582234; Nile tilapia (*Oreochromis niloticus)* ESR2, 4098200; Nile tilapia (*Oreochromis niloticus)* ESR1, 4098198; Nile tilapia (*Oreochromis niloticus)* ESR2b, 92090686; Mozambique tilapia (*Oreochromis mossambicus)* ESR2a, AM284391; Mozambique tilapia (*Oreochromis mossambicus)* ESR1, AM284390; Marine medaka (*Oryzias javanicus)* ESR2, 60101767; Marine medaka (*Oryzias javanicus)* ESR1, 60101765; Medaka (*Oryzias latipes)* ER, 6451939; Fathead minnow (*Pimephales promelas*) ESR2, 48525995; Fathead minnow (*Pimephales promelas)* ESR1, 55139373; Roach minnow (*Rutilus rutilus)* ESR, 59805062; Atlantic salmon (*Salmo salar)* ESR1, 74422192; Atlantic salmon (*Salmo salar)* ESR2, 40846403; Gilthead seabream (*Sparus auratus)* ESR1, 115313957; Gilthead seabream (*Sparus auratus)* ESR2b, 49523737; Gilthead seabream (*Sparus auratus)* ESR2, 4838541; Mozambique tilapia (*Oreochromis mossambicus)* ESR2b, EU140820

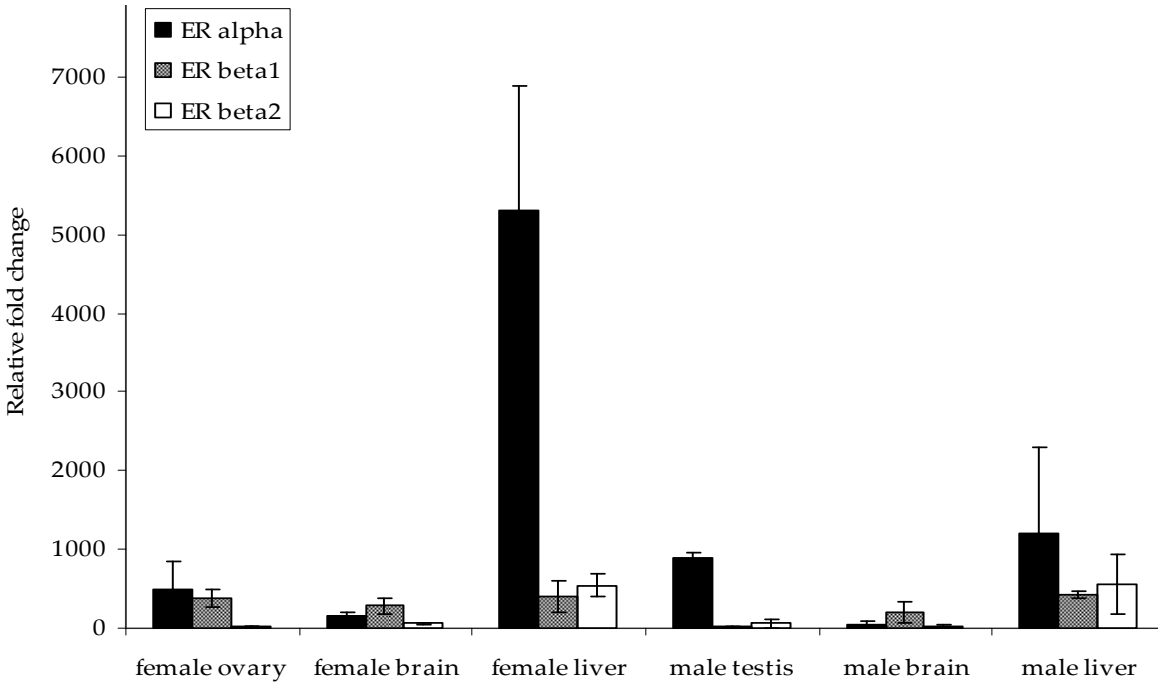


**Figure 4** Quantitative gene expression of ESR1, ESR2a and ESR2b amongst gonadal, brain and liver tissues in adult *O. mossambicus*. Relative fold change has been calculated against 20 dpf juvenile whole body homogenates (n = 20).

*Gender specific gene analysis*

Gender specific dimorphic expression patterns were found for liver samples of adult specimens in ESR1 at $p<0.05$ (Mann-Whitney U). No significant difference between male and female samples were found for ESR2a and ESR2b in liver or gonadal samples, nor in any other tissue compared for either of the ESR isoforms.

*Temporal expression*

Temporal up- and down regulation of ESR1 in *O. mossambicus* revealed significant up regulation at 50-60 dpf and significant down regulation again thereafter (Figure 5). Expression of ESR1 remains low again until 95 dpf when another significant increase occur which decrease again at 105 dpf. Hereafter basal levels was measured of ESR1 until 110 dpf.

Relative to the 5 dpf samples, ESR2a increased significantly at 20 dpf ($p<0.05$, Mann-Whitney U). Hereafter, our data revealed basal levels of ESR2a but include much variation. At 60 dpf significantly low levels were measured where after another increase occurred, peaking at 75 dpf.

ESR2b mRNA increased significantly for the first time at 20 dpf, and continued to be present at an elevated level until 45 dpf significant decrease occur, remaining low with much variation and no significant increase or decrease.

When the juvenile data for ESR1 is compared to adult females, juveniles at 60 dpf are maintaining similar levels of the gene than female ovaries, but ~10 times less than female liver tissue. As for the ESR2 isoforms, juveniles expressed ESR2a at ~ 50 times less than both ovarian and liver tissue, and ESR2b again at similar levels than female ovaries, but ~150 times less than female liver.

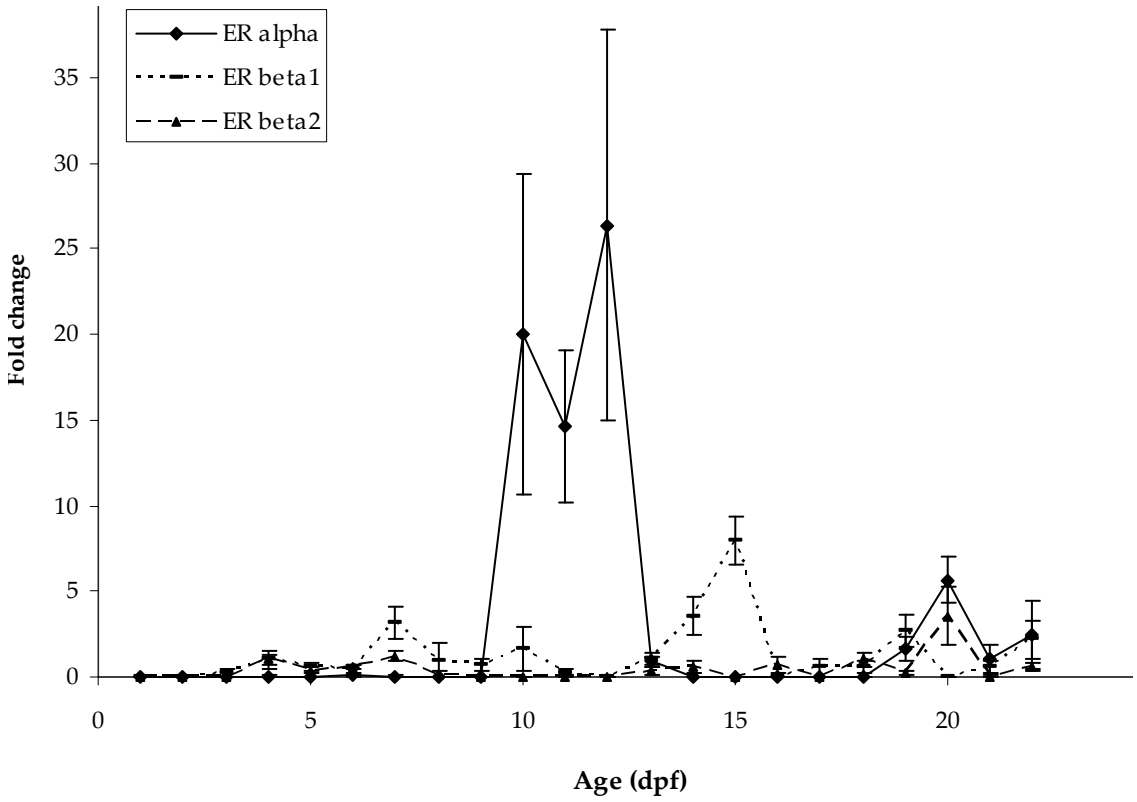


**Figure 5** Real-time PCR quantification of ESR1, ESR2a and ESR2b mRNA from whole body homogenates during the period 5 dpf to 110 dpf.

## DISCUSSION

Assessing the biologic consequences of chronic multigenerational exposures to endocrine disrupting compounds (EDCs) is a complex challenge. Consequently, some aspects of the endocrine system are studied in a piece meal fashion (Kime 1998; Anway & Skinner 2006; Segner *et al.* 2006; Hiramatsu *et al.* 2007; Hecker *et al.* 2007). EDCs known to have estrogenic effects, are modeled and screened for by using *in vivo* and *in vitro* bioassays or a combination thereof (McLachlan 2001; Brown *et al.* 2003; Jacobs 2004) which are most often absolute or quantitative responses by estrogen-responsive genes (Chapter 1). Most of these genes are regulated by $E_2$ via the genomic pathway whereby the ligand binds to it specific nuclear receptor, ESR. To understand and monitor xenoestrogenic effects *in vivo*, it is thus imperative to fully characterize the normal expression of ESR in non-induced animals in order to make informed conclusions with regards to the expression levels of such estrogen responsive genes.

Three ESR subtypes have previously been cloned from several fish species including the tilapiines *O. niloticus* (Chang et al., 1999; Wang et al., 2005) and *O. aureus* (le Roux et al., 1993; Tan et al., 1996). The current study confirmed the presence of these three ESR paralogues in *O. mossambicus*. Typically vertebrate-like (Urushitani *et al.* 2003; Wang *et al.* 2005; Greytak & Callard 2007), ESR in *O. mossambicus* segregates firstly into two subclades (ESR1 and ESR2, Figure 3). Wang *et al.* (2005) reported a third ESR subtype, ESR2b for *O. niloticus* that was consequently confirmed for Mozambique tilapia in this study.

The duplication of these genes are believed to be the result of polyploidization or genome duplication amongst ancestral aquatic vertebrates (Wang *et al.* 2005). After duplication of the ancestral ESR1 gene, the coding sequences of ESR2b accumulated novel mutations at a greater rate than ESR2a as is indicated by patterns of amino acid divergence in other teleosts (Hawkins *et al.* 2000). *In situ* hybridization of ESR1, ESR2a and ESR2b in Atlantic croaker hypothalamus illustrated different patterns of expression (Hawkins *et al.* 2000), which, in addition to differential expression in rats (Osterlund *et al.* 1998), suggests distinctive neuroendocrine roles. Quantified ESR expression levels in brain of the *O. mossambicus* (Figure 4) for each of the isoforms reported in this study supports this hypothesis. Extensive work have been done on differential expression of ESR isoforms in the brain tissue of human and other primate and its implications discussed (Osterlund & Hurd 2001; Perez *et al.* 2004; Manthey &

Behl 2006; Gioiosa *et al.* 2007; Brann *et al.* 2007). Evidently, when ESR is expressed in brain tissue, ESR1 may modulate neural cell populations involved in automatic and reproductive neuroendocrine functions as well as emotional interpretation and processing, whereas ESR2 is suggested to play a role in cognition, non-emotional memory and motor functions (Balthazart *et al.* 2006). Published results further suggest that $E_2$ can modulate behavior and functions mediated by the amygdale and hypothalamus via differentially regulated ESR subtypes (Osterlund *et al.* 1998). In fish however, the specific functions/modulation effects of the isoforms are still largely unclear, but studies in this regard may help to shed light on the dual nature (Figure 2, Chapter 1) of estrogen pathways in vertebrates (Falkenstein *et al.* 2000).

The ESR forms part of the nuclear receptor superfamily (Evans 1988). ESR has been studied extensively in mammalian models and to a lesser extent in other vertebrates, including teleosts (Wang *et al.* 2005; Meucci & Arukwe 2006; Martyniuk *et al.* 2007; Greytak & Callard 2007). The level of ESR transcription itself is known to be generally under control of $E_2$ and up- or down regulation is tissue specific (Tsai & O'Malley 1994; Arnold *et al.* 1996; Tan *et al.* 1996). In oviparous species, the hepatic ESR concentration is markedly increased by $E_2$, – not surprising since the liver is one of the main target organs for estrogens during the adult life of teleosts (Tata 1976; Ding *et al.* 1990). The current study supports this issue as within male and female adults, highest expression of ESR1 was found in the liver, with the only sexual dimorphic pattern in the liver samples. The other two isoforms did not reveal such dimorphism (Figure 4) and is in agreement of results found in zebrafish (*Danio rerio*) and trout (*Oncorhynchus mykiss*) confirming that the expression of ESR1 is robustly stimulated by $E_2$ treatment *in vivo* (Pakdel *et al.* 2000; Menuet *et al.* 2004). In the light of this, these results further suggest that ESR1 may very well be the main receptor responsible for ligand binding in the liver, which is in turn the only target for *vtg* transcription as shown in the splicing-related results in Chapter 2. This is however not exclusive to the expression of other isoforms as in both male and female livers, measured low but detectable levels were measured of both ESR2a and ESR2b. Furthermore, the elevated expression of ESR1 in both the gonads of males and females in this study reveal a definite genomic pathway (Figure 2, Chapter 1), only or in part by $E_2$ in these tissues. As no dimorphism was found between male and female of either of the ESR2a or ESR2b isoforms in

the gonads, the data suggest that during the reproductive cycle of adults when $E_2$ levels fluctuate, ESR2 isoforms have distinct implication on hepatic functions as was found by Menuet et al. (2004) and Sabo-Attwood et al. (2004). In the brain of male and female adults, ESRs were all very low expressed which points to a possible non-genomic pathway to occur in the brain as has also been suggested by others (Osterlund & Hurd 2001; Balthazart *et al.* 2004).

Expression of ERs during ontogeny in fish has been reported to start soon after fertilization – in zebrafish as early as 48 hours post-fertilization for all isoforms of ESR (Bardet *et al.* 2002). Such early expression of ESR is feasible in the light of maternally inherited $E_2$ in embryos which only diminishes after the onset of gonadal differentiation (Hines *et al.* 1999) in tilapia. Hereafter, $E_2$ remains low until after ovarian development which is discussed by Martyniuk et al (2007) to be influenced partially by temperature as *cyp19b* and ESRs (Wang & Tsai 2000; Tsai *et al.* 2000) are reported to be expressed increasingly at higher temperatures (Blazquez *et al.* 2001; Piferrer *et al.* 2005). It therefore seems likely that differential expression of ESRs in response to environmental signals is a important factor contributing to gonadal and brain development (Martyniuk *et al.* 2007). $E_2$ function via this genomic pathway is therefore dependent on the availability of ESR which is subsequently implicated in estrogenic endocrine disruption. In the present study all ESR isoforms' expression is initiated at 20 dpf. ESR transcripts are shown to be maternally transferred but rapidly degraded post-fertilization in killifish, and ESR1 transcripts is selectively expressed by preovulatory oocytes in contrast with mRNA of either of the ESR2 isoforms (Greytak & Callard 2007). In the present study for all three ESR revealed very low expression during the 5 to 15 dpf window. The relatively low expression of ESR2b in the ovary (which includes unfertilized eggs) therefore suggests that ESR2b found in juveniles is made *de novo*, and not been maternally transferred, which agrees with the situation in killifish (Greytak & Callard 2007). In comparison, ESR1 is being upregulated as of stage 20 dpf in *O. mossambicus*. Gonads in Tilapia are known to start to differentiate around 21 dpf (Nakamura *et al.* 1998; D'Cotta *et al.* 2001; Chang *et al.* 2005) but as our histology (Chapter 3) determined, this differentiation is only somatically, and no distinguished germ cells are present. These were only identified in selected cases at 40 dpf. Therefore, and in addition to Nakamura et al. (1998), I postulate the liver of the different sexes may react differentially to sex specific stimuli (hormones and

temperature) and therefore would be induced in females and to a lesser degree in males.

The absence of a dimorphic expression profile in ESR2b during the complete period I investigated indicates that this gene may possibly not be under gender specific regulation which is supported by the absence of ESR2a or ESR2b induction by $E_2$ in livers of other teleosts (Menuet *et al.* 2004; Sabo-Attwood *et al.* 2004).

*Correlation with vtg*

On account of the expected functional relationship of ESR1 in hepatic tissue of adult specimens with vitellogenin production, I tested for correlation between the various ESR isoforms and VTG quantified transcript levels in liver (Table 3).

**Table 2** Spearman Rank Order Correlation values for O. mossambicus liver samples. Bold values are significant at $p < 0.05$

| | *vtg* | ESR1 | ESR2a | ESR2b |
|---|---|---|---|---|
| *vtg* | - | | | |
| ESR1 | 0.035961 | - | | |
| ESR2a | **-0.304725** | -0.073659 | - | |
| ESR2b | **-0.311848** | 0.009092 | **0.323365** | - |

In hepatic tissue of adult O. *mossambicus*, I found a significant ($p<0.05$) correlation of *vtg* with ESR2a and ESR2b transcripts. Vitellogenin is known to have functions additional to egg yolk formation in teleosts (Tata 1976; Norberg *et al.* 1989; Ding *et al.* 1994). Therefore it is not surprising that *vtg* expression in hepatic tissue correlates well with ESR2b expression. However, in the light of the previous discussion, not ESR2 isoforms, but ESR1 is expected to correlate with *vtg* expression. Excluding males from this correlation did not change the results (not shown). A possible explanation may be that females tested (n = 9) may be at various stages in the vitellogenic cycle, during which it is known for a female to have varying levels of vitellogenin. I suggest that ESR1 may be under direct

regulation of $E_2$ which also induce *vtg* expression at the appropriate time in the vitellogenic cycle. However, at stages of low *vtg* expression in the liver of females, *vtg* may be down regulated in turn by other transcription regulators as is discussed in Chapter 2. I further propose that ligand binding to ESR2b might be the pathway whereby basal levels of *vtg* are maintained over and above the gender dependant regulation of *vtg* via ESR1, however not $E_2$ regulated. On the contrary, a study on largemouth bass (*Micropterus salmoides*) illustrated in adults that elevated *vtg* and $E_2$ levels was correlated with up regulated ESR1 transcription and to a lesser extent with ER$\gamma$ (ESR2b) whereas ESR2 (ESR2a) remained unchanged during the upregulation of *vtg* and $E_2$ (Sabo-Attwood *et al.* 2004). Conversely, the present study and another on feral adult *O. mossambicus* found no correlation between ESR1 and *vtg*, with the latter also not correlating ESR2 (isoform not distinguished) and *vtg* in an estrogen polluted river (Park *et al.* 2007), questioning the current theory that ESR gene expression is induced in an isoform specific manner by xenoestrogens (Meucci & Arukwe 2006; Greytak & Callard 2007). This challenge researchers in the toxicogenomics arena with the question of mode of action by $E_2$ with regards to which method is being used to monitor $E_2$ presence in aquatic systems. Subsequent studies therefore on *O. mossambicus* may provide a tool at another level to monitor estrogenic exposure.

**CONCLUSION**

Vitellogenesis as endocrine disruption endpoint in oviparous species has been used extensively for the past two decades to report $E_2$ activity in aquatic systems. But as I approach to understand the mechanisms of action estrogens incorporates, modern research on this matter is challenged with contradicting results between several species when this nuclear ligand receptor (ESR) is concerned. Overall, this study confirms the basal expression of ESR2a and ESR2b during temporal development, with a clear upregulation of ESR1 during the time of gonadal differentiation. In addition quantitative results support the inducing effect of ESR1 by $E_2$ in adult specimens. This study therefore underlines the necessity to firstly characterize the behaviour of ESR isoforms at transcriptional and translational levels and secondly to investigate other pathways such as the suggested "non-genomic" pathway of estrogens proposed in recent literature for specific species under investigation before inferring any estrogenic endocrine disrupting effects.

## REFERENCE LIST


**Altschul S.F., Madden T.L., Schaffer A.A., Zhang J., Zhang Z., Miller W. & Lipman D.J.**, 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research.* 25, 3389-3402.

**Anway M.D. & Skinner M.K.**, 2006. Epigenetic Transgenerational Actions of Endocrine Disruptors. *Endocrinology.* 147, s43-s49.

**Arnold S.F., Collins B.M., Robinson M.K., Guillette J. & McLachlan J.A.**, 1996. Differential interaction of natural and synthetic estrogens with extracellular binding proteins in a yeast estrogen screen. *Steroids.* 61, 642-646.

**Balthazart J., Cornil C.A., Taziaux M., Charlier T.D., Baillien M. & Ball G.F.**, 2006. Rapid changes in production and behavioral action of estrogens. *Neuroscience.* 138, 783-791.

**Balthazart J., Baillien M., Cornil C.A. & Ball G.F.**, 2004. Preoptic aromatase modulates male sexual behavior: slow and fast mechanisms of action. *Physiology & Behavior.* 83, 247-270.

**Bardet P., Horard B., Robinson-Rechavi M., Laudet V. & Vanacker J.**, 2002. Characterization of oestrogen recepors in zebrafish (*Danio rerio*). *Journal of Molecular Endocrinology.* 28, 153-163.

**Blazquez M., Felip A., Zanuy S., Carrillo M. & Piferrer F.**, 2001. Critical period of androgen-inducible sex differentiation in a teleost fish, the European sea bass. *Journal of Fish Biology.* 58, 342-358.

**Brann D.W., Dhandapani K., Wakade C., Mahesh V.B. & Khan M.M.**, 2007. Neurotrophic and neuroprotective actions of estrogen: Basic mechanisms and clinical implications. *Steroids.* 72, 381-405.

**Brown A.R., Riddle A.M., Cunningham N.L., Kedwards T.J., Shillabeer N. & Hutchinson T.H.**, 2003. Predicting the Effects of Endocrine Disrupting Chemicals on Fish Populations. *Human and Ecological Risk Assessment.* 9, 761-788.

**Bustin S.** 2002. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *Journal of Molecular Endocrinology.* 29, 23-39.

**Chang X., Kobayashi T., Senthilkumaran B., Kobayashi-Kajura H., Sudhakumari C.C. & Nagahama Y.**, 2005. Two types of aromatase with different encoding genes, tissue distribution and developmental expression in Nile tilapia (*Oreochromis niloticus*). *General and Comparative Endocrinology.* 141, 101-115.

**Chenna R., Sugawara H., Koike T., Lopez R., Gibson T.J., Higgins D.G. & Thompson J.D.**, 2003. Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Research.* 31, 3497-3500.

**D'Cotta H., Fostier A., Guiguen Y., Govoroun M. & Baroiller J.F.**, 2001. Aromatase plays a key role during normal and temperature-induced sex differentiation of tilapia *Oreochromis niloticus*. *molecular reproduction and development.* 59, 265-276.

**Ding J.L., Ho B., Valotaire Y., LeGuellec K., Lim E.H., Tay S.P. & Lam T.J.**, 1990. Cloning, characterisation and expression of vitellogenin gene of *Oreochromis aureus* (Teleostei, Cichlidae). *Biochemistry International.* 20, 843-852.

**Ding J.L., Lim E.H. & Lam T.J.**, 1994. Cortisol-induced hepatic vitellogenin mRNA in *Oreochromis aureus* (Steindachner). *General and Comparative Endocrinology.* 96, 276-287.

**Evans R.** 1988. The steroid and thyroid hormone receptor superfamily. *Science.* 240, 889-895.

**Falkenstein E., Tillmann H.C., Christ M., Feuring M. & Wehling M.**, 2000. Multiple Actions of Steroid Hormones - A Focus on Rapid, Nongenomic Effects. *Pharmacological Reviews.* 52, 513-556.

**Felsenstein J.** 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution.* 39, 783-791.

**Gioiosa L., Fissore E., Ghirardelli G., Parmigiani S. & Palanza P.**, 2007. Developmental exposure to low-dose estrogenic endocrine disruptors alters sex differences in exploration and emotional responses in mice. *Hormones and Behavior.* 52, 307-316.

**Greene G., Gilna P., Waterfield M., Baker A., Hort Y. & Shine J.**, 1986. Sequence and expression of human estrogen receptor complementary DNA. *Science.* 231, 1150-1154.

**Greytak S.R. & Callard G.V.**, 2007. Cloning of three estrogen receptors (ER) from killifish (*Fundulus heteroclitus*): Differences in populations from polluted and reference environments. *General and Comparative Endocrinology.* 150, 174-188.

**Hall T.** 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids, Symposium Series.* 41, 95-98.

**Hawkins M.B., Thornton J.W., Crews D., Skipper J.K., Dotte A. & Thomas P.**, 2000. Identification of a third distinct estrogen receptor and reclassification of estrogen receptors in teleosts. *Proceedings of the National Academy of Sciences U.S.A.* 97, 10751-10756.

**Hecker M., Thomas Sanderson J. & Karbe L.**, 2007. Suppression of aromatase activity in populations of bream (*Abramis brama*) from the river Elbe, Germany. *Chemosphere.* 66, 542-552.

**Higgins D., Thompson J., Gibson T., Thompson J., Higgins D. & Gibson T.**, 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research.* 22, 4673-4680.

**Hines G.A., Boots L.R., Wibbels T. & Watts S.A.**, 1999. Steroid Levels and Steroid Metabolism in Relation to Early Gonadal Development in the TilapiaOreochromis niloticus(Teleostei: Cyprinoidei). *General and Comparative Endocrinology.* 114, 235-248.

**Hiramatsu N., Cheek A.O., Sullivan C.V., Matsubara T. & Hara A.**, 2007. Vitellogenesis and endocrine disruption. In Mommsen,T.P. & Moon,T.W. *Biochemical and Molecular biology of fishes*. Elsevier Science BV,Amsterdam, pp 431-471.

**Jacobs M.N.** 2004. In silico tools to aid risk assessment of endocrine disrupting chemicals. *Toxicology.* 205, 43-53.

**Kime D.** 1998. *Endocrine disruption in fish*. Kluwer Academic Press. Boston .

**Kubista M., Andrade J.M., Bengtsson M., Forootan A., Jonak J., Lind K., Sindelka R., Sjoback R., Sjogreen B. & Strombom L.**, 2006. The real-time polymerase chain reaction. *Molecular Aspects of Medicine.* 27, 95-125.

**Kuiper G.G., Enmark E., Pelto-Huikko M., Nilsson S. & Gustafsson J.A.**, 1996. Cloning of a novel estrogen receptor expressed in rat prostate and ovary. *Proceedings of the national academy of sciences.* 93, 5925-5930.

**Manthey D. & Behl C.**, 2006. From structural biochemistry to expression profiling: Neuroprotective activities of estrogen. *Neuroscience.* 138, 845-850.

**Martyniuk C., Gallant N., Marlatt V., Wiens S., Woodhouse A. & Trudeau V.**, 2007. Current Perspectives on 17beta-Estradiol action and nuclear Estrogen Receptors (ER) in Teleost fish. In*Fish Physiology*.pp 625-663.

**McDonnell D.P. & Norris J.D.**, 2002. Connections and Regulation of the Human Estrogen Receptor. *Science.* 296, 1642-1644.

**McLachlan J.A.** 2001. Environmental Signaling: What Embryos and Evolution Teach Us About Endocrine Disrupting Chemicals. *Endocrine Reviews.* 22, 319-341.

**Menuet A., Le Page Y., Torres O., Kern L., Kah O. & Pakdel F.**, 2004. Analysis of the estrogen regulation of the zebrafish estrogen receptor (ER) reveals distinct effects of ERalpha, ERbeta1 and ERbeta2. *Journal of Molecular Endocrinology.* 32, 975-986.

**Meucci V. & Arukwe A.**, 2006. Transcriptional modulation of brain and hepatic estrogen receptor and P450arom isotypes in juvenile Atlantic salmon (*Salmo salar*) after waterborne exposure to the xenoestrogen, 4-nonylphenol. *Aquatic Toxicology.* 77, 167-177.

**Nakamura M., Kobayashi T., Chang X.T. & Nagahama Y.**, 1998. Gonadal sex differentiation in teleost fish. *Journal of Experimental Zoology.* 281, 362-372.

**Nilsson S., Makela S., Treuter E., Tujague M., Thomsen J., Andersson G., Enmark E., Pettersson K., Warner M. & Gustafsson J.A.**, 2001. Mechanisms of Estrogen Action. *Physiological Reviews.* 81, 1535-1565.

**Norberg B., Bjornsson B.T., Brown C.L., Wichardt U.P., Deftos L.J. & Haux C.**, 1989. Changes in plasma vitellogenin, sex steroids, calcitonin, and thyroid hormones related to sexual maturation in female brown trout (Salmo trutta). *General and Comparative Endocrinology.* 75, 316-326.

**Osterlund M., Kuiper G.J.M., Gustafsson J.A. & Hurd Y.L.**, 1998. Differential distribution and regulation of estrogen receptor-[alpha] and -[beta] mRNA within the female rat brain. *Molecular Brain Research.* 54, 175-180.

**Osterlund M.K. & Hurd Y.L.**, 2001. Estrogen receptors in the human forebrain and the relation to neuropsychiatric disorders. *Progress in Neurobiology.* 64, 251-267.

**Pakdel F., Metivier R., Flouriot G. & Valotaire Y.**, 2000. Two Estrogen Receptor (ER) Isoforms with Different Estrogen Dependencies Are Generated from the Trout ER Gene. *Endocrinology.* 141, 571-580.

**Park C.B., Takemura A., Aluru N., Park Y.J., Kim B.H., Lee C.H., Lee Y.D., Moon T.W. & Vijayan M.M.**, 2007. Tissue-specific suppression of estrogen, androgen and glucocorticoid receptor gene expression in feral vitellogenic male Mozambique tilapia. *Chemosphere.* 69, 32-40.

**Pearce S. & Jordan V.**, 2004. The biological role of estrogen receptors alpha and beta in cancer. *Critical Reviews in Oncology/Hematology.* 50, 3-22.

**Pearson K.** 1896. Regression, heredity, and panmixia. *Philosophical Transactions of the Royal Society of London.* 187, 253-318.

**Perez S., Sendera T.J., Kordower J.H. & Mufson E.J.**, 2004. Estrogen receptor alpha containing neurons in the monkey forebrain: lack of association with calcium binding proteins and choline acetyltransferase. *Brain Research.* 1019, 55-63.

**Pfaffl M.W.** 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research.* 29, e45.

**Piferrer F., Blazquez M., Navarro L. & Gonzalez A.**, 2005. Genetic, endocrine, and environmental components of sex determination and differentiation in the European sea bass (*Dicentrarchus labrax* L.). *General and Comparative Endocrinology.* 142, 102-110.

**Rutledge R. & Côte C.**, 2003. Mathematics of quantitative kinetic PCR and the application of standard curves. *Nucleic Acids Research.* 31, E93.

**Sabo-Attwood T., Kroll K.J. & Denslow N.D.**, 2004. Differential expression of largemouth bass (Micropterus salmoides) estrogen receptor isotypes alpha, beta, and gamma by estradiol. *Molecular and Cellular Endocrinology.* 218, 107-118.

**Saitou N. & Nei M.**, 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution.* 4, 406-425.

**Segner H., Eppler E. & Reinecke M.**, 2006. The imact of environmental hormonally active substances on the endocrine and immune systems of fish. In Reinecke,M., Zaccone,G. & Kapoor,B. *Fish endocrinology*. Science publishers,Enfield, pp 809-865.

**Spearman C.** 1904. "General intelligence," objectively determined and measured. *American Journal of Psychology.* 15, 201-293.

**Tan N.S., Lam T.J. & Ding J.L.**, 1996. The first contiguous estrogen receptor gene from a fish, *Oreochromis aureus*: evidence for multiple transcripts. *Molecular and Cellular Endocrinology.* 120, 177-192.

**Tata J.R.** 1976. The expression of the vitellogenin gene. *Cell.* 9, 1-14.

**Traupe T., Stettler C.D., Li H., Haas E., Bhattacharya I., Minotti R. & Barton M.**, 2007. Distinct Roles of Estrogen Receptors {alpha} and {beta} Mediating Acute Vasodilation of Epicardial Coronary Arteries. *Hypertension.* 49, 1364-1370.

**Tsai C.Γ., Wang L.Γ., Chang C.Γ. & Kao C.Γ.**, 2000. Effects of Gonadal Steroids on Brain Serotonergic and Aromatase Activity During the Critical Period of Sexual Differentiation in Tilapia, *Oreochromis mossambicus*. *Journal of Neuroendocrinology.* 12, 894-898.

**Tsai M.J. & O'Malley B.W.**, 1994. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annual Reviews of Biochemistry.* 63, 451-486.

**Urushitani H., Nakai M., Inanaga H., Shimohigashi Y., Shimizu A., Katsu Y. & Iguchi T.**, 2003. Cloning and characterization of estrogen receptor [alpha] in mummichog, Fundulus heteroclitus. *Molecular and Cellular Endocrinology.* 203, 41-50.

**Vasudevan N. & Pfaff D.W.**, 2007. Membrane-Initiated Actions of Estrogens in Neuroendocrinology: Emerging Principles. *Endocrine Reviews.* 28, 1-19.

**Walter P., Green S., Greene G., Krust A., Bornert J.M., Jeltsch J.M., Staub A., Jensen E., Scrace G., Waterfield M. & Chambon P.**, 1985. Cloning of the Human Estrogen Receptor cDNA. *Proceedings of the national academy of sciences.* 82, 7889-7893.

**Wang D., Senthilkumaran B., Sudhakumari C.C., Sakai F., Matsuda M., Kobayashi Tokimasa, Yoshikuni M. & Nagahama Y.**, 2005. Molecular cloning, gene expression and charaterization of the third estrogen receptor of the Nile tilapia, *Oreochromis niloticus*. *Fish physology and biochemistry.* 31, 255-266.

**Wang L.H. & Tsai C.L.**, 2000. Temperature affects the development of central neurotransmitter systems of tilapia, *Oreochromis mossambicus*. *Neuroscience Letters.* 285, 95-98.

**Zar J.** 1996. *Biostatistical analysis*. Prentice Hall. New Jersey, USA .

# General conclusion

Several synthetic chemicals, termed xenoestrogens, have shown proof to interact as agonists with the estrogen receptor (ESR) to elicit biological responses similar to those of natural estrogens, such as 17β-Estradiol ($E_2$). In turn, $E_2$ itself can only be aromatized from androgens by Cytochrome P450 19 (*cyp19*). As I approached to study quantitative expression of genes related to endocrine disruption, I chose the product most often used in estrogenic endocrine disruption, vitellogenin (VTG). The gene coding for VTG (*vtg*) is known to be particularly responsive to $E_2$ (or any xenobiotic form thereof) in oviparous animals (Chapter1 and 2).

Building a platform from which Southern Africa can monitor estrogenic disruption in the freshwater aquaculture species *Oreochromis mossambicus*, the present study reports the partial cloning of *vtg* and 3 kb of 5'flanking area. Subsequently, cloning (in part or in full) of two isoforms of *cyp19* and 3 isoforms of ESR was included, which in itself is known to be responsive to $E_2$. Resulting sequences from these candidate genes provides in addition to its academic knowledge, the opportunity to develop sound quantitative protocols by which transcription can be quantified (Chapters 2-4). A recent study (Davis *et al.* 2007) reported three different isoforms for *vtg* in this species. The present study (Chapter 2) however clearly illustrates splice variants to occur in tissues other than liver, but those fragments revealed the retention of introns which indeed code for stop codons when translated in frame. For the purpose of translating vitellogenin protein, those are considered non-functional transcripts which I refer to as "immature" mRNA. Therefore the results presented by Davis et al (2007) is not supported in this study, and further investigation may shed light on this issue. In addition, this study has reported for the first time the matter of "immature" mRNA for *vtg* which occurred in tissues other than liver of *O. mossambicus*, and may provide an explanation for contradicting results reported for *vtg* in studies to date.

Temporal expression of *vtg*, *cyp19* (a and b) and ESRs revealed basal levels of all genes at 20 dpf. The data further suggest on the matter of $E_2$ induction, that even though *vtg* may be inducible (by exogenous estrogens, Chapter 2), it may possibly be suppressed by factors not determined in this study. Why? *Vtg* expression was low and not even upregulated during the time of gonadal development as was indicated by histological results (Chapter 3).

Conversely, *cyp19a* revealed significant increase of transcript levels around 80 dpf, and ESR2a around 75 dpf. ESR1 illustrated severe increase during the time of onset of gonadal differentiation - ~55 dpf. As the liver of juvenile fish by this time is "equipped" to induce *vtg* transcription, we did not measure that. Secondly, the argument of suppression of *vtg,* is supported by results in adult tissue specificity. ESR1 in teleosts is known to be under the strict control of $E_2$ (Menuet *et al.* 2004; Sabo-Attwood *et al.* 2004). It would be expected that especially in adult gonads dimorphism would be apparent, which was not the case. Amongst un-induced males and females, a dimorphic expression pattern was measured instead in liver samples, supporting the possible induction by $E_2$. However, as this dimorphism was only reported in liver samples and not in gonads which is the major site of $E_2$ synthesis another hypothesis may be presented. ESR1 may be highly under the regulation of $E_2$, but ESR2a is hereby suggested to be the major role player in *vtg* regulation by $E_2$ – a theory further supported by data published for goldfish (Soverchia *et al.* 2005) and rainbow trout (Leanos-Castaneda & Van Der Kraak 2007).

In terms of EDC monitoring, the results in this study provide a method to measure estrogenic disruption in both the natural environment and controlled experimental studies, using the confirmed primer sets for quantitative *vtg* expression and providing reference values for such monitoring.

In conclusion, this study present, in addition to the academic results discussed and reported in chapters 2-4, a sound method to monitor estrogenic disruption at a transcriptional level in tier I screens in the endocrine disruption community of Southern Africa, using a native species, responsive to low and high concentrations of $E_2$ without mortality.

In using juveniles for this purpose project managers can use increased numbers of animals, reduce analysis duration and benefit by not having to deal with sex dimorphism.

## REFERENCE LIST

**Davis L.K., Hiramatsu N., Hiramatsu K., Reading B.J., Matsubara T., Hara A., Sullivan C.V., Pierce A.L., Hirano T. & Grau E.G.**, 2007. Induction of Three Vitellogenins by 17beta-Estradiol with Concurrent Inhibition of the Growth Hormone-Insulin-Like Growth Factor 1 Axis in a Euryhaline Teleost, the Tilapia (*Oreochromis mossambicus*). *Biology of Reproduction.* 77, 614-625.

**Leanos-Castaneda O. & Van Der Kraak G.**, 2007. Functional characterization of estrogen receptor subtypes, ER[alpha] and ER[beta], mediating vitellogenin production in the liver of rainbow trout. *Toxicology and Applied Pharmacology*. 224, 116-125.

**Menuet A., Le Page Y., Torres O., Kern L., Kah O. & Pakdel F.**, 2004. Analysis of the estrogen regulation of the zebrafish estrogen receptor (ER) reveals distinct effects of ERalpha, ERbeta1 and ERbeta2. *Journal of Molecular Endocrinology*. 32, 975-986.

**Sabo-Attwood T., Kroll K.J. & Denslow N.D.**, 2004. Differential expression of largemouth bass (Micropterus salmoides) estrogen receptor isotypes alpha, beta, and gamma by estradiol. *Molecular and Cellular Endocrinology*. 218, 107-118.

**Soverchia L., Ruggeri B., Palermo F., Mosconi G., Cardinaletti G., Scortichini G., Gatti G. & Polzonetti-Magni A.M.**, 2005. Modulation of vitellogenin synthesis through estrogen receptor beta-1 in goldfish (*Carassius auratus*) juveniles exposed to 17-[beta] estradiol and nonylphenol. *Toxicology and Applied Pharmacology*. 209, 236-243.