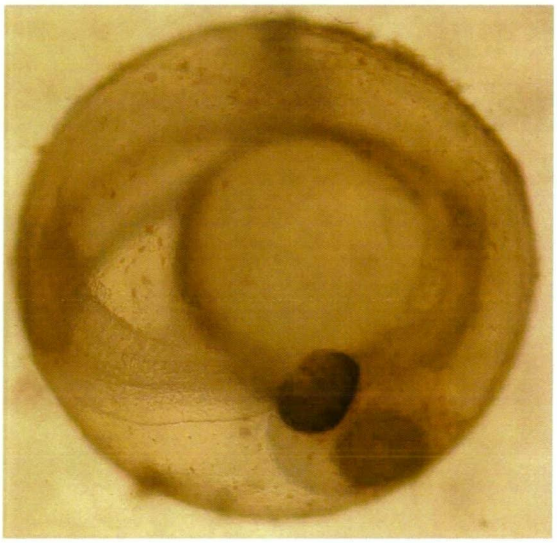
Molecular investigations on sex determination and differentiation pathways in the common carp, *Cyprinus carpio*



By Megan L. Barney

B. Aqua (Hons)

University of Tasmania

Submitted in fulfilment of the
requirements of the degree of
Doctor of Philosophy

May, 2010

Declaration

This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution. To the best of my knowledge all material previously published or written by another person is acknowledged in the text of the thesis.

This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution. To the best of my knowledge the thesis does not contain any material previously published or written by another person except where due acknowledgement is made.

Megan Louise Barney ..... Date 31/05/2010

Authority of Access

This thesis is not to be made available for loan or copying for three years following the date this statement was signed. Following that time the thesis may be made available for loan and limited copying in accordance with the *Copyright Act 1968*.

Megan Louise Barney ..... Date 31/05/2010

Cover Picture: M.L. Barney, *Cyprinus carpio* embryo

Abstract

Common carp is a highly important species, with significant aquaculture production and frequent use for fish biology and aquaculture research. In Australia, New Zealand and the US, common carp are declared as an invasive pest causing damage to endemic ecosystems. This thesis aims to advance our understanding of the molecular pathway of sex determination and differentiation in common carp with a view to facilitate the development of genetic control mechanisms in this species. The objective was addressed by the molecular cloning of six key genes involved in sexual development, 3 each critical for female and male development. Spatial expression of these genes was analysed in adult tissues and the onset and timing of expression was determined during early ontogeny and through larval development at two temperatures (20 and 25 °C) allowing investigation of the effect of temperature on expression and final sex ratios.

The study confirms that there are two isoforms of the cytochrome P450 aromatase gene in the species, namely the ovarian (*cyp19a*) and brain (*cyp19b*). Based on the level of CYP19 expression, the brain was found to be the main site of aromatase synthesis, contributed predominantly by the brain isoform. Also expressed highly in the brain were both isoforms of SRY (sex-determining region Y)-box 9 (*sox9*) and forkhead box L2 *(foxL2)*. Within the gonad, *cyp19a* and *foxL2* were predominantly expressed in the ovary while *doublesex* and *mab-3* related transcription factor 1 (*dmrt1*) was primarily expressed in the testis. Ontogenic expression indicated that *cyp19a* and *sox9a* were maternally inherited. Female critical *cyp19a* showed sexually dimorphic expression only in fish larger than 20 mm, with warmer conditions (25 °C) suppressing expression and suggesting a male-skewed final sex ratio. This indicates that differential expression maybe a result of sex differentiation rather than a cause.

Conversely, expression of *cyp19b* peaked prior to hatching possibly indicating that sexual differentiation occurs first in the brain, before the gonads are present. Expression of *dmrt1*, critical for male development, peaked soon after fertilisation in the 25 °C treatment indicating a role early in the sex-determining pathway. Peak expression of *sox9* genes and *foxL2* occurred prior to hatch, with consequent expression failing to show any sexually dimorphic expression, suggesting that these genes play a role in early larval development in the species, but not sex differentiation.

This thesis found *cyp19a* and *dmrt1* to be accurate markers of either ovarian or testis differentiation respectively. The ability to influence the expression of these genes may result in manipulation of sex ratios of common carp, and other fish. This would be of benefit to both pest control, where population sex bias can result in extinction, and also aquaculture, where monosex populations can improve production efficiency. By developing a greater understanding of sex determination and differentiation teleost fish it is possible to gain further insights into the evolution of sex determination in all vertebrate species.

Table of Contents

List of Tables

List of Figures

Acknowledgments

I would like to thank my supervisors Dr. Jawahar Patil and Prof. Chris Carter for giving me the opportunity to undertake this PhD and for their continued guidance, support and encouragement. Your large efforts in the final stages of thesis preparation, while contending with personal deadlines and major upheaval, are much appreciated.

I would especially like to thank Rasanthi for her friendship and assistance in the lab. Your support and knowledge were essential for the development of my real-time assays. Your encouragement (especially for knock-off drinks) has helped me through this PhD.

Thanks also to the CSIRO Carp group for your assistance in the lab and ongoing support and interest. Thanks especially to Janina for your friendship and enforcing morning coffee breaks!

Thanks to the team at K&C Fisheries for their assistance collecting broodstock, maintaining and sampling the larval trials over the many months. Thank you to Keith, Cate and Piggy for your friendship and for welcoming me into your home for several weeks at a time, and enduring the 5 hourly sampling though the night.

Thanks also to Chris and the boys on the Inland Fisheries Service Carp Team for suppling fish when needed and for hunting down females when they could. Your efforts and Christmas parties won't be forgotten. I wish you all the best with the future success of carp removal from the Tasmanian lakes.

Thanks to my friends in both Launceston in Hobart for your ongoing interest and encouragement. Your ongoing friendship bespite my absence is invaluable.

I wish to thank my family for their ongoing encouragement and support thoughout my many years of study. Thank you for putting up with my stress and moods. Thanks also for always welcoming me home with good food, good wine and good company.

Finally I would like to thank Justin for your encouragement and for supporting me, despite me moving to the opposite end of the state. You have provided a light at the end of many tunnels and your encouragement and distractions are always welcome.

Co-Authorship

Chapters 3, 4, 6, 7 and 8 were prepared as scientific manuscripts as identified below and on the first page of each chapter. In all instances the candidate had the primary responsibility. The supervisors contributed to the experimental design and implementation of the research program, data analysis, interpretation of results and manuscript preparation.

Chapter 3: Barney, M.L. (70%), Patil, J.G., Gunasekera, R.M. and Carter, C.G. (2008). Distinct cytochrome P450 aromatase isoforms in the common carp (*Cyprinus carpio*): Sexual dimorphism and onset of ontogenetic expression. Gen. Comp. Endocrinol., 156 (3), 499-508.

Chapter 4: Barney, M.L (70%), Patil, J.G. and Carter, C.G. Expression of Cytochrome P450 Aromatase through the period of sexual differentiation in the common carp (*Cyprinus carpio*) reared at two temperature regimes. In preparation.

Chapter 6: Barney, M.L (80%) andand Patil, J.G. Cloning and expression of *foxL2* in the common carp (*Cyprinus carpio*): Sexual dimorphism and developmental expression. In preparation.

Chapter 7: Barney, M.L (80%) and Patil, J. Cloning and expression of *dmrt1* in the common carp (*Cyprinus carpio*): Sexual dimorphism and expression during development. In preparation.

Chapter 8: Barney, M.L (80%) andand Patil, J.G. *Sox9* genes in the common carp (*Cyprinus carpio)*: Cloning and expression in adults and during development. In preparation.

The contribution of each co-author was as follows:

- Patil, J.G. (CSIRO) was the primary supervisor and assisted with general supervision of all aspects of this thesis. This includes expremental design, interpretation of data and proof reading manuscripts.
- Carter, C.G. (UTas) mainly assisted with general supervision and with proof reading manuscripts.
- Gunasekera, R. (CSIRO) assisted with development of the real-time assays and undertook cloning of the *cyp19a* promoter.

We the undersigned agree with the above stated 'proportion of work undertaken' for each of the above published (or in preparation) peer-reviewed manuscripts contributing to this thesis.

Co-Supervisor:
Dr Jawahar Patil

Co-Supervisor:
Professor Chris Carter

Head of School:
Professor Chad Hewitt

List of Abbreviations

AA	amino acids
AhR	arylhydrocarbon receptor
AI	Aromatase Inhibitor
ANOVA	Analysis of Variance
AP	Adapter primer
ARE	Androgen response element
ARNT	AhR/nuclear translocation factor
B(a)P	benzo(a)pyrene
bp	base pairs
C/EBP	CCAAT enhancer binding protein
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CRE	cAMP response elements
CSIRO	Commonwealth Scientific and Industrial Research Organisation
C_T	Cycle Threshold
Cyp19	Cytochrome P450, family 19 (aromatase)
DCP	Daughterless Carp Project
ddpf	degree days post fertilisation
ddph	degree days post hatch
DM	Doublesex Mab-3
DMO	DM domain ovarian specific
DMRT1	doublesex and Mab-3-related transcription factor
DMY	DM-related transcription factor Y
DNA	Deoxyribonucleic acid
dpf	days post fertilisation
dph	days post hatch
E2	Estrogen
EDTA	Ethylenediaminetetraacetic acid
ERE	Estrogen response element
FAO	Food and Agriculture Organisation of the United Nations
FH	Fork head
FoxL2	winged helix/forkhead transcription factor L2
GSD	Genetic Sex Determination
GSP	Gene Specific Primer
IACRC	Invasive Animal Co-operative Research Centre
kb	kilo base
Mab-3	male aberrant factor 3
MNE	Mean Normalised Error
NBRE	NGFI-B/Nur77 binding site
NGF1/Nu77	nerve growth factor inducible-B
NGSP	Nested Gene Specific Primer
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction

RACE	Random Amplification of cDNA Ends
RNA	Ribonucleic acid
RT-PCR	Reverse Transcription PCR
SE	Standard Error
SNK	Student-Newman-Kuels
Sox9	SRY-box 9
SRY	Sex determing region Y
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TL	Total length
TSD	Temperature Sex Determination
UPM	Universal Primer Mix
WT1	Wilm tumor 1

CHAPTER 1

General Introduction

Chapter 1 - General Introduction

1.1 Introduction to common carp, Cyprinus carpio

Common or European carp, *Cyprinus carpio*, is a teleost (bony) fish belonging to the order Cypriniformes and family Cyprinidae. The species is recognised by small eyes, thick lips with two barbels at each corner of the mouth, large scales and strongly serrated spines in the dorsal and anal fins. Colour can vary, but is often olive green to silvery grey dorsally, fading to silvery yellow on the belly. Small common carp can be confused with goldfish, *Carassius auratus*, however, the latter has no barbels on the corners of the mouth.

The common carp is native to the Black, Caspian and Aral Sea drainages of western Asia from where it spread east into Siberia and Asia and west into Europe during the Pleistocene-Holocene transition. Over the last 200 hundred years it was translocated or introduced to throughout the world for the purposes of establishing new fisheries and has consequently now established on all continents except Antarctica (Berg 1964, cited in Balon, 1995).

Kirpitchnikov (1967) recognised four subspecies of carp: *C. carpio carpio* (Europe), *C. c. aralensis* (Central Asia), *C. c. haematopterus* (East Asia) and *C. c. viridiviolaceus* (southeast Asia). However, in the few cases where populations from different geographical regions were examined two genetically distinct groups- European and East Asian- could be detected (Brody et al., 1979; Gross et al., 2002; Kohlmann and Kersten, 1999; Paaver, 1983) supporting the existence of two subspecies, *C. c. carpio* and *C. c. haematopterus*, formerly distinguished on the basis of morphological differences ((Balon, 1995; Kirpitchnikov, 1967, 1999). The degree

of differentiation supports the subspecies status of *C. c. carpio* assigned to European carp and *C. c. haematopterus* assigned to Asian carp but does not justify a separate subspecies status (*C. c. aralensis*) for the Central Asian carp. European and Central Asian carp are closely related and there is evidence for a single origin of present day European domesticated and wild/feral carp from a common ancestor with Central Asian carp (Kohlmann et al., 2003). The species investigated in the current study are of the European strain, *C. c. carpio* and will be hereafter referred to as common carp.

1.1.1 Common carp as an aquaculture species

Common carp is an important freshwater aquaculture finfish species, probably with the earliest domestication records among fishes, going back approximately 4000 years with a book called *Kwai Sin Chak Shik* written during the Sung Dynasty in A.D. 1243, describing the transportation and trading of 'carp' fry in bamboo baskets (Balon, 1995, 2004). According to the Food and Agriculture Organisation (FAO) (2007) common carp had the 4th highest production of all aquaculture species (including fish, crustaceans and molluscs) with over 3.1 million tonnes produced in 2006. The majority of commercial common carp production is from polyculture in China, with additional intensive pond and tank culture in Europe and Israel and semi-intensive pond polyculture in India (Carter, 2006).

1.1.2 Common carp as a research species

As well as a food source, the common carp has been used extensively in fish biology and aquaculture research (for review see Horvàth and Orbàn, 1995; Hulata, 1995). Common carp is believed to have evolved from allotetraploidization (species hybridization) and many loci are still expressed in duplicate (David et al., 2003; Larhammar and Risinger, 1994). Its chromosome number (2n=100) is twice that of other Cyprinidae, and its DNA content is higher (David et al., 2003; Ohno et

al., 1967). Additional evidence for carp tetraploidy is that many enzymes are expressed in duplicate (Engel et al., 1971; Ferris and Whitt, 1977). Enzymes which appear to be expressed as one variant only are assumed to have another that has become a pseudogene. Using the proportion of lost duplicate loci to estimate the time elapsed since the tetraploidization event, it is suggested that tetraploidy in carp is more ancient than in salmonids with carps found to express fewer duplicate loci than tetraploid salmoid fishes (Engel et al., 1971). Using c-myc genes the tetraploidization event of common carp is estimated to have taken place 58 MYA (Zhang et al., 1995). Other duplicated common carp genes suggest a more recent divergence time of less than 16 MYA (Larhammar and Risinger, 1994). Microsatellite analysis found two distinct modes in distribution of differences between paralogs, suggesting one whole-genome duplication (about 12 MYA) and a more recent wave of segmental duplications (between 2.3 and 6.8 MYA)(David et al., 2003). It is therefore likely that common carp and goldfish derived from a shared ancestor after the tetraploidisation event as goldfish have the same number of chromosomes (Ojima et al., 1966) and can form hybrids with common carp (Hubbs, 1955; Hume et al., 1983).

Larhammar and Risinger (1994) state that the absence of multiple forms of some of the proteins could be due to complete absence of electrophoretically detectable differences between duplicate loci because of a much shorter time since tetraploidization. The coding capacity at both common carp loci seems to be maintained and both loci give rise to somatotropin mRNA as cDNA clones which have been isolated from both loci (Chao et al, 1989, Koren et al., 1989). Furthermore, it was found that numerous insertions/deletions have occurred in the 3'UT but not in the coding region, indicating that there is still a selection against mutations that would alter the reading frame. These characteristics make cyprinid

species an interesting evolutionary model for examining tetraploidisation, with a role for polyploidisation in speciation and diversification.

As well as being important in aquaculture, common carp are often used as a sentinel species in studies investigating potential alterations to the function of the endocrine system of humans and wildlife as a result of chemical exposure (Lavado et al., 2004; Moens et al., 2007; Moens et al., 2006).

Segregation and sex-reversal studies also widely use common carp, however genomic information is limited; currently only 2262 nucleotide and 2325 protein sequences are available for common carp in GenBank, compared to 171,119 nucleotides and 71,687 proteins for zebrafish, *Danio rerio* (14/07/09 http://www.ncbi.nlm.nih.gov/). As a popular aquaculture species, with established methods of artificial spawning and rearing, common carp is a traditional subject for chromosome set manipulation studies since it has high fecundity and unique morphological genetic markers such as genes for scale cover types and colour mutations (Gomelsky, 2003; Horvàth and Orbàn, 1995).

1.1.3 Common carp as a pest species

In parts of Australasia and America common carp are an introduced species widely regarded as a pest and a threat to endemic ecology (Koehn, 2004; Zambrano et al., 2006). In Australia, they were first introduced over 100 years ago (Brown, 1996) and there have been at least three separate introduction of distinct strains or genetic-types (Kailola et al., 1993) including Koi, a colourful variety of the same species (Davis et al., 1999). It was not until the early 1960's that the 'Boolarra' strain, that was originally introduced into the Gippsland waterbodies and subsequently translocated

into the Murray-Darling basin, is believed to have caused an upsurge in common carp abundance becoming very widely distributed in little more than 10 years (Shearer and Mulley, 1978). Common carp are now widely distributed throughout south-eastern Australia including Tasmania (IFS, 2004) (Figure 1.1).

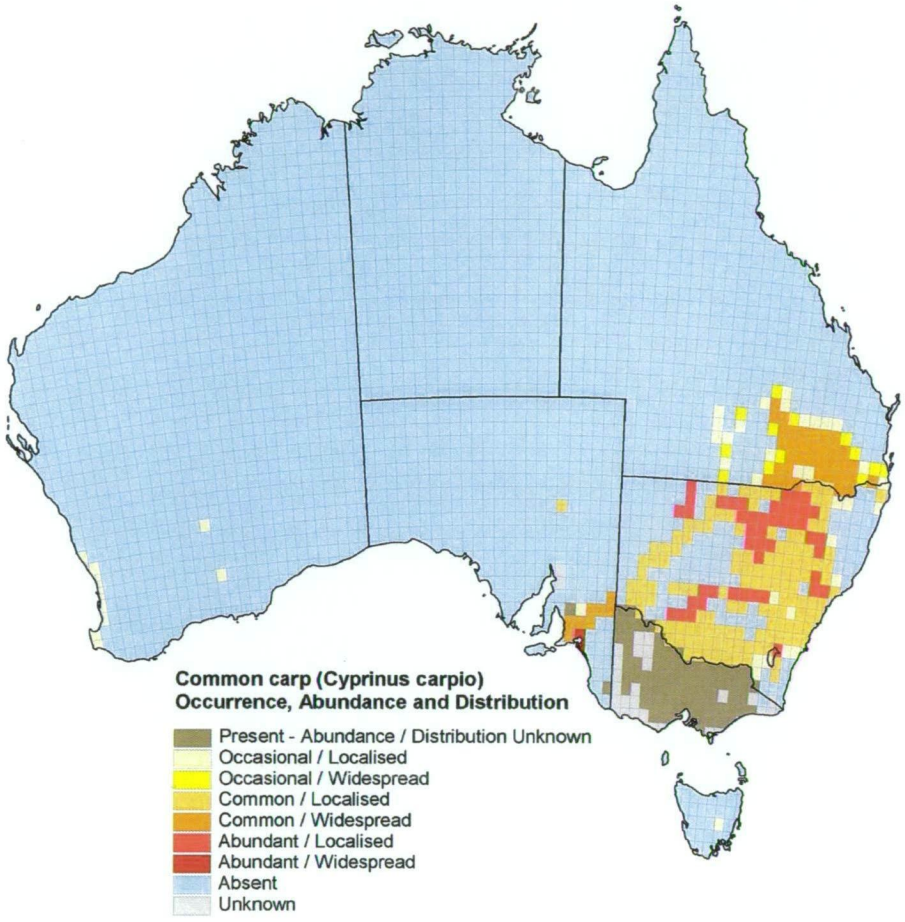


Figure 1.1 Common carp (*Cyprinus carpio*) occurrence, abundance and distribution in Australia

(Courtesy IA CRC, 2008)

Their adaptability and tolerance to poor water quality allow common carp to inhabit a wide range of conditions and dominate the fish communities of the southern Murray-Darling Basin (Gehrke et al., 1995; Harris and Gehrke, 1997). The abundance of native fish populations and species diversity in the Murray-Darling Basin has reportedly declined over the last century (Bomford and Tilzey, 1997; Harris and Gehrke, 1997; Reid et al., 1997) with some species now locally extinct in

parts of their distribution (Cadwallader and Gooley, 1984). In contrast, common carp have undergone a rapid expansion in range and abundance throughout this region since their introduction in the 1960's (Koehn et al., 2000). The life-history of common carp is one characterised by flexibility, with long breeding seasons (up to 9 months), the ability to spawn multiple times each year (Smith and Walker, 2004) and with high fecundity (Sivakumaran et al., 2003). Floodplains and slow flowing pool-type habitats appear to be the preferred spawning habitat (Sivakumaran et al., 2003; Stuart and Jones, 2006). Male and female common carp are long-lived (up to 28 years) and mature relatively early compared with similar-sized native fish (Brown et al., 2005).

Common carp also have significant impacts on aquatic systems, causing extensive habitat and water quality degradation and altering composition, abundance and diversity of macrophytes as well as diversity of macroinvertebrates (Fletcher et al., 1985; Miller and Crowl, 2006; Roberts et al., 1995) as well as increasing water turbidity and nutrient concentrations (Driver et al., 2005; King, 1997). There is still some debate however as to what extent common carp are the cause of major disturbances in freshwater systems and to what extent they are a response to disturbance, after which they may gain opportunistic dominance (Harris and Gehrke, 1997). Common carp also pose an economic threat to industries that depend on pristine water quality and aquatic habitats, including domestic and irrigation water, agriculture, tourism and commercial and recreational fisheries (Adams, 2008).

A suite of techniques has been implemented to control the abundance and environmental impacts of common carp. These include commercial and recreational harvest, environmental rehabilitation, water level manipulation to sabotage

spawning, biomanipulation (stocking with predatory fish), exclusion with screens or barriers, poisoning and biological control, bioacoustics and bubble barriers, and genetic manipulation (Berg et al., 1997; Gervai et al., 1980; Koehn et al., 2000; Propst and Gido, 2004; Shields, 1958; Stuart and Jones, 2006; Stuart et al., 2006; Taylor et al., 2005).

Several methods of common carp management have been discussed in Australia (Roberts and Tilzey, 1996) including employment of a daughterless trait, a heritable sex ratio manipulation mechanism predicted to cause localised extinction of populations (Hamilton, 1967; Werren et al., 1981). The Murray-Darling Basin Commission is supporting the Austral-Asian Invasive Animal Co-operative Research Centre and its research partner CSIRO Marine Research to undertake the Daughterless Carp Project (DCP) as a tool to control common carp in the basin. The DCP focuses on genetically manipulating the population sex ratio of common carp by introducing exclusively male-bearing fish. Through the use of gene technology, it is hoped to introduce multiple copies of a 'daughterless' gene into common carp which would be periodically released into the wild. Copies of the gene are carried by the males, and introgressed though the pest population, resulting in male bias of population sex ratios and ultimately resulting in drastic reduction of female offspring in the population and potentially leading to localised extinction of pest populations. The research described in the current thesis is part of this project as it explores potential candidate genes (Section 1.4.3) in the sex determination pathway of common carp that may be targeted to manipulate sex ratios. At the start of this research none of these genes had previously been explored in common carp.

The primary gene target for the 'daughterless' approach is the ovarian isoform of P450 aromatase gene (*cyp19a)* due to its essential role in estrogen synthesis and ovarian development (see section 1.4.3.1). Estrogen is required for many functions and in many tissues, it is therefore important to know where *cyp19a* is locally expressed, and co-expressed with the brain isoform, to determine potential undesired or compensatory effects of 'switching off' this gene. However, this information was not available in the literature and the current thesis not only identifies both ovarian and brain aromatase isoforms but also demonstrates tissue- and sex-specific expression in adults and onset of timing. As aromatase is the primary target for the DCP, addition effort was made to clone and identify the promoter region of both aromatase isoforms to determine potential regulatory element involved in transcription. *FoxL2* is one such regulatory element of aromatase (section 1.4.3.2 and Chapter 6). This thesis describes, for the first time, the cloning and expression of this gene in adult and developing common carp. In addition, cloning and the expression profiles of *dmrt1* (section 1.4.3.3 and Chapter 7) and *sox9* (section 1.4.3.4 and Chapter 8) are reported; both factors involved in male development.

Exploring the molecular pathways that lead to ovary and testis development in teleost fish will result in a greater understanding of sex determination and differentiation in all vertebrate species. More specifically this allows greater understanding of a potential 'Achilles heel' or vulnerable aspects of sexual development that may be used to control sex ratio and in turn, pest species. Examples of this would be controlling phenotypic sex by inducing or repressing expression of critical genes involved in development of either females (*cyp19a* -see Chapter 4) or males (*dmrt1* -see Chapter 7). More generally, the ability to manipulate sex ratios of common carp, and other fish, is of benefit to both pest control, where population sex

bias can result in extinction, and also aquaculture, where monosex populations can improve production efficiency.

This introductory chapter will summarise what is understood of sex determination and differentiation mechanisms in fish, with particular focus on gonochoristic species including the common carp. Also reviewed are candidate genes of interest that have also been explored in this thesis to elucidate their role in sex determination and their potential as candidates for developing daughterless technology.

1.2 Conserved sex-determining pathways and mechanisms

In mammals, there is greater knowledge of the molecular mechanisms of sex determination and differentiation (Brennan and Capel, 2004) allowing investigation into other vertebrate systems through comparative studies. Comparative studies between whole genomes of mammals and fish have shown significant similarities (Aparicio et al., 1995; Postlethwait et al., 2000) suggesting that some sex differentiation genes of the regulatory hierarchy may be conserved (*foxL2* for example Baron et al., 2005a).

In contrast to many developmental processes, sex-determining mechanisms show no clear evolutionary conservation between phyla. For example the mammalian male-inducing master gene, SRY (sex-determining region Y), seems to be specific to mammals (Capel, 2000). As there is no sex-specific SRY homologue in birds or any reptile, SRY is unlikely to have been the original sex-determining gene in the common ancestor of all higher vertebrates.

However, it has been shown that sex chromosomes of even widely divergent groups now appear to have changed very little over the last 300+ million years. Even independently derived sex chromosomes seem to have followed the same set of evolutionary rules; male heterogamety (XX female:XY male system-typified by mammals), female heterogamety (ZW female:ZZ male system- in birds and snakes), as well as a variety of genetic and environmental sex determining systems (found in teleosts and reptiles) (Graves and Shetty, 2001). The sex-determining pathway seems to be extremely conserved, although the control of the genes in this pathway is vested in different elements. Furthermore it has been suggested that sex-determining genes can be made redundant, and replaced by control at another step of a conserved sex-determining pathway. The possibility of several genes acting as a sex switch may have led to the evolution of new sex-determining/chromosome systems. Sex-determining systems in fish appear to be at a primitive stage of evolution (Charlesworth, 2004; Nanda et al., 1992; Solari, 1994; Thorgaard, 1977), but collectively represent a wide variety of systems ranging from monogenic to polygenic systems. Moreover the phenotypic sex is known to be influenced or manipulated by environmental factors such as temperature and exogenous treatment of sex steroids. Therefore, knowledge of the relationships between sex-determining genes, sex steroids and environmental factors will provide critical information to understand animal sex determination and sex differentiation in general and particularly to elucidate the conserved mechanisms that operate behind sex determination in vertebrates.

Genetic mechanisms which trigger sex determination appear to be diverse in non-mammalian vertebrates, especially in teleost species that exhibit male heterogamety, female heterogamety, temperature- and behaviour-dependent systems (Devlin and

Nagahama, 2002; Godwin et al., 2003; Matsuda, 2003; Nakamura et al., 1998). However, studies indicate that many downstream gene products of sex determination genes are functionally similar in diverse species. Many steroidogenic pathways and genes that are important in sex determination in mammals are also conserved in other vertebrates and show gonad-specific expression during the period of sex determination (Matsuda, 2003).

Despite a range of different mechanisms, morphological development of the gonads in all vertebrate groups appears to have been conserved through evolution. The basic structure of the testis is highly conserved among vertebrates, and strong parallels in invertebrates such as the fly, *Drosophila melanogaster (Drosophila)* suggest that the mechanistic pathways that control the architecture and cellular development of the organ are also conserved (Capel, 2000). For these reasons, information about molecular pathways of gonadogenesis in other animals may provide valuable insights into the mammalian system.

Investigating the coordinated interaction between the transcription and hormonal factors of sex determination may be important to understand the mechanisms that lead to testicular and ovarian differentiation. Among the vertebrates, fish exhibiting a wide range of gonadal differentiation types including gonochorism and hermaphroditism will continue to provide an excellent model for the comparative study of sex determination and gonadal sex differentiation. Greater understanding of these processes in fish will broaden our understanding of vertebrate sex determination and differentiation, and will have important applications for fisheries and aquaculture (Devlin and Nagahama, 2002). For example, elucidation of a sex-determining gene would contribute toward reliable production of monosex fish for increased yields in aquaculture and development of pest control technologies.

1.3 Sex determination and differentiation in fish

The expression of sex is governed by two processes: sex determination (for the genetic or genotypic sex) and sex differentiation (for the gonadal or phenotypic sex). Sex determination refers to the mechanisms that direct sex differentiation, whereas sex differentiation refers to the development of testis or ovaries from undifferentiated or bipotential gonad (Hayes, 1998). Differentiation also includes sexually influenced development of morphology, behaviour and biochemical secondary sex characteristics.

Sexual dimorphism is a common trait among vertebrates (Morrish and Sinclair, 2002; Sinclair et al., 2002; Zarkower, 2001) and is achieved by complex regulatory cascades that direct an early bipotential embryo down either a male or female path of sexual development (Devlin and Nagahama, 2002; Swain and Lovell-Badge, 1999). Although detailed morphological information on sexual dimorphism has been well documented in many species of fish, the molecular pathways of sex determination and differentiation are relatively unknown (Devlin and Nagahama, 2002; Penman and Piferrer, 2008; Piferrer and Guiguen, 2008).

It appears that mechanisms of primary sex determination evolve rapidly with initial sex-determining cues at the top of the regulatory hierarchy differing greatly between species (Zarkower, 2001). This is particularly evident in fish, which due to the great number of species (c. 31,100) and the large variety of aquatic environments inhabited, display extreme diversity in modes of reproduction and sex-determining systems, even among closely related species (Bernard, 2005; Devlin and Nagahama, 2002; Froese and Pauly, 2008).

1.3.1 Types of sex determination

In teleost fish, sex determination is influenced by a balance of genetic, steroidogenic and/or environmental processes (Devlin and Nagahama, 2002). The interactions between these factors are the primary control directing the course of sex differentiation and the associated physiological processes supporting gonadal development and function. Many fish have genetic systems that direct gonadal differentiation during early development and maintain the differentiated state throughout adulthood, or in the case of sequential hermaphrodites, until the pattern of gene expression changes and sex reversal occurs in response to age or environmental cues (Sadovy and Shapiro, 1987). Known types of genetic sex determination in fish are: male (XX/XY) and female (ZZ/ZW) heterogamy, such as in medaka, *Oryzias latipes* (Matsuda, 2005) and the lizardfish, *Trachinocephalus myops*, (Ueno et al., 2001) respectively; multiple sex chromosomes (X, Y, Z, W) as in platyfish, *Xiphophorus maculatus* (Volff and Schartl, 2001); polygenic sex determination (postulated for zebrafish (Lawrence et al., 2008) and pufferfish; fugu, *Takifugu rubripes* and Tetraodon, *Tetraodon nigroviridis* (Li et al., 2002)); and autosomal genes which can override the sex-determining activity of the primary sex chromosomes, as seen in swordtails, *Xiphophorus Poeciliidae* (Devlin and Nagahama, 2002). Although morphologically distinct sex chromosomes cannot be identified in common carp (Kirpichnikov, 1981), sex determination is thought to be of the XX/XY system with conventional diploid offspring yielding 1:1 sex ratios (Cherfas et al., 1994a; Komen et al., 1992; Manzoor Ali and Satyanarayana Rao, 1989).

Environmental influences have an impact on sex determination. Temperature is a modulator of sex determination in a number of species, including channel catfish,

Ictalurus punctatus (Patino et al., 1996), pejerrey *Odontesthes bonariensis* (Strüssmann et al., 1996a; Strüssmann et al., 1996b), with a specific temperature range inducing sex-specific development. Dissolved oxygen levels have also been suggested to alter sex ratio, with hypoxia leading to male-biased populations of zebrafish (Shang et al., 2006). Another factor found to influence phenotypic sex in some species is pH, where high and low pH usually result in female and male-biased offspring, respectively (Baroiller et al., 1999; Rubin, 1985).

In highly evolved heterogametic systems, the mode of gonadal differentiation is expected to be gonochoristic, however sex chromosomes have also been reported in a few hermaphroditic species (Devlin and Nagahama, 2002). Environmental factors causing sex inversion in hermaphroditic fish have been extensively reviewed (e.g. Baroiller et al., 1999; Devlin and Nagahama, 2002; Godwin et al., 2003; Ross, 1990). However, there is limited knowledge on the genetic mechanisms that regulate hermaphrodism, and it is unknown if primary sex determination is indeed regulated by genetic or environmental factors. A study on mangrove rivulus, *Rivulus marmoratus* indicates that temperature can influence sex determination as well as sex-inversion in hermaphroditic fish, with low temperature increasing the occurrence of primary males (5-35%) and increased temperatures induced sex-inversion (ie. secondary male production) (Harrington, 1967, 1968).

1.3.2 Types of gonadal differentiation

Teleostean fish demonstrate diversity in reproduction, with different modes of gonadal differentiation including gonochorism, hermaphroditism, and unisexuality (Breder and Rosen, 1966; Price, 1984; Yamamoto, 1969). Temperature, social cues,

and other factors are capable of redirecting the gonadal development (Devlin and Nagahama, 2002; Nakamura et al., 1998).

Gonochoristic fish develop as either males or females and retain the same sex throughout adulthood (Devlin and Nagahama, 2002; Yamamoto, 1969). There are two types of gonochorism, differentiated and undifferentiated. In differentiated gonochorists, ovarian and testicular differentiation proceeds directly from the undifferentiated gonad (e.g. common carp, *Cyprinus carpio* (Komen et al., 1992) and European seabass, *Dicentrarchus labrax* (Blázquez et al., 1998)), while undifferentiated gonochorists pass during development through a non-functional female phase when the gonads of all individuals, irrespective of the genetic sex, display primary stage oocytes (e.g. zebrafish, *Danio rerio* (Takahashi, 1977) and Sumatra barb, *Barbus tetrazona*). Later on, the population differentiates into males and females in approximately equal proportions. However, social factors such as density and relative size of juveniles in a cohort has been found to influence sex-determination in some species (Francis and Barlow, 1993; Francis, 1984).

Hermaphrodite fish function as both sexes, either simultaneously/synchronous or sequentially, at some time during their life. *Synchronous* hermaphrodites have the ability to produce male and female gametes simultaneously (e.g. *Serranus* sp. (Oliver, 1991, 1997; Petersen, 1990; Pressley, 1981) or *Gobiidae* (Cole, 1990; St. Mary, 1998), while *sequential* hermaphrodites undergo sex-change from male-female (protoandry) (e.g. Gilthead seabream, *Sparus aurata* (Brusle' Sicard and Fourcault, 1997) or female-male (protogyny) (e.g. wrasse, *Thalassoma bifasciatum* (Shapiro and Rasotto, 2009) during adulthood (reviewed by Devlin and Nagahama,

2002). There is yet another set of species that exhibit bidirectional sex reversal (Munday et al., 1998).

1.4 Molecular mechanisms of sex determination and differentiation in fish

1.4.1 Molecular mechanisms of sex determination

A complex series of interacting biochemical processes are involved in the sex determination pathway. At some point early in the cascade a primary sex-determining gene in activated, resulting in a commitment to the production of either male or female gametes (Devlin and Nagahama, 2002; Wilkins, 1995). In genetic systems, mechanisms range from purely polygenic controls, to those with dominant sex-determining factors mixed with autosomal controls, to highly evolved sex chromosomes with heterogametic (XY) males or heterogametic (ZW) females (Bull, 1983). In worms and flies, sex is determined by the ratio of X chromosomes to autosomes (Cline and Meyer, 1996; Parkhurst and Meneely, 1994). In mammals, sex is determined genetically by the presence or absence of a Y chromosome (Capel, 2000; Swain and Lovell-Badge, 1999).

Fishes provide a rich material for studying the evolution of sex chromosomes. Although sex determination in most fishes is likely regulated by genetic factors, relatively few teleosts have karyotypically distinct sex chromosomes (Arkhipchuk, 1995). In most species, the sex chromosomes are still in early stages of differentiation, and do not show distinct differences in length or gene content (Lee et al., 2004). Both XY and WZ gonosomal systems have evolved repeatedly in various groups of fishes (Devlin and Nagahama, 2002). However, additional autosomal loci also contribute to sex determination in many species (Kosswig, 1964). Master genes have been characterised for mammals and some invertebrates (Raymond et al., 1998;

Sinclair et al., 1990). However, identifying sex-determining loci in fish has been less successful, with no apparent sex chromosomes and no sex-linked loci or molecular markers discovered in popular fish models, such as zebrafish, fugu and Tetraodon (Li et al., 2002; Schartl, 2004), suggesting polygenic sex determination.

Within the pathway of genetic systems, certain components, or combinations of components, may become dominant in influencing the direction of sex determination such that environmental factors have little influence. In polygenic systems, the sex of a particular individual will be determined by the strength of the genetic factors it receives from its parents with some genes directing ovarian and others testicular development. Over time, one component may gain such influence over the direction of the pathway that other genetic loci do not override its effects. If this occurs, sex will be determined by a simple, single locus genetic system, and sex chromosomes may develop (Devlin and Nagahama, 2002).

Species such as medaka (Matsuda et al., 2002) and platyfish, *Xiphophorus* (Schultheis et al., 2006) have been shown to possess sex chromosomes and have a well defined sex-determining region on the Y chromosome. However, only in medaka has the primary sex-determining gene been revealed in the sex-determining region. This gene encodes a conserved DM-domain protein, DMY (Matsuda et al., 2002; Nanda et al., 2002). DMY is a new doublesex/mab-3 gene most closely related to DMRT1 (Kondo et al., 2003). DMY expressed specifically in the somatic (sertoli) cells surrounding the primordial germ cells prior to morphological sex differentiation (Nagahama, 2005). Additionally, the DMY protein is localised to the sertoli cell nuclei continuously from embryonic stages to adulthood, suggesting that it is an important regulator during early testicular differentiation and is required to maintain

the differentiated state of the testis (Nagahama, 2005). Loss/gain function studies provide strong evidence that DMY is the master sex-determining gene in this species; with naturally sex reversed XY females showing mutations affecting DMY, and transgenic studies with a genomic DMY fragment showed that transgenic XX fish develop into males, in some cases fully fertile (Nagahama, 2005).

Sequence comparison showed that DMY had greatest homology with DMRT1, which in vertebrates has a conserved role in male gonadal development (Lutfalla et al., 2003; Nanda et al., 2002). It is suggested that DMY originated from a duplicate copy of autosomal *dmrt1*, that in a new genetic environment acquired a sex-determining function (Matsuda, 2005). However it may not represent a general sex-determining gene in fishes as it has not been found in a distant species of medaka or any other fish species studied (Kondo et al., 2003). Some strains of medaka with uncompromised sex-reversed XX males, indicate that autosomal factors can over-ride the role of DMY as a male determinant (Nanda et al., 2003). This highlights that the genes at the top of the sex-determining cascade leading to males and females are highly variable in vertebrates and particularly fish.

The platyfish is another interesting model for sex determination study, as it belongs to the Poeciliidae family which displays diverse genetic sex-determining systems ranging from simple heterogametic, to polyfactoral sex determination (Volff and Schartl, 2001). With three different but homologous sex chromosomes (X, Y, Z), platyfish can be XY or YY males and XX, XW or YW females (Volff and Schartl, 2001). These sex chromosomes appear to be an early stage of differentiation as there are no karyomorphological differences and viable YY males. This multiple sex chromosome system may be explained by a) male determining genes exist on all

gonosomes but only the Y-linked allele/s are expressed, with X and W alleles repressed by autosomal factors or b) a dosage dependant mechanism, with a varying copy numbers of the sex-determining genes on each sex chromosome (Volff and Schartl, 2001). While the sex-determining loci in platyfish remain unknown, recent molecular analysis of the Y chromosome's sex-determining region has revealed a number of potential candidate genes for this species, however all gene candidates identified so far have been detected on both the X and the Y, reflecting the poor degree of differentiation of the gonosomes and their probable recent origin (Böhne et al., 2008; Schultheis et al., 2006; Volff et al., 2003). Even if it can not be excluded that some genes are functional only on one type of sex chromosome, this contrasts with the situation observed for the master sex-determining genes in medaka (*DMY*) and mammals (*SRY*), which are specific to one sex chromosomes, the Y. This further emphasises the recent evolution of primary sex-determining genes in fishes. None of the genes identified so far in the Y sex-determining region of the platyfish are similar to known sex-determining genes such as *dmrt* (*DMY* or *doublesex*) or HMG-domain genes (*SRY* or *sox9*) described in other organisms (Schultheis et al., 2006).

Although a greater understanding of sex-determining loci is being developed as more species of fish are being examined, the timing and influence of these sex-determining mechanisms on sex steroid production and consequent gonadal differentiation remain unclear.

1.4.2 Molecular mechanisms of gonadal sex differentiation

Although much is known about the process of sex differentiation in fish, the precise mechanisms underpinning sex differentiation as well as those involved in primary sex-determination remain undefined (Devlin and Nagahama, 2002). Fish gonadal sex differentiation exhibits much diversified strategies and physiological regulations than

found in mammals (Baron and Guiguen, 2003). The differentiation of the bipotential gonad to either testis or ovaries relies on the regulation of the steroidogenic pathway. In lower vertebrates, sex steroid (estrogens and androgens) play a critical role in gonadal differentiation (Devlin and Nagahama, 2002; Strüssmann and Nakamura, 2002). This is evident from functional sex reversal reported in a number of species following exogenous administration of sex steroid hormones (for review see Cheshenko et al., 2008; Piferrer, 2001; Tzchori et al., 2004). In common carp it is possible to produce meiotic gynogens (Komen et al., 1988), mitotic gynogens (Cherfas et al., 1993b), hybrid gynogens (Cherfas et al., 1994b), induced triploids (Cherfas et al., 1993a), hybrid triploids (Wu et al., 1993), mitotic androgens (Bongers et al., 1994), hybrid androgens (Cherfas et al., 1994b), mitotic tetraploids (Cherfas et al., 1993b), bred tetraploids (Cherfas et al., 1994b), and hybrid tetraploids (Cherfas et al., 1994b). However, the mechanism of action of these steroids on gonad sex differentiation is not well understood.

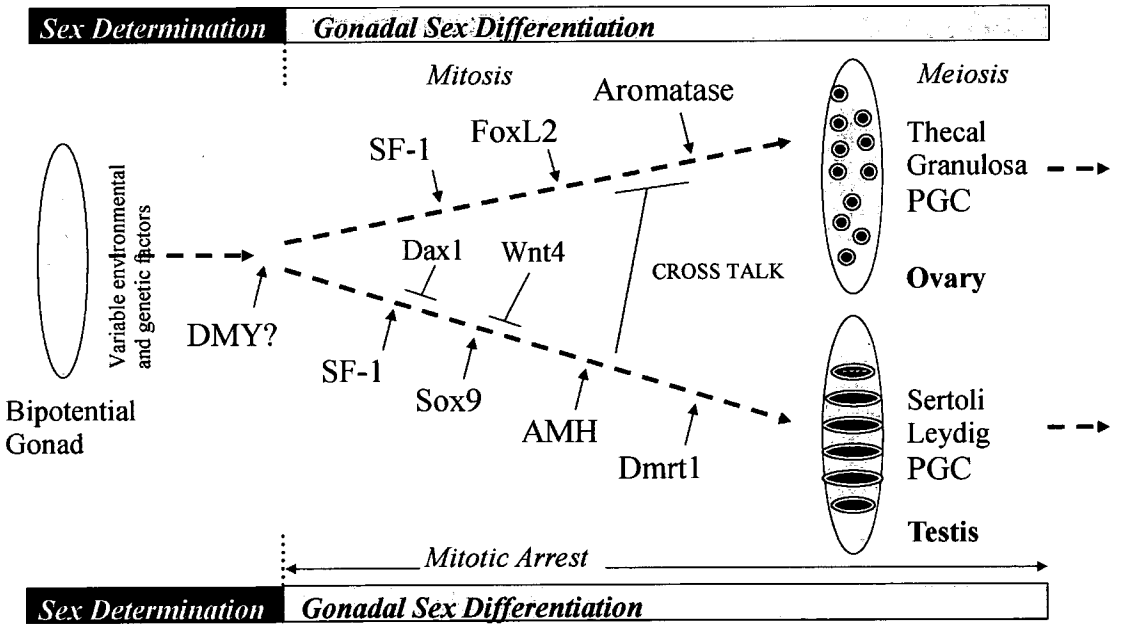


Figure 1.2 Putative mechanisms of fish sex determination and differentiation.

(Arrow, activation; bars, repression)

Unlike sex-determining systems, the genes involved in gonadal differentiation appear to be relatively conserved (Figure 1.2). Genes such as steroidogenic factor-1 (*SF-1* or *Ad4BP*), *cyp19*, *foxL2*, *AMH*, *sox9* and *dmrt1* that have been identified as factors in mammalian sex differentiation are largely conserved across fish and other vertebrates studied, albeit some species specific differences are exhibited. The large majority of these molecules had not been isolated, let alone characterised in the common carp. Therefore, with the view to understand these mechanisms, as well as facilitate the manipulation of sex in the species, the isolation, characterisation, molecular analysis and potential interaction of four key genes (inclusive of isoforms), and their response to temperature cues was analysed in this study. This represents two genes that may have a role in ovarian (aromatase and *foxL2*) and two in testicular (*dmrt1* and *sox9*) differentiation.

1.4.3 Candidate genes

1.4.3.1 Aromatase

The hormonal balance between estrogens and androgens appears crucial in the process of sex differentiation in developing teleost fish. This balance relies on the availability and activity of steroid synthesizing enzymes, and in particular cytochrome P450 aromatase complex expressed by the *cyp19* gene (Fenske and Segner, 2004). In the majority of mammals, there is expression of only a single functional *cyp19* gene. Conversely, two genetically distinct *cyp19* isozymes with different catalytic activity and with distinct or partially overlapping expression patterns have been identified in many teleost species including goldfish (Callard and Tchoudakova, 1997), zebrafish (Chiang et al., 2001a; Kishida and Callard, 2001; Trant et al., 2001); mosquitofish, *Gambusia holbrooki* (Patil et al., 2004); Atlantic halibut, *Hippoglossus hippoglossus* (van Nes et al., 2005) and Nile tilapia (Harvey et

al., 2003). These isozymes are termed *cyp19a* and *cyp19b* for ovarian and brain P450 aromatase, respectively, based on their exclusive or predominant expression in these tissues. The existence of two paralogous genes is suggested to be due to the occurrence of genome duplication early in the teleost linage (Taylor et al., 2003).

Commonly, *cyp19* is considered to be closely involved in the process of sexual differentiation in fish, based both on the profiles of the enzyme expression and activity around the reproductive cycle and on the observations that treatment with aromatase activators or inhibitors can lead to skewed sex ratios (Devlin and Nagahama, 2002; von Hofsten and Olsson, 2005). Female-male sex reversal in many fish after blocking of aromatase with inhibitors or treatment with estrogen receptor antagonists, before or during sex differentiation, indicates estrogen signaling is required for early ovarian differentiation (Baroiller et al., 1999; Guiguen et al., 1999; Nakamura et al., 1998; Piferrer et al., 1994). In male tilapia, steroidogenic enzymes were not detected until after gonadal differentiation, unlike in female fry (Nakamura et al., 2000) indicating a greater role of steroid hormones in ovarian differentiation than testis.

The expression of *cyp19* genes is regulated by transcription factors and response elements located in the promoter region. Analysis of the promoter region of aromatase genes in fish shows the presence of binding sites for the piscine equivalent to many mammalian transcription factors implicated in the cascade of sex determination and differentiation. While transcription factors appear to be conserved among vertebrates, response elements appear to have adapted to facilitate localized expression of paralogous genes. For example, in fish there is a unique presence of an estrogen responsive element (ERE) motif in the *cyp19b* promoter suggesting that

brain aromatase is mainly involved in estrogen-mediated neural estrogen synthesis. Because neural aromatase activity and *cyp19b* mRNA are upregulated by estrogen in the adult goldfish brain and zebrafish embryo, it is tempting to speculate that control is exerted directly on these EREs. No EREs are found within a region 356 bp upstream of the predominant brain exon 1a of the zebra finch aromatase (Ramachandran et al., 1999), nor has an ERE been recognized in the human or mouse brain-specific promoter region (Honda et al., 1999; Honda et al., 1994). Thus, estrogen effects on neural aromatase expression in avian and mammalian brain must take place through other regulators or transcription factors.

Although the two aromatase isoforms have similar gene structures in the coding region, the binding regions of SF-1 and SRY/*sox9*, which are sex-determining factors in mammals, are present in the 5′ flank region of *cyp19a* but not *cyp19b* of tilapia (Chang et al., 2005), zebrafish (Kazeto et al., 2001; Tong and Chung, 2003) and goldfish (Tchoudakova et al., 2001). This data indicates that *cyp19a* plays a decisive role in sex differentiation of these species. However, in medaka the SF-1 region has been identified in both isoforms (Kuhl et al., 2005; Tanaka et al., 1995). SF-1 is an important *cis*-element that regulates expression of several steroidogenic enzymes in gonads and adrenals (Hatano et al., 1994) and has shown expression dependant on *cyp19* in diverse vertebrates (Crews et al., 2001; Lynch et al., 1993). During gonadal differentiation in mammals (Ikeda et al., 1994), turtle, *Trachemys scripta* (Fleming et al., 1999), and rainbow trout, *Oncorhynchus mykiss* (Baron and Guiguen, 2003), SF-1 is up-regulated in males and down-regulated in females. However, in alligator, *Alligator mississippiensis* (Western et al., 2000), American bullfrog, *Rana catesbeiana* (Mayer et al., 2002), chicken (Smith et al., 1999b) and tilapia (Nagahama, 2005) expression is similar between sexes or even greater in ovaries. As

the pattern of expression is highly variable between species, it is likely that the interaction between SF-1 and other co-factors may cause sex-specific regulation of aromatase, as is implicated by the paradoxical actions of gonadotropins on *cyp19a* gene (Yoshiura et al. (2003).

1.4.3.2 FoxL2

Another transcription factor that shows sexually dimorphic expression corresponding to ovarian aromatase is *foxL2,* a member of the winged helix/forkhead family of transcription factors known to be involved in ovarian development granulosa cell differentiation, and thus the proper maintenance of ovarian function (Cocquet et al., 2003; Loffler et al., 2003; Ottolenghi et al., 2005; Pisarska et al., 2004; Schmidt et al., 2004; Uda et al., 2004; Yao, 2005). It is the earliest known sexually dimorphic marker, expressed in the somatic cells during early development and later in granulosa cells surrounding the oocytes (Cocquet et al., 2002). *FoxL2* has been found to correlate with the level of *cyp19* expression in a diverse range of species, including medaka (Nakamoto et al., 2006), tilapia (Yoshiura et al., 2003), rainbow trout (Baron et al., 2005b), Japanese flounder, *Paralichthys olivaceus* (Yamaguchi et al., 2007), wrinkled frog, *Rana rugosa* (Oshima et al., 2008), chicken (Govoroun et al., 2004), turtle (Ramsey et al., 2007) and mammals (Pannetier et al., 2006).

Interestingly, rainbow trout has two genetically independent paralogue genes, *foxL2a* and *b* (Baron et al., 2004; Vizziano-Cantonnet et al., 2008; Vizziano et al., 2007). *FoxL2a* expression patterns in rainbow trout are similar to that observed for the mammalian *foxL2* gene. However, *foxL2b* is specific to somatic cells of the ovary, and is expressed later than *foxL2a.* This is perhaps an example of specialised function of duplicated genes where other species have only maintained one functional isoform. Inferring from the genomic sequences from teleost species for

which whole genome sequences are available, it appears that *foxl2* is duplicated in many more teleosts (Baron et al., 2004; Vizziano-Cantonnet et al., 2008; Vizziano et al., 2007).

It appears that both *foxL2* and SF-1 activate *cyp19* expression by binding directly to the promoter, with *foxL2* enhancing SF-1 activated transcription by forming a heterodimer (Nagahama, 2005). Sex reversal studies show that *in vivo* expression in female tilapia of a transgenic dominant-negative-mutant-*foxL2* construct reduced aromatase expression causing ovaries to degenerate and testicular tissue to develop with complete sex-reversal resulting in viable males (Nagahama, 2005). Sex reversal experiments in rainbow trout resulted in *foxL2* expression in the ovaries of sex-reversed XY genetic males following estrogen treatment, while female-male sex reversal with an aromatase inhibitor resulted in greatly reduced *foxL2* expression (Baron et al., 2004). This suggests either a positive feedback loop, possibly via estrogen, or male-promoting factors in the masculinised gonad such as *dmrt1*, may influence *foxL2* transcription.

1.4.3.3 Dmrt1

In higher vertebrates, the female pathway is generally considered the default in the absence of any male inducing factor (*e.g.,* SRY). In humans, a sex reversing syndrome associated with deletions of chromosome 9p is pinpointed to a critical region at the end of the short arm, and the *dmrt1* was cloned from this interval (Raymond et al., 1998). Since this human chromosome is equivalent to the bird Z chromosome, it is not surprising to find that *dmrt1* maps to the chicken Z (Nanda et al., 1999). With no copy on the W, there is a dosage difference of two copies (male) to one (female). The gene is expressed specifically in the testis at about the same time as *sox9* in both mice and chickens, so is obviously an ancient component of the

vertebrate sex-determining pathway (Smith et al., 1999a). In fact, the name of this gene is derived from its relatedness to two transcriptional factors involved in sex determination in invertebrates, *Drosophila doublesex* and the nematode *mab-3* gene. The DM-domain genes are the first to show sexually dimorphic expression across both vertebrate and invertebrate phyla (Smith et al., 1999a). With *dmrt1* associated with sex determination for perhaps a billion years making it a likely candidate for the bird/reptile sex-determining gene (Graves and Shetty, 2001).

In fish, *dmrt1* is an autosomal gene thought to function downstream in the pathway of genes regulating testis differentiation due to its expression before and during testis differentiation and development (Brunner et al., 2001; Marchand et al., 2000; Yamaguchi et al., 2006), rather than determination. *Dmrt1* may also play a role in female gonadal development as low expression was observed in differentiating ovaries of zebrafish (Guo et al., 2005), rainbow trout (Marchand et al., 2000) and rice feild eel, *Monopterus albus* (Huang et al., 2005).

Dmrt1 expression in tilapia suggests a role in early testis differentiation (Guan et al., 2000) and also an involvement in regulating *foxL2* and aromatase expression (Ijiri et al., 2008; Nagahama, 2005). A transgenic study demonstrated that over-expression of *dmrt1* in the gonads of XX fry caused a reduction in aromatase expression resulting in degeneration of ovaries and development of testicular tissue, with complete sex reversal in some individuals (Nagahama, 2005). The mechanism of this action was explored using luciferase assays, suggesting that DMRT1 bound to SF-1 itself, repressing SF-1 activated transcription of aromatase (Nagahama, 2005).

Whilst the early research focused on the testis inducing characteristics of *dmrt1*, a more recent study reported a female-specific DM domain gene (DMO) directing ovarian differentiation in tilapia (Guan et al., 2000). This suggests strong evolutionary parallels between invertebrate and vertebrate sex-determining mechanisms as the *Drosophila doublesex* gene regulates somatic sexual differentiation in both sexes (Burtis and Baker, 1989). Interestingly, an SRY-consensus site is present in the 5' upstream region of *dmrt1,* but not in the 5' flanking region of *DMO* in tilapia. This suggests one of the upstream regulatory genes of *dmrt1* could be an SRY-like gene from the Y chromosome which probably upregulates *dmrt1* expression to promote testis formation. In females, without the Y chromosome, *dmrt1* is either not upregulated or possibly repressed by an unknown ovary determining factor, thus blocking testis formation. In XX males, Guan et al. (2000) found environmental or hormonal influences compensate for the absence of a signal from the Y chromosome which results in XX-sex reversal and upregulation of *dmrt1* expression.

An outstanding candidate for the first master sex-determining gene in a teleost fish, medaka, is a recent duplicate of *dmrt1* gene, referred to as *DMY* (Matsuda et al., 2002). *DMY* is located on the Y chromosome and is expressed exclusively in XY embryos. This gene is therefore thought to be the initiating switch in male sex determination (Mank et al., 2006). Although this gene has not been isolated in other species, it is highly likely that similar sex-determining genes have developed in different species over the course of evolution. Such genes may zygotically direct female and others male development, alternatively, sex of an individual may be determined by the strength of genetic factors received from parents (Devlin and Nagahama, 2002).

1.4.3.4 Sox9

Failed attempts to isolate a teleostean equivalent to the mammalian testis determining factor SRY have identified a *SRY*-related gene *sox9* (*SRY*-like HMG-box 9) in fish, which is involved in mammalian sex determination down-steam of SRY (Morrish and Sinclair, 2002). To date, *sox9* has been identified in many different species, namely human (Foster et al., 1994), mouse (Kanai and Koopman, 1999; Wright et al., 1995), chicken (da Silva et al., 1996), lizard (Choudhary et al., 2000), frog (Takase et al., 2000), turtle (Spotila et al., 1998), alligator (Western et al., 1999) and several fish species, thereby demonstrating its conserved role in evolution.

Two *sox9* genes, *sox9a* and *sox9b*, have been found in several teleost fish; zebrafish (Chiang et al., 2001b), three-spined stickleback, *Gasterosteus aculeatus* (Klüver et al., 2005), fugu (Koopman et al., 2004), medaka (Yokoi et al., 2002), rice field eel (Zhou et al., 2003) and rainbow trout (Takamatsu et al., 1997). Investigations on these fish showed that not only have the genes been duplicated, but also their functions have been partitioned in lineage-specific ways. Interestingly, in zebrafish, *sox9a* is a putative regulator of the gonadal expression of an SF-1 homologue (von Hofsten and Olsson, 2005). However based on expression pattern studies in medaka and rice field eel indicate that the function of *sox9* genes in cartilage development (chondrogenesis) is conserved in fish, but its role in male sex determination and differentiation may not be (Yokoi et al., 2002; Zhou et al., 2003). In zebrafish, *sox9a* is expressed in adult testis while *sox9b* is expressed in adult ovary (Chiang et al., 2001b). However, in adult medaka, *sox9a* was expressed predominantly in the ovary and much less expressed in the testis than *sox9b* (Klüver et al., 2005).

The combined expression pattern of the two *sox9* genes in zebrafish and three-spined stickleback correspond approximately to that of the single *sox9* in mouse, indicative of a partitioning of ancestral sub-functions (Chiang et al., 2001b; Cresko et al., 2003; Yan et al., 2002). This split expression pattern of *sox9a/b* may merely mimic an ancestral role of a single SOX9 which functioned in both testis and ovary. This is seen in the expression of *sox9* in the differentiated ovary in other species, including human and frog (Hanley et al., 2000; Takase et al., 2000). However, the predominant expression of *sox9* genes in the differentiated ovary appears to be fish-specific.

Extending present knowledge of the relationships between these candidate genes in common carp sex differentiation should help to create a better understanding of animal sex determination and sex differentiation in general, and to elucidate the conserved vertebrate sex-determining mechanisms.

1.5 Aims and hypotheses of thesis

The overall aim of this study is to enhance our understanding of the mechanisms underpinning sex determination and differentiation in common carp. To address this aim the following experiments were undertaken:

1) select, clone and characterise candidate genes in sex determination and differentiation
2) determine sex- and tissue- specific expression of candidate genes
3) examine timing and onset of candidate genes and expression patterns through gonadal differentiation
4) explore the effect of two different rearing temperatures on gene expression and resultant sex ratios

5) Analyse potential interactions of candidate genes and consequences of these interactions

Thesis chapters are written in the form of manuscripts for submission to peer-reviewed journals. Consequently there is some repetition of information in introduction sections of the aromatase Chapters 3 and 4. In addition, Chapter 2 provides a materials and methods chapter that covers sample collection of adult tissues and embryonic/larval fish. General techniques used for cloning and sequencing genes and phylogenetic analysis are covered in this chapter with specific details found in each respective chapter. Methods used for preparing samples for expression analysis and real-time PCR and data analysis are also found in this chapter.

Chapters 3-8 outline the isolation, characterisation and expression studies and are stand alone chapters which have been published or are under internal review in preparation for submission to peer-reviewed journals.

Chapter 3 identifies two aromatase isoforms and examines the hypothesis that aromatase plays a role in sex determination and differentiation in common carp. Sexually dimorphic expression was shown in adult fish and an initial larval rearing study identified maternal inheritance and expression through early ontogeny. Larval incubation at 20 and 25 °C examined the hypothesis that higher temperature inhibits aromatase transcription. This study was published in General and Comparative Endocrinology (Barney et al., 2008). Due to poor larval survival in this rearing trial, a second artificial spawning was carried out in the following year.

In Chapter 4, aromatase expression though the gonadal differentiation stage was examined from a second larval rearing trial, which again had incubation at 20 and 25 °C. Following the development of the gonad, remaining fish were allowed to grow to at least 5 cm, the size at which the differentiated gonad can be seen by eye and dissected for staining to determine the sex of individuals. This chapter examined the hypothesis that higher temperature inhibits aromatase transcription resulting in a male-skewed final sex ratio.

To further explore the role and influencing factors of aromatase in common carp sex differentiation, the promoter regions of both brain and ovarian isoforms were identified and analysed in Chapter 5. This addressed the hypothesis that these different promoter regions interact with different transcription factors that enable specialised function.

Chapter 6, 7 and 8 examine the isolation, characterisation and expression of *foxL2*, *sox9* and *dmrt1* through larval and gonadal development stage. The individuals sampled in this study were also used for aromatase expression analysis allowing the relationship between these genes to be explored.

In Chapter 6, expression of *foxL2* was examined in adults and through larval development and gonadal differentiation at two temperature regimes. This chapter addressed the hypothesis that *foxL2* acts as a precursor or transcriptional factor for aromatase expression.

Chapter 7 explores the expression of *sox9*, a conserved gene involved in cartilage formation (chondrogenesis) and male-development in higher vertebrates. This

chapter examines expression of co-orthologues (*sox9a* and *b*) in adult tissues and also through embryonic and larval development, again at two temperature regimes. This chapter addresses the hypothesis the *sox9* genes are involved in chondrogenesis and sex determination in common carp.

In Chapter 8 expression of *dmrt1*, another male-determining gene is examined in adult common carp and through development at two temperatures. The hypothesis for this chapter is that *dmrt1* is involved in male sex differentiation in common carp. Finally, Chapter 9 is a general discussion specifically with the aim of examining the roles of aromatase, *foxL2*, *sox9* and *dmrt1* in sexual development and places them in the larger sex differentation pathway of common carp and other teleosts.

1.6 References

Adams, S., 2008. Vertebrate Pest Animal Management Strategy for the Central West Catchment (NSW), http://www.cw.cma.nsw.gov.au/pdf/Reports/CWCMA_Report_vertebratepestanimalstrategy_report_jun08.pdf.

Aparicio, S., Morrison, A., Gould, A., Gilthorpe, J., Chaudhuri, C., Rigby, P., Krumlauf, R., Brenner, S., 1995. Detecting conserved regulatory elements with the model genome of the Japanese puffer fish, Fugu rubripes. Proc. Natl. Acad. Sci. USA 92, 1684–1688.

Arkhipchuk, V.V., 1995. Role of chromosomal and genome mutations in the evolution of bony fishes. Hydrobiol., 31, 55-65.

Balon, E.K., 1995. Origin and domestication of the wild carp, Cyprinus carpio: from Roman gourmets to the swimming flowers. Aquaculture, 129, 3-48.

Balon, E.K., 2004. About the oldest domesticates among fishes. J. Fish Biol., 65, 1 27.

Barney, M.L., Patil, J.G., Gunasekera, R.M., Carter, C.G., 2008. Distinct cytochrome P450 aromatase isoforms in the common carp (Cyprinus carpio): Sexual dimorphism and onset of ontogenic expression. Gen Comp Endocrinol, 156, 499-508.

Baroiller, J.-F., Guiguen, Y., Fostier, A., 1999. Endocrine and environmental aspects of sex differentiation in fish. Cell Mol Life Sci, 55, 910-931.

Baron, D., Batista, F., Chaffaux, S., Cocquet, J., Cotinot, C., Cribiu, E., De Baeree, E., Guiguen, Y., Jaubert, F., Pailhoux, E., Pannetier, M., Vaiman, D., Vigier, B., Veitia, R., Fellous, M., 2005a. Foxl2 gene and the development of the

ovary: a story about goat, mouse, fish and woman. Reprod, Nutr, Dev, 45, 377-382.

Baron, D., Cocquet, J., Xia, X., Fellous, M., Guiguen, Y., Veitia, R.A., 2004. An evolutionary and functional analysis of FoxL2 in rainbow trout gonad differentiation. J Mol Endocrinol, 33, 705-715.

Baron, D. & Guiguen, Y., 2003. Gene expression during gonadal sex differentation in rainbow trout (Orcorhynchus mykiss): from candidate genes studies to high thoughout genomic approach. Fish Physiol Biochem, 28, 119-123.

Baron, D., Haulgatte, R., Fostier, A., Guiguen, Y., 2005b. Large-Scale Temporal Gene Expression Profiling During Gonadal Differentiation and Early Gametogenesis in Rainbow Trout. Biol Reprod, 73, 959-966.

Berg, S., Jeppesen, E., Søndergaard, M., 1997. Pike (Esox lucius L.) stocking as a biomanipulation tool 1. Effects on the fish population in Lake Lyng, Denmark. Hydrobiologia, 342-343, 311-318.

Bernard, J., 2005. Particularities of reproduction and oogenesis in teleost fish compared to mammals. Reprod. Nutr. Dev., 45, 261-279.

Blázquez, M., Zanuy, S., Carrillo, M., Piferrer, F., 1998. Structural and functional effects of early exposure to estradiol-17β and 17α-ethynylestradiol on the gonads of the gonochoristic teleost Dicentrarchus labrax. Fish Physiol Biochem, 18, 37-47.

Böhne, A., Schultheis, C., Zhou, Q., Froschauer, A., Schmidt, C., Selz, Y., Braasch, I., Ozouf-Costaz, C., Dettai, A., Ségurens, B., Couloux, A., Bernard-Samain, S., Chilmonczyk, S., Gannouni, A., Madani, K., Brunet, F., Galiana-Arnoux, D., Schartl, M., Volff, J.-N., 2008. Identification of new gene candidates on the sex chromosomes of the platyfish Xiphophorus maculatus. Cybium, 32, 69-71.

Bomford, M. & Tilzey, R., 1997. Pest management principles for European carp. In:. J. Roberts & R. Tilzey (Eds.), Controlling carp: exploring the options for Australia, CSIRO Land and Water. pp. 9-20.

Bongers, A.B.J., in't Veld, E.P.C., Abo-Hashema, K., Bremmer, I.M., Eding, E.H., Komen, J., Richter, C.J.J., 1994. Androgenesis in common carp (Cyprinus carpio L.) using UV irradiation in a synthetic ovarian fluid and heat shocks. Aquaculture, 122, 119-132.

Breder, C.M. & Rosen, D.E., 1966. Modes of reproduction in fishes Natural History Press, Garden City, NY.

Brennan, J. & Capel, B., 2004. One tissue, two fates: molecular genetic events that underlie testis versus ovary development. Nat Rev Genet, 5, 509-521.

Brody, T., Kirsht, D., Parag, G., Wohlfarth, G., Hulata, G., Moav, R., 1979. Biochemical genetic comparison of the Chinese and European races of the common carp. Anim. Blood Grps. Biochem. Genet., 10, 141–149.

Brown, P., 1996. Carp in Australia. Fishfacts No. 4. NSW Fisheries: Sydney.

Brown, P., Sivakumaran, K.P., Stoessel, D., Giles, A., 2005. Population biology of carp (Cyprinus carpio L.) in the mid-Murray River and Barmah forest Wetlands, Australia. Mar. Freshwater Res., 56, 1151–1164.

Brunner, B., Hornung, U., Shan, Z., Nanda, I., Kondo, M., Zend-Ajusch, E., Haaf, T., Ropers, H.-H., Shima, A., Schmid, M., Kalscheuer, V.M., Schartl, M., 2001. Genomic Organization and Expression of the Doublesex-Related Gene Cluster in Vertebrates and Detection of Putative Regulatory Regions for DMRT1. Genomics, 77, 8-17.

Brusle′ Sicard, S. & Fourcault, B., 1997. Recognition of sex-inverting protandric Sparus aurata: ultrastructural aspects. J. Fish Biol., 50, 1094– 1103.

Bull, J.J., 1983. Evolution of Sex Determinating Mechanisms. Benjamin/Cummings Publishing Company, Inc., Menlo Park, CA.

Burtis, K.C. & Baker, B.S., 1989. Drosophila doublesex gene controls somatic sexual differentiation by producing alternatively spliced mRNAs encoding related sex-specific polypeptides. Cell, 56, 997-1010.

Cadwallader, P.L. & Gooley, G.J. 1984 Past and present distributions and translocations of Murray cod Maccullochella peeli, and trout cod M. macquariensis (Pisces: Percichthyidae) in Victoria. In Proceedings of the Royal Society of Victoria, Vol. 96, pp. 33–43.

Callard, G.V. & Tchoudakova, A., 1997. Evolutionary and functional significance of two CYP19 genes differentially expressed in brain and ovary of goldfish. J Steroid Biochem Mol Biol, 61, 387-392.

Capel, B., 2000. The battle of the sexes. Mech Dev, 92, 89-103.

Carter, C.G., 2006. Aquaculture of Finfish and Shellfish: Principles and Applications. In: Y.H. Hui (Eds.), Handbook of Food Science, Technology, and Engineering, Vol. 1, Taylor & Francis, Boca Raton. pp. 37-1 to 37-14.

Chao, S.-C., Pan, F.-M., Chang, W.-C., 1989. Purification of carp growth hormone and cloning of the complementary DNA. Biochim Biophys Acta, 1007, 233-236.

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). Gen Comp Endocrinol, 141, 101-115.

Charlesworth, B., 2004. Sex determination: Primitive Y chromosomes in fish. 14, R745-R747.

Cherfas, N.B., Gomelsky, B.I., BenDom, N., Peretz, Y., Hulata, G., 1994a. Assessment of triploid common carp (Cyprinus carpio L.) for culture. Aquaculture, 127.

Cherfas, N.B., Gomelsky, B.I., Emelyanova, O.V., Recoubratsky, A.V., 1994b. Induced diploid gynogenesis and polypioidy in crucian carp, Carassius auratus gibelio (Bloch) x common carp, Cyprinus carpio L., hybrids. Aquacult. Fish. Mgmt, 25, 943–954.

Cherfas, N.B., Gomelsky, B.I., Peretz, Y., Ben Dom, N., Hulata, G., Moav, B., 1993a. Induced gynogenesis and polypioidy in the Israeli common carp line DOR-70. Israel J. Aquacult., 45, 59–72.

Cherfas, N.B., Hulata, G., Kozinsky, O., 1993b. Induced diploid gynogenesis and polypioidy in ornamental (koi) carp, Cyprinus carpio L. 2. Timing of heat shock during the first cleavage. Aquaculture, 111.

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. Gen Comp Endocrinol, 155, 31-62.

Chiang, E.F.-L., Yan, Y.-L., Guiguen, Y., Postlethwait, J., Chung, B., 2001a. Two cyp19 (P450 aromatase) genes on duplicated zebrafish chromosomes are expressed in ovary or brain. Mol Biol Evol, 18, 542-550.

Chiang, E.F.L., Pai, C.-I., Wyatt, M., Yan, Y.-L., Postlethwait, J., Chung, B.-c., 2001b. Two Sox9 genes on duplicated Zebrafish chromosomes: Expression of similar transcription activators in distinct sites. Dev Biol, 231, 149-163.

Choudhary, B., Ganesh, S., Raman, R., 2000. Evolutionary conservation of the gene Cvsox9 in the lizard, Calotes versicolor, and its expression during gonadal differentiation. Dev. Genes Evol., 210, 250-257.

Cline, T. & Meyer, B., 1996. Vive la difference: males vs females in flies vs worms. Annu Rev Genet, 30, 637–702.

Cocquet, J., De Baere, E., Gareil, M., Pannetier, M., Xia, X., Fellous, M., Veitia, R.A., 2003. Structure, evolution and expression of the FOXL2 transcription unit. Cytogen. Genome Res., 101, 206-211.

Cocquet, J., Pailhoux, E., Jaubert, F., Servel, N., Xia, X., Pannetier, M., De Baere, E., Messiaen, L., Cotinot, C., Fellous, M., Veitia, R.A., 2002. Evolution and expression of FOXL2. J. Med. Genet., 39, 916-921.

Cole, K.S., 1990. Patterns of gonad structure in hermaphroditic gobies Teleostei Gobiidae. Environ. Biol. Fishes 28, 125–142.

Cresko, W.A., Yan, Y.-L., Baltrus, D.A., Amores, A., Singer, A., Rodríguez-Marí, A., Postlethwait, J.H., 2003. Genome duplication, subfunction partitioning, and lineage divergence: Sox9 in stickleback and zebrafish. Dev. Dynam., 228, 480-489.

Crews, D., Fleming, A., Willingham, E., Baldwin, R., Skipper, J.K., 2001. Role of steroidogenic factor 1 and aromatase in temperature-dependent sex determination in the red-eared slider turtle. J Exp Zool, 290, 597-606.

da Silva, S.M., Hacker, A., Harley, V., Goodfellow, P., Swain, A., Lovell-Badge, R., 1996. Sox9 expression during gonadal development implies a conserved role for the gene in testis differentiation in mammals and birds. Nat Genet, 14, 62-68.

David, L., Blum, S., Feldman, M.W., Lavi, U., Hillel, J., 2003. Recent duplication of the common carp (Cyprinus carpio L.) genome as revealed by analyses of microsatellite loci. Mol Biol Evol, 20, 1425-1434.

Davis, K.M., Dixon, P.I., Harris, J.H., 1999. Allozyme and mitochondrial DNA analysis of carp, Cyprinus carpio L., from south-eastern Australia. Mar. Freshwater Res., 50, 253-60.

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.

Driver, P.D., Closs, G.P., Koen, T.B., 2005. The effects of size and density of carp (Cyprinus carpio L.) on water quality in an experimental pond. Archiv für Hydrobiologie, 163, 117-131.

Engel, W., Faust, J., Wolf, U., 1971. Isoenzyme polymophism of the sorbitol dehyrogenase and the NADP-dependant isocitrate dehydogenases in the fish family Cyprinidae. Anim. Blood Groups Biochem. Genet., 2, 127-133.

FAO, 2007. The state of world fisheries and aquaculture, 2006, Rome, http://www.fao.org/docrep/009/A0699e/A0699e00.htm.

Fenske, M. & Segner, H., 2004. Aromatase modulation alters gonadal differentiation in developing zebrafish (Danio rerio). Aquat Toxicol, 67, 105-126.

Ferris, S.D. & Whitt, G.S., 1977. The evolution of duplicate gene expression in the carp (Cyprinus carpio). Experientia, 33, 1299-1301.

Fleming, A., Wibbels, T., Skipper, J.K., Crews, D., 1999. Developmental expression of Steroidogenic Factor 1 in a turtle with temperature-dependent sex determination. Gen Comp Endocrinol, 116, 336-346.

Fletcher, A.R., Morison, A.K., Hume, D.J., 1985. Effects of carp, Cyprinus carpio L., on communities of aquatic vegetation and turbidity of waterbodies in the lower Goulburn River basin. Aust. J. Mar. Freshwat. Res., 36, 311-327.

Foster, J.W., Dominguez-Steglich, M.A., Guioli, S., Kwok, C., Weller, P.A., Stevanovic, M., Weissenbach, J., Mansour, S., Young, I.D., Goodfellow, P.N., Brook, J.D., Schafer, A.J., 1994. Campomelic dysplasia and autosomal sex reversal caused by mutations in an SRY-related gene. Nature, 372.

Francis, R. & Barlow, G., 1993. Social control of primary sex differentation in the Midas Cichlid. PNAS, 90, 10673-10675.

Francis, R.C., 1984. The effects of bidirectional selection for social dominance on agonistic behaviour and sex ratios in the paradise fish (Macropodus opercularis). Behaviour, 90.

Froese, R. & Pauly, D.E. 2008 FishBase, World Wide Web electronic publication. Available at www.fishbase.org, version (12/2008).

Gehrke, P.C., Brown, P., Schiller, C.B., Moffat, D.B., Bruce, A.M., 1995. River regulation and fish communities in the Murray-Darling River System, Australia. Regul. River., 11.

Gervai, J., Pater, S., Nagy, A., Horvath, L., Csenyi, V., 1980. Induced triploidy in carp, Cyprinus carpio L. J. Fish Biol., 17, 667-671.

Godwin, J., Luckenbach, A., Borski, R.J., 2003. Ecology meets endocrinology: environmental sex determination in fishes. Evol. Dev., 5, 40-49.

Gomelsky, B., 2003. Chromosome set manipulation and sex control in common carp: a review. Aquatic Living Resources, 16, 408-415.

Govoroun, M.S., Pannetier, M., Pailhoux, E., Cocquet, J., Brillard, J.-P., Couty, I., Batellier, F., Cotinot, C., 2004. Isolation of chicken homolog of the FOXL2 gene and comparison of its expression patterns with those of aromatase during ovarian development. Dev. Dynam., 231, 859-870.

Graves, J.A.M. & Shetty, S., 2001. Sex from W to Z: Evolution of vertebrate sex chromosomes and sex determining genes. J Exp Zool, 290, 449-462.

Gross, R., Kohlmann, K., Kersten, P., 2002. PCR-RFLP analysis of the mitochondrial ND-3/4 and ND-5/6 gene polymorphisms in the European and East Asian subspecies of common carp (Cyprinus carpio L.). Aquaculture Research, 204, 507-516.

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). Biochem Biophys Res Commun, 272, 662-666.

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

Guo, Y., Cheng, H., Huang, X., Gao, S., Yu, H., Zhou, R., 2005. Gene structure, multiple alternative splicing, and expression in gonads of zebrafish Dmrt1. Biochem Biophys Res Commun, 330, 950-957.

Hamilton, W., 1967. Extraordinary sex ratios. Science, 156, 477–488.

Hanley, N.A., Hagan, D.M., Clement-Jones, M., Ball, S.G., Strachan, T., Salas-Cortes, L., McElreavey, K., Lindsay, S., Robson, S., Bullen, P., Ostrer, H., Wilson, D.I., 2000. SRY, SOX9, and DAX1 expression patterns during human sex determination and gonadal development. Mech Dev, 91, 403–407.

Harrington, R., 1967. Environmentally controlled induction of primary male gonochorists from eggs of the self-fertilizing hermaphroditic fish, Rivulus marmoratus. Poey. Biol. Bull., 132, 174-199.

Harrington, R., 1968. Delimination of the thermolabile phenocritical period if sex determination and differentation in the ontogeny of the normally hermaphoriditic fish, Rivulus marmoratus. Poey. Physiol. Zool., 41, 447-460.

Harris, J.H. & Gehrke, P.C., 1997. Fish and rivers in stress—the NSW rivers survey. Cronulla and Canberra, NSW Fisheries Office of Conservation and the Cooperative Research Centre for Freshwater Ecology. pp.298.

Harvey, S.C., Kwon, J.Y., Penman, D.J., 2003. Physical mapping of the brain and ovarian aromatase genes in the Nile Tilapia, Oreochromis niloticus, by fluorescence in situ hybridization. Animal Genetics, 34, 62-64.

Hatano, O., Takayama, K., Imai, T., Waterman, M.R., Takakusu, A., Omura, T., Morohashi, K., 1994. Sex-dependent expression of a transcription factor, Ad4BP, regulating steroidogenic P-450 genes in the gonads during prenatal and postnatal rat development. Development, 120, 2787-2797.

Hayes, T.B., 1998. Sex determination and primary sex mechanisms in amphibian: genetic and developmental mechanisms. J Exp Zool, 281, 373-399.

Honda, S., Harada, N., Abe-Dohmae, S., Takagi, Y., 1999. Identification of cis-acting elements in the proximal promoter region for brain-specific exon 1 of the mouse aromatase gene. Brain Res. Mol. Brain Res., 66, 122-132.

Honda, S., Harada, N., Takagi, N., 1994. Novel Exon 1 of the Aromatase Gene Specific for Aromatase Transcripts in Human Brain. Biochem Biophys Res Commun, 198, 1153-1160.

Horvàth, L. & Orbàn, L., 1995. Genome and gene manipulation in the common carp. Aquaculture, 129, 157-181.

Huang, X., Guo, Y., Shui, Y., Gao, S., Yu, H., Cheng, H., Zhou, R., 2005. Multiple alternative splicing and differential expression of dmrt1 during gonad transformation of the rice field eel. Biol Reprod, 73, 1017-1024.

Hubbs, C.L., 1955. Hybridisation between fish species in nature. Syst. Zool., 4, 1-20.

Hulata, G., 1995. A review of genetic improvement of the common carp (Cyprinus carpio L.) and other cyprinids by crossbreeding, hybridization and selection. Aquaculture, 129, 143-155.

Hume, D.J., Fletcher, A.R., Morison, A.K., 1983. Interspecific hybridization between carp (Cyprinus carpio L.) and golfish (Carassius auratus L.) from Victorian Waters. J. Mar. Fresh. Res., 34, 915-919.

IFS, 2004. Carp Management Program Report, Lakes Crescent and Sorell, Tasmania, Australia.

Ijiri, S., Kaneko, H., Kobayashi, T., Wang, D.-S., Sakai, F., Paul-Prasanth, B., Nakamura, M., Nagahama, Y., 2008. Sexual dimorphic expression of genes in gonads during early differentiation of a teleost fish, the Nile tilapia Oreochromis niloticus. Biol Reprod, 78, 333-341.

Ikeda, Y., Shen, W.H., Ingraham, H.A., Parker, K.L., 1994. Developmental expression of mouse steroidogenic factor-1, an essential regulator of the steroid hydroxylases. Mol Endocrinol, 8, 654-662.

Kailola, P.J., Williams, M.J., Stewart, P.C., Reichelt, R.E., McNee, A., Grieve, C., 1993. Australian Fisheries Resources. Bureau of Resource Sciences and Fisheries Research and Development Corporation, Canberra.

Kanai, Y.A. & Koopman, P., 1999. Structural and functional characterization of the mouse Sox9 promoter, implications for campomelic dysplasia. Hum. Mol. Genet., 8, 691–696.

Kazeto, Y., Ijiri, S., Place, A., Zohar, Y., Trant, J.M., 2001. The 5'-flanking regions of CYP19A1 and CYP19A2 in zebrafish. Biochem Biophys Res Commun, 288, 503-508.

King, A.J., Robertson, A.I. & Healey, M.R. , 1997. Experimental manipulations of the biomass of introduced carp (Cyprinus carpio) in billabongs. I. Impacts on water-column properties. Mar. Freshwater Res., 48, 435–443.

Kirpichnikov, V.S., 1981. Genetic bases of fish selection. Springer-Verlag, Berlin, pp. 410.

Kirpitchnikov, V.S., 1967. Homologous hereditary variation and evolution of wild common carp Cyprinus carpio L. Genetika (Moscow), 8, 65-72 (in Russian).

Kirpitchnikov, V.S., 1999. Genetics and breeding of common carp (Review by Billard, R., Repérant, J., Rio, J.P., Ward, R.), INRA, Paris.

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.

Klüver, N., Kondo, M., Herpin, A., Mitani, H., Schartl, M., 2005. Divergent expression patterns of Sox9 duplicates in teleosts indicate a lineage specific subfunctionalization. Dev. Genes Evol., 215, 297-305.

Koehn, J.D., 2004. Carp (Cyprinus carpio) as a powerful invader in Australian waterways. Freshwater Biol., 49, 882-894.

Koehn, J.D., Brumley, A., Gehrke, P., 2000. Managing the impacts of carp Bureau of Rural Sciences, Department of Agriculture, Fisheries and Forestry - Australia, Canberra.

Kohlmann, K., Gross, R., Murakaeva, A., Kersten, P., 2003. Genetic variability and structure of common carp (Cyprinus carpio) populations throughout the distribution range inferred from allozyme, microsatellite and mitochondrial DNA markers. Aquat. Living Resour. , 16, 421-431.

Kohlmann, K. & Kersten, P., 1999. Genetic variability of German and foreign common carp (Cyprinus carpio L.) populations. Aquaculture, 173, 435-445.

Komen, J., de Boer, P., Richter, C.J.J., 1992. Male sex reversal in gynogenetic XX females of common carp (Cyprinus carpio L.) by a recessive mutation in a sex-determining gene. J. Hered., 83, 431-434.

Komen, J., Duynhouwer, J., Richter, C.J.J., Huisman, E.A., 1988. Gynogenesis in common carp (Cyprinus carpio L.).1. Effects of genetic manipulation of sexual products and incubation conditions of eggs. Aquaculture, 69, 227-239.

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. Curr Biol, 13, 416-420.

Koopman, P., Schepers, G., Brenner, S., Venkatesh, B., 2004. Origin and diversity of the Sox transcription factor gene family: genome-wide analysis in Fugu rubripes. Gene, 328, 177-186.

Koren, Y., Sarid, S., Ber, R., Daniel, V., 1989. Carp growth hormone: molecular cloning and sequencing of cDNA. Gene, 77, 309-315.

Kosswig, C., 1964. Polygenic sex determination. Experientia, 20.

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

Larhammar, D. & Risinger, C., 1994. Molecular genetic aspects of tetraploidy in the common carp, Cyprinus carpio. Mol. Phylogenet. Evol., 3, 59-68.

Lavado, R., Thibaut, R., Raldúa, D., Martín, R., Porte, C., 2004. First evidence of endocrine disruption in feral carp from the Ebro River. Toxicol Appl Pharmacol, 196, 247-257.

Lee, B.Y., Hulata, G., Kocher, T.D., 2004. Two unlinked loci controlling the sex of blue tilapia (Oreochromis aureus). Heredity, 92, 543-549.

Li, Y., Hill, J.A., Yue, G.H., Chen, F., Orban, L., 2002. Extensive search does not identify genomic sex markers in Tetraodon nigroviridis. J. Fish Biol., 61, 1314-1317.

Loffler, K.A., Zarkower, D., Koopman, P., 2003. Etiology of Ovarian Failure in Blepharophimosis Ptosis Epicanthus Inversus Syndrome: FOXL2 Is a Conserved, Early-Acting Gene in Vertebrate Ovarian Development. Endocrinology, 144, 3237-3243.

Lutfalla, G., Crollius, H.R., 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. J Mol Evol, 57, S148-S153.

Lynch, J.P., Lala, D.S., Peluso, J.J., Luo, W., Parker, K.L., White, B.A., 1993. Steroidogenic factor 1, an orphan nuclear receptor, regulates the expression of the rat aromatase gene in gonadal tissues. Mol Endocrinol, 7, 776-786.

Mank, J.E., Promislow, D.E.L., Avise, J.C., 2006. Evolution of alternative sex-determining mechanisms in teleost fishes. Biol. J. Linn. Soc., 87, 83-93.

Manzoor Ali, P.K.M. & Satyanarayana Rao, G.P., 1989. Growth improvement in carp, Cyprinus carpio (Linnaeus), sterilized with 17alpha-methyltestosterone. Aquaculture, 76, 157-167.

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. BBA-Gene Struct. Expr., 1493, 180-187.

Matsuda, M., 2003. Sex determination in fish: Lessons from the sex-determining gene of the teleost medaka, Oryzias latipes. Develop. Growth Differ., 45, 397-403.

Matsuda, M., 2005. Sex determination in the teleost medaka, Oryzias latipes. Annu Rev Genet, 39, 293-307.

Matsuda, M., Nagahama, Y., Shinomiya, A., Sato, T., Matsuda, C., Kobayashi, T., Morrey, C.E., Shibata, N., Asakawa, S., Shimuzu, 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.

Mayer, L.P., Overstreet, S.L., Dyer, C.A., Propper, C.R., 2002. Sexually dimorphic expression of steroidogenic factor 1 (SF-1) in developing gonads of the American bullfrog, Rana catesbeiana. Gen Comp Endocrinol, 127, 40-47.

Miller, S.A. & Crowl, T.A., 2006. Effects of common carp (Cyprinus carpio) on macrophytes and invertebrate communities in a shallow lake. Freshwater Biol., 51, 85-94.

Moens, L.N., Smolders, R., van der Ven, K., van Remortel, P., Del-Favero, J., De Coen, W.M., 2007. Effluent impact assessment using microarray-based analysis in common carp: A systems toxicology approach. Chemosphere, 67, 2293-2304.

Moens, L.N., van der Ven, K., Van Remortel, P., Del-Favero, J., De Coen, W.M., 2006. Expression profiling of endocrine-disrupting compounds using a customized Cyprinus carpio cDNA microarray. Toxicol Sci, 93, 298-310.

Morrish, B.C. & Sinclair, A.H., 2002. Vertebrate sex determination: many means to an end. Reproduction, 124, 447-457.

Munday, P.L., Caley, M.J., Jones, G.P., 1998. Bi-directional sex change in a coral-dwelling goby. Behav. Ecol. Sociobiol., 43, 371-377.

Nagahama, Y., 2005. Molecular mechanisms of sex determination and gonadal sex differentiation in fish. Fish Physiol Biochem, 31, 105-109.

Nakamoto, M., Matsuda, M., Wang, D.-S., Nagahama, Y., Shibata, N., 2006. Molecular cloning and analysis of gonadal expression of Foxl2 in the medaka, Oryzias latipes. Biochem Biophys Res Commun, 344, 353-361.

Nakamura, M., Kobayashi, T., Chang, X.-T., Nagahama, Y., 1998. Gonadal sex differentiation in teleost fish. J Exp Zool, 281, 362-372.

Nakamura, M., Kobayashi, T., Yoshiura, Y., Nagahama, Y. 2000 Role of endogenous steroid hormones on gonadal sex differentation in fish. In Proceedings of the 6th International Symposium of the Reproductive Physiology of Fish (eds B. Norberg, O.S. Kjesbu, G.L. Taranger, E. Andersson & S.O. Stefansson), pp. 247-249, Bergen, Norway.

Nanda, I., Hornung, U., Kondo, M., Schmid, M., Schartl, M., 2003. Common spontaneous sex-reversed XX males of the medaka Oryzias latipes. Genetics, 163, 245-251.

Nanda, I., Kondo, M., Hornung, U., Asakawa, S., Winkler, C., Shimizu, A., Shan, Z., Haaf, T., Shimizu, N., Shima, A., Schmid, M., Schartl, M., 2002. A duplicated copy of DMRT1 in the sex-determining region of the Y chromosome of the medaka, Oryzias latipes. P. Natl. Acad. Sci. USA, 99, 11778-11783.

Nanda, I., Schartl, M., Feichtinger, W., Epplen, J.T., Schmid, M., 1992. Early stages of sex chromosome differentiation in fish as analysed by simple repetitive DNA sequences. Chromosoma, 101, 301-310.

Nanda, I., Shan, Z., Schartl, M., Burt, D.W., Koehler, M., Nothwang, H., Grutzner, F., Paton, I.R., Windsor, D., Dunn, I., Engel, W., Staeheli, P., Mizuno, S., Haaf, T., Schmid, M., 1999. 300 million years of conserved synteny between chicken Z and human chromosome 9. Nat Genet, 21, 258–259.

Ohno, S., Muramoto, J., Christian, L., Atkin, N.B., 1967. Diploid-tetraploid relationship among Old World members of the fish family Cyprinidae. Chromosoma (Berl.), 23, 1-9.

Ojima, J., Hitotsumachi, S., Makino, S., 1966. Cytogenetic studies in lower vertebrates. I: A preliminary report on the chromosomes of the funa (Carassius aurarus) and the goldfish. Proc. Jpn. Acad., 42, 62-66.

Oliver, A.S., 1991. Hermaphrodite sex hormones: in vitro secretion of steroid hormones from the ovotestis of the teleost Serranus subligarius. Am. Zool., 31, 67A.

Oliver, A.S., 1997. Size and density dependent mating tactics in the simultaneously hermaphroditic seabass Serranus subligarius (Cope, 1870). Behaviour, 134, 563–594.

Oshima, Y., Uno, Y., Matsuda, Y., Kobayashi, T., Nakamura, M., 2008. Molecular cloning and gene expression of Foxl2 in the frog Rana rugosa. Gen Comp Endocrinol, 159, 170-177.

Ottolenghi, C., Omari, S., Garcia-Ortiz, J.E., Uda, M., Crisponi, L., Forabosco, A., Pilia, G., Schlessinger, D., 2005. Foxl2 is required for commitment to ovary differentiation. Hum Mol Genet, 14, 2053-2062.

Paaver, T., 1983. Biochemical genetics of the common carp, Cyprinus carpio L. Valgus, Tallinn (in Russian).

Pannetier, M., Fabre, S., Batista, F., Kocer, A., Renault, L., Jolivet, G., Mandon-Pepin, B., Cotinot, C., Veitia, R., Pailhoux, E., 2006. FOXL2 activates P450 aromatase gene transcription: towards a better characterization of the early steps of mammalian ovarian development. J Mol Endocrinol, 36, 399-413.

Parkhurst, S. & Meneely, P., 1994. Sex determination and dosage compensation: lessons from flies and worms. Science, 264.

Patil, J., Davidson, S., Beyer, J., Thresher, R. 2004 Molecular cloning, and ontogenic and tissue expression of brain and ovarian P450 aromatase genes in the mosquitofish Gambrusia holbrooki. In 5th International Symposium on Fish Endocrinology, University Jaume I of Castellon, Spain.

Patino, R., Davis, K.B., Schoore, J.E., Uguz, C., Stussmann, C.A., Parker, N.C., Simco, B.A., Goudie, C.A., 1996. Sex differentiation of Channel catfish gonads: Normal development and effects of temperature. J Exp Zool, 276-218.

Penman, D.J. & Piferrer, F., 2008. Fish gonadogenesis. Part I: Genetic and environmental mechanisms of sex determination. Reviews in Fisheries Science, 16, 16 - 34.

Petersen, C.W., 1990. Variation in reproductive success and gonadal allocation in the simultaneous hermaphrodite Serranus fasciatus. Oecologia, 83.

Piferrer, F., 2001. Endocrine sex control strategies for the feminization of teleost fish. Aquaculture, 197, 229-281.

Piferrer, F. & Guiguen, Y., 2008. Fish gonadogenesis. Part II: Molecular biology and genomics of sex differentiation. Reviews in Fisheries Science, 16, 35 - 55.

Piferrer, F., Zanuy, S., Carrillo, M., Solar, I.I., Devlin, R.H., Donaldson, E.M., 1994. Brief treatment with an aromatase inhibitor during sex differentiation causes chromosomally female salmon to develop as normal, functional males. J Exp Zool, 270, 255-262.

Pisarska, M.D., Bae, J., Klein, C., Hsueh, A.J.W., 2004. Forkhead L2 is expressed in the ovary and represses the promoter activity of the steroidogenic acute regulatory gene. Endocrinology, 145, 3424-3433.

Postlethwait, J.H., Woods, I.G., Ngo-Hazelett, P., Yan, Y.-L., Kelly, P.D., Chu, F., Huang, H., Hill-Force, A., Talbot, W.S., 2000. Zebrafish comparative genomics and the origins of vertebrate chromosomes. Genome Res, 10, 1890-1902.

Pressley, P.H., 1981. Pair formation and joint territoriality in a simultaneous hermaphrodite: the coral reef fish Serranus tigrinus. . Z. Tierpsychol., 56, 33–46.

Price, D.J., 1984. Genetics of sex deterimination in fishes - A brief overview. In: G.W. Potts & R.J. Wootton (Eds.), Fish Reproduction, Academic Press, Plymouth, Devon. pp. 77-89.

Propst, D.L. & Gido, K.B., 2004. Responses of native and nonnative fishes to natural flow regime mimicry in the San Juan River. trans. Amer. Fish. Soc., 133, 922-931.

Ramachandran, B., Schlinger, B.A., Arnold, A.P., Campagnoni, A.T., 1999. Zebra finch aromatase gene expression is regulated in the brain through an alternate promoter. Gene, 240, 209-216.

Ramsey, M., Shoemaker, C., Crews, D., 2007. Gonadal expression of Sf1 and aromatase during sex determination in the red-eared slider turtle (Trachemys scripta), a reptile with temperature-dependent sex determination. Differentiation, 75, 978-991.

Raymond, C.S., Shamu, C.E., Shen, M.M., Seifert, K.J., Hirsch, B., Hodgkin, J., Zarkower, D., 1998. Evidence for evolutionary conservation of sex-determining genes. Nature, 391, 691-695.

Reid, D.D., Harris, J.H., Chapman, D.J., 1997. NSW Inland Commercial Fishery Data Analysis. FRDC Project No. 94/027.

Roberts, J., Chick, A., Oswald, L., Thompson, P., 1995. Effect of carp, Cyprinus carpio L., an exotic benthivorous fish, on aquatic plants and water quality in experimental ponds. Mar. Freshwater Res., 46, 1171-1180.

Roberts, J. & Tilzey, R., 1996. Controlling carp. Exploring the options for Australia. In: (Eds.), CSIRO Land and Water, Griffith. pp. 129.

Ross, R.M., 1990. The evolution of sex-change mechanisms in fishes. Env. Biol. Fishes, 29, 81-93.

Rubin, D.A., 1985. Effect of pH on sex ratio in cichlids and a poecillid (Teleostei). Copeia, 233-235.

Sadovy, Y. & Shapiro, D.Y., 1987. Criteria for the diagnosis of hermaphroditism in fishes. Copeia, 1, 136-156.

Schartl, M., 2004. A comparative view on sex determination in medaka. Mech Dev, 121, 639-645.

Schmidt, D., Ovitt, C.E., Anlag, K., Fehsenfeld, S., Gredsted, L., Treier, A.-C., Treier, M., 2004. The murine winged-helix transcription factor Foxl2 is required for granulosa cell differentiation and ovary maintenance. Development, 131, 933-942.

Schultheis, C., Zhou, Q., Froschauer, A., Nanda, I., Selz, Y., Schmidt, C., Matschl, S., Wenning, M., Veith, A.-M., Naciri, M., Hanel, R., Braasch, I., Dettai, A., Bohne, A., Ozouf-Costaz, C., Chilmonczyk, S., Segurens, B., Couloux, A., Bernard-Samain, S., Schmid, M., Schartl, M., Volff, J.-N., 2006. Molecular analysis of the sex-determining region of the Platyfish Xiphophorus maculatus. Zebrafish, 3, 299-309.

Shang, E.H.H., Yu, R.M.K., Wu, R.S.S., 2006. Hypoxia affects sex differentiation and development, leading to a male-dominated population in Zebrafish (Danio rerio). Environ Sci Technol, 40, 3118-3122.

Shapiro, D.Y. & Rasotto, M.B., 2009. Sex differentiation and gonadal development in the diandric, protogynous wrasse, Thalassoma bifasciatum (Pisces, Labridae). J. Zool., 230, 231– 245.

Shearer, K.D. & Mulley, J.C., 1978. The introduction and distribution of the carp, Cyprinus carpio Linnaeus, in Australia. Mar. Freshwater Res., 29, 551-563.

Shields, J.T., 1958. Experimental control of carp reproduction through water drawdowns in Fort Randall reservoir, South Dakota. Trans. Amer. Fish. Soc., 87, 23-33.

Sinclair, A., Smith, C., Western, P., McClive, P. 2002 A comparative analysis of vertebrate sex determination. In The Genetics and Biology of Sex Determination (ed D. Chadwick, Goode, J.), Vol. 244, pp. 102-114. Novartis Foundation.

Sinclair, A.H., Berta, P., Palmer, M.S., Hawkins, J.R., Griffiths, B.L., Smith, M.J., Foster, J.W., Frischauf, A.-M., Lovell-Badge, R., Goodfellow, P.N., 1990. A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. Nature, 346, 240-245.

Sivakumaran, K.P., Brown, P., Stoessel, D., Giles, A., 2003. Maturation and Reproductive Biology of Female Wild Carp, Cyprinus carpio, in Victoria, Australia. Environ. Biol. Fish., 68, 321-332.

Smith, B.B. & Walker, K.F., 2004. Spawning dynamics of common carp in the River Murray, South Australia, shown by macroscopic and histological staging of gonads. J. Fish Biol., 64, 336-354.

Smith, C.A., McClive, P.J., Western, P.S., Reed, K.J., Sinclair, A.H., 1999a. Evolution: Conservation of a sex-determining gene. Nature, 402, 601-602.

Smith, C.A., Smith, M.J., Sinclair, A.H., 1999b. Expression of Chicken Steroidogenic Factor-1 during Gonadal Sex Differentiation. Gen Comp Endocrinol, 113, 187-196.

Solari, A.J., 1994. Sex chromosomes and sex determination in fishes. In: A.J. Solari (Eds.), Sex chromosomes and sex determination in vertebrates, Boca Raton; CRC Press. pp.

Spotila, L.D., Spotila, J.R., Hall, S.E., 1998. Sequence and expression analysis of WT1 and Sox9 in the red-eared slider turtle, Trachemys scripta. J Exp Zool, 281, 417-427.

St. Mary, C.M., 1998. Characteristic gonad structure in the gobiid genus Lythrypnus with comparisons to other hermaphroditic gobies. Copeia, 1998, 720– 724.

Strüssmann, C.A., Moriyama, S., Hanke, E.F., Calsina Cota, J.C., Takashima, F., 1996a. Evidence of thermolabile sex determination in pejerrey. J. Fish Biol., 48, 643-651.

Strüssmann, C.A. & Nakamura, M., 2002. Morphology, endocrinology, and environmental modulation of gonadal sex differentiation in teleost fishes. Fish Physiol Biochem, 26, 13-29.

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

Stuart, I.G. & Jones, M., 2006. Large, regulated forest floodplain is an ideal recruitment zone for non-native common carp (Cyprinus carpio L.). Mar. Freshwater Res., 57, 337–347.

Stuart, I.G., Williams, A., McKenzie, J., Holt, T., 2006. Managing a migratory pest species: a selective trap for common carp. N. AM. J. Fish. Manage., 26, 888-893.

Swain, A. & Lovell-Badge, R., 1999. Mammalian sex determination: a molecular drama. Genes Dev, 13, 755-767.

Takahashi, H., 1977. Juvenile hermaphroditism in the zebrafish, Brachydanio rerio. Bull. Fac. Fish. Hokkaido Univ., 28, 57-65.

Takamatsu, N., Kanda, H., Ito, M., Yamashita, A., Yamashita, S., Shiba, T., 1997. Rainbow trout SOX9: cDNA cloning, gene structure and expression. Gene, 202, 167-170.

Takase, M., Noguchi, S., Nakamura, M., 2000. Two Sox9 messenger RNA isoforms: Isolation of cDNAs and their expression during gonadal development in the frog Rana rugosa. FEBS Lett, 466.

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). J. Biochem-Tokyo, 117, 719-725.

Taylor, J.S., Braasch, I., Frickey, T., Meyer, A., Van de Peer, Y., 2003. Genome duplication, a trait shared by 22,000 species of ray-finned fish. Genome Res, 13, 382-390.

Taylor, R.M., Pegg, M.A., Chick, J.H., 2005. Response of bighead carp to a bioacoustic behavioural fish guidance system. Fisheries Manag. Ecol., 12, 283-286.

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. J. Steroid Biochem., 78, 427-439.

Thorgaard, G.H., 1977. Heteromorphic sex chromosomes in male rainbow trout. Science, 196, 900-902.

Tong, S.-K. & Chung, B.-c., 2003. Analysis of zebrafish cyp19 promoters. J. Steroid Biochem., 86, 381-386.

Trant, J.M., Gavassa, S., Ackers, J., Chung, B.-C., Place, A.R., 2001. Developmental expression of cytochrome P450 aromatase genes (CYP19a and CYP19b) in zebrafish fry (Danio rerio). J Exp Zool, 290, 475-483.

Tzchori, I., Zak, T., Sachs, O., 2004. Masculinization of genetic females of the common carp (Cyprinus carpio L.) by dietary administration of an aromatase inhibitor. Israeli Journal of Aquaculture-Bamidgeh, 56, 239-246.

Uda, M., Ottolenghi, C., Crisponi, L., Garcia, J.E., Deiana, M., Kimber, W., Forabosco, A., Cao, A., Schlessinger, D., Pilia, G., 2004. Foxl2 disruption causes mouse ovarian failure by pervasive blockage of follicle development. Hum Mol Genet, 13, 1171-1181.

Ueno, K., Ota, K., Kobayashi, T., 2001. Heteromorphic sex chromosomes of lizardfish (Synodontidae): focus on the ZZ-ZW1W2 system in Trachinocephalus myops. Genetica, 111, 133-142.

van Nes, S., Moe, M., Andersen, O., 2005. Molecular characterization and expression of two cyp19 (P450 aromatase) genes in embryos, larvae and adults of Atlantic halibut (Hippoglossus hippoglossus). Mol Reprod Dev, 72, 437-449.

Vizziano-Cantonnet, D., Baron, D., Mahe, S., Cauty, C., Fostier, A., Guiguen, Y., 2008. Estrogen treatment up-regulates female genes but does not suppress all

early testicular markers during rainbow trout male-to-female gonadal trans-differentiation. J Mol Endocrinol, 41, 277-288.

Vizziano, D., Randuineau, G., Baron, D., Cauty, C., Guiguen, Y., 2007. Characterization of early molecular sex differentiation in rainbow trout, Oncorhynchus mykiss. Dev. Dynam., 236, 2198-2206.

Volff, J.-N., Körting, C., Froschauer, A., Zhou, Q., Wilde, B., Schultheis, C., Selz, Y., Sweeney, K., Duschl, J., Wichert, K., Altschmied, J., Schartl, M., 2003. The xmrk oncogene can escape nonfunctionalization in a highly unstable subtelomeric region of the genome of the fish xiphophorus. Genomics, 82, 470-479.

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

von Hofsten, J. & Olsson, P.-E., 2005. Zebrafish sex determination and differentiation: Involvement of FTZ-F1 genes. Reprod. Biol. Endocrin., 3, 63.

Werren, J.H., Skinner, S.W., Charnov, E.L., 1981. Paternal inheritance of a daughterless sex ration factor. Nature, 293, 467-468.

Western, P.S., Harry, J.L., Graves, J.A.M., Sinclair, A.H., 1999. Temperature-dependent sex determination: Upregulation of SOX9 expression after commitment to male development. Dev. Dynam., 214, 171-177.

Western, P.S., Harry, J.L., Graves, J.A.M., Sinclair, A.H., 2000. Temperature-dependent sex determination in the American alligator: expression of SF1, WT1 and DAX1 during gonadogenesis. Gene, 241, 223-232.

Wilkins, A.S., 1995. Moving up the hierarchy: a hypothesis on the evolution of a genetic sex determination pathway. BioEssays, 17, 71-77.

Wright, E., Hargrave, M.R., Christiansen, J., Cooper, L., Kun, J., Evans, T., Gangadharan, U., Greenfield, A., Koopman, P., 1995. The Sry-related gene Sox9 is expressed during chondrogenesis in mouse embryos. Nat Genet, 9, 15-20.

Wu, C., Chen, Y.R., Liu, X., 1993. An artificial multiple triploid carp and its biological characteristics. Aquaculture, 111, 255–262.

Yamaguchi, A., Lee, K.H., Fujimoto, H., Kadomura, K., Yasumoto, S., Matsuyama, M., 2006. Expression of the DMRT gene and its roles in early gonadal development of the Japanese pufferfish Takifugu rubripes. Comp. Biochem. Phys. D, 1, 59-68.

Yamaguchi, T., Yamaguchi, S., Hirai, T., Kitano, T., 2007. Follicle-stimulating hormone signaling and Foxl2 are involved in transcriptional regulation of aromatase gene during gonadal sex differentiation in Japanese flounder, Paralichthys olivaceus. Biochem Biophys Res Commun, 359, 935-940.

Yamamoto, T., 1969. Sex differentiation. In: W.S. Hoar & D.J. Randall (Eds.), Fish Physiology: Reproduction and growth, bioluminescence, pigments and poisons, Vol. III, Academic Press, New York. pp. 117-175.

Yan, Y.L., Miller, C.T., Nissen, R.M., Singer, A., Liu, D., Kirn, A., Draper, B., Willoughby, J., Morcos, P.A., Amsterdam, A., Chung, B.C., Westerfield, M., Haffter, P., Hopkins, N., Kimmel, C., Postlethwait, J.H., Nissen, R., 2002. A

zebrafish Sox9 gene required for cartilage morphogenesis. Development, 129, 5065–5079.

Yao, H.H.-C., 2005. The pathway to femaleness: current knowledge on embryonic development of the ovary. Mol Cell Endocrinol, 230, 87-93.

Yokoi, H., Kobayashi, T., Tanaka, M., Nagahama, Y., Wakamatsu, Y., Takeda, H., Araki, K., Morohashi, K., Ozato, K., 2002. Sox9 in a teleost fish, medaka (Oryzias latipes): Evidence for diversified function of Sox9 in gonad differentiation. Mol Reprod Dev, 63, 5-16.

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 interaction1. Biol Reprod, 68, 1545-1553.

Zambrano, L., Martínez-Meyer, E., Menezes, N., Townsend Peterson, A., 2006. Invasive potential of common carp (Cyprinus carpio) and Nile tilapia (Oreochromis niloticus) in American freshwater systems. Can. J. Fish. Aquat. Sci., 63, 1903-1910.

Zarkower, D., 2001. Establishing sexual dimorphism: Conservation amidst diversity? Nat Rev Genet, 2, 175-185.

Zhang, H., Okamoto, N., Ikeda, Y., 1995. Two c-myc genes from a tetraploid fish, the common carp (Cyprinus carpio). Gene, 153, 231-236.

Zhou, R., Liu, L., Guo, Y., Yu, H., Cheng, H., Huang, X., Tiersch, T.R., Berta, P., 2003. Similar gene structure of two Sox9a genes and their expression patterns during gonadal differentiation in a teleost fish, rice field eel (Monopterus albus). Mol Reprod Dev, 66, 211-217.

CHAPTER 2

General Materials and Methods

Chapter 2 - General Materials and Methods

2.1 Fish

2.1.1 Source

Fish were sourced from both Tasmania and Victoria. In Tasmania, fish were collected from Lakes Crescent and Sorell (42° 15' S, 147° 17' E) and supplied by the Inland Fisheries Service Tasmania after collection as part of their ongoing eradication program. In Victoria, mature adults were collected from the Avon River, approximately 21 km from the aquaculture facility in (38° 01'08 S, 147° 12' E) and transported with the assistance of K&C Fisheries. The 'Boolara' strain of common carp is known to inhabit both these areas (Shearer and Mulley, 1978).

2.1.2 Fish breeding and larval rearing

Breeding of wild caught fish and larval rearing was carried out at an aquaculture facility (K&C Fisheries, Sale, Victoria, Australia). Fish were taken from the Avon River, where the water temperature was ~26 °C and transported in 1000 L tanks with aeration. Eggs were stripped from Ovaprim (Syndel Laboratories Inc.) induced fish using previously established protocols (Patil et al., unpublished). Eggs from 2-3 females were covered with milt from 2-3 males and water (~23 °C) was added to initiate fertilisation and chorion hardening. After 10-15 min, grasses were run through the egg mixture to allow adhesion of the eggs. Egg covered grasses were then randomly allocated to pre-prepared 1000 L rearing tanks maintained at either 20 or 25 °C.

2.1.3 Sample collection

2.1.3.1 Samples from adult fish

Samples of brain, pituitary, gonad, visceral fat, liver, eye and optic nerve were collected from 3 male and 3 female wild caught adult common carp, *Cyprinus carpio*, (1.37 kg ± 0.25) from Lake Sorell, Tasmania, Australia. Fish were anaesthetised with a fatal dose of 2-phenoxyethanol and killed with a sharp blow to the head. Tissues were immediately removed by dissection, weighed and stored in RNAlater (QIAGEN Pty. Ltd.) according to manufacturer's instructions.

2.1.3.2 Sampling of developmental stages

For embryonic analysis 30 unfertilised eggs or embryos were sampled every 5 h until hatch, after which the sample size was reduced to 15 larvae at 10 h intervals until day 7. Sampling continued at time points corresponding to different stages of gonadal development identified by Beyer (2004) with the expected period of molecular sex differentiation occurring between day 40 and 70. The term degree days post hatch (ddph) was used to take into account the affect of temperature and age when analysing expression levels of post-hatch (ddph) aromatase (Chapter 5), and post-fertilisation (ddpf) levels of *foxL2* (Chapter 6), *dmrt1* (Chapter 7) and *Sox9* genes (Chapter 8). The term degree day (day x °C) allows correct scaling for the physiology that drives exothermic growth and development as these factors are proportional to the time spent at temperature (Neuheimer and Taggart, 2007).

Samples were stored in RNAlater (QIAGEN Pty. Ltd.) at 4 °C until transported back to the laboratory. At each sampling point the total length (TL) was recorded.

All animal experiments were approved by the Animal Ethics Committee at the University of Tasmania (Approval A0008839).

2.2 Molecular Biology

2.2.1 Oligonucleotides

Oligonucleotide primers were designed using Primer Express software (Applied Biosystems) and synthesised by Sigma-Proligo (Sigma-Aldrich Pty. Ltd.) See specific chapters for primer details.

2.2.2 RNA extraction, reverse transcription and cloning of cDNA

Total RNA was extracted from ovary and brain using TRIzol (Invitrogen Australia Pty. Ltd.) according to the manufacturer's instructions. Gel electrophoresis and spectrophotometry were used to determine RNA quality and quantity.

The common carp genes of interest were amplified, cloned and sequenced using rapid amplification of cDNA ends (RACE) using the BD SMART RACE cDNA Amplification Kit (Clontech, Takara Bio, Japan) according to the manufacturer's instructions. Confirmation of the 5'- end of the cloned genes was carried out by nested 5'-RACE with gene specific primers (GSP) and Universal Primer Mix (UPM) (primary PCR) and nested gene specific primers (NGSP) and Nested Universal Primer (NUP) (nested PCR). Both primary and nested PCR reactions consisted of a master mix containing a final concentration of 1X BD Advantage 2 PCR buffer, 0.2 mM dNTP mix and 1X BD Advantage 2 Polymerase Mix. To each master mix for primary PCR, 2.5 μL of first-stand cDNA was added with 1X UPM and 1 μM GSP in a final volume of 50 μL. The 25 cycle amplification procedure consisted of cycling at 94 °C for 30 s, 68 °C for 30 s and 72 °C for 3 min. Once complete, 5 μL of the primary PCR product was diluted into 245 μL of Tricine-EDTA buffer for nested PCR. The nested PCR reaction consisted of the above master mix with 5 μl of the

diluted primary PCR product and 1 μM of both primers NGSP and NUP to replace first-strand cDNA and primary PCR primers. The amplification procedure for nested PCR was 20 cycles with cycling conditions as described above.

The fragments amplified by 5'- and 3'-RACE were cloned, purified and sequenced to identify cloning of the desired gene. Clones containing the appropriate fragments were then purified and ligated using Quick Ligation Kit (New England Biolabs Inc.) into pCR®2.1-TOPO® cloning vector (Invitrogen Australia Pty. Ltd.). Successful ligation was initially determined by restriction enzyme digestion followed by purification and sequencing.

2.2.3 Sequencing and phylogenetic analysis

Sequencing of cDNA was done on both strands of double-stranded DNA using the BigDye TerminatorV3.1 Sequencing Kit (Applied Biosystems) and DyeEx 2.0 Spin Kit for Dye-Terminator Removal (QIAGEN Pty. Ltd.) according to manufacturer's instructions, followed by automated sequencing (ABI PRISM 3100 Genetic Analyzer, Applied Biosystems). Complete sequencing was carried out with universal primers M13F and M13R (Sigma-Proligo), complimentary to the vector, and sequential primers when necessary for complete sequence coverage. Nucleotide sequences were analysed using SeqManII, Lasergene 7 (DNASTAR Inc.). Deduced amino acid sequences for deduced common carp genes were aligned by ClustalW algorithm (Thompson et al., 1994) together with respective sequences reported for other teleosts and higher vertebrates. Molecular phylogenetic analysis was performed using Neighbor-Joining method (Saitou and Nei, 1987) with 5000 bootstraps in MEGA version 3.1 (Kumar et al., 2004).

2.2.4 Expression analysis

2.2.4.1 Sample preparation and RNA extraction of adult tissues

Adults selected for expression analysis were sexually mature, according to gonad somatic index evaluation and condition of the gonad (Gupta, 1975; Jhingran and Pullin, 1988; Smith and Walker, 2004). All RNA extractions were carried out with kits from QIAGEN Pty. Ltd. Total RNA was extracted using the RNeasy Lipid Kit according to manufacturer's instructions. To process larger samples (~1 g) the RNeasy Mini Lipid protocol was scaled up by proportionally increasing the volume of QIAzol Lysis Buffer and chloroform for use with the Maxi Kit columns.

Tissues were prepared differently for homogenisation depending on the nature of the tissue. For eye tissue, much of the fatty tissue surrounding the eye was surgically removed, followed by dissection of the eye in two sections, the outer corneal half (cornea and lens) (outer eye) and the inner retinal-half (inner eye). Approximately ~1 g of tissue from each eye section was used for RNA extraction. For all other tissues the following masses were used; brain ~100 mg, liver ~30 mg and other tissues ~50 mg. To examine spatial expression within the brain, each was dissected into four sections (A-D) (Figure 2.1). Sections of brain that were larger than 100 mg were separated into two tubes for homogenisation and extraction, with the final eluates being combined. For smaller sections of the brain and the pituitary, as much tissue as possible was taken.

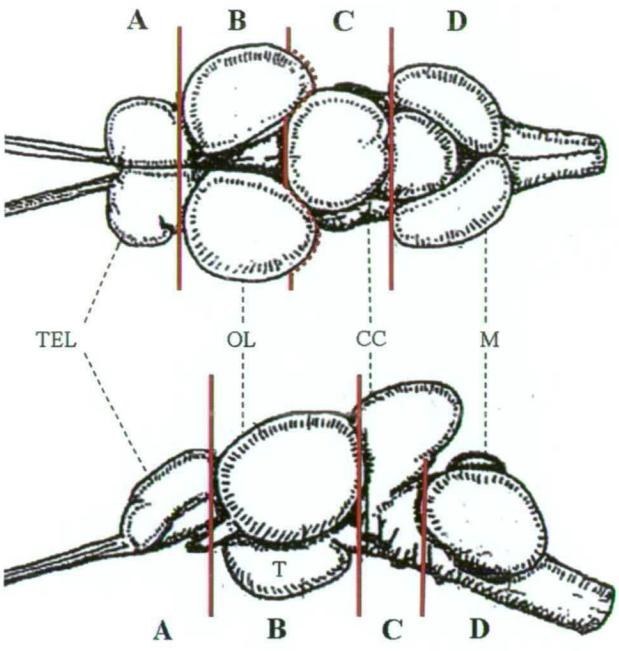


Figure 2.1 Dorsal and lateral view of common carp brain sections used for RNA extraction. Sections incorporate; A: Telencephalon (TEL); B: Optic Lobe (OL), Thalamus/ Hypothalamus (T); C: Cerebellum (CC); D: Medulla (M) (adapted from Kotrschal et al., 1991).

Homogenisation of tissues was carried out with a Tissuelyser (QIAGEN Pty. Ltd.) at 30 Hz using 2 cycles of 3 min, checking progress of homogenisation and alternating tube position after the first cycle. For the eye, each section was homogenised with 10 mL of QIAzol Lysis Buffer (QIAGEN Pty. Ltd.) in the Tissuelyser 10 mL grinding jars with 20 mm stainless steel ball. The homogenate was then transferred by pipette to a 50 mL tube for further processing. For all other tissues the appropriate mass was homogenised in 1 mL of Lysis Buffer. For fibrous tissues (optic nerve and testis) 5 mm steel balls were used for complete homogenisation. Subsequent steps followed the manufacturer's instructions with the use of RNase-Free DNase set (QAIGEN Pty. Ltd.). Gel electrophoresis and spectrophotometry were used to determine RNA quality and quantity.

Synthesis of cDNA was carried out using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) with the addition of RNase Inhibitor

(Applied Biosystems) according to manufacturer's instructions. An equal volume of RNA (~1 μg) was added to each 10 μL aliquot of master mix. A thermocycler program consisted of 25 °C for 10 min, 37 °C for 120 min, 85 °C for 5 s and final holding step at 4 °C. Resulting cDNA samples were quantified by spectrophotometer and stored at -20 °C until use.

2.2.4.2 Sample preparation and RNA extraction of development stages

Samples of embryological stages were pooled (n=5) for RNA extraction, hatched larvae were processed individually. RNA extraction was carried out using the RNeasy Lipid Mini Kit using 5 mm steel balls for homogenising samples with the Tissuelyser as previously described. The High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used for cDNA synthesis using ~1 μg of RNA.

2.2.4.3 Real-time assay

Real-time PCR was carried out on a 7500 Real Time PCR Machine (Applied Biosystems) with SYBR Green fluorescent label using *β-actin* as an endogenous control (Patil and Gunasekera, 2008, Sawyer et al., 2006; van Nes et al., 2005). Gene specific primers were designed for each isoform (see specific chapters) and tested for specificity and efficiency. For aromatase and *sox9* genes specificity of primers was tested by both standard- and real-time PCR by pairing both primer combinations with both cloned isoforms as templates. A melting curve analysis was performed for all genes to check specificity and identity of real-time PCR products. As PCR amplification efficiencies can vary between tissues (due to inhibitors etc.), the optimum efficiency was determined for each primer pair on its respective tissue (ovary- *cyp19a* and *foxL2*; brain- *cyp19b*; testis; *dmrt1* and *sox9* genes). The efficiency values for each primer pair and the average efficiency value for *β-actin*

(from amplification in brain and gonad) were used in subsequent analysis. These values can be found in the specific chapters. Prior to quantification, RNA samples were analysed for possible genomic DNA contamination. Samples were considered genomic DNA-free when RNase digested aliquots failed to amplify following Real Time PCR using *β-actin* primers. Any DNA contaminated RNA samples were subject to a second, on-column DNase treatment to remove traces of genomic DNA. For each embryological stage, three pooled samples were analysed and for hatchlings up to eight (8) individuals at each time point were analysed.

Real-time analysis was carried out with a final reaction volume of 25 µL; using 50-56 ng/µL cDNA, 1x SYBR® Green PCR Master Mix (Applied Biosystems) and primers at a final concentration of 100 nM. Real-time assays were carried out on separate plates for each tissue type, where possible, to minimise technical variability. Each sample was run in triplicate for each of the three genes of interest. Cycling parameters were as follows: 50 °C for 2 min, 95 °C for 10 min, then 40 cycles of 95 °C of 5 s and 60 °C for 1 min. For embryonic samples in Chapter 3, the PCR program was extended to 45 cycles to allow detection of small levels of expression. Dissociation was carried out by one cycle of 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s.

2.2.5 Data analysis

Real-time PCR data were compiled and collected using SDS 3.1.1 software (Applied Biosystems). Cycle threshold (C_T) was calculated automatically using the SDS software with a manual baseline set at 3-15 cycles. Acceptability of each triplicate reaction was set at C_T standard error (SE) of 0.7, as calculated by the SDS software. For samples that failed this test, triplicate data was analysed for an outlier or the

sample was repeated. Data was analysed using *Qgene*, a publicly available Exel script package (Muller et al., 2002), using the efficiency values determined earlier for each of the genes. Data from each sample was averaged and shown as mean normalised gene expression (MNE) ± SE. Statistical analysis was performed using SPSS 11.0.0 (SPSS Inc., Chicago, 2001). Data were first tested for normality, square root transformed to normalise where necessary and subsequently analysed by univariate analysis of variance (ANOVA) followed by the Student-Newman-Kuels (SNK) method for pairwise multiple comparisons. Student's *t* test was used to compare expression of genes within a particular tissue and sex-specific differences of genes in respective tissues. Significance was set at $P < 0.05$.

2.3 References

Beyer, J., 2004. Early gonad development in the common carp, *Cyprinus carpio (L.)* at 20 °C and 25 °C. Honours Thesis, University of Tasmania, Launceston.

Gupta, S., 1975. The development of carp gonads in warm water aquaria. J. Fish Biol., 7, 775-782.

Jhingran, V.G. & Pullin, R.S.V., 1988. A hatchery manual for the Common, Chinese and Indian Major carps. Asian Development Bank and ICLARM, Manila.

Kotrschal, K., Brandstatter, R., Gomahr, A., Junger, H., Palzenberger, M., Zaunreiter, M., 1991. Brain and sensory systems. In: I.J. Winfeild & J.S. Nelson (Eds.), Cyprinid Fishes: Systematics, biology and exploitation, Chapman and Hall, London. pp. 284-331.

Kumar, S., Tamura, K., Nei, M., 2004. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Briefings in Bioinformatics, 5, 150-163.

Muller, P.Y., Janovjak, H., Miserez, A.R., Dobbie, Z., 2002. Processing of gene expression data generated by quantitative real-time RT-PCR. BioTechniques, 32, 1372-1379.

Neuheimer, A.B. & Taggart, C.T., 2007. The growing degree-day and fish size-at-age: the overlooked metric. Can. J. Fish. Aquat. Sci., 64, 375-385.

Patil, J.G. & Gunasekera, R.M., 2008. Tissue and sexually dimorphic expression of ovarian and brain aromatase mRNA in the Japanese medaka (Oryzias latipes): Implications for their preferential roles in ovarian and neural differentiation and development. Gen Comp Endocrinol, 158, 131-137.

Saitou, N. & Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol, 4, 406-425.

Sawyer, S., 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. Gen Comp Endocrinol, 147, 108-117.

Shearer, K.D. & Mulley, J.C., 1978. The introduction and distribution of the carp, *Cyprinus carpio* Linnaeus, in Australia. Mar. Freshwater Res., 29, 551-563.

Smith, B.B. & Walker, K.F., 2004. Spawning dynamics of common carp in the River Murray, South Australia, shown by macroscopic and histological staging of gonads. J. Fish Biol., 64, 336-354.

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

van Nes, S., Moe, M., Andersen, O., 2005. Molecular characterization and expression of two *cyp*19 (P450 aromatase) genes in embryos, larvae and adults of Atlantic halibut (*Hippoglossus hippoglossus)*. Mol Reprod Dev, 72, 437-449.

CHAPTER 3

Distinct cytochrome P450 Aromatase isoforms in the common carp (*Cyprinus carpio*): Sexual dimorphism and onset of ontogenic expression

Adapted from Barney, M.L., Patil, J.G., Gunasekera, R.M. and Carter, C.G. 2008. Distinct cytochrome P450 aromatase isoforms in the common carp (*Cyprinus carpio*): Sexual dimorphism and onset of ontogenetic expression. Gen. Comp. Endocrinol., 156 (3), 499-508.

Chapter 3 - Distinct cytochrome P450 Aromatase isoforms in the common carp (Cyprinus carpio): Sexual dimorphism and onset of ontogenic expression

3.1 Abstract

Cytochrome P450 aromatase (CYP19) is a key enzyme in the steroidogenic pathway that catalyses the conversion of testosterone to estrogen, and therefore is thought to influence gonadal sex differentiation. In an effort to understand the role of this enzyme in ovarian differentiation, cDNA was isolated encoding the two distinct isoforms, ovarian and brain (termed *cyp19a* and *cyp19b* respectively) of adult common carp, *Cyprinus carpio*. The cloned cDNA for *cyp19a* had an open reading frame (ORF) of 518 amino acid residues, in contrast to *cyp19b* with an ORF of 511 amino acids. Sequence and phylogenetic analysis showed that these CYP19 isoforms were orthologous with previously described *cyp19a* and *cyp19b* from other teleosts. Quantitative real-time PCR indicated that both isoforms are expressed in adult ovary and brain, with predominant expression of *cyp19a* in the ovary and *cyp19b* in the brain. The major aromatase expressing tissue was found to be the brain, with greatest *cyp19b* expression in the anterior quarter (telencephalon) in both sexes. The gonad showed sexually dimorphic expression of both genes and dimorphic expression of *cyp19a* in the cerebellum and the liver. Ontogenic expression showed that only the ovarian aromatase transcript is inherited maternally, with lower expression observed through early larval development under warmer rearing conditions. The differential and overlapping expression suggests these two aromatase genes have different roles in reproductive physiology.

3.2 Introduction

The hormonal balance between estrogens and androgens is crucial to sex differentiation in teleost fish. It relies on availability and activity of steroid synthesising enzymes, in particular the cytochrome P450 aromatase complex (P450arom). Biosynthesis of estrogens occurs in all classes of vertebrates (Simpson et al., 2002) and the importance of P450arom has been shown in many teleosts, with genetically female fish developing into phenotypic males when aromatase activity is inhibited during development (e.g. Tzchori et al., 2004). Although estrogen is generally regarded as a circulating hormone derived from the gonads, P450arom is expressed in the brain and other estrogenic tissues, where it regulates the spatio-temporal quantity of estrogen available for activating steroidogenic pathways (McEwen and Alves, 1999).

Most higher vertebrates have a single *cyp19* gene with multiple promoter elements that regulate tissue-specific expression with aromatase expression occurring in both gonads and in the brain (reviewed by Simpson et al., 2002). However, in teleosts, gonadal and neural expression of *cyp19* is regulated by two distinct aromatase isoforms (*cyp19a* and *cyp19b*; ovary and brain isoforms) (Goto-Kazeto et al., 2004). These isozymes have different catalytic activity (Zhao et al., 2001) and distinct or partially overlapping expression patterns are reported in other teleost species, including members of Cypriniformes - zebrafish, *Danio rerio* (Kishida and Callard, 2001; Trant et al., 2001) and goldfish, *Carassius auratus* (Gelinas et al., 1998; Tchoudakova and Callard, 1998). Menuet et al. (2005) identified brain aromatase as a critical gene whose disruption is likely to affect the functioning of the whole reproductive axis. Generally, sexual determination is under weak genetic control in

most fish, with sexual differentiation largely influenced by hormonal and/or environmental cues.

Biochemical pathways involving many different proteins (e.g. transcriptional factors, steroidogenic enzymes, receptors, etc.) control sex differentiation and reproduction in fishes. The structure and function of such proteins and other macromolecules are dramatically influenced by temperature. Therefore, temperature fluctuations as are encountered by fish in natural habitats could alter sex determination and differentiation pathways and influence resulting sex ratios (Devlin and Nagahama, 2002). Higher temperature was suggested to cause suppression of P450arom expression resulting in a skew toward male development in Japanese flounder, *Paralichthys olivaceus* (Kitano et al., 1999) and Nile tilapia, *Oreochromis niloticus* (D'Cotta et al., 2001). It is anticipated that temperature would have variable effects on sex differentiation depending on the genetic background and developmental stability of different species and strains (Devlin and Nagahama, 2002). In zebrafish temperature influenced phenotypic sex through stress on various cell types, resulting in oocyte apoptosis and thereby promoting testicular formation (Uchida et al., 2002). As well as being an important food fish , common carp are often used as a sentinel species in studies investigating potential alterations to the function of the endocrine system of humans and wildlife as a result of chemical exposure (Lavado et al., 2004; Moens et al., 2007; Moens et al., 2006). Such studies frequently use aromatase activity as a measure of endocrine disruption (Greytak et al., 2005; Hinfray et al., 2006; Lavado et al., 2004; Villeneuve et al., 2007). However little is known about the basal levels of aromatase activity or the role of aromatase in the endocrine pathway of common carp.

The current study observed early development at 20 °C and 25 °C, temperatures within the observed natural spawning range of 15-28 °C (Fernandez-Delgado, 1990; Shields, 1957; Swee and McCrimmon, 1966). The selected rearing temperatures also correspond to the temperature range over which spawning is observed in Australia where common carp are considered a pest species (Smith, 2005).

To address the role of aromatase in sex differentiation in the common carp, two aromatase isoforms (ovary- and brain-derived) were cloned and their expression profile in key adult tissues determined. Expression of both genes is analysed through early embryonic development at high and low temperatures to determine maternal inheritance, initial zygotic expression and effect of temperature on its biosynthesis.

3.3 Material and Methods

3.3.1 Sample collection

Breeding, larval rearing and sampling procedures were as previously described (Chapter 2).

3.3.2 Molecular biology

General molecular biology techniques and sample preparation procedures were as previously described (Chapter 2).

3.3.2.1 Oligonucleotides

Oligonucleotide primers used for cloning and real-time assays are listed in Table 3.1. Ovarian aromatase primer, Ar1F, was designed based on ovarian aromatase sequence of zebrafish (AF226620). Confirmation of 5'end was carried out with gene-specific primer, cOAromGSP_1R and Carp270R. Primers for cloning brain aromatase were designed by aligning protein sequences of brain aromatase from goldfish (CAU18974), roach, *Rutilus rutilus* (AB190292) and zebrafish (AF183908).

Multiple alignments were performed for identification of conserved domains using BlockMaker (Henikoff et al., 1995) and degenerate PCR primers (cBrArom1.F and cBrAromA.R) designed in highly conserved, aromatase-specific region using CODEHOP (Rose et al., 2003). Incidentally this forward primer was the consensus sequence of goldfish and roach. Complete sequencing of the 3' end of the cloned brain aromatase was carried out with the gene-specific primer, cBrArom2.F.

Real Time PCR primers for brain aromatase, cBrArom3.F and cBrAromB.R and ovarian aromatase primers cOvArom1.F and cOvAromA.R were designed according to the requirements of SYBR Green Chemistry (Applied Biosystems) and also to be isoform specific. The common carp *ß-actin* cDNA was deduced based on the genomic DNA sequence (accession number: M24113) and specific primers cBActin4.F and cBActinD.R were selected and designed to serve as endogenous control in the relative quantitative PCR assay.

Table 3.1 Primers for cloning and real-time PCR expression analysis of *cyp19a* and *cyp19b* in common carp.

Primer Name	Sequence (5' - 3')	Amplicon
Ar1F	CCATCGATATCCGTTCTTATGGCAGGTGA	3'-RACE
cOAromGSP_1R	GATGAGAGTTTCTCACCGTTGATCCAGACC	5'-RACE
Carp270R	AGACCAAATGAACCGACAGTAAGACAG	5'-RACE nested
cbrarom1.F	ACCTCATCCTGAACATCGGACGCATGCACA	3'-RACE
cbraromA.R	ACGCAGGCCCGCGGGCCRCARCCRAA	5'-RACE
cBrArom2.F	TGGTTGCTCATCCTCATGTCGTTCCG	3'-RACE sequencing
cBrArom3.F	ATGATGGAGCAGGTCGTCAAG	Real-Time PCR
cBrAromB.R	TCAACGCCATCAACGTTACC	Real-Time PCR
cOvArom1.F	GGTGCCCAAGACAATGTATATGG	Real-Time PCR
cOvAromA.R	TTGTCCGATGGTGTCTGATGG	Real-Time PCR
cBActin4.F	CCCATCGAGCACGGTATTG	Real-Time PCR
cBActinD.R	AAGGTGTGATGCCAGATCTTCTC	Real-Time PCR

3.3.2.2 Real-time assay

Real-time assay procedures were as previously described (Chapter 2). The efficiency values for aromatase primers and the average efficiency value for *β-actin* (from amplification in brain and gonad) were used in subsequent analysis. These values were 1.87 for ovarian aromatase, 1.88 for brain aromatase and 1.80 for *β-actin*, with correlation coefficients of 0.99, 0.99 and 0.98 for ovarian-, brain-aromatase and *β-actin* respectively.

3.3.3 Data analysis

General data analysis procedures were previously described (Chapter 2).

3.4 Results

3.4.1 Cloning and Phylogenetic analysis

Two distinctly different aromatase genes were isolated from the ovary and brain. The cloned cDNA for *cyp19a* (EU375455) was 1807 bp in length with an open reading frame (ORF) of 518 amino acids (AA) (schematic diagram in Appendix), with 94% identity to the subsequently listed common carp *cyp19a* (ABF82249) and 69-92% identity with other teleost forms of *cyp19a*. In contrast, the *cyp19b* cDNA (EU375456) was a 2846 bp fragment with an ORF of 511 AA (schematic diagram in Appendix), which had 68-96% identity with other brain-derived teleost aromatase proteins and 61-65% identity with teleost ovary-derived aromatase proteins. The current common carp *cyp19a* shared only 56% identity with the common carp *cyp19b* protein and 43-69% with other brain-derived teleost aromatase.

The resulting tree of the deduced amino acid sequence from the ORF demonstrates that the two paralogue isozymes are located within each of the two main clades corresponding to brain- and ovary-derived aromatase present in teleosts (Figure 3.1)

with common carp aromatase isoforms showing highest identity with the corresponding goldfish form and more distantly zebrafish and roach.

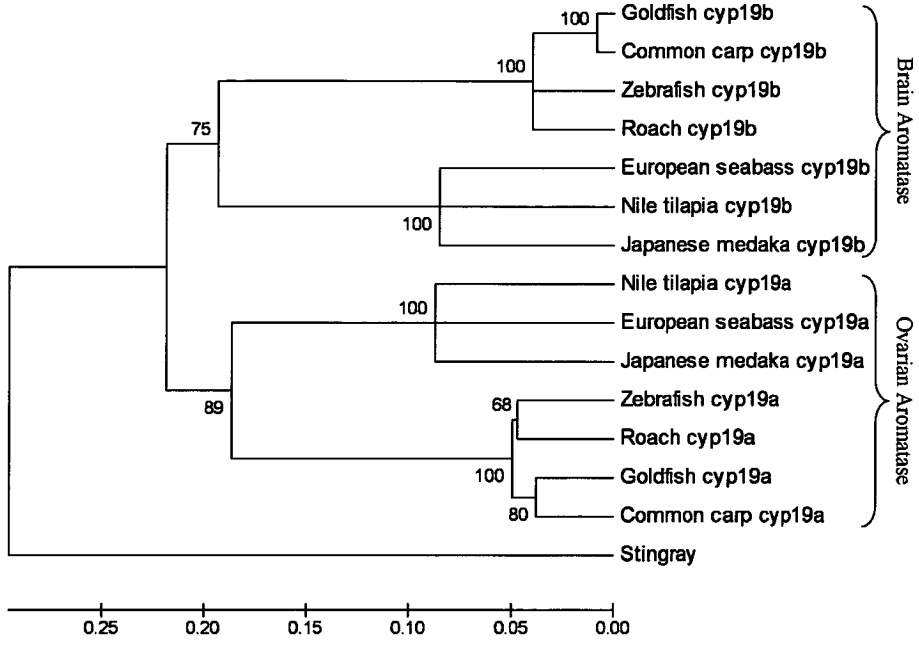


Figure 3.1 Phylogenetic tree of vertebrate aromatase proteins.

Deduced amino acid sequences of P450arom were used from zebrafish, *Danio rerio,* brain (AF183908) and ovary (AF226620), goldfish, *Carassius auratus*, brain (CAU18974) and ovary (AB009336), Japanese medaka, *Oryzias latipes,* brain (AY319970) and ovary (D82968), European seabass, *Dicentrarchus labrax,* brain (AY138522) and ovary (AJ298290), Nile Tilapia, *Oreochromis niloticus*, brain (AF306786) and ovary (TNU72071), Roach, *Rutilus rutilus*, brain (AB190292) and ovary (AB190291) with Atlantic stingray, *Dasyatis sabina* (AF097513) as an outgroup.

Sequence alignment demonstrated three highly conserved regions corresponding to I-helix region, aromatase specific conserved region and heme-binding region (data not shown). Comparison of the amino acid identity of the two common carp aromatase isoforms demonstrated 90% identity in the I-helix region, with 73 and 74% identity in the aromatase-specific and heme-binding regions respectively. Comparison of both isoforms with cyprinids (roach, zebrafish and goldfish) and perciforms (Nile tilapia, European seabass and Japanese medaka) showed greatest identity in the I-helix region (87-100%). Overall, greatest identity (91-100%) was

observed in all regions when comparing corresponding aromatase isoforms of common carp and other cyprinids.

3.4.2 Tissue and sex specific distribution of cyp19a and cyp19b mRNA in adult common carp

Examination of each aromatase isoforms within its respective tissue demonstrated predominant, but not exclusive expression. Ovary was the predominant *cyp19a* expressing tissue with *cyp19b* expressed only at basal levels (Figure 3.2). However, brain was the major aromatase expressing tissue in common carp, with *cyp19b* contributing to the bulk of the activity, with little contribution from *cyp19a*. In the ovary, expression of *cyp19a* was 32-fold higher than *cyp19b* ($t(4)=5.501$, $P<0.005$), where as both isoforms were expressed at equally low levels in the testis (data not shown). Differential expression of the two isoforms was seen in all brain sections and the pituitary of both sexes with significantly more *cyp19b* than *cyp19a*. The largest difference in expression levels was observed in Brain A of both sexes, with 4800-fold higher *cyp19b* in males ($t(4)= 9.612$, $P<0.005$) and 1500-fold in females ($t(4)= 14.483$, $P<0.005$). The smallest difference seen in Brain C of both sexes, 75-fold higher *cyp19b* in males ($t(4)= 5.135$, $P<0.05$) and 90-fold in females ($t(4)= 3.241$, $P<0.05$). Male liver was the only other tissue to display significantly different expression of aromatase genes with only *cyp19a* expressed ($t(4)= 13.898$, $P<0.000$) (Figure 3.2), with similar levels of both aromatase genes detected in the livers of females (data not shown).

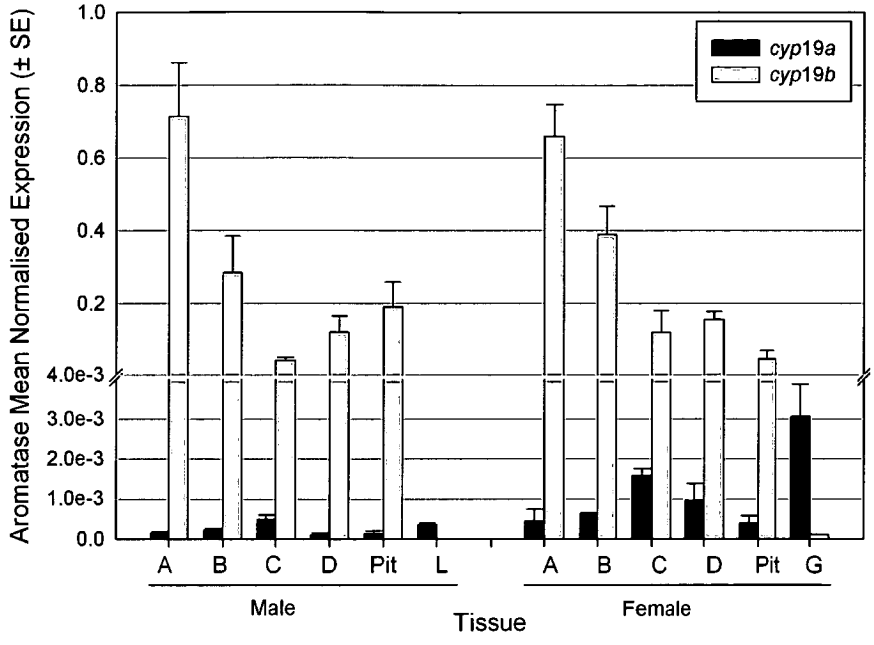


Figure 3.2 Expression of both aromatase genes within tissues showing differential expression. Each bar represents the mean normalised expression (±SE; n=3). X-axis labels: A-D = brain sections (see Fig. 1), Pit = Pituitary, G = Gonad, L = Liver. Student's *t* test was used for pairwise comparisons of a both aromatase genes within a given tissue.

Tissue- and sex-specific expressions of the two isoforms are summarised in Figure 3.3. In females *cyp19a* expression was highest in the ovary (F=7.424, P <0.000) (Figure 3.3a). In males Brain C (cerebellum) and testis showed predominant *cyp19a* expression (F=9.691, P <0.000), although expression was significantly lower than that found in the respective female tissue (see sex-specific differences below). Hepatic expression of *cyp19a* was observed in males with low expression levels similar to that found in the optic lobe (Brain B). In females, a significantly higher expression of *cyp19a* was observed in Brain C when compared to same tissue in males, but was not significantly different than expression in the ovary. All other sections of the brain and also the liver had similar expression levels of *cyp19a* ranging between 4- (liver) and 8-fold (pituitary) lower than in the ovary. In both sexes levels of *cyp19a* in the eye sections, optic nerve and fat were very low.

To discern spatial patterns of *cyp19b* expression within the brain, expression levels in the four brain sections and pituitary were subject to statistical analysis. In both sexes *cyp19b* expression differed significantly between brain regions (males, F=11.077, P <0.001; females, F=12.963, P <0.001), with Brain A expressing the highest levels (Figure 3.3b).

Sex-specific differences of both genes were found within the gonad, with females having 7-fold greater expression of *cyp19a* (t(4)=4.059, P <0.05) and 7-fold lower expression of *cyp19b* (t(4)=43498, P <0.05) when compared to the testis (Figure 3.3). Sexually dimorphic expression of *cyp19a* was also observed in Brain B (t(4)=7.435, P <0.005), Brain C (t(4)=5.376, P <0.005) and Brain D (t(4)=2.833, P <0.05) with females expressing 3-fold greater in Brain B and C and 9-fold more in Brain D, when compared to the same regions of male brain.

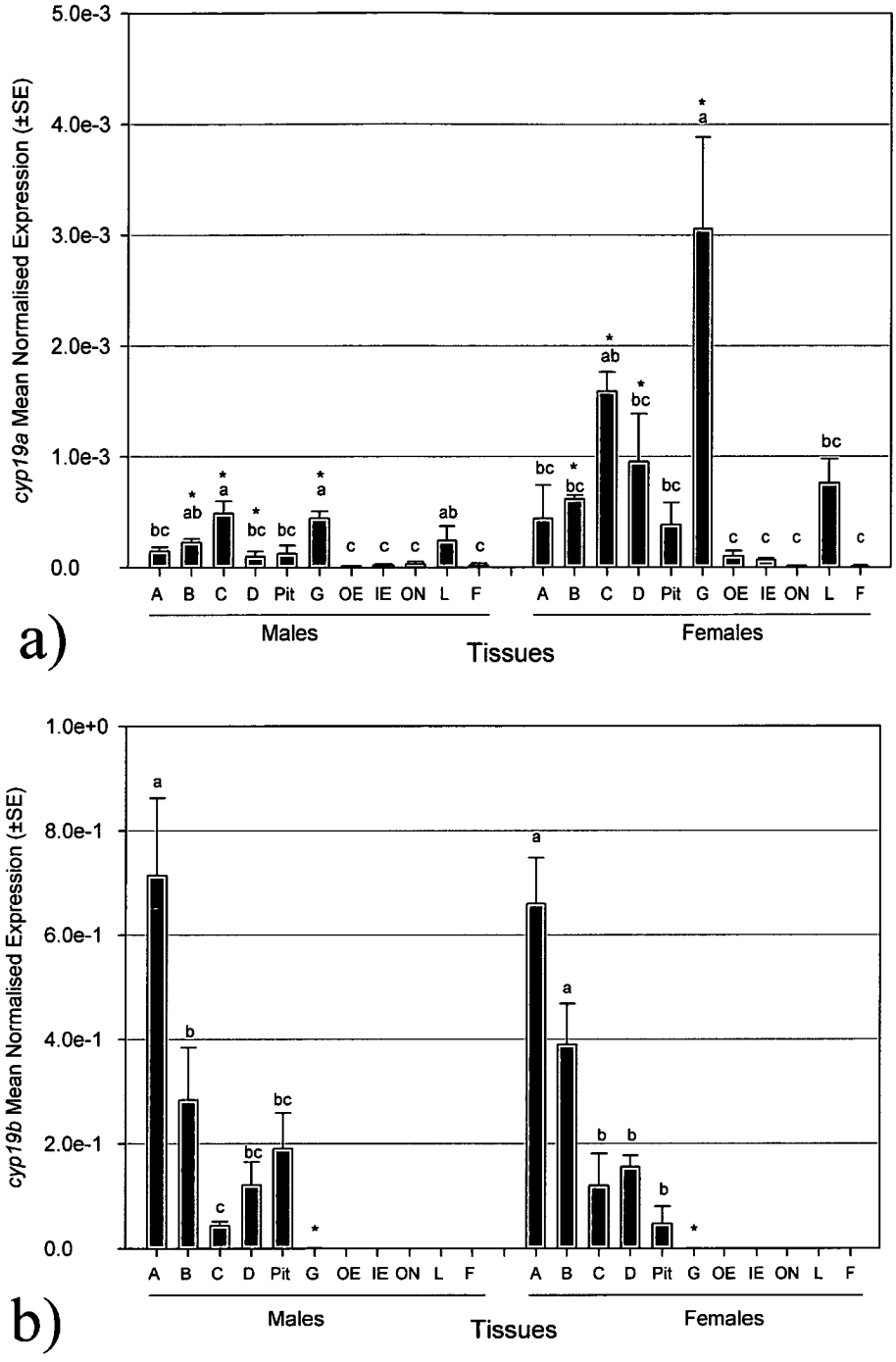


Figure 3.3 Tissue- and sex-specific differences in adult common carp of a) *cyp19a* and b) *cyp19b* expression.

Each bar represents the mean normalised expression (±SE; n=3). X-axis labels: A-D = brain sections (see Figure 2.1), Pit = Pituitary, G = Gonad, OE = Outer Eye, IE = Inner Eye, ON = Optic Nerve, L = Liver, F = Fat. One-way ANOVA was used to analyse data by tissue type within each sex, followed by SNK analysis to determine which means differed significantly ($P < 0.001$), as indicated by letters (a-c). Student's t test was used for pairwise comparisons of a given tissue between sex, and significant differences ($P < 0.05$) are labelled with asterisks (*). Note scale difference in Figure 3.3a and b.

3.4.3 Differential expression of cyp19a and cyp19b mRNA during early development

Temporal changes in *cyp19a* and *cyp19b* expression during early development were quantified by real-time PCR up to 295 hpf in the 20 °C treatment and 175 h at 25 °C (Figure 3.4). Low survival rates in the 25 °C group restricted sample sizes. Statistical analysis of *cyp19b* expression in the 25 °C treatment group was only carried out on the last four time points during larval development as expression was below the detectable limit in the earlier developmental stages.

A high level of *cyp19a* was observed in unfertilised eggs, however this was 3-fold lower than the level expressed in the whole ovary ($F=17.358$, $P<0.014$) (Figure 3.3 and Figure 3.4).

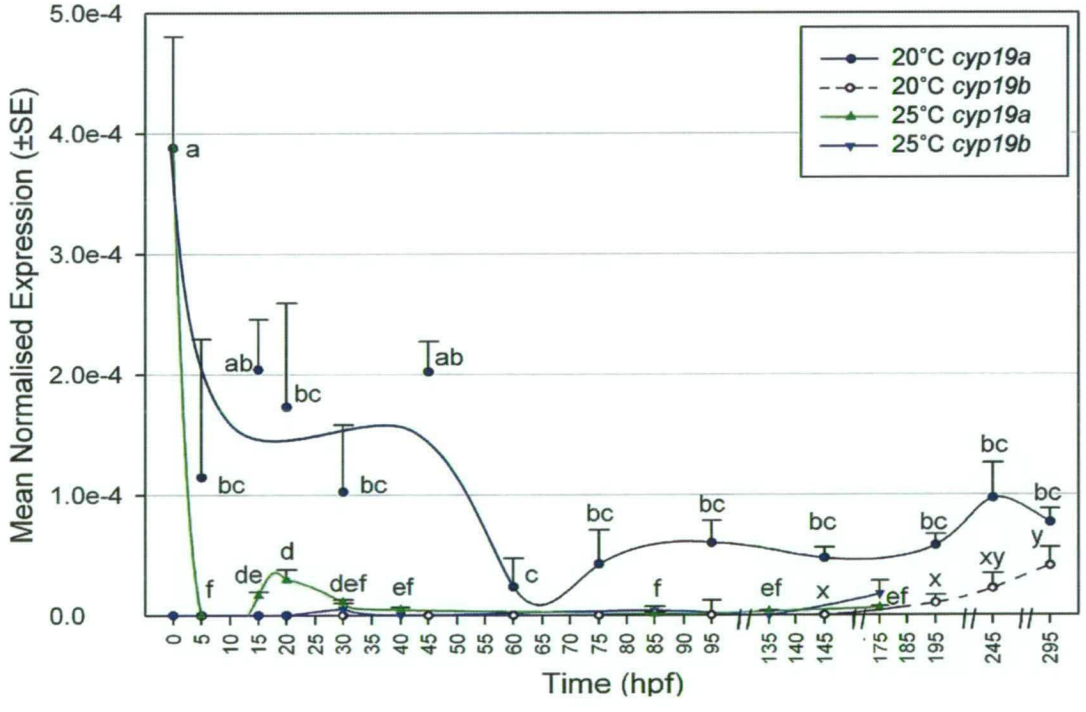


Figure 3.4 Temporal expression of *cyp19a* and *b* in embryos and larvae reared at 20 or 25 °C. One-way ANOVA was used to analyse data for temperature specific differences, followed by SNK analysis to determine which means differed significantly ($P<0.01$), as indicated by letters (20 °C- a-f, 25 °C- x-y). Line shown for 20 °C *cyp19a* expression used to display observed trend.

Over time there were significant changes in *cyp19a* expression in both the 20 °C (F=3.843, P <0.000) and the 25 °C treatment (F=61.180, P <0.000). In the 20 °C treatment, following fertilisation there was a 3-fold drop in *cyp19a* expression at morula stage which was sustained through gastrulation and early somite development at 45 hpf. This was followed by a further 9-fold drop at 60 hpf while undergoing segmentation and developing into a late somite stage embryo. This low level of expression was maintained through hatching (95 hpf) and early larval development up to 295 hpf. In contrast, the 25 °C treatment expression dropped dramatically after fertilisation and was not detectable after 5 hpf coinciding with late blastula stage. A significant increase was seen after 15 hpf with a small peak at 20 hpf correlating with early/mid somite development. Expression of *cyp19a* in the 25 °C treatment again decreased after 30 hpf, correlating with segmentation and remained low through hatching (75 hpf) and early larval development to 175 hpf.

Expression of *cyp19b* in unfertilised eggs and through embryonic development in both temperature treatments was undetectable up to mesolarvae stage at 195 hpf. In the 20 °C treatment expression of *cyp19b* steadily increased 4-fold from 195 to 295 hpf (F=5.088, P <0.007) (Figure 3.4).

3.5 Discussion

CYP19 is an essential enzyme catalysing the conversion of androgen into estrogens, a key biosynthetic step associated with sex differentiation (Cooke et al., 1998), gonadal development/function (Conley and Hinshelwood, 2001) and sexual behaviour (Lephart, 1996) in vertebrates. In all teleosts examined to date, at least two CYP19 loci that code for two distinct isoforms of the P450arom enzyme have been isolated– ovary-derived, *cyp19a* and brain-derived, *cyp19b*. This study reports

isolation of the complete cDNA encoding both isoforms in the common carp along with quantification of their expression in adult tissues, as well as preliminary examination on influence of temperature on their expression during ontogeny.

3.5.1 Phylogenetics

The nucleotide sequence for *cyp19a* identified in the current study had 94% identity to the subsequently listed common carp *cyp19a* (ABF82249). This slight variation may be attributed to intra-species variation or sequencing variables. As in other teleost species, the current study found the brain-derived aromatase form (2.8 kb) was larger than the ovary-derived transcript (1.8 kb). For example in other cyprinids; goldfish *cyp19b* is 3.0 kb (Gelinas et al., 1998) vs. *cyp19a* 1.9 kb (Tchoudakova and Callard, 1998), and zebrafish *cyp19b* is 4.4 kb vs. *cyp19a* 2.1 kb (Kishida and Callard, 2001).

Phylogenetic analysis demonstrated that the common carp brain-derived aromatase shared greater homology with other teleost brain forms than the common carp ovary-derived aromatase, which in turn was homologous with other ovarian types. This supports a shared ancestry of the two separated genes and the existence of two paralogous genes suggested to be due to the occurrence of genome duplication early in the teleost linage (Taylor et al., 2003). This early differentiation of two separate aromatase genes is evident through high homology of aromatase isoforms within the cyprinid and perciform species compared in the current study. Not surprisingly, within cyprinids, both common carp aromatase genes share a much higher homology with the respective genes of goldfish than with zebrafish.

Structural analysis of common carp aromatase proteins with other teleosts demonstrates high homology with the three conserved domains, 1) I-helix, 2) aromatase-specific and 3) heme-binding region. From the amino acid alignment, greatest identity was seen in the I-helix region. This conservation could reflect findings of a human aromatase study which found the I-helix region to be the active site of aromatase with a bend that is the important in establishing optimum fit and most efficient alignment of the C19 substrate (Graham-Lorence et al., 1991). Considering the essential role of this region, it is not surprising that it remains highly conserved among species.

3.5.2 Tissue-specific and sexually dimorphic expression in adults

3.5.2.1 Gonad

Across all non-neural tissues the greatest aromatase expression was observed in the ovary, with *cyp19a* being the dominant isoform. Nonetheless, as observed in many other teleosts, the level of *cyp19a* expression in the ovary was far lower than *cyp19b* levels observed in brain tissues. Within the ovary however, *cyp19b* was also expressed but at a level 30-fold lower than ovary-derived form. This observation is consistent with all other teleost species studied, where estrogen in the ovary is predominately or exclusively supplied by *cyp19a* expression. The higher expression of *cyp19a* in ovary compared to testis contributes to ovarian development due to subsequent high aromatase activity and synthesis of estrogens (Devlin and Nagahama, 2002). In the male gonad overlapping expression of both aromatase genes was observed. Interestingly, expression of both genes was found to be sexually dimorphic in the gonad with males having lower *cyp19a* and higher expression of *cyp19b* than females, zebrafish show a similar pattern (Sawyer et al., 2006; Trant et al., 2001).

Overlapping aromatase expression in gonads of both sexes is seen in other species including zebrafish (Trant et al., 2001), seabass (Blázquez and Piferrer, 2004) and wrasse, *Halichoeres tenuispinis* (Choi et al., 2005). Testicular expression of the aromatase genes may indicate a role of aromatase in gonadal-estrogen synthesis in the testis. Nunez and Applebaum (2006) suggested estrogen may be no less important in male fishes and noted that estradiol has been detected in the plasma of male teleosts with some studies describing seasonal fluctuations of plasma estradiol. In adult Atlantic croaker, *Micropogonias undulates, cyp19a* expression in ovary and testis were comparable, with the suggestion that estrogens disrupt Leydig cell steroidogenesis by inhibiting enzymes that lead to the synthesis of testosterone (Nunez and Applebaum, 2006). Therefore, low *cyp19a* expression may be required in developing testis to minimize estrogen-dependent inhibition of androgen synthesis, necessary for germ cell development. Low level expression in testis may also explain the non detection of aromatase in developed testis of Atlantic halibut, *Hippoglossus Hippoglossus* (van Nes et al., 2005), Japanese flounder (Kitano et al., 1999) and Nile tilapia (Kwon et al., 2001) as assayed by a less sensitive RT-PCR technique. Variations in reported expression levels may be partially attributed to enhanced sensitivity of newly developed or improved techniques.

3.5.2.2 Brain and pituitary

As in other teleost species studied to date, the brain was determined as having the highest aromatase expression, with *cyp19b* contributing to bulk of the activity. Although *cyp19b* expression was more abundant, both aromatase genes were detected in the common carp brain. High brain aromatase activity levels in adult teleosts have been suggested to play a role in continuous neurogenesis, migration, sexual plasticity, and regeneration of the brain (Forlano et al., 2001; Gelinas et al., 1998). In both sexes, *cyp19b* expression was greatest in the anterior quarter of the

brain (telencephalon- Brain A) and also the optic lobe/thalamus (Brain B) in females. This agrees with findings in goldfish where aromatase is concentrated in reproductive control centres of basal forebrain, especially in the telencephalic lobes, and in the hypothalamus/preoptic area, with lower levels of activity present in virtually all other regions of the brain, including basal midbrain, optic lobes, cerebellum, medulla and spinal cord (Callard et al., 1993). An elevated level of expression in the anterior quarter is also supported by Pellegrini et al. (2005) who demonstrated that aromatase activity is restricted to radial glial cells in three other fish species. These radial glial cells were found to be abundant in the telencephalon, preoptic area, and mediobasal hypothalamus, with scattered positive cells in mid- and hindbrain. Therefore the observed patterns of *cyp19b* expression in brain of common carp in this study appears to be in general agreement with the known patterns of radial glial cell distribution in the brain of teleosts. This may imply an important role for aromatase and locally produced estrogen in developmental, reproductive and behavioural processes, as has been shown to be the case in mammals (Conley and Hinshelwood, 2001). Interestingly, the observed areas of higher aromatase activity are also known to be populated with gonadotropin-releasing hormone (GnRH-I) neurons that are responsible for the control of gonadal maturation in teleost species (Lethimonier et al., 2004). The localised production of estrogen in the brain and pituitary in the fish may also have important roles in regulating gonadotropin directly or indirectly, indicating an involvement of *cyp19b* in the brain-pituitary-gonadal axis (Goto-Kazeto et al., 2004) as suggested in the channel catfish, *Ictalurus punctatus* (Kazeto et al., 2003). With this in mind it is interesting to note that sexually dimorphic expression of *cyp19b* was not found in the current study. This conflicts with observations in pejerrey, *Odontesthes bonariensis* (Strobl-Mazzulla et al., 2005), zebrafish (Goto-Kazeto et al., 2004) and Atlantic halibut (Matsuoka et al.,

2006) where males displayed higher expression of *cyp19b* in the anterior and midbrain areas compared to females. These differences across species and between sexes could be due to sex-linked seasonal variations of *cyp19b* expression associated with reproductive cycles, as aromatase expression has been shown to vary according to reproductive status of individual fish (Kazeto et al., 2003; Nunez and Applebaum, 2006). Furthermore, the Tasmanian fish used in the current study were collected during the local spawning season between September 2006 and January 2007, where severe drought conditions combined with catchment management practices limited the availability of suitable spawning sites. Therefore one cannot rule out the possible influence of prevailing environmental factors and habitat availability on the observed results. Through endocrine disruption studies, aromatase is known to be an environmentally sensitive gene. Therefore further investigation into inter-annual variation of expression may elucidate impacts of environmental factors, such as temperature, on fish reproduction in general.

In contrast to *cyp19b*, expression of *cyp19a* in the brain sections of males and females was comparatively low, and displayed a different pattern of expression. In the brain *cyp19a* was expressed at a comparatively low level. Although expression was consistent across female brain sections, in males greatest expression was observed in the cerebellum (Brain C). Chiang et al. (2001a) also reported low expression of *cyp19a* in the zebrafish brain, with expression mainly concentrated in the hypothalamic area. In the common carp brain, female dominant sexually dimorphic expression of *cyp19a* was seen in optic lobe/thalamus (Brain B), cerebellum (Brain C) and medulla (Brain D). In Atlantic halibut *cyp19a* expression was higher in the female brain and the isolated pituitary than in the male (van Nes et al., 2005). This sexually dimorphic expression of *cyp19a,* but not *cyp19b* may

suggest a sex-specific role of *cyp19a* in the female brain of common carp, perhaps related to mating or mating behaviour. This may be further implicated by sexually dimorphic anatomical findings in cyprinids with females having larger cerebellum and smaller olfactory bulbs (Kotrschal et al., 1998). In a recent study on common carp, Lastein et al. (2006) described for the first time how fish single bulbar neurons decipher, on a sex-related basis, the information provided by sex pheromones. Callard et al. (1993) implicated a role of neuroestrogen in the integration, transmission and/or processing of visual information in goldfish. Further studies into common carp mating behaviour and pheromone reception may elucidate sex-specific and neural roles for *cyp19a*.

3.5.2.3 Liver

Notable expression of aromatase was also observed in the liver, with both genes detected equally in females and only *cyp19a* observed in males. Neither aromatase gene demonstrated sexually dimorphic expression in the liver, indicating equally low expression in both sexes. The liver is generally considered a non-steroidogenic tissue in vertebrates, although aromatase mRNA was detected in human fetal and adult liver (Conley and Hinshelwood, 2001) and is well documented in rats and a few fish species (Piferrer and Blazquez, 2005). In fish the liver is one of the main targets for gonadal-derived estrogen action (Piferrer and Blazquez, 2005). It has further been suggested that the presence of hepatic aromatase mRNA expression and enzymatic activity during the period of peak vitellogenesis in some species could serve as an extra-gonadal source of estrogen in vitellogenic females (Gonzalez and Piferrer, 2003). Detection of aromatase in male liver in the current study may further support the possibility that estrogen is also required in males for normal testicular development and function or it could just be a case of ectopic expression. Interestingly however, brain aromatase mRNA expression has not been observed in

the liver of goldfish (Gelinas et al., 1998), zebrafish (Kishida and Callard, 2001), Nile tilapia (Kwon et al., 2001) and rainbow trout (Dalla Valle et al., 2002). In contrast, Sundaray (2005) studied only *cyp19b* and found that it was expressed in the liver of the wrasse, *Pseudolabrus sieboldi*. Further studies are required to determine if differences in hepatic aromatase expression relate to reproductive cycle or species specific differences.

3.5.2.4 Co-expression of aromatase genes in gonad and brain

The co-expression of aromatase genes in the brain and gonad found in the present study has been documented in other fish species such as Nile tilapia (Chang et al., 2005), rainbow trout, *Oncorhynchus mykiss* (Dalla Valle et al., 2002), and zebrafish (Kishida and Callard, 2001; Piferrer and Blazquez, 2005). However, conflicting results have also been presented in zebrafish by Chaing et al. (2001a), where only *cyp19b* could be detected in neural tissues. Interestingly, in the closely related goldfish, exclusive expression of *cyp19a* has been found in the ovary but both genes have been detected in the brain (Gelinas et al., 1998; Tchoudakova and Callard, 1998). Differences in mRNA expression of the two aromatase genes may be explained by species specific differences related to further gene specialisation, gonadal development and reproductive strategy, individual fish differences, age or detection method employed. Further studies are needed to determine the role of the aromatase isoforms in the testis and the ovarian-derived isoform in the brain. Determining whether the two isoforms are co-expressed in a localised fashion in the brain or have unique distributions may elucidate specialised function.

3.5.2.5 Maternal inheritance and ontogenic expression

Temporal changes in *cyp19a* and *cyp19b* expression during early development of a single egg batch were quantified by real-time PCR up to 325 hpf in the 20 °C treatment and 185 h at the 25 °C, falling short of the predicted period of molecular gonadal sex differentiation between 900-1000 hpf as determined by a preliminary morphology study (Beyer et al., unpublished). Poor survival to hatching, particularly in 25 °C treatment group, resulted in premature termination of the experiments. To our knowledge, this is the first time that both aromatase genes have been examined through early development in the common carp and also with the consideration of the effect of temperature in any teleost species.

In many animal species, maternally inherited mRNAs and proteins are used to program the earliest stages of development but are degraded by the mid-blastula transition, allowing genetic control of development to be regulated by zygotically synthesised transcripts (Pedersen, 1998). Expression of *cyp19a* was observed in unfertilised common carp eggs, however, the level of expression was lower than that observed in the whole ovary, consistent with findings in zebrafish (Sawyer et al., 2006). The observed temporal pattern of *cyp19a* in unfertilised eggs and embryos at both temperatures are consistent with maternal transfer and subsequent degradation with little or no zygotic expression during the experimental period. In the 25 °C treatment, expression was not detectable at 5 hpf, possibly implying that the transcript is processed and dissipated at a much faster rate at a higher temperature. Alternatively the higher temperature may have an inhibitory effect on biosynthesis and zygotic transcription of the gene, potentially leading to suppression of ovarian differentiation. It has been previously documented that increased rearing temperature resulted in masculinisation of genetically female goldfish (Goto-Kazeto et al., 2006).

Expression of *cyp19b* in unfertilised eggs and through embryonic development was undetectable at both temperatures, suggesting that the transcript is not inherited maternally, unlike *cyp19a*. This conflicts with findings in zebrafish where a 10-fold enrichment of *cyp19b* was seen from whole ovary to unfertilised egg (Sawyer et al., 2006). However, in the present study initiation of zygotic expression may have been observed late in the 20 °C treatment. These results show unique maternal inheritance and zygotic expression patterns of both aromatase genes. In a recent paper, Sawyer et al. (2006) detected both aromatase isoforms in unfertilised egg using real-time PCR with SYBR green chemistry (as in the current study), with a subsequent decrease between fertilisation and onset of embryonic transcription at the blastula stage (3-5 hpf). This differs from another zebrafish study using more sensitive Taqman real-time PCR, where neither aromatase isoform were detected until after hatching with subsequent sexually dimorphic *cyp19b* expression, suggesting that the brain-form of aromatase dictates phenotypic sex differentiation, if not sex determination (Trant et al., 2001). However, according to Chiang et al. (2001a) *cyp19a* plays a decisive role in sex differentiation based on the presence of binding regions for sex-determining factors in mammals, in the 5′ flanking region of Nile tilapia, zebrafish and goldfish *cyp19a* but not *cyp19b*. Thus, further study is required to determine the function of aromatase during embryonic and larval development. The current study demonstrates a unique view of aromatase maternal inheritance and effect of temperature on its biosynthesis in a teleost species.

3.6 Conclusion

In summary, two aromatase isoforms were identified in the common carp, brain- and ovary-derived. As in other species, aromatase expression was greatest in the neural tissues with *cyp19b* (brain-derived) expression higher in the anterior quarter of the

brain with neuro-anatomical localisation found to overlap with brain areas related to reproduction. Similarly, *cyp19a* was predominantly expressed in the ovary; however expression of both genes were found in all major tissues tested. Analysis of unfertilised eggs and embryonic development demonstrated that only the ovarian aromatase mRNA is maternally inherited, with higher rearing temperature resulting in dramatically reduced transcription. Further investigations will help to determine if temperature causes increased processing and dissipation, or inhibition of biosynthesis. An extended experimental period would also determine aromatase expression through gonadal differentiation and determine the effect of temperature on sex ratio.

3.7 Acknowledgements

Support and assistance provided by CSIRO, Invasive Animal Cooperative Research Centre, Inland Fisheries Service Tasmania and K&C Fisheries. Editorial assistance from R.E. Thresher and M. Sellars is much appreciated. This project received funding support from the Fisheries Research and Development Corporation.

3.8 *References*

Blázquez, 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)*. Mol Cell Endocrinol, 219, 83-94.

Callard, G.V., Drygas, M., Gelinas, D., 1993. Molecular and cellular physiology of aromatase in the brain and retina. J Steroid Biochem Mol Biol, 44, 541-547.

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*). Gen Comp Endocrinol, 141, 101-115.

Chiang, E.F.-L., Yan, Y.-L., Guiguen, Y., Postlethwait, J., Chung, B., 2001. Two cyp19 (P450 aromatase) genes on duplicated zebrafish chromosomes are expressed in ovary or brain. Mol Biol Evol, 18, 542-550.

Choi, J.Y., Park, J.G., Jeong, H.B., Lee, Y.D., Takemura, A., Kim, S.J., 2005. Molecular cloning of cytochrome P450 aromatases in the protogynous wrasse, *Halichoeres tenuispinis*. Comp. Biochem. Physiol., B, 141, 49-59.

Conley, A. & Hinshelwood, M., 2001. Mammalian aromatases. Reproduction, 121, 685-695.

Cooke, B., Hegstrom, C.D., Villeneuve, L.S., Breedlove, S.M., 1998. Sexual differentiation of the vertebrate brain: Principles and mechanisms. Front. Neuroendocrinol., 19, 323-362.

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*. Mol Reprod Dev, 59, 265-276.

Dalla Valle, L., Ramina, A., Vianello, S., Belvedere, P., Colombo, L., 2002. Cloning of two mRNA variants of brain aromatase cytochrome P450 in rainbow trout (*Oncorhynchus mykiss* Walbaum). J Steroid Biochem Mol Biol, 82, 19-32.

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.

Fernandez-Delgado, C., 1990. Life history patterns of the common carp, *Cyprinus carpio*, in the estuary of the Guadalquivir River in south-west Spain. Hydrobiologia, 206, 19-28.

Forlano, P.M., Deitcher, D.L., Myers, D.A., Bass, A.H., 2001. Anatomical distribution and cellular basis for high levels of aromatase activity in the brain of teleost fish: aromatase enzyme and mRNA expression identify glia as source. J Neurosci, 21, 8943-8955.

Gelinas, D., Pitoc, G.A., Callard, G.V., 1998. Isolation of a goldfish brain cytochrome P450 aromatase cDNA. Mol Cell Endocrinol, 138, 81-93.

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. Gen Comp Endocrinol, 132, 223-230.

Goto-Kazeto, R., Abe, Y., Masai, K., Yamaha, E., Adachi, S., Yamauchi, K., 2006. Temperature-dependent sex differentiation in goldfish: Establishing the temperature-sensitive period and effect of constant and fluctuating water temperatures. Aquaculture, 254, 617-624.

Goto-Kazeto, R., Kight, K.E., Zohar, Y., Place, A.R., Trant, J.M., 2004. Localization and expression of aromatase mRNA in adult zebrafish. Gen Comp Endocrinol, 139, 72-84.

Graham-Lorence, S., Khalil, M.W., Lorence, M.C., Mendelson, C.R., Simpson, E.R., 1991. Structure-function relationships of human aromatase cytochrome P-450 using molecular modeling and site-directed mutagenesis. J Biol Chem, 266, 11939-11946.

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. Aquat Toxicol, 71, 371-389.

Henikoff, S., Henikoff, J.G., Alford, W.J., Pietrokovski, S., 1995. Automated construction and graphical presentation of protein blocks from unaligned sequences. Gene, 163, GC17-GC26.

Hinfray, N., Palluel, O., Turies, C., Cousin, C., Porcher, J.M., Brion, F., 2006. Brain and gonadal aromatase as potential targets of endocrine disrupting chemicals in a model species, the zebrafish (*Danio rerio*). Environ Toxicol, 21, 332-337.

Kazeto, Y., Goto-Kazeto, R., Place, A.R., Trant, J.M., 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 Physiol Biochem, 28, 29-32.

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.

Kitano, T., Takamune, K., Kobayashi, T., Nagahama, Y., Abe, S., 1999. Suppression of P450 aromatase gene expression in sex-reversed males produced by rearing genetically female larvae at a high water temperature during a period of sex differentiation in the Japanese flounder (*Paralichthys olivaceus*). J Mol Endocrinol, 23, 167-176.

Kotrschal, K., Van Staaden, M.J., Huber, R., 1998. Fish brains: evolution and environmental relationships. Reviews in Fish Biology and Fisheries, 8, 373-408.

Kwon, J.Y., McAndrew, B.J., Penman, D.J., 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*). Mol Reprod Dev, 59, 359-370.

Lastein, S., Hamdani, E.H., Doving, K.B., 2006. Gender distinction in neural discrimination of sex pheromones in the olfactory bulb of Crucian carp, *Carassius carassius*. Chemical Senses, 31, 69-77.

Lavado, R., Thibaut, R., Raldúa, D., Martín, R., Porte, C., 2004. First evidence of endocrine disruption in feral carp from the Ebro River. Toxicol Appl Pharmacol, 196, 247-257.

Lephart, E.D., 1996. A review of brain aromatase cytochrome P450. Brain Research Reviews, 22, 1-26.

Lethimonier, C., Madigou, T., Munoz-Cueto, J.-A., Lareyre, J.-J., Kah, O., 2004. Evolutionary aspects of GnRHs, GnRH neuronal systems and GnRH receptors in teleost fish. Gen Comp Endocrinol, 135, 1-16.

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. Comp. Biochem. Physiol., B, 144, 128-135.

McEwen, B.S. & Alves, S.E., 1999. Estrogen actions in the central nervous system. Endocrine Reviews, 20, 279-307.

Menuet, A., Pellegrini, E., Brion, F., Gueguen, M.-M., Anglade, I., Pakdel, F., Kah, O., 2005. Expression and estrogen-dependent regulation of the zebrafish brain aromatase gene. J Comp Neurol, 485, 304-320.

Moens, L.N., Smolders, R., van der Ven, K., van Remortel, P., Del-Favero, J., De Coen, W.M., 2007. Effluent impact assessment using microarray-based

analysis in common carp: A systems toxicology approach. Chemosphere, 67, 2293-2304.

Moens, L.N., van der Ven, K., Van Remortel, P., Del-Favero, J., De Coen, W.M., 2006. Expression profiling of endocrine-disrupting compounds using a customized *Cyprinus carpio* cDNA microarray. Toxicol Sci, 93, 298-310.

Nunez, B.S. & Applebaum, S.L., 2006. Tissue- and sex-specific regulation of CYP19A1 expression in the Atlantic croaker (*Micropogonias undulatus*). Gen Comp Endocrinol, 149, 205-216.

Pedersen, R.A., 1998. Early mammalian embryogenesis. In: E. Knobil, J.D. Neill, L.L. Ewing, G.S. Greenwald, C.L. Markert & D.W. Pfaff (Eds.), The Physiology of Reproduction, Vol. 1, Raven Press, New York. pp. 187-230.

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

Piferrer, F. & Blazquez, M., 2005. Aromatase distribution and regulation in fish. Fish Physiol Biochem, 31, 215-226.

Rose, T., Henikoff, J., Henikoff, S., 2003. CODEHOP (COnsensus-DEgenerate Hybrid Oligonucleotide Primer) PCR primer design. Nucleic Acids Res, 31, 3763-3766.

Sawyer, S., 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. Gen Comp Endocrinol, 147, 108-117.

Shields, J.T., 1957. Experimental control of carp reproduction through water drawdowns in Fort Randall reservoir, South Dakota. Transactions of the American Fisheries Society, 87, 23-32.

Simpson, E.R., Clyne, C., Rubin, G., Boon, W.C., Robertson, K., Britt, K., Speed, C., Jones, M., 2002. Aromatase - A brief overview. Annu Rev Physiol, 64, 93-127.

Smith, B.B., 2005. The state of the art: a synopsis of information on common carp (*Cyprinus carpio*) in Australia. Final Technical Report Series No. 77, prepared by the South Australian Research and Development Institute (Aquatic Sciences), Adelaide.

Strobl-Mazzulla, P.H., Moncaut, N.P., López, G.C., Miranda, L.A., Canario, A.V.M., Somoza, G.M., 2005. Brain aromatase from pejerrey fish (*Odontesthes bonariensis*): cDNA cloning, tissue expression, and immunohistochemical localization. Gen Comp Endocrinol, 143, 21–32.

Sundaray, J., Ohta, K., Yamaguchi, A., Kitano, T., Matsuyama, M., 2005. Isolation, cloning, sequencing of brain type aromatase and its expression in male and female Wrasse, *Pseudolabrus sieboldi*. Fish Physiol Biochem, 31, 137-141.

Swee, U.B. & McCrimmon, H.R., 1966. Reproductive biology of the carp, *Cyprinus carpio* L., in Lake St. Lawrence, Ontario. Transactions of the American Fisheries Society 95, 372-380.

Taylor, J.S., Braasch, I., Frickey, T., Meyer, A., Van de Peer, Y., 2003. Genome duplication, a trait shared by 22,000 species of ray-finned fish. Genome Res, 13, 382-390.

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.

Trant, J.M., Gavassa, S., Ackers, J., Chung, B.-C., Place, A.R., 2001. Developmental expression of cytochrome P450 aromatase genes (CYP19a and CYP19b) in zebrafish fry (*Danio rerio*). J Exp Zool, 290, 475-483.

Tzchori, I., Zak, T., Sachs, O., 2004. Masculinization of genetic females of the common carp (*Cyprinus carpio* L.) by dietary administration of an aromatase inhibitor. Israeli Journal of Aquaculture-Bamidgeh, 56, 239-246.

Uchida, D., Yamashita, M., Kitano, T., Iguchi, T., 2002. Oocyte apoptosis during the transition from ovary-like tissue to testes during sex differentiation of juvenile zebrafish. J Exp Biol, 205, 711-718.

van Nes, S., Moe, M., Andersen, O., 2005. Molecular characterization and expression of two *cyp*19 (P450 aromatase) genes in embryos, larvae and adults of Atlantic halibut (*Hippoglossus hippoglossus)*. Mol Reprod Dev, 72, 437-449.

Villeneuve, D.L., Larkin, P., Knoebl, I., Miracle, A.L., Kahl, M.D., Jensen, K.M., Makynen, E.A., Durhan, E.J., Carter, B.J., Denslow, N.D., Ankley, G.T., 2007. A graphical systems model to facilitate hypothesis-driven ecotoxicogenomics research on the teleost brain-pituitary-gonadal axis. Environ Sci Technol, 41, 321-330.

Zhao, J., Mak, P., Tchoudakova, A., Callard, G., Chen, S., 2001. Different catalytic properties and inhibitor responses of the goldfish brain and ovary aromatase isozymes. Gen Comp Endocrinol, 123, 180-191.

CHAPTER 4

Expression of Cytochrome P450 Aromatase through the period of sexual differentiation in the common carp, *Cyprinus carpio* (L) reared under two temperature regimes.

Adapted from Barney, M.L., Patil, J.G. and Carter, C.G. Expression of Cytochrome P450 Aromatase through the period of sexual differentiation in the common carp (*Cyprinus carpio*) reared under two temperature regimes. In preparation.

Chapter 4 - Expression of Cytochrome P450 Aromatase through the period of sexual differentiation in the common carp (*Cyprinus carpio*) reared under two temperature regimes.

4.1 Abstract

Cytochrome P450 aromatase (CYP19) is a key enzyme in the steroidogenic pathway that catalyses the conversion of testosterone to estrogen, and is therefore likely to influence gonadal sex differentiation. In the common carp, *Cyprinus carpio,* only the ovarian aromatase transcript (*cyp19a*) appears to be inherited maternally, and it also exhibits reduced levels of expression at higher temperatures. Consequently this study followed the expression of both ovarian (*cyp19a*) and brain (*cyp19b*) aromatase genes over the critical period of sex differentiation- from hatch to 138 and 118 days post fertilisation (dpf) in larvae reared at 20 and 25 °C, respectively. The brain isoform (*cyp19b*) exhibited two spikes in expression at about 250 and 350 ddph at both temperatures. In contrast, expression of *cyp19a* at 20 °C peaked at 250 ddph but not until approximately 2500 ddph at 25 °C. Thus, the *cyp19a* isoform, but not *cyp19b,* showed temperature sensitivity. Cluster analysis of *cyp19a* expression in relation to larval total length (TL) identified three patterns that correlated to putative gonadal sex: male, female and undifferentiated. Differences in *cyp19a* expression levels in putative males (low expression) and females (high expression) could only be detected after the larvae had exceeded 20 mm TL at which size morphological sex differentiation occurs in this species. Elevated temperature (25 °C) resulted in reduced expression levels of *cyp19a*, a reduction in the number of larvae expressing high levels of *cyp19a* and suggests a reduction in the proportion of females in the treatment group. Collectively the results suggested that temperature may play a role in sex differentiation cascade in common carp.

4.2 Introduction

The availability and activity of steroid synthesising enzymes, in particular the cytochrome P450 aromatase complex (P450arom) controls the hormonal balance between estrogens and androgens which in turn plays a crucial role in sex differentiation in teleosts (Devlin and Nagahama, 2002). Biosynthesis of estrogens occurs in all classes of vertebrates (Simpson et al., 2002) and the importance of P450arom has been shown in many teleosts (Guiguen et al., 2009; Piferrer and Blazquez, 2005). For example, genetically female fish develop into phenotypic males when aromatase activity is inhibited during development (e.g. Tzchori et al., 2004).

Most higher vertebrates have a single *cyp19* gene with multiple promoter elements that regulate tissue-specific expression with aromatase expression occurring in both gonads and in the brain (reviewed by Simpson et al., 2002). However, in teleosts, gonadal and neural expression of *cyp19* is regulated by two distinct aromatase isoforms (*cyp19a* and *cyp19b*; ovary and brain isoforms) that have different catalytic activity (Zhao et al., 2001). In teleosts these isoforms have distinct or partially overlapping expression patterns, this includes common carp, *Cyprinus carpio* (Chapter 3 / Barney et al., 2008) and other Cypriniformes, zebrafish, *Danio rerio* (Kishida and Callard, 2001; Trant et al., 2001) and goldfish, *Carassius auratus* (Gelinas et al., 1998; Tchoudakova and Callard, 1998).

The role of *cyp19a*, and thus estrogens in ovarian differentiation and maturation has been demonstrated through inhibition of *cyp19a* activity during the key period of sex differentiation resulting in genotypic females developing as phenotypic males in a number of fish species (Fenske and Segner, 2004; Guiguen et al., 1999; Kwon et al., 2000; Piferrer et al., 1994). Although *cyp19a* is clearly implicated in ovarian

differentiation (Piferrer and Guiguen, 2008), the brain is the major aromatase expressing tissue in teleosts including common carp (Chapter 3 / Barney et al., 2008). The high abundance of *cyp19b* in the teleost brain is thought to be related to the capacity of the teleost brain for continued growth throughout life (Kishida and Callard, 2001). A recurrent question has been whether the teleost brain is the first site of aromatisation during ontogeny and whether changes in *cyp19b* are involved in gonadal sex differentiation in gonochoristic fish (Piferrer and Guiguen, 2008).

Sex differentiation in fish is known to be influenced by hormonal and/or environmental cues (Devlin and Nagahama, 2002). In many species temperature is the main environmental determinant of sex during early development (Bull, 1983; Pieau, 1996). Biochemical pathways that control sex differentiation and reproduction in fishes involve many different proteins (e.g. transcriptional factors, steroidogenic enzymes, receptors). The structure and function of such proteins and other macromolecules are dramatically influenced by temperature- fluctuations of which, as encountered by fish in natural habitats, could alter sex determination and differentiation pathways and influence resulting sex ratios (Devlin and Nagahama, 2002). High temperatures down regulate the expression of aromatase or transcriptional factors responsible for the expression of the aromatase during sexual differentiation in fish (Baroiller and D'Cotta, 2001; D'Cotta et al., 2001; Tsai et al., 2003).

Cyp19a is thermally sensitive and temperature perturbation during the critical period of gonad development are thought to disrupt genetic sex differentiation patterns (D'Cotta et al., 2001; Kitano et al., 1999; Kwon et al., 2002; van Nes and Andersen, 2006). Higher temperature is suggested to cause suppression of aromatase expression

preventing estradiol biosynthesis resulting in testicular differentiation (Guiguen et al., 1999; Kitano et al., 1999; Kwon et al., 2001; Uchida et al., 2002). Variations in environmental temperature alter the sex ratio in fish populations (Conover and Kynard, 1981) and increasing temperature invariably results in an increase in the number of males in thermosensitive fish species (Ospina-Alvarez and Piferrer, 2008). The effect of temperature on sex ratio in the common carp has not previously been examined, however studies suggest that higher temperatures should skew sex ratios toward males (Nagy et al., 1981). Here, methyl-testosterone treated female genotypic carp at 20 °C resulted in non-sex reversed females and hermaphodites while at 25 °C seemed to increase the effectiveness of the hormone, resulting in males and undifferentiated fry, suggesting that the higher temperature assisted the development of testicular tissue (Nagy et al., 1981).

As well as being an important food fish (FAO, 2007), common carp are often used as a sentinel species in studies investigating potential alterations to the function of the endocrine system of humans and wildlife as a result of chemical exposure (Lavado et al., 2004; Moens et al., 2007; Moens et al., 2006). Such studies frequently use aromatase activity as a measure of endocrine disruption (Greytak et al., 2005; Hinfray et al., 2006; Lavado et al., 2004; Villeneuve et al., 2007). However little is known about levels of aromatase activity during development or the role of aromatase in the endocrine pathway of common carp.

The current study aimed to track early development at 20 °C and 25 °C, temperatures within the observed natural spawning range of 15-28 °C (Fernandez-Delgado, 1990; Shields, 1957; Swee and McCrimmon, 1966). The selected rearing temperatures also correspond to the temperature range over which spawning is observed in Australia

where common carp are considered a pest species (Smith, 2005). To further address the role of aromatase in sex differentiation in the common carp, the present study also examined the expression of both genes through larval development at high (25 °C) and low (20 °C) temperatures to determine the role of these genes in gonadal differentiation and the effect of temperature on sex ratio, presumably through altered aromatase expression.

4.3 Material and Methods

4.3.1 Sample collection

Breeding, larval rearing and sampling procedures were as previously described (Chapter 2).

4.3.2 Molecular biology

General molecular biology techniques and sample preparation procedures were as previously described (Chapter 2).

Real-time PCR primers used to quantify the expression of *cyp19a*, *cyp19b* and the internal control (*ß-actin*) were identical to those previously described in Chapter 3 / Barney et al. (2008).

4.3.3 Data analysis

General data analysis procedures were previously described (Chapter 2).

This chapter also used cluster analysis using TL of individuals and MNE of *cyp19a* to estimate individuals that were undifferentiated or putative females/males. This analysis followed the assumption that the (1) the timing of sex differentiation is more dependent on size than age in the species and (2) *cyp19a* expression is a suitable marker of ovarian differentiation, with higher levels in differentiating females (Blázquez et al., 2008). Both variables (TL and *cyp19a* expression) were z-score

transformed to standardise values before K-cluster analysis to split all data from both temperatures into 3 clusters (undifferentiated and putative females or males).

4.3.4 Sex ratio determination

Following termination of the expression experiment (138 and 118 dpf at 20 and 25 °C respectively) remaining larva were maintained at their experimental temperature regime until they had exceeded 5 cm TL at which they were sampled and fixed in formalin to determine the phenotypic sex. Individuals were weighed, measured and 2 samples of gonad removed, stained and a tissue squash prepared for microscopic examination. Two methods of staining were employed for identifying and validation of the phenotypic sex; 1) Diff Quik Stain (Lab Aids, Narrabeen, NSW, Australia), and 2) aceto-carmine stain (Guerrero and Shelton, 1974).

4.4 Results

4.4.1 Growth

Total length (TL) of hatched larva was measured at the time of sampling. At both temperatures TL increased steadily over time (20 °C- $F=48.805$, $P<0.000$, 25 °C- $F=102.197$, $P<0.000$) (Figure 4.1). At the higher temperature (25 °C) the growth rate was significantly higher ($F=115.176$, $P>0.000$), with a mean total length of 47.75 ± 3.43 mm after 118 dpf compared to 31.13 ± 3.43 mm at 20 °C after 138 dpf.

4.4.2 Expression of cyp19a and b during larval and gonadal differentiation

Temporal changes in *cyp19a* and *cyp19b* expression during larval development were quantified by real-time PCR from hatching, 3.5 and 2.5 dpf, up to 138 and 118 dpf in both 20 °C and 25 °C treatment groups respectively (Figure 4.1).

Temporal changes in *cyp19a* expression were observed at both temperatures over the developmental period (20 °C- $F = 4.215$, $P<0.000$; 25 °C- $F= 4.549$, $P<0.000$). At

hatching *cyp19a* expression was low ($9.69e^{-6} \pm 4e^{-6}$) in the 20 °C treatment, followed by a 10-fold increase at 250 ddph at which time larva were 6.67 ± 0.22 mm TL. Expression was then relatively stable until a second significant increase at 1530 ddph (TL 88 ± 1.8 mm) reaching expression levels of $1.27e^{-4}$ (± $3.2e^{-5}$) and remained around this level during rest of the sampling period.

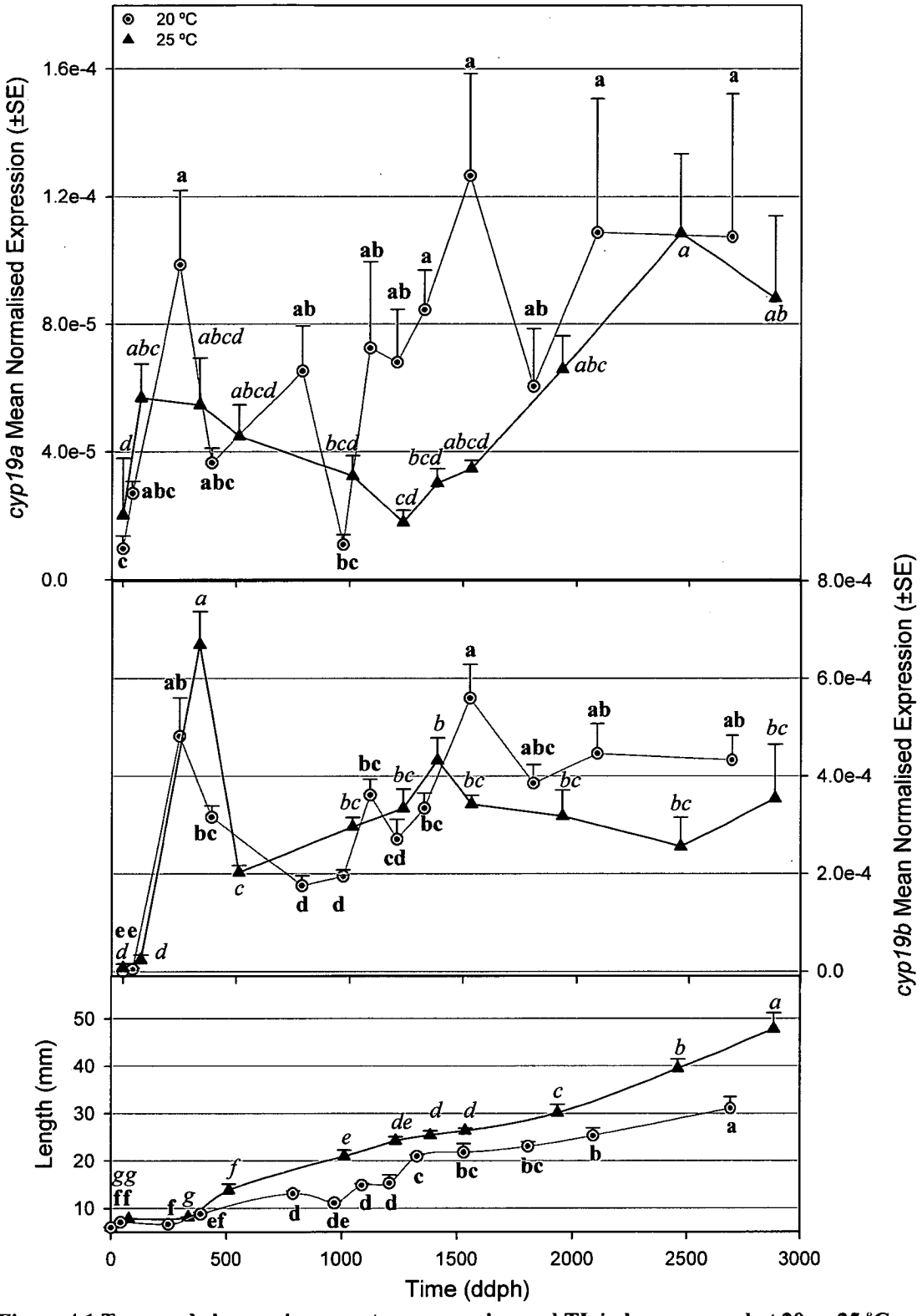


Figure 4.1 Temporal changes in aromatase expression and TL in larvae reared at 20 or 25 °C. One-way ANOVA was used to analyse data for temperature specific differences, followed by SNK analysis to determine which means differed significantly ($P < 0.01$), as indicated by letters (20 °C- bold; 25 °C- italic). Note scale difference for expression levels.

At 25 °C, *cyp19a* expression at hatch was also low ($2.02e^{-5}$ ±$1.78e^{-5}$) but 2-fold higher than observed at 20 °C ($9.69e^{-6}$ ±$4.0e^{-6}$). At 78 ddph (TL 7.81 ±0.06 mm) expression of *cyp19a* had increased 3 fold and remained at about this level until a second larger increase in expression at 2462.5 ddpf (TL 39.50 ±1.94 mm). Beyond ~1500 ddph a large standard error (SE) in *cyp19a* was observed and indicated an increase in inter-individual variation.

Closer examination of the raw data suggested that most individuals had reached a TL of greater than 20 mm (size at which sex differentiation is known to occur in common carp) by this period. In order to examine putative sex related differences, the expression data for each of the individuals larger than 20 mm was plotted on a scatter graph (Figure 4.2). Irrespective of the temperature, the data was suggestive of two groups for which expression was high (putative female) or low (putative male) (i.e. above and below a MNE of about $1.0e^{-4}$ respectively). The proportion of individuals showing low expression at 25 °C was 91% and significantly higher than 70% at 20 °C (Chi squared= 5.941, P>0.015).

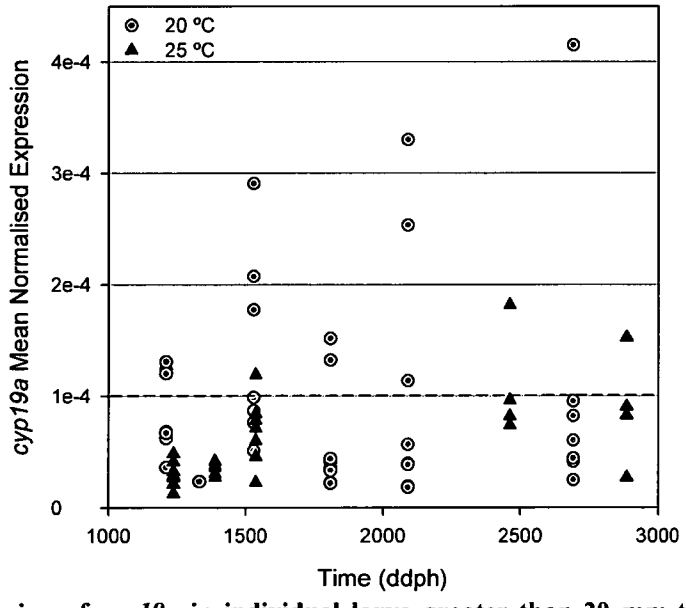


Figure 4.2 Expression of *cyp19a* in individual larva greater than 20 mm total length at two temperatures (● 20 °C and ▲25 °C).

Individuals with expression levels above a MNE of 1e-4 (dashed line) represent putative females and those below putative males.

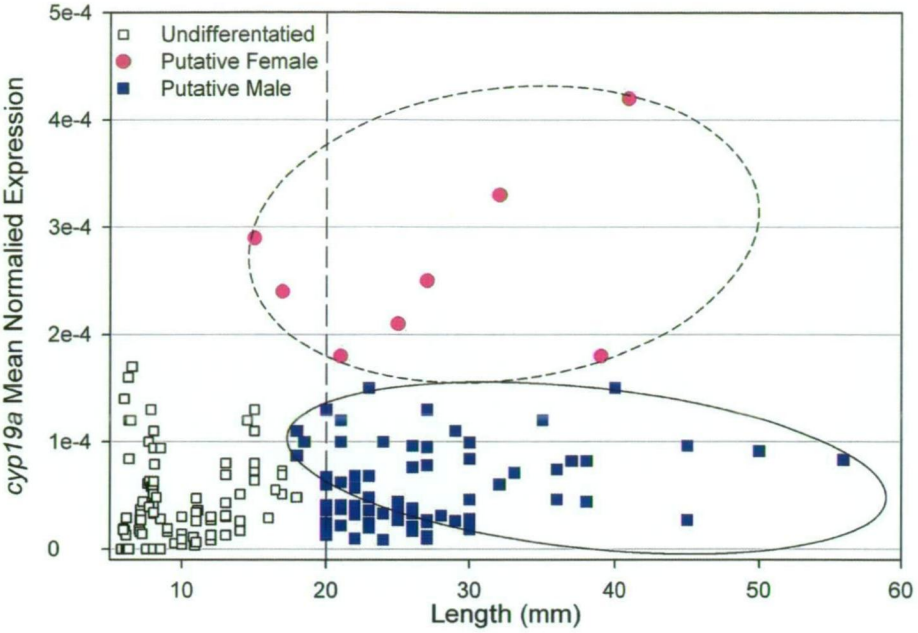


Figure 4.3 Putative females and males as determined by cluster analysis with size and MNE of *cyp19a* in larval common carp.

Ovarian differentiation detectable by histology at 20 mm TL (dashed line). Undifferentiated larvae indicated by □; putative females ● (dashed oval) and males ■ (solid oval).

Further cluster analysis of *cyp19a* expression of all larvae in relation to TL segregated the data into three different clusters or groups (Figure 4.3). The majority of larvae below 20 mm TL clustered into one single group with low expression. This group potentially consists of undifferentiated individuals. However in larva exceeding 20 mm TL, the data segregated into two groups that showed low and high expression representing putative males and females respectively.

At both temperatures there were significant temporal changes in *cyp19b* expression (Figure 4.1; 20 °C- $F= 49.359$, $P<0.000$; 25 °C- $F= 35.954$, $P<0.000$). At 20 °C levels of *cyp19b* expression was undetected at the time of hatch and was first detected at 42.5 ddph (MNE $4.78e^{-6} \pm 2.72e^{-6}$) when the larvae had reached a TL of 7.12 ± 0.03 mm. By 250 ddph expression increased by 100-fold and then gradually decreased to around $2.0e^{-4}$ at 790 ddph and remained at about this level until 1330

ddph (TL 21.0 ± 0.38 mm). A second peak in *cyp19b* expression was observed at 1530 ddph ($5.60e^{-4}$ ± $6.92e^{-5}$) and remained at about this level until 2690 ddph (TL 31.12 ± 2.47 mm). A similar trend was observed in the 25 °C group, where the expression of *cyp19b* was undetectable at hatch and was low (>$2.5e^{-5}$) up to 78 ddph. At 337.5 ddph (TL 8.15 ± 0.08 mm) there was a 27-fold increase in *cyp19b* with expression observed at a higher level ($6.70e^{-4}$ ± $6.78e^{-5}$) than in the corresponding peak at 20 °C. After which, expression of *cyp19b* at 25 °C reduced to ~$2.0e^{-4}$ at 512.5 ddph (13.81 ± 1.34 mm TL) and remained around this level with a second smaller peak ($4.33e^{-4}$ ± $4.58e^{-5}$) observed at 1387.5 ddph (25.43 ± 0.93 mm TL). Expression was maintained around this level for the remaining experimental period.

4.4.3 Sex ratios from different temperature treatments

At the time of terminating the experiment (354 dpf) only 16 individuals remained in the 25 °C treatment and 52 individuals (39 of which were >5 cm) in the 20 °C treatment. Sex was determined morphologically using 2 staining methods for all 25 °C individuals, with an equal number randomly taken of 20 °C individuals. At 20 °C there were 44.7 % males compared with 68.8% at 25 °C (Figure 4.4).

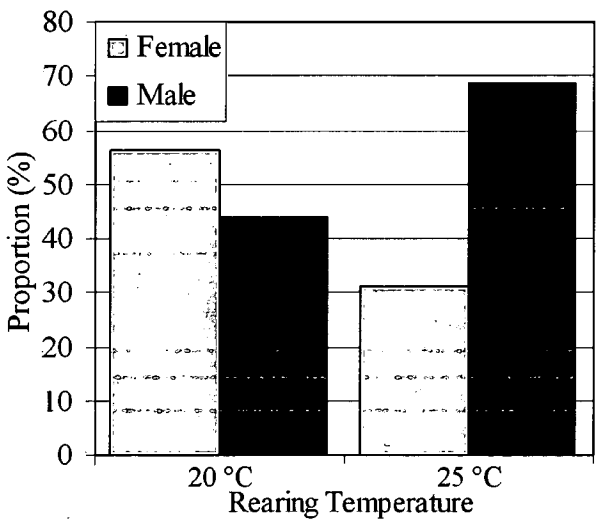


Figure 4.4 Percentage of male and female common carp (≥ 5 cm TL) in groups reared at 20 °C and 25 °C.

(Pearson Chi-Square= 2.032, P=0.154)

4.5 Discussion

Estrogens are involved in various aspects of sexual differentiation, gonadal growth and development (Lange et al., 2003; Simpson et al., 2002). By converting androgens to estrogens, aromatase (CYP19) plays an important role in the molecular mechanisms of sex determination and differentiation of lower vertebrates including fishes (Bogart, 1987; Simpson et al., 1994). In this study expression of aromatase (ovarian and brain isoforms) was examined during the period of larval and gonadal development. Zygotic expression of both isoform was found to peak following hatch, with predominant aromatase expression (*cyp19b*) in the brain. Expression of *cyp19a* showed sexual dimorphism in larvae exceeding 20mm TL, and therefore can serve as a molecular marker for ovarian differentiation in the species. Expression of *cyp19a* also appeared to be reduced by warmer temperature (25 °C), conceivably resulting in repression of ovarian differentiation leading to sex ratio bias.

In common carp, it was previously shown that only the ovarian aromatase (*cyp19a*) transcript is inherited maternally (Chapter 3 / Barney et al., 2008), with lower expression observed through early larval development under warmer rearing conditions. By design the current study extended the sampling period to encompass the period of critical sex differentiation, but did not include the early embryonic stages. Nevertheless, a similar trend in *cyp19a* expression was observed in the current study, all nine overlapping sample points verified. Specifically, *cyp19a* expression increased immediately after hatching, expression was suppressed at 25 °C compared with a higher peak in expression at 20 °C. This observation confirms that an elevated temperature in common carp represses expression of *cyp19a*. Similar observations have been reported in zebrafish (Uchida et al., 2004), Japanese flounder, *Paralichthys olivaceus* (Kitano et al., 1999) and Nile tilapia, *Oreochromis*

niloticus (D'Cotta et al., 2001; Kwon et al., 2000). In Atlantic halibut, *Hippoglossus hippoglossus,* warmer temperatures were again found to decrease expression of *cyp19a* while also increasing *cyp19b* expression (van Nes and Andersen, 2006).

A previous study in common carp (Beyer, 2004) reported that length is a good indicator of sex differentiation. Similarly, in sea bass, sex differentiation is more dependent on length than on age (Blázquez et al., 2008). Evidence in the current study suggests that individuals exceeding 20 mm TL showed sexually dimorphic expression of *cyp19a* with high (putative female) and low (putative males) expressing groups, at both temperatures. Several other studies have demonstrated that *cyp19a* expression is sexually dimorphic in sexually differentiated juveniles and adult fish (Chapter 3 / Barney et al., 2008; Kwon et al., 2001; Patil and Gunasekera, 2008). Therefore *cyp19a* expression serves as a molecular marker of ovarian differentiation in the species as has also been shown in other teleosts such as Nile tilapia (Kwon et al., 2001), Atlantic halibut (van Nes et al., 2005), pejerrey, *Odontesthes bonariensis* (Fernandino et al., 2008), zebrafish (Chiang et al.) and medaka, *Oryzias latipes* (Patil and Gunasekera, 2008). Further clustering of *cyp19a* expression data into one single low expressing group and two (a low and high expressing) groups above 20 mm TL, suggests that gonadal differentiation in common carp occurs around this size (20 mm TL). A similar conclusion was made previously employing histological techniques (Beyer, 2004).

In the previous study (Chapter 3 / Barney et al., 2008), zygotic expression of the *cyp19b* was slightly delayed compared to that of *cyp19a*, this was also observed in the 25 °C treatment of the current study. This is in contrast to the 20 °C treatment were zygotic expression of both isoforms occurs at about the same time, as seen in

medaka (Patil and Gunasekera, 2008). Both these patterns of expression differ from observations in other species such as zebrafish (Sawyer et al., 2006) and goldfish (Callard et al., 2001) wherein the onset of *cyp19b* expression precedes that of *cyp19a*. This observation together with the maternal inheritance of *cyp19a* (Chapter 3 / Barney et al., 2008) suggests that it plays an important role in early growth and neural differentiation in the species; at least until the *cyp19b* transcription is activated. Once activated the zygotic expression of *cyp19b* expression peaked at 250 ddph in both temperature treatments to a level well above the highest *cyp19a* expression at the corresponding temperatures. At this stage, larvae in the 25 °C treatment were pigmented and feeding exogenously while the 20 °C larvae were developing at a slower rate showing little sign of exogenous feeding. Therefore the absence of a temperature related difference in the timing of *cyp19b* expression despite the difference in growth is very intriguing. Due to the intermittent nature of sampling, it is possible that the 25 °C treatment may have peaked earlier than 250 ddph and perhaps even at a higher level as seen in Atlantic halibut (van Nes and Andersen, 2006) and Mozambique tilapia, *Oreochromis mossambicus* (Tsai et al., 2003) where brain-type aromatase (*cyp19b*) increased in warmer incubation temperatures. However this seems unlikely to be the case in common carp given that temperature related difference were not observed during rest of the experimental period.

Nevertheless based on the higher levels of *cyp19b* expression, the brain is the primary site of aromatisation during larval development and adulthood in common carp as found in most teleost fish, including zebrafish (Kishida and Callard, 2001) and medaka (Patil and Gunasekera, 2008). Not only does the brain develop earlier than the gonads but also, in most fish the brain show high levels of

17β-hydroxysteroid dehydrogenase activity, the enzyme responsible for testosterone synthesis, which in turn, becomes the primary substrate for estrogen synthesis mediated by aromatase (González and Piferrer, 2002; Pasmanik and Callard, 1985).

Although the brain is the primary site of aromatase expression, it still remains unclear whether changes in *cyp19b* are involved in gonadal sex differentiation in gonochoristic fish. Evidence in hermaphroditic fish suggests that changes in some neurotransmitters, gonadotropin-releasing hormone, or gonadotrophins are correlated with the process of sex change (Francis, 2004), and *cyp19b* has been identified as a critical gene whose disruption is likely to affect the functioning of the whole reproductive axis (Menuet et al., 2005). It has been hypothesised that temperature could act directly on the *cyp19b* gene to modify the sex differentiation pathway during embryogenesis. This "brain sexualisation" is consistent with the presence of a brain rudiment (late neurula stage at approximately 20 hpf) prior to the first sign of gonad formation (PGC's identified at time of hatching), thus leading to the hypothesis that sexual differentiation takes place in the brain prior to the gonad (Arnold, 2004; Francis, 1992). In Mozambique tilapia, expression of both brain aromatase and estrogen receptors were differentially regulated according to the temperature and the developmental period suggesting a relation with the brain-sex differentiation (Tsai et al., 2007). Although whole larvae were examined in the current carp study, in Atlantic halibut higher expression levels of aromatase genes were observed in the brain at an earlier developmental stage than for gonads also suggesting that sexual differentiation may begin in the brain prior to the gonad (Matsuoka et al., 2006). In European sea bass, *Dicentrarchus labrax*, and zebrafish (Blázquez and Piferrer, 2004; Trant et al., 2001) *cyp19b* is suggested to dictate sex differentiation displaying dimorphic expression at the time of histological gonadal

differentiation. Although sex-specific expression of *cyp19b* was not observed in the current study, the earlier peak of *cyp19a* expression suggests that it may play a role in sex differentiation via the brain-hypothalamic-gonadal axis.

Sex ratios and sex differentiation of various freshwater or marine species, from temperate or tropical habitats can be influenced by environmental factors (Baroiller and D'Cotta, 2001) and water temperature is the factor most often identified (Baroiller and D'Cotta, 2001; Bull, 1983). The influence of temperature on phenotypic sex determination has been demonstrated in many diverse and commercially important teleost species including Paralichthyd flounders (Kitano et al., 1999; Luckenbach et al., 2003) Atlantic halibut (van Nes and Andersen, 2006), barfin flounder, *Verasper moseri* (Goto et al., 1999), European sea bass (Mylonas et al., 2005), and Nile tilapia (Bezault et al., 2007; Rougeot et al., 2007; Rougeot et al., 2008). Thermal effects occur during a critical period and reportedly mediated through the aromatase expression (Ospina-Álvarez and Piferrer, 2008). The molecular mechanisms of thermosensitivity have been addressed in two species, Nile tilapia and Japanese flounder, where aromatase gene expression is down-regulated by masculinizing temperature treatments (Baroiller and D'Cotta, 2001). In zebrafish, high water temperatures depleted aromatase activity in all-female ovary and induced ovarian apoptosis and differentiation of spermatogonia, resulting in 100% masculinisation (Uchida et al., 2004). Apoptosis is also suggested to be involved in the temperature effects on the gonads in pejerrey species (Ito et al., 2003; Strüssmann et al., 1998). It is anticipated that temperature would have variable effects on sex differentiation depending on the genetic background and developmental stability of different species and strains (Devlin and Nagahama, 2002).

Although the data is preliminary an increased percentage of males at warmer rearing conditions are suggested in the current study. A male biased sex ratio would be in agreement with earlier observations and predictions of Nagy et al. (1981) in the species. Similar results were observed in a closely related cyprinid the goldfish (Goto-Kazeto et al., 2006). This suggests that sex determination in common carp is not only genetic but may also be subject to environmental influence through temperature and potentially mediated via ovarian aromatase gene expression amongst others. Further experiments on sex ratio are required to determine the extent temperature affects phenotypic sex in common carp. Considering the very wide environmental tolerances and the widespread populations of common carp it seems unlikely that the temperatures examined here could have a significant effect on sex differentiation as a whole. This property however, may make common carp an interesting species to examine the adaptive sex ratio theory (Charnov and Bull, 1977), where it is suggested that populations at different latitudes compensate for differences in thermal environment and seasonality by adjusting the response of sex ratio to temperature, and by altering the level of environmental as opposed to genetic control.

The mechanism connecting environmental temperature and aromatase expression still remain unclear, although recent evidence in the European sea bass suggests that high temperature effects could be mediated by methylation of specific CpG sites in the *cyp19a* promoter (Piferrer and Guiguen, 2008). It was earlier proposed that the molecular mechanisms of TSD was based on the temperature regulation of a thermosensitive factor directly or indirectly involved in the regulation of aromatase expression and/or enzyme activity (Pieau, 1996). The steroidogenic factor-1 (SF-1) has been proposed as the thermosensitive regulatory candidate. This transcription

factor plays essential roles at multiple levels of the reproductive axis as it regulates the expression of steroidogenic enzymes (reviewed by Morohashi and Omura, 1996; Parker and Schimmer, 1997). In the red-eared slider turtle, *Trachemys scripta,* SF-1 expression in the gonads increased and decreased at male and female producing temperatures, respectively (Fleming et al., 1999). Further studies demonstrated that estrogen treatment downregulated SF-1 in this species and caused development of females (Crews et al., 2001). Intriguingly, SF-1 responsive elements were identified in the 5' flanking region of the cyp19a gene, but not in cyp19b, of zebrafish, goldfish and Nile tilapia (Callard et al., 2001; Chang et al., 2005; Tong and Chung, 2003). To further understand the effects of temperature on mechanisms of sex differentiation and determination, further studies on the thermosensitivity of other factors, including SF-1 and ovarian aromatase are warranted.

4.6 Conclusion

In the current study it is proposed that sex-related changes in ovarian *(cyp19a)* aromatase expression occurred in larva exceeding 20 mm TL, and after the gonad had begun to morphological development. The present study demonstrated an effect of temperature on expression of *cyp19a* but not *cyp19b* and provided preliminary data suggesting that common carp may exhibit thermolability in the phenotypic expression of sex. However, given the wide distribution of common carp in both temperate and tropical habitats, it seems unlikely that the effect of temperature on aromatase activity is enough to alter sex ratio consistently.

4.7 References

Arnold, A.P., 2004. Sex chromosomes and brain gender. Nat Rev Neurosci, 5, 701-708.

Barney, M.L., Patil, J.G., Gunasekera, R.M., Carter, C.G., 2008. Distinct cytochrome P450 aromatase isoforms in the common carp (*Cyprinus carpio*): Sexual

dimorphism and onset of ontogenic expression. Gen Comp Endocrinol, 156, 499-508.

Baroiller, J.F. & D'Cotta, H., 2001. Environment and sex determination in farmed fish. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 130, 399-409.

Beyer, J., 2004. Early gonad development in the common carp, *Cyprinus carpio (L.)* at 20 °C and 25 °C. Honours Thesis, University of Tasmania, Launceston.

Bezault, E., Clota, F., Derivaz, M., Chevassus, B., Baroiller, J.-F., 2007. Sex determination and temperature-induced sex differentiation in three natural populations of Nile tilapia (*Oreochromis niloticus*) adapted to extreme temperature conditions. Aquaculture, 272, S3-S16.

Blázquez, M., González, A., Papadaki, M., Mylonas, C., Piferrer, F., 2008. Sex-related changes in estrogen receptors and aromatase gene expression and enzymatic activity during early development and sex differentiation in the European sea bass (*Dicentrarchus labrax*). Gen Comp Endocrinol, 158, 95-101.

Blázquez, 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)*. Mol Cell Endocrinol, 219, 83-94.

Bogart, M.H., 1987. Sex determination: A hypothesis based on steroid ratios. J Theor Biol, 128, 349-357.

Bull, J.J., 1983. Evolution of sex determinating mechanisms. Benjamin/Cummings Publishing Company, Inc., Menlo Park, CA.

Callard, G.V., Tchoudakova, A., Kishida, M., Wood, E., 2001. Differential distribution, developmental programming, estrogen regulation and promoter characteristics of *cyp19* genes in teleost fish. J Steroid Biochem Mol Biol, 79, 305-314.

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*). Gen Comp Endocrinol, 141, 101-115.

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

Chiang, E.F.-L., Yan, Y.-L., Guiguen, Y., Postlethwait, J., Chung, B., 2001. Two cyp19 (P450 aromatase) genes on duplicated zebrafish chromosomes are expressed in ovary or brain. Mol Biol Evol, 18, 542-550.

Conover, D.O. & Kynard, B.E., 1981. Environmental sex determination: Interaction of temperature and genotype in a fish. Science, 213, 577-579.

Crews, D., Fleming, A., Willingham, E., Baldwin, R., Skipper, J.K., 2001. Role of steroidogenic factor 1 and aromatase in temperature-dependent sex determination in the red-eared slider turtle. J Exp Zool, 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*. Mol Reprod Dev, 59, 265-276.

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.

FAO, 2007. The state of world fisheries and aquaculture, 2006, Rome, http://www.fao.org/docrep/009/A0699e/A0699e00.htm.

Fenske, M. & Segner, H., 2004. Aromatase modulation alters gonadal differentiation in developing zebrafish (*Danio rerio*). Aquat Toxicol, 67, 105-126.

Fernandez-Delgado, C., 1990. Life history patterns of the common carp, *Cyprinus carpio*, in the estuary of the Guadalquivir River in south-west Spain. Hydrobiologia, 206, 19-28.

Fernandino, J.I., Hattori, R.S., Shinoda, T., Kimura, H., Strobl-Mazzulla, P.H., Strussmann, C.A., Somoza, G.M., 2008. Dimorphic expression of *dmrt1* and cyp19a1 (ovarian aromatase) during early gonadal development in pejerrey, *Odontesthes bonariensis*. Sex Dev, 2, 316-24.

Fleming, A., Wibbels, T., Skipper, J.K., Crews, D., 1999. Developmental expression of Steroidogenic Factor 1 in a turtle with temperature-dependent sex determination. Gen Comp Endocrinol, 116, 336-346.

Francis, R.C., 1992. Sexual lability in teleosts: Developmental factors. The Quarterly Review of Biology, 67, 1-18.

Francis, R.C., 2004. Sexual lability in teleosts. Q. Rev. Biol., 67, 1-18.

Gelinas, D., Pitoc, G.A., Callard, G.V., 1998. Isolation of a goldfish brain cytochrome P450 aromatase cDNA. Mol Cell Endocrinol, 138, 81-93.

González, A. & Piferrer, F., 2002. Characterization of aromatase activity in the sea bass: effects of temperature and different catalytic properties of brain and ovarian homogenates and microsomes. J Exp Zool, 293, 500-510.

Goto-Kazeto, R., Abe, Y., Masai, K., Yamaha, E., Adachi, S., Yamauchi, K., 2006. Temperature-dependent sex differentiation in goldfish: Establishing the temperature-sensitive period and effect of constant and fluctuating water temperatures. Aquaculture, 254, 617-624.

Goto, R., Mori, T., Kawamata, K., Matsubara, T., Mizuno, S., Adachi, S., Yamauchi, K., 1999. Effects of temperature on gonadal sex determination in barfin flounder *Verasper moseri*. Fisheries Science (Japan), 65, 884-887.

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. Aquat Toxicol, 71, 371-389.

Guerrero, R.D. & Shelton, W.L., 1974. An aceto-carmine squash method for sexing juvenile fishes. The Progressive Fish-Culturist, 36, 56-56.

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

Guiguen, Y., Fostier, A., Piferrer, F., Chang, C.F., 2009. Ovarian aromatase and estrogens: a pivotal role for gonadal sex differentiation and sex change in fish. Gen Comp Endocrinol, In press, corrected proof.

Hinfray, N., Palluel, O., Turies, C., Cousin, C., Porcher, J.M., Brion, F., 2006. Brain and gonadal aromatase as potential targets of endocrine disrupting chemicals in a model species, the zebrafish (*Danio rerio*). Environ Toxicol, 21, 332-337.

Ito, L.S., Yamashita, M., Strüssmann, C.A., 2003. Histological process and dynamics of germ cell degeneration in pejerrey *Odontesthes bonariensis* larvae and juveniles during exposure to warm water. J. Exp. Zool. A. Comp. Exp. Biol., 297, 169–179.

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.

Kitano, T., Takamune, K., Kobayashi, T., Nagahama, Y., Abe, S., 1999. Suppression of P450 aromatase gene expression in sex-reversed males produced by rearing genetically female larvae at a high water temperature during a period of sex differentiation in the Japanese flounder (*Paralichthys olivaceus*). J Mol Endocrinol, 23, 167-176.

Kwon, J.Y., Haghpanah, V., Kogson-Hurtado, L.M., McAndrew, B.J., Penman, D.J., 2000. Masculinization of genetic female Nile tilapia (*Oreochromis niloticus*) by dietary administration of an aromatase inhibitor during sexual differentiation. J Exp Zool, 287, 46-53.

Kwon, J.Y., McAndrew, B.J., Penman, D.J., 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*). Mol Reprod Dev, 59, 359-370.

Kwon, J.Y., McAndrew, B.J., Penman, D.J., 2002. Treatment with an aromatase inhibitor suppresses high-temperature feminization of genetic male (YY) Nile tilapia. Journal of Fish Biology, 60, 625-636.

Lange, I.G., Hartel, A., Meyer, H.H.D., 2003. Review: Evolution of oestrogen functions in vertebrates. J Steroid Biochem Mol Biol, 83, 219-226.

Lavado, R., Thibaut, R., Raldúa, D., Martín, R., Porte, C., 2004. First evidence of endocrine disruption in feral carp from the Ebro River. Toxicol Appl Pharmacol, 196, 247-257.

Luckenbach, J.A., Godwin, J., Daniels, H.V., Borski, R.J., 2003. Gonadal differentiation and effects of temperature on sex determination in southern flounder (*Paralichthys lethostigma)*. Aquaculture, 216, 315-327.

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. Comp. Biochem. Physiol., B, 144, 128-135.

Menuet, A., Pellegrini, E., Brion, F., Gueguen, M.-M., Anglade, I., Pakdel, F., Kah, O., 2005. Expression and estrogen-dependent regulation of the zebrafish brain aromatase gene. J Comp Neurol, 485, 304-320.

Moens, L.N., Smolders, R., van der Ven, K., van Remortel, P., Del-Favero, J., De Coen, W.M., 2007. Effluent impact assessment using microarray-based analysis in common carp: A systems toxicology approach. Chemosphere, 67, 2293-2304.

Moens, L.N., van der Ven, K., Van Remortel, P., Del-Favero, J., De Coen, W.M., 2006. Expression profiling of endocrine-disrupting compounds using a customized *Cyprinus carpio* cDNA microarray. Toxicol Sci, 93, 298-310.

Morohashi, K.I. & Omura, T., 1996. Ad4BP/SF-1, a transcription factor essential for the transcription of the steroidogenic cytochrome P450 genes and for the establishment of the reproductive function. FASEB J, 10, 1569-1577.

Mylonas, C.C., Anezaki, L., Divanach, P., Zanuy, S., Piferrer, F., Ron, B., Peduel, A., Atia, I.B., Gorshkov, S., Tandler, A., 2005. Influence of rearing temperature during the larval and nursery periods on growth and sex differentiation in two Mediterranean strains of *Dicentrarchus labrax*. J. Fish Biol., 67, 652-668.

Nagy, A., Bercsenyi, M., Csani, V., 1981. Sex reversal in carp (*Cyprinus carpio*) by oral administration of methyltestosterone. Can. J. Fish. Aquat. Sci., 38, 725-728.

Ospina-Alvarez, N. & Piferrer, F., 2008. Temperature-dependent sex determination in fish revisited: Prevalence, a single sex ratio response pattern, and possible effects of climate change. PLoS ONE, 3, e2837.

Parker, K.L. & Schimmer, B.P., 1997. Steroidogenic factor 1: A key determinant of endocrine development and function. Endocr Rev, 18, 361-377.

Pasmanik, M. & Callard, G.V., 1985. Aromatase and 5α-reductase in the teleost brain, spinal cord and pituitary gland. Gen Comp Endocrinol, 60, 244-251.

Patil, J.G. & Gunasekera, R.M., 2008. Tissue and sexually dimorphic expression of ovarian and brain aromatase mRNA in the Japanese medaka (*Oryzias latipes*): Implications for their preferential roles in ovarian and neural differentiation and development. Gen Comp Endocrinol, 158, 131-137.

Pieau, C., 1996. Temperature variation and sex determination in reptiles. BioEssays, 18, 19-26.

Piferrer, F. & Blazquez, M., 2005. Aromatase distribution and regulation in fish. Fish Physiol Biochem, 31, 215-226.

Piferrer, F. & Guiguen, Y., 2008. Fish gonadogenesis. Part II: Molecular biology and genomics of sex differentiation. Reviews in Fisheries Science, 16, 35 - 55.

Piferrer, F., Zanuy, S., Carrillo, M., Solar, I.I., Devlin, R.H., Donaldson, E.M., 1994. Brief treatment with an aromatase inhibitor during sex differentiation causes chromosomally female salmon to develop as normal, functional males. J Exp Zool, 270, 255-262.

Rougeot, C., Prignon, C., Kanfitine, S.Y., Ngouana Kengne, C.V., Mélard, C., 2007. Sex determination in Nile tilapia, *Oreochromis niloticus*: Effect of high temperature during embryogenesis on sex ratio and sex differentiation pathway. Aquaculture, 272, S306-S307.

Rougeot, C., Prignon, C., Ngouana Kengne, C.V., Mélard, C., 2008. Effect of high temperature during embryogenesis on the sex differentiation process in the Nile tilapia, *Oreochromis niloticus*. Aquaculture, 276, 205-208.

Sawyer, S., 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. Gen Comp Endocrinol, 147, 108-117.

Shields, J.T., 1957. Experimental control of carp reproduction through water drawdowns in Fort Randall reservoir, South Dakota. Transactions of the American Fisheries Society, 87, 23-32.

Simpson, E.R., Clyne, C., Rubin, G., Boon, W.C., Robertson, K., Britt, K., Speed, C., Jones, M., 2002. Aromatase - A brief overview. Annu Rev Physiol, 64, 93-127.

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

Smith, B.B., 2005. The state of the art: a synopsis of information on common carp (*Cyprinus carpio*) in Australia. Final Technical Report Series No. 77, prepared by the South Australian Research and Development Institute (Aquatic Sciences), Adelaide.

Strüssmann, C.A., Saito, T., Takashima, F., 1998. Heat-induced germ cell deficiency in the teleosts *Odontesthes bonariensis* and *Patagonina hatcheri*. Comp. Biochem. Physiol. A, 199, 637-644.

Swee, U.B. & McCrimmon, H.R., 1966. Reproductive biology of the carp, *Cyprinus carpio* L., in Lake St. Lawrence, Ontario. Transactions of the American Fisheries Society 95, 372-380.

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.

Tong, S.-K. & Chung, B.-c., 2003. Analysis of zebrafish *cyp19* promoters. J. Steroid Biochem., 86, 381-386.

Trant, J.M., Gavassa, S., Ackers, J., Chung, B.-C., Place, A.R., 2001. Developmental expression of cytochrome P450 aromatase genes (CYP19a and CYP19b) in zebrafish fry (*Danio rerio*). J Exp Zool, 290, 475-483.

Tsai, C.-L., Chang, S.L., Wang, L.H., Chao, T.Y., 2003. Temperature influences the ontogenetic expression of aromatase and oestrogen receptor mRNA in the developing Tilapia (*Oreochromis mossambicus*) brain. J Neuroendocrinol, 15, 97-102.

Tsai, C., Wang, L., Shiue, Y., Chao, T., 2007. Influence of temperature on the ontogenetic expression of neural development-related genes from developing Tilapia brain expressed sequence tags. Mar Biotechnol, 9, 243-261.

Tzchori, I., Zak, T., Sachs, O., 2004. Masculinization of genetic females of the common carp (*Cyprinus carpio* L.) by dietary administration of an aromatase inhibitor. Israeli Journal of Aquaculture-Bamidgeh, 56, 239-246.

Uchida, D., Yamashita, M., Kitano, T., Iguchi, T., 2002. Oocyte apoptosis during the transition from ovary-like tissue to testes during sex differentiation of juvenile zebrafish. J Exp Biol, 205, 711-718.

Uchida, D., Yamashita, M., Kitano, T., Iguchi, T., 2004. An aromatase inhibitor or high water temperature induce oocyte apoptosis and depletion of P450 aromatase activity in the gonads of genetic female zebrafish during sex-reversal. Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology, 137, 11-20.

van Nes, S. & Andersen, O., 2006. Temperature effects on sex determination and ontogenetic gene expression of the aromatases *cyp19a* and *cyp19b*, and the estrogen receptors *esr1* and *esr2* in Atlantic halibut (*Hippoglossus Hippoglossus)*. Mol Reprod Dev, 73, 1481-1490.

van Nes, S., Moe, M., Andersen, O., 2005. Molecular characterization and expression of two *cyp*19 (P450 aromatase) genes in embryos, larvae and adults of Atlantic halibut (*Hippoglossus hippoglossus)*. Mol Reprod Dev, 72, 437-449.

Villeneuve, D.L., Larkin, P., Knoebl, I., Miracle, A.L., Kahl, M.D., Jensen, K.M., Makynen, E.A., Durhan, E.J., Carter, B.J., Denslow, N.D., Ankley, G.T., 2007. A graphical systems model to facilitate hypothesis-driven ecotoxicogenomics research on the teleost brain-pituitary-gonadal axis. Environ Sci Technol, 41, 321-330.

Zhao, J., Mak, P., Tchoudakova, A., Callard, G., Chen, S., 2001. Different catalytic properties and inhibitor responses of the goldfish brain and ovary aromatase isozymes. Gen Comp Endocrinol, 123, 180-191.

CHAPTER 5

Promoter Analysis of Cytochrome P450 Aromatase genes in the common carp (*Cyprinus carpio*).

Chapter 5 - Promoter Analysis of Cytochrome P450 Aromatase genes in the common carp (*Cyprinus carpio*).

5.1 Abstract

The involvement of aromatase in sex differentiation by catalysing the conversion of androgens into estrogens is well documented. Two aromatase genes were previously described in the common carp, *Cyprinus carpio*, a highly valuable aquaculture species as well as an invasive pest. To determine the theoretical molecular basis underlying the specific activation or repression of brain and ovarian aromatase gene expression during sex differentiation and in response to exogenous estradiol, this study isolated the 5'-flanking fragment of these genes and characterized their potential promoter sequences. The promoters show conserved binding sites for cAMP response elements (CRE) and putative binding sites for transcription factors belonging to the Sox and forkhead families. While half-estrogen response elements (ERE) and half-androgen response element (ARE) were found in both promoters, full ERE binding sites were unique to the promoter of the brain isoform (*cyp19b*). Conversely, binding sites for steroidogenic factor-1 (SF-1) were unique to the ovarian aromatase (*cyp19a*) promoter. These unique binding sites indicate that these two isoforms are regulated by different transcriptional factors which will affect or facilitate tissue specific expression.

5.2 Introduction

Cytochrome P450 aromatase (CYP19) is a key enzyme in the steroidogenic pathway that catalyses the conversion of testosterone to estrogen, and therefore influences gonadal sex differentiation. Biosynthesis of estrogens occurs in all classes of vertebrates with most higher vertebrates having a single *cyp19* gene with multiple

promoter elements that regulate tissue-specific expression with aromatase expression occurring in both gonads and in the brain (reviewed by Simpson et al., 2002). However, in teleosts, gonadal and neural expression of *cyp19* is regulated by two distinct aromatase isoforms (*cyp19a* and *cyp19b*; ovary and brain isoforms respectively). The existence of two paralogous genes is suggested to be due to the occurrence of genome duplication early in the teleost linage (Taylor et al., 2003) with partitioning of initial functions of each gene in two different tissues.

The expression of *cyp19* genes is regulated by transcription factor binding sites and response elements located in the promoter region. Analysis of the promoter region of aromatase genes in fish shows the presence of binding sites for the piscine equivalent to many mammalian transcription factors implicated in the sex determination and differentiation cascade. While transcription factors appear to be conserved among vertebrates, response elements appear to have adapted to facilitate localised expression of paralogous genes.

The promoter region of piscine *cyp19* genes have been cloned in numerous gonochoristic species including; medaka, *Oryzias latipes* (Kuhl et al., 2005; Tanaka et al., 1995), goldfish, *Carassius auratus* (Callard et al., 2001; Tchoudakova et al., 2001), zebrafish, *Danio rerio* (Kazeto et al., 2001), channel catfish, *Ictalurus punctatus* (Kazeto and Trant, 2005; Tong and Chung, 2003), Nile tilapia, *Oreochromis niloticus* (Chang et al., 2005), gilthead seabream, *Sparus aurata* (Wong et al., 2006), European seabass, *Dicentrarchus labrax* (Galay-Burgos et al., 2006), grey mullet, *Mugil cephalus* (Nocillado et al., 2007), mummichog, *Fundulus heteroclitus* (Dong et al., 2008) and hermaphroditic species barramundi, *Lates calcarifer* (protrandry), humpback grouper, *Cromileptes altiveli* (protogyny), and

broad-barred goby, *Gobiodion histrio* (bi-directional) (Gardner et al., 2005). This has helped to place *cyp19* in a broader context in fish reproduction with the finding that potential *cis*-acting elements in the *cyp19* genes of hermaphroditic and gonochoristic fish are the same.

Sex differentiation in fish is extremely complex, involving environmental as well as genetic factors and underlying mechanisms vary even between quite closely related species (reviewed in Baroiller and D'Cotta, 2001; Devlin and Nagahama, 2002). The regulation of teleost *cyp19* expression at the transcriptional level by endocrine disrupting chemicals have been recently and extensively reviewed (Cheshenko et al., 2008). Sex differentiation in many fish, including those with well-defined genetic sex-determining systems, can be influenced by temperature (for example, Craig et al., 1996). These effects may be mediated at least in part through changes in aromatase activity and expression as a result of promoter characteristics and functionality.

Two distinct *cyp19* genes are found in common carp with differential expression in numerous tissues (Chapter 3 / Barney et al., 2008). To investigate the molecular basis of tissue-specific expression of these genes, this study isolated the promoter regions and analysed potential binding and transcription sites.

5.3 Material and Methods

5.3.1 Sample collection

For promoter analysis a sample of liver was taken from an adult female common carp collected from Lake Sorell, Tasmania as previously described in Chapter 2. This fish was also used for expression analysis in Chapters 3, 6, 7 and 8.

5.3.2 Genomic DNA extraction

Genomic DNA was extracted from fresh liver tissue by first grinding to a powder under liquid nitrogen. The sample was then incubated overnight at 37 °C in a Proteinase K digestion buffer (Proteinase K (20 mg/mL), 50 mM Tris HCl, pH 8.0, 100 mM EDTA, 100 mM NaCl, 1% SDS). This was followed by the addition of 4.3 µl of 100mg/ml RNase and a second incubation at 37 °C for 1 hour. The samples were extracted once each using phenol, phenol/chloroform/isoamyl alcohol (25:1:24) and finally chloroform/isoamyl alcohol (24:1). DNA was precipitated using molecular grade absolute ethanol. Pellets were washed in 70% ethanol, dried, and resuspended in sterile dH$_2$O.

5.3.3 Construction of DNA libraries

Cloning of *cyp19* promoter regions was carried out using the GenomeWalker Universal Kit (Clontech, Palo Alto, CA, USA). Construction of GenomeWalker libraries was carried out according to the manufacturer's instructions. The genomic DNA (2.5 µg) was digested at 37 °C overnight with one of four different restriction enzymes (80 units of *Dra*I, *Eco*RV, *Pvu*II, or *Stu*I), and the digested blunt end DNAs were purified by phenol extraction and ethanol precipitation. Adapter ligation reactions (4 µL of digested, purified DNA, 1.9 µL of GenomeWalker Adapter, 1.6 µL 10X ligation buffer and 0.5 µL T4 DNA Ligase) were incubated overnight at 16 °C. Reactions were stopped by incubating at 70 °C for 5 min and then 72 µL TE (10/1 pH 7.5) added to each tube and mixed gently.

5.3.4 Genome walking in libraries

Gene specific primers (GSP) were designed based on the cDNA sequences of ovarian (EU375455) and brain (EU375456) isoforms, according to the manufacturer's instructions and are listed in Table 5.1.

Table 5.1 Gene specific primers used for genome walking aromatase promoters

Primer Name	Sequence 5' - 3'	Amplicon
GSP1A	TCAGGAGGTCCAGAGGAGCCTCACTGAGAT	*cyp19a primary walk*
GSP2A	CCACAAGGCTGAAGAAGTTCACCTGCCATA	*cyp19a nested walk*
GSP1B	CCTCTTTAGATCTGCATCCGCTTCATTTTG	*cyp19b primary walk*
GSP2B	CAGACTTGCCGTTTACATGCTCCCATCA	*cyp19b nested walk*

Sufficient primary PCR master mix was prepared, containing; 40 µL deionised water, 5 µL 10X Advantage 2 PCR Buffer, 1 µL dNTP (10 mM each), 1 µL Adapter Primer 1 (AP1) and 1 µL Advantage 2 Polymerase Mix (50X). For each DNA library, 1 µL of GSP1 (10 µM) and 1 µL of library template were added to the PCR master mixes. Water replaced the template for the negative control reaction. Cycling parameters were as follows: 7 cycles; 94 °C for 25 sec, 72 °C for 3 min, followed by 32 cycles of 94 °C for 25 sec, 67 °C for 3 min and an additional 7 min at 67 °C after the final cycle. Primary PCR products were analysed on 1.5% agarose/EtBr gel.

Each primary PCR product and the negative control were diluted 1:50 with deionised water. The secondary PCR master mix was as above with Adapter Primer 2 (AP2) instead of AP1. For each nested PCR reaction, 1 µL of GSP2 (10 µM) was added to the master mix and 1 µL of diluted primary PCR product added to the respective tube including the negative control. Cycling parameters were as follows: 5 cycles; 94 °C for 25 sec, 72 °C for 3 min, followed by 20 cycles of 94 °C for 25 sec, 67 °C for 3 min and an additional 7 min at 67 °C after the final cycle. Secondary PCR products were again examined on gel.

5.3.5 Sequencing

Libraries that displayed large single products (>3 kb) were then cloned and purified. Sequencing procedure was as previously described (Chapter 2). Fragments were confirmed to be correct promoter regions by designing proof-reading primers that were when paired with the original GSP1, cloned a confirmatory overlap at the start of the coding region of the respective gene. Nucleotide sequences were analysed using SeqManII, Lasergene 7 (DNASTAR Inc.) and a full length promoter regions was constructed in BioEdit (Hall, 1999).

5.3.6 Promoter analysis

The putative transcription initiation site of common carp *cyp19a* and *b* was determined by 5' RACE-PCR, as already described (Chapter 3 / Barney et al., 2008). Potential transcription factor binding sites were identified by MatInspector (http://www.genomatix.de) and TFSEARCH (http://www.cbrc.jp), promoter analysis tools available on the internet, and also compared with previously published sequences using BLAST (http://www.ncbi.nlm.nih.gov/).

5.4 Results

The 5'-flanking regions of the *cyp19* genes were isolated by a PCR-based genomic walking strategy using the Universal GenomeWalker Kit and gene-specific primers GSP1A, GSP2A and GSP1B, GSP2B. All the four genomic libraries yielded specific amplifications of the ovarian aromatase promoter with sizes ranging from ~250 bp to ~3 kb. The largest product (~3 kb) obtained from the *StuI* library was sequenced. Specific amplifications of the brain aromatase promoter were yielded from three libraries, *EcoRV*, *StuI* and *PvuII*, with sizes ranging from ~1.2 kb to ~5.2 kb. The largest product (~5.2 kb) from the *EcoRV* library was sequenced. Following

sequencing the ovarian (Figure 5.1) and brain (Figure 5.2) 5' flanking regions were determined to be 3179 bp and 5209 bp respectively.

Both the sequenced promoter regions had the highest identity with the respective region for goldfish. The carp *cyp19a* promoter was 88% similar to goldfish *cyp19a,* clone 3 (AF324897) over a 673 bp section. For *cyp19b* the homology was higher, with 97% identity over an 801 bp region of goldfish *cyp19b*, clone 2 (AF324894).

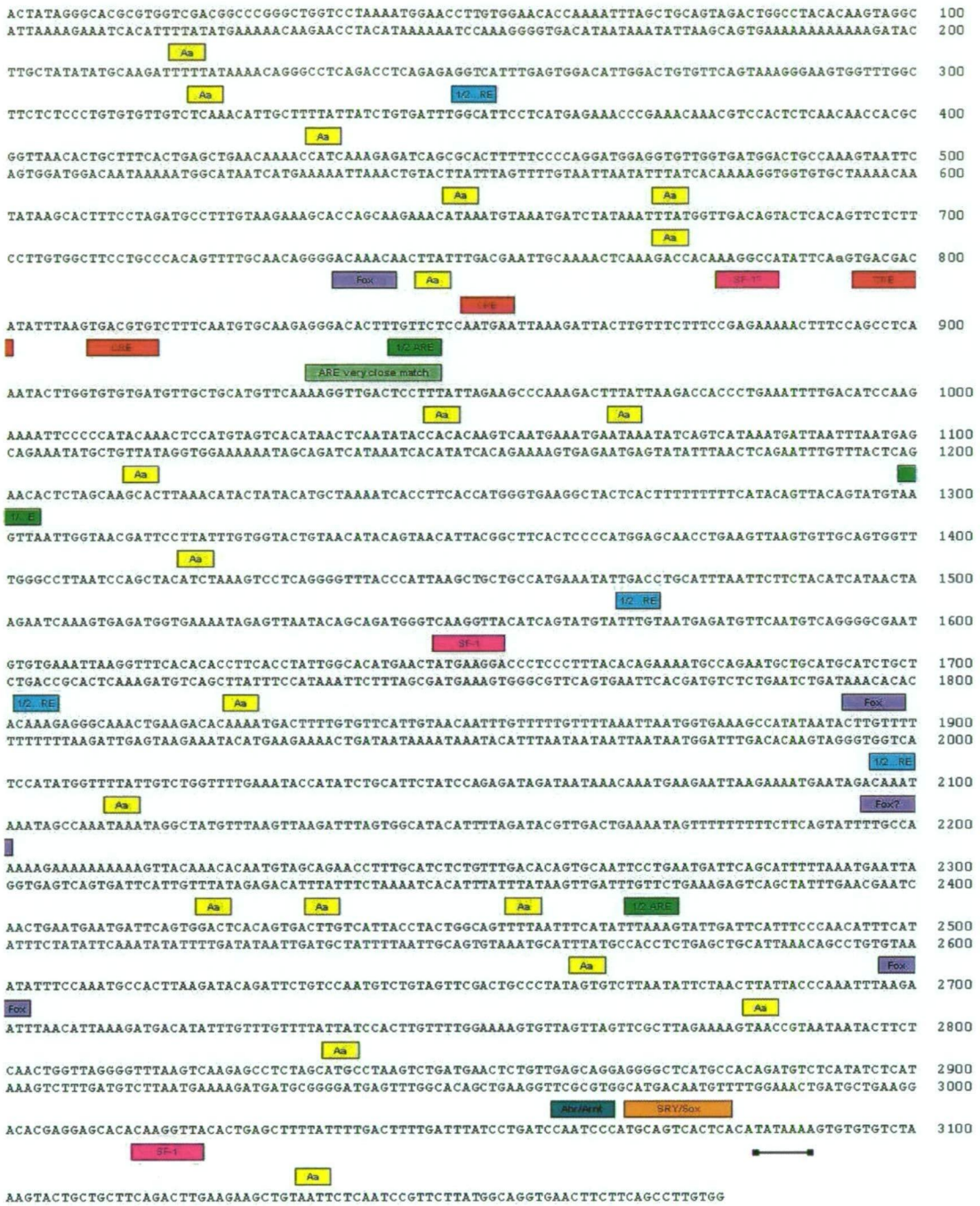


Figure 5.1 Sequenced 5' flanking region of common carp *cyp19a* gene.

Each of the putative binding sites are marked below the sequence and boxed. The TATA box is underlined. Yellow – Aα/Aβ; Aqua- Full/half ERE; Purple- Fox; Red- CRE; Lime- Full/half ARE; Pink- SF-1; Orange- SRY/Sox; Pale Orange- iSRY/iSox; Green- Ahr/Arnt; Blue- NBRE.

Figure 5.2 Sequenced 5' flanking region of common carp *cyp19b* gene.

Each of the putative binding sites are marked below the sequence and boxed. The TATA box is underlined. Yellow – Aα/Aβ; Aqua- Full/half ERE; Purple- Fox; Red- CRE; Lime- Full/half ARE; Pink- SF-1; Orange- SRY/Sox; Pale Orange- iSRY/iSox; Green- Ahr/Arnt; Blue- NBRE.

Table 5.2 Selected binding sites / transcription factors predicted from common carp aromatase promoters.

Binding Site/ Transcription Factor	*cyp19a*	*cyp19b*
Forkhead	4	3
CRE	3	2
SRY	0	10
iSRY	0	1
SRY/Sox	1	2
iSRY/Sox	0	3
Aα	20	14
Ab	0	1
ERE	0	2
1/2 ERE	4	1
1/2 ARE	3	7
SF-1	3	0
Ahr/ Arnt	1	0
WT-1	0	0

Several binding sites were identified in both promoters and included estrogen receptor element (ERE) half sites, androgen receptor (ARE) half sites, cyclic-AMP responsive element (CRE) like sites, a CCAAT enhancer binding protein (C/EBP) recognition sites, a TATA box as well as several potential regulatory elements (Table 5.2). Among these putative regulatory elements, 3 putative SF-1 recognition sites were identified in the *cyp19a* promoter but none in the 5.2 kb region of *cyl9b* promoter. Recognition sites of arylhydrocarbon receptor (AhR) were also found to be unique to *cyp19a*. Conversely, two full ERE sites and eleven SRY (sex-determining region Y) binding sites (one inverted) were also found to be unique to the *cyp19b* promoter. Shared SRY/Sox binding sites were mainly found in the *cyp19b* promoter, with five recognised sites (three inverted), while only one site was predicted in the *cyp19a* promoter. Interestingly, the half-ERE site predicted in *cyp19b* is actually included in a consensus nerve growth factor inducible-B (NGFI-B/Nur77) binding site (NBRE). Also uniquely present in the *cyp19b* promoter was a single copy of a core sequences found in transcriptionally active regions of the mouse brain-specific exon I promoter, Ab region (CCCCT), while another similar region, Aα (TTAAT) (Honda et al., 1999), was common to both aromatase promoters. There were no

Wilm tumor 1 (WT1) binding sites in the common carp *cyp19* promoters. Other interesting sites that were predicted were in both *cyp19* promoters include heat shock factors (8 in *cyp19a* and 12 in *cyp19b*) and hypoxia inducible factors (1 in *cyp19a* and 4 *cyp19b*).

5.5 Discussion

The promoter sequences of the *cyp19* gene are highly conserved in mammals, with a 90% similarity among, human, mouse and bovine ovarian-specific promoter regions and likewise for the brain-specific ones (Honda et al., 1996; Jenkins et al., 1993; Shen et al., 1994). Teleost *cyp19* promoters are less than 40% similar to the mammalian promoter, but show about 40–52% similarity to other teleost promoters (Tong and Chung, 2003).

Interestingly potential *cis*-acting elements in the *cyp19* genes of gonochoristic fish are the same as in hermaphrodites (Gardner et al., 2005). Despite the evolution of two distinct *cyp19* genes in fishes, similarity in gene structure and splicing mechanism is conserved within the vertebrate phylum.

Differential expression of two distinct *cyp19* genes occurs in numerous tissues of common carp and other teleosts. Tissue-specific expression is effected by a combination of transcriptional factors located in the 5'-flanking end or promoter of each gene. By determining potential activation or response site in promoter regions it is possible to predict factors that may influence expression and place these genes in larger endocrine pathway.

This study found TATA boxes in both common carp *cyp19* promoters as found in most other species (Simpson et al., 1997). As the brain and ovarian aromatase isoforms in teleosts originated from a single ancestral gene it is not surprising that many binding and transcription sites are shared. One of these shared elements is the forkhead (FH) gene family. A member of this family is the winged helix/forkhead transcription factor gene 2 (*foxL2*), one of the very few ovarian differentiation genes known in vertebrates (Baron et al., 2005). This transcription factor has recently been shown to activate, with SF-1 (see below), the transcription of *cyp19a* in Nile tilapia by binding to a specific motif present in the promoter region of the *cyp19a* gene (Wang et al., 2007). The *foxL2* consensus sequence also found in the *cyp19a* promoters of European seabass, *Dicentrarchus labrax* (Galay-Burgos et al., 2006), and Japanese flounder, *Paralichthys olivaceus* (Yamaguchi et al., 2007). Guiguen et al. (2009) also reported the presence of FOX recognition 7-bp motif (50-G/A-T/C-C/A-A-A-C/T-A-30) (Kaufmann and Knöchel, 1996) in the *cyp19a* promoter sequences of rainbow trout, *Oncorhynchus mykiss* [sequence ID: AB210815 (Kanda et al., 2006)] and gilthead seabream, *Sparus aurata* [sequence ID: AY779630 (Wong et al., 2006)]. Another common factor in both *cyp19* promoters was the CRE binding protein which can induce *cyp19* expression and in turn estrogen production in fish (Tong and Chung, 2003). As aromatase is responsible for the conversion of androgens to estrogens it is not surprising that half-ARE were also found in both *cyp19* promoter regions.

Although the two aromatase isoforms have similar gene structures in the coding region and shared binding sites in the promoter, there are several elements that are unique to either isoform. Of special interest within the context of gonadal sex differentiation is the finding that in most fish, *cyp19a* promoter sequences contain

binding motifs for SF-1, half-ERE, potential SRY box (sox), CRE, GATA binding proteins and WT1. All these factors are actually known to be important players in the sex differentiation cascade of vertebrates (Brennan and Capel, 2004). However, GATA binding sites (data not shown), SRY/Sox sites (see below) and previously mentioned CRE sites were present in both common carp *cyp19* promoters, while no WT1 sites were found in either promoter. This may indicate some species or lineage specific divergence.

The binding region of SF-1 was found in the 5'-flanking region of *cyp19a* but not *cyp19b* of common carp. Similar observations have been made in zebrafish (Tong and Chung, 2003), goldfish (Callard et al., 2001) and Nile tilapia (Chang et al., 2005). However, in medaka the SF-1 region has been identified in both isoforms (Kuhl et al., 2005; Tanaka et al., 1995). SF-1 is an important *cis*-element that regulates expression of several steroidogenic enzymes in gonads and adrenals (Hatano et al., 1994) and has shown expression dependant on *cyp19* in diverse vertebrates (Crews et al., 2001; Lynch et al., 1993). As the pattern of expression is highly variable between species, it is likely that the interaction between SF-1 and other co-factors may cause sex-specific regulation of aromatase (Yoshiura et al., 2003). In the human and rat ovary, binding of the SF-1 protein to specific target sequences of the ovary-specific promoter have been implicated in the control of cAMP-induced CYP19 expression (Lynch et al., 1993). Although SF-1 has also been described in the brain promoter of the human aromatase gene (Honda et al., 1999), SF-1 knockout mice continue to express aromatase in the brain, suggesting that it is not essential for aromatase expression in the mouse brain (Shinoda et al., 1995). This then explains why either the SF-1 binding site was retained in the *cyp19a* promoter or lost in the *cyp19b* promoter. However among these putative transcription factors

potentially acting as cis-elements to regulate the *cyp19a* gene, only a few of them have been experimentally demonstrated to directly regulate fish *cyp19a*. Among those, SF-1 has been shown to regulate the transcription *cyp19a* in rainbow trout (Kanda et al., 2006).

Another unique site in the *cyp19a* promoter is the recognition sequence for aryl hydrocarbon receptor (AhR/nuclear translocation factor (ARNT) heterodimer (Tchoudakova et al., 2001) also present in the goldfish *cyp19a* promoter (Callard et al., 2001). This site is activated by xenobiotics, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and benzo(a)pyrene (B(a)P) (Cheshenko et al., 2007), inducing apoptosis in cells (Puga et al., 2002) and suppressing the activation of *cyp19* (Dasmahapatra et al., 2000) therefore having a role in the disruption of steroidogenesis and other reproductive parameters in natural fish population through environmental pollutants.

The co-binding site for SRY/*Sox9*, sex-determining factors in mammals, are present in the 5′ flanking region of *cyp19*a but not *cyp19*b of tilapia (Chang et al., 2005), zebrafish (Kazeto et al., 2001; Tong and Chung, 2003) and goldfish (Tchoudakova et al., 2001). However in the current study these binding sites were found in both common carp *cyp19* promoters. The specific SRY binding site was found to be specific to the *cyp19b* promoter. This may imply that different fish species regulate expression of the two aromatase isoforms in a species specific manner, perhaps influenced by the unique physiology and habitat.

In the *cyp19b* promoter there is a unique presence of the ERE motif. This and the high expression of *cyp19b* in the brain (Chapter 3 / Barney et al., 2008) suggest that

cyp19b is mainly involved in estrogen-mediated neural estrogen synthesis. Conversely the *cyp19a* promoter only contains half-ERE sites, thus this lack of full EREs may prevent or restrict an auto-regulatory positive feedback loop brought about by the product of its activity (E_2). As neural aromatase activity and *cyp19b* mRNA are upregulated by estrogen in the adult goldfish brain and zebrafish embryo, it is tempting to speculate that control is exerted directly on these EREs (Callard et al., 2001). No EREs are found within the brain-specific promoter region of zebra finch (Ramachandran et al., 1999), human (Honda et al., 1994) or mouse (Honda et al., 1999). Thus, estrogen effects on neural aromatase expression in avian and mammalian brain must take place through other regulators or transcription factors.

Other interesting binding sites that were predicted in both common carp promoters include hypoxia inducible factors and heat shock factors. It was earlier proposed that the molecular mechanisms of temperature-dependant sex determination (TSD) were based on the temperature regulation of either (a) the expression of heat shock proteins (hsp) which are involved in the binding of estrogens to estrogen receptors, or (b) a thermosensitive factor directly or indirectly involved in the regulation of aromatase expression and/or enzyme activity (Pieau, 1996). A relationship between temperature and *cyp19a* in common carp has been suggested wherein higher temperatures cause faster processing and dissipation of maternal transcripts in embryos (Chapter 3) or may suppress expression during larval development (Chapter 4). It is therefore likely that temperature directly influences the transcription, presumably mediated through the heat shock binding domains. However this could also be indirectly mediated via the unique presence of SF-1 in the promoter of *cyp19a*, as SF-1 is also known to be temperature sensitive (reviewed by Morohashi and Omura, 1996; Parker and Schimmer, 1997).

5.6 Conclusion

The promoter regions of the two distinct *cyp19* isoforms found in common carp have several shared bindings sites that may be remnants of a single ancestral *cyp19*. Although the data presented here are based on *in silico* prediction, it is interesting that each promoter has unique binding sites which may explain their regulation or predominant expression in either the ovary (*cyp19a*) or brain (*cyp19b*). Whilst the sub-functioning of ovarian and brain aromatase seems conserved across all teleosts, there appears to be subtle differences in their regulation among different species of teleosts.

5.7 References

Barney, M.L., Patil, J.G., Gunasekera, R.M., Carter, C.G., 2008. Distinct cytochrome P450 aromatase isoforms in the common carp (*Cyprinus carpio*): Sexual dimorphism and onset of ontogenic expression. Gen Comp Endocrinol, 156, 499-508.

Baroiller, J.F. & D'Cotta, H., 2001. Environment and sex determination in farmed fish. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 130, 399-409.

Baron, D., Batista, F., Chaffaux, S., Cocquet, J., Cotinot, C., Cribiu, E., De Baeree, E., Guiguen, Y., Jaubert, F., Pailhoux, E., Pannetier, M., Vaiman, D., Vigier, B., Veitia, R., Fellous, M., 2005. *Foxl2* gene and the development of the ovary: a story about goat, mouse, fish and woman. Reprod, Nutr, Dev, 45, 377-382.

Brennan, J. & Capel, B., 2004. One tissue, two fates: molecular genetic events that underlie testis versus ovary development. Nat Rev Genet, 5, 509-521.

Callard, G.V., Tchoudakova, A., Kishida, M., Wood, E., 2001. Differential distribution, developmental programming, estrogen regulation and promoter characteristics of *cyp19* genes in teleost fish. J Steroid Biochem Mol Biol, 79, 305-314.

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*). Gen Comp Endocrinol, 141, 101-115.

Cheshenko, K., Brion, F., Le Page, Y., Hinfray, N., Pakdel, F., Kah, O., Segner, H., Eggen, R.I.L., 2007. Expression of zebra fish aromatase *cyp19a* and *cyp19b* genes in response to the ligands of estrogen receptor and aryl hydrocarbon receptor. Toxicol Sci, 96, 255-267.

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. Gen Comp Endocrinol, 155, 31-62.

Craig, J.K., Foote, C.J., Wood, C.C., 1996. Evidence for temperature-dependent sex determination in sockeye salmon (*Oncorhynchus nerka*). Can. J. Fish. Aquat. Sci., 53, 141-147.

Crews, D., Fleming, A., Willingham, E., Baldwin, R., Skipper, J.K., 2001. Role of steroidogenic factor 1 and aromatase in temperature-dependent sex determination in the red-eared slider turtle. J Exp Zool, 290, 597-606.

Dasmahapatra, A.K., Wimpee, B.A.B., Trewin, A.L., Wimpee, C.F., Ghorai, J.K., Hutz, R.J., 2000. Demonstration of 2,3,7,8-tetrachlorodibenzo-p-dioxin attenuation of P450 steroidogenic enzyme mRNAs in rat granulosa cell in vitro by competitive reverse transcriptase-polymerase chain reaction assay. Mol Cell Endocrinol, 164, 5-18.

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.

Dong, W., Wang, L., Thornton, C., Scheffler, B.E., Willett, K.L., 2008. Benzo(a)pyrene decreases brain and ovarian aromatase mRNA expression in *Fundulus heteroclitus*. Aquat Toxicol, 88, 289-300.

Galay-Burgos, M., Gealy, C., Navarro-Martín, L., Piferrer, F., Zanuy, S., Sweeney, G.E., 2006. Cloning of the promoter from the gonadal aromatase gene of the European sea bass and identification of single nucleotide polymorphisms. Comp. Biochem. Physiol., A, 145, 47-53.

Gardner, L., Anderson, T., Place, A.R., Dixon, B., Elizur, A., 2005. Sex change strategy and the aromatase genes. The Journal of Steroid Biochemistry and Molecular Biology, 94, 395-404.

Guiguen, Y., Fostier, A., Piferrer, F., Chang, C.F., 2009. Ovarian aromatase and estrogens: a pivotal role for gonadal sex differentiation and sex change in fish. Gen Comp Endocrinol, In press, corrected proof.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. . Nucl. Acids Symp. Ser. , 41, 95-98.

Hatano, O., Takayama, K., Imai, T., Waterman, M.R., Takakusu, A., Omura, T., Morohashi, K., 1994. Sex-dependent expression of a transcription factor, Ad4BP, regulating steroidogenic P-450 genes in the gonads during prenatal and postnatal rat development. Development, 120, 2787-2797.

Honda, S., Harada, N., Abe-Dohmae, S., Takagi, Y., 1999. Identification of cis-acting elements in the proximal promoter region for brain-specific exon 1 of the mouse aromatase gene. Brain Res. Mol. Brain Res., 66, 122-132.

Honda, S., Harada, N., Takagi, N., 1994. Novel exon 1 of the aromatase gene specific for aromatase transcripts in human brain. Biochem Biophys Res Commun, 198, 1153-1160.

Honda, S., Harada, N., Takagi, Y., 1996. The alternative exons 1 of the mouse aromatase cytochrome P-450 gene. Biochim Biophys Acta, 1305, 145-150.

Jenkins, C., Michael, D., Mahendroo, M., Simpson, E., 1993. Exon-specific northern analysis and rapid amplification of cDNA ends (RACE) reveal that the proximal

promoter II (PII) is responsible for aromatase cytochrome P450 (CYP19) expression in human ovary. Mol Cell Endocrinol, 97, R1-R6.

Kanda, H., Okubo, T., Omori, N., Niihara, H., Matsumoto, N., Yamada, K., Yoshimoto, S., Ito, M., Yamashita, S., Shiba, T., Takamatsu, N., 2006. Transcriptional regulation of the rainbow trout CYP19a gene by FTZ-F1 homologue. J Steroid Biochem Mol Biol, 99, 85-92.

Kaufmann, E. & Knöchel, W., 1996. Five years on the wings of fork head. Mech Dev., 57, 3-20.

Kazeto, Y., Ijiri, S., Place, A., Zohar, Y., Trant, J.M., 2001. The 5'-flanking regions of *CYP*19A1 and *CYP*19A2 in zebrafish. Biochem Biophys Res Commun, 288, 503-508.

Kazeto, Y. & Trant, J.M., 2005. Molecular biology of channel catfish brain cytochrome P450 aromatase (CYP19A2): cloning, preovulatory induction of gene expression, hormonal gene regulation and analysis of promoter region. J Mol Endocrinol, 35, 571-583.

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

Lynch, J.P., Lala, D.S., Peluso, J.J., Luo, W., Parker, K.L., White, B.A., 1993. Steroidogenic factor 1, an orphan nuclear receptor, regulates the expression of the rat aromatase gene in gonadal tissues. Mol Endocrinol, 7, 776-786.

Morohashi, K.I. & Omura, T., 1996. Ad4BP/SF-1, a transcription factor essential for the transcription of the steroidogenic cytochrome P450 genes and for the establishment of the reproductive function. FASEB J, 10, 1569-1577.

Nocillado, J.N., Elizur, A., Avitan, A., Carrick, F., Levavi-Sivan, B., 2007. Cytochrome P450 aromatase in grey mullet: cDNA and promoter isolation; brain, pituitary and ovarian expression during puberty. Mol Cell Endocrinol, 263, 65-78.

Parker, K.L. & Schimmer, B.P., 1997. Steroidogenic factor 1: A key determinant of endocrine development and function. Endocr Rev, 18, 361-377.

Pieau, C., 1996. Temperature variation and sex determination in reptiles. BioEssays, 18, 19-26.

Puga, A., Xia, Y., Elferink, C., 2002. Role of the aryl hydrocarbon receptor in cell cycle regulation. Chem. Biol. Interact., 141, 117.

Ramachandran, B., Schlinger, B.A., Arnold, A.P., Campagnoni, A.T., 1999. Zebra finch aromatase gene expression is regulated in the brain through an alternate promoter. Gene, 240, 209-216.

Shen, P., Campagnoni, C.W., Kampf, K., Schlinger, B.A., Arnold, A.P., Campagnoni, A.T., 1994. Isolation and characterization of a zebra finch aromatase cDNA: in situ hybridization reveals high aromatase expression in brain. Brain Res. Mol. Brain Res., 24, 227-237.

Shinoda, K., Lei, H., Yoshii, H., Nomura, M., Nagano, M., Shiba, H., Sasaki, H., Osawa, Y., Ninomiya, Y., Niwa, O., Morohashi, K.-I., Li, E., 1995. Developmental defects of the ventromedial hypothalamic nucleus and pituitary gonadotroph in the FTZ-F1 disrupted mice Dev. Dyn., 204, 22-29.

Simpson, E.R., Clyne, C., Rubin, G., Boon, W.C., Robertson, K., Britt, K., Speed, C., Jones, M., 2002. Aromatase - A brief overview. Annu Rev Physiol, 64, 93-127.

Simpson, E.R., Zhao, Y., Agarwal, V.R., Michael, M.D., Bulun, S.E., Hinshelwood, M.M., Graham-Lorence, S., Sun, T., Fisher, C.R., Qin, K., Mendelson, C.R., 1997. Aromatase expression in health and disease. Recent Prog Horm Res, 52, 185–213.

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*). J. Biochem-Tokyo, 117, 719-725.

Taylor, J.S., Braasch, I., Frickey, T., Meyer, A., Van de Peer, Y., 2003. Genome duplication, a trait shared by 22,000 species of ray-finned fish. Genome Res, 13, 382-390.

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. J. Steroid Biochem., 78, 427-439.

Tong, S.-K. & Chung, B.-c., 2003. Analysis of zebrafish *cyp19* promoters. J. Steroid Biochem., 86, 381-386.

Wang, D.-S., Kobayashi, T., Zhou, L.-Y., Paul-Prasanth, B., Ijiri, S., Sakai, F., Okubo, K., Morohashi, K.-i., Nagahama, Y., 2007. Foxl2 up-regulates aromatase gene transcription in a female-specific manner by binding to the promoter as well as interacting with Ad4 binding brotein/Steroidogenic Factor 1. Mol Endocrinol, 21, 712-725.

Wong, T.T., Ijiri, S., Zohar, Y., 2006. Molecular biology of ovarian aromatase in sex reversal: complementary DNA and 50-flanking region isolation and differential expression of ovarian aromatase in the gilthead seabream (*Sparus aurata*). Biol Reprod, 74, 857-864.

Yamaguchi, T., Yamaguchi, S., Hirai, T., Kitano, T., 2007. Follicle-stimulating hormone signaling and Foxl2 are involved in transcriptional regulation of aromatase gene during gonadal sex differentiation in Japanese flounder, *Paralichthys olivaceus*. Biochem Biophys Res Commun, 359, 935-940.

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 interaction1. Biol Reprod, 68, 1545-1553.

CHAPTER 6

Cloning and expression of *foxL2* in the common carp (*Cyprinus carpio*): Sexual dimorphism and developmental expression.

Barney, M.L. and Patil, J.G. Expression of *foxL2* in the common carp (*Cyprinus carpio*): Sexual dimorphism and developmental expression. In preparation.

Chapter 6 – Cloning and expression of *foxL2* in the common carp (*Cyprinus carpio*): Sexual dimorphism and developmental expression.

6.1 Abstract

A *foxL2* cDNA was cloned from common carp ovary by RT-PCR and subsequent RACE. Alignment of this sequence with known *foxL2* sequences from vertebrates confirmed the conservation of the FOXL2 protein sequences, especially within the forkhead domain region. Real-time PCR revealed that *foxL2* is expressed in the common carp brain (B), pituitary (P) and gonads (G), reflecting its involvement in B–P–G axis, with sexually dimorphic expression in the gonads with predominant expression in the ovary. During development *foxL2* expression was unaffected by temperature, with peak expression prior to hatch and no sex-specific expression during the first 138 days of development. It is proposed that *foxL2* plays a role in early sex differentiation in common carp with sex-specific differences in expression after formation of the ovaries.

6.2 Introduction

The gene cascade leading to the female specific expression of ovarian aromatase (*cyp19a*), and the subsequent feminisation of the indifferent gonads, is poorly understood in fish. Among the putative upstream regulators of fish *cyp19a*, the winged helix/forkhead transcription factor gene 2 (*foxL2*), a member of the forkhead (FH) gene family, is of special interest. This is because it is one of the very few ovarian differentiation genes that has been conserved across vertebrate evolution with female-specific expression in the differentiating ovary of mammals (Baron et al., 2005), birds (Govoroun et al., 2004), and fish (Baron et al., 2004; Nakamoto et al., 2006; Wang et al., 2007). During early human development, *foxL2* plays a crucial

role in female reproduction, especially in differentiation and proliferation of granulosa cells, ovarian development, and maintenance of ovarian function by regulating the transcription of certain target genes (Cocquet et al., 2003; Loffler et al., 2003; Pisarska et al., 2004; Yao, 2005). *FoxL2* is recognized as the earliest sexually dimorphic marker of ovarian determination and differentiation in mammals because of its expression in the early genital ridge and its inhibitory action on the male differentiation pathway (Ottolenghi et al., 2005).

Mouse, chicken, and turtle represent three phylogenetically distant vertebrate groups which possess different mechanisms of sex determination, however all express *foxL2* in the early ovaries around the time of sex determination and exhibit sexually dimorphic expression (Loffler et al., 2003). This suggests that *foxL2* is a highly conserved early regulator of vertebrate ovarian differentiation. In teleost fish, *foxL2* expression was found to be sexually dimorphic in Nile tilapia, *Oreochromis niloticus* (Wang et al., 2007; 2004), rainbow trout, *Oncorhynchus mykiss* (Baron et al., 2004), and Japanese medaka, *Oryzias latipes* (Nakamoto et al., 2006), hence supporting the hypothesis that *foxL2* plays an important role in ovarian differentiation in teleosts as well. In medaka, *foxL2* is known to be involved in the differentiation of the granulosa cells (Nakamoto et al., 2006) and in Nile tilapia expression begins early during differentiation of the gonads and persists until adulthood (Wang et al., 2004). Interestingly, some teleosts such as rainbow trout, Tetraodon, *Tetraodon nigroviridis* and fugu, *Takifugu rubripes*, are reported to have two genetically independent paralogue genes, *foxL2a* and *b* (Baron et al., 2004; Vizziano-Cantonnet et al., 2008; Vizziano et al., 2007). A divergent paralog of *foxL2* has also been determined from the genome of zebrafish (NCBI accession BC162838). In rainbow trout the *foxL2a* expression is similar to that observed for the mammalian *foxL2* gene, while *foxL2b* is

specific to somatic cells of the ovary, and is expressed later than *foxL2a* (Baron et al., 2005).

Further investigations in mammals indicate that *foxL2* activates *cyp19* gene transcription by direct binding to the promoter (Pannetier et al., 2006). In a diverse range of species *foxL2* has been found to correlate positively with the level of aromatase (*cyp19*) expression, these species including Nile tilapia (Yoshiura et al., 2003), rainbow trout (Baron et al., 2005), Japanese flounder, *Paralichthys olivaceus* (Yamaguchi et al., 2007), wrinkled frog, *Rana rugosa* (Oshima et al., 2008), chicken (Govoroun et al., 2004; Loffler et al., 2003), goat (Pannetier et al., 2006), turtle (Ramsey et al., 2007) and mouse (Loffler et al., 2003).

The *foxL2* is located upstream of *cyp19* in the sex differentiation pathway and is considered the earliest known gene exhibiting sexually dimorphic expression patterns in ovarian somatic cells (Baron et al., 2005; Nakamoto et al., 2006). However, it is unknown how *foxL2* regulates sex differentiation, especially in regard to its regulation by other genes in the pathway. To address its role in sex differentiation in the common carp, a *foxL2* homologue was cloned and its expression profile determined in key adult tissues. Expression was also analysed through embryonic and larval development at high and low temperatures to determine maternal inheritance, initial zygotic expression, expression through the period of sex differentiation and the effect of temperature on its biosynthesis.

6.3 Material and Methods

6.3.1 Sample collection

Breeding, larval rearing and sampling procedures were as previously described (Chapter 2).

6.3.2 Molecular biology

General molecular biology techniques and sample preparation procedures were as previously described (Chapter 2).

6.3.2.1 Oligonucleotides

Oligonucleotide primers used for cloning and real-time assays are listed in Table 6.1. The design of cloning primers, cFoxl2.F1 and R1, was based on the gene-specific conserved Fork Head domain. Real-time primers, cFoxl2_F5 and cFoxl2_R3, were designed according to the requirements of SYBR Green Chemistry (Applied Biosystems) and also to be isoform specific. The common carp *ß-actin* specific primers cBActin4.F and cBActinD.R (Chapter 3 / Barney et al., 2008) were used as an endogenous control in the relative quantitative PCR assay.

Table 6.1 Primers for cloning and real-time PCR expression analysis of *foxL2* in common carp.

Primer Name	Sequence 5' - 3'	Amplicon
cFoxl2.R1	ACGCCGGATCCAGGGTCCAATAGTTG	5' RACE
cFoxl2.F1	TAGCTATGTGGCGCTGATTGCGATGG	3' RACE
cFoxl2_R2	GCCTTCACGCGGAACTTTAATGAAGC	5' Nested RACE
cFoxl2_F3	GGAGAGCGCGGAAAAGAGACTGACCCTC	3' Nested RACE
cFoxl2_F5	AACGCGGTGAAGAAAGAAGAGTT	RT PCR
cFoxl2_R3	GACGCATCCACCTTCTCAGAAC	RT PCR
cBActin4.F	CCCATCGAGCACGGTATTG	RT PCR
cBActinD.R	AAGGTGTGATGCCAGATCTTCTC	RT PCR

6.3.3 Real-time analysis

Real-time assay procedures were as previously described (Chapter 2). The efficiency value for *foxL2* primers (2.02), and the previously determined efficiency value for *β-actin* (1.80) (Chapter 3 / Barney et al., 2008) were used in subsequent analysis. Correlation coefficients were 0.99 and 0.98 for *foxL2* and *β-actin* respectively.

6.3.4 Data analysis

General data analysis procedures were previously described (Chapter 2).

6.4 ***Results***

6.4.1 Cloning and phylogenetic analysis

FoxL2 was cloned from common carp ovary tissue and the full length cDNA was 1351 bp with an open reading frame (ORF) of 292 AA (schematic diagram in Appendix). The nucleotide sequence was found to have 83% identity to a predicted nucleotide sequence similar to zebrafish *foxL2* (XM_001922826). Examination of the common carp FoxL2 protein found that it shared 68-83% identity with other teleostean FoxL2 proteins and contained a conserved Forkhead (FH) also known as "winged helix" transcription factor domain of 77 AA. This FH domain was found to have 6 DNA binding sites and within this highly conserved region the common carp sequence was found to have 98% and 97% identity to zebrafish (NP_001038717) and humans (AAH19896) respectively.

A phylogenetic tree was generated including common carp, teleostean and select tetrapods Foxl2 protein sequences (Figure 6.1). The teleostean FoxL2 proteins form a large clade with Cypriniformes and Siluriformes (common carp, zebrafish, *Danio rerio*, and southern catfish, *Silurus meridionalis*) lying close together.

The FoxL2b proteins for rainbow trout and zebrafish formed a separate clade located between chicken and amphibians. Partial FoxL2b proteins were identified from the

medaka and three-spined stickleback, *Gasterosteus aculeatus*, genomes but were not included in the tree.

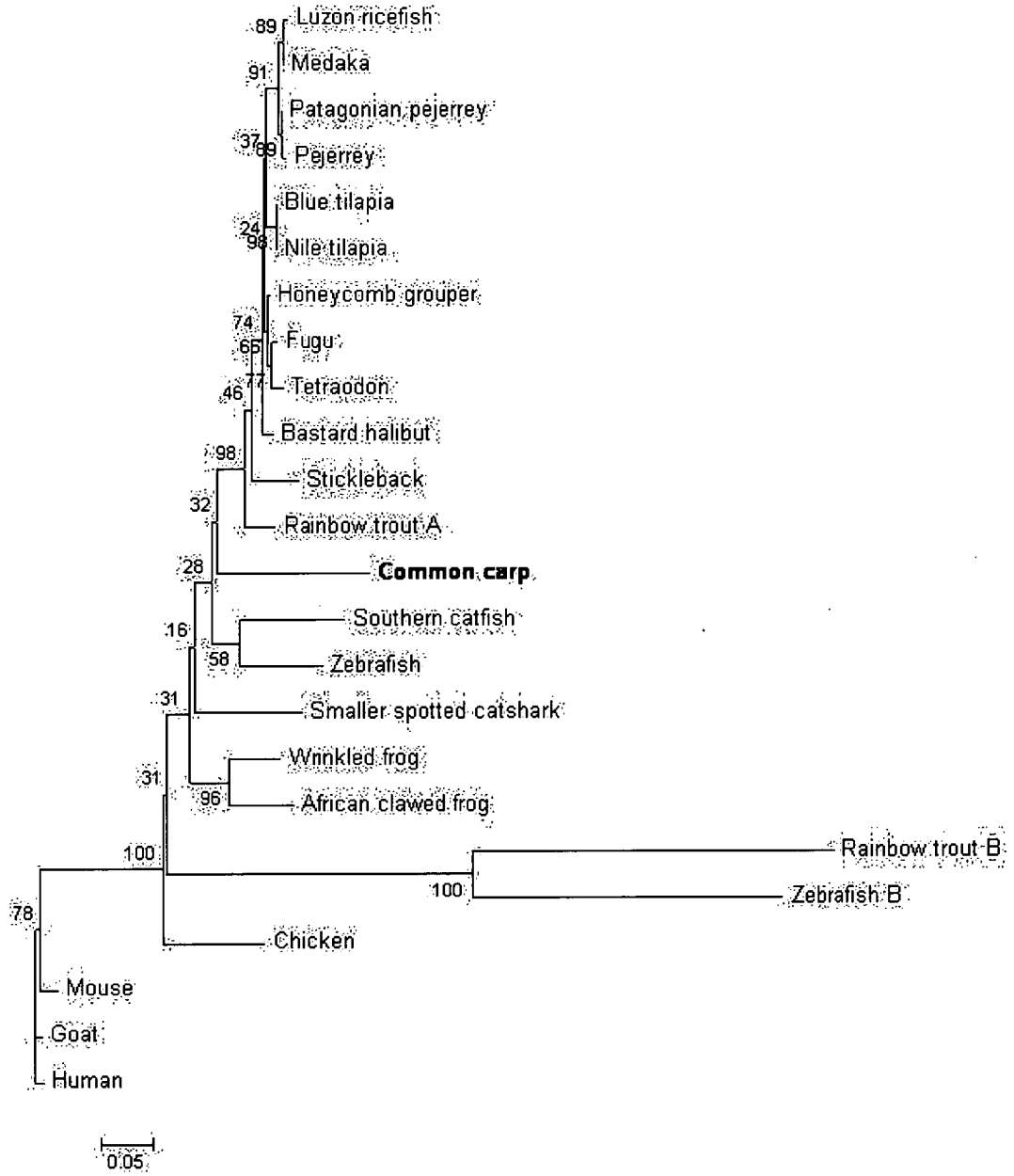


Figure 6.1 Phylogenetic tree of available FoxL2 proteins.

Deduced protein sequences from the following species were used for alignment; Japanese flounder, *Paralichthys olivaceus* (AB303854), Medaka, *Oryzias latipes* (AB252055), Luzon ricefish, *Oryzias luzonensis* (AB428665), Honeycomb grouper, *Epinephelus merra* (EU555180), Southern catfish, *Silurus meridionalis* (EF015396), Zebrafish, *Danio rerio* (NM_001045252 and *foxL2b*-BC162838), Blue tilapia, *Oreochromis aureus* (AM232738), Nile tilapia, *Oreochromis niloticus* (AM232737), Pejerrey, *Odontesthes bonariensis* (EU864151), Patagonian pejerrey, *Odontestes hatcheri* (FJ548572), Smaller spotted catshark, *Scyliorhinus canicula* (EF522837), Rainbow trout, *Oncorhynchus mykiss* (AY507927 & *foxL2b*-AY507926), Tetraodon, *Tetraodon nigroviridis* predicted from Ensembl transcript ENSTNIT00000017555 (CAAE01014974), Three-spined stickleback, *Gasterosteus*

aculeatus (from ENSGACT00000007467), fugu, *Takifugu rubripes* (from ENSTRUT00000019818), African clawed frog, *Xenopus laevis* (AB372218), Wrinkled frog, *Rana rugosa* (AB372103), Chicken (AY487165), mouse (AF522275), goat (AY112725), Human (DQ016609). Tree rooted by Human FOXL2.

6.4.2 Tissue and sex-specific expression of foxL2 mRNA in adult common carp

Tissue- and sex-specific expressions of *foxL2* is summarised in Figure 6.2. In females, highest expression was observed in the ovary and in the medulla (Brain D) ($F=27.465$, $P<0.000$), similar expression was found in the optic lobe (Brain B), and expression was 2-fold lower in the telencephalon and cerebellum (Brain A and C). The pituitary had the lowest *foxL2* expression of the neural tissues, 5-fold lower than in the medulla, and the liver was observed to express the least *foxL2* of all female tissues. In males the cerebellum (Brain C) showed the highest *foxL2* expression ($F=10.684$, $P<0.000$) with similar levels expressed in the telencephalon and medulla (Brain A and D) and 1.5-fold lower expression in the cerebellum (Brain C). The pituitary and testis showed similar expression levels of *foxL2*, 5-fold lower than expression in the telencephalon of males, and no expression was detected in the male liver.

A sex-specific difference in *foxL2* was found between the gonad, with females having 7-fold greater expression ($t(4) = 74.366$, $P < 0.001$) when compared to the testis (Figure 6.2).

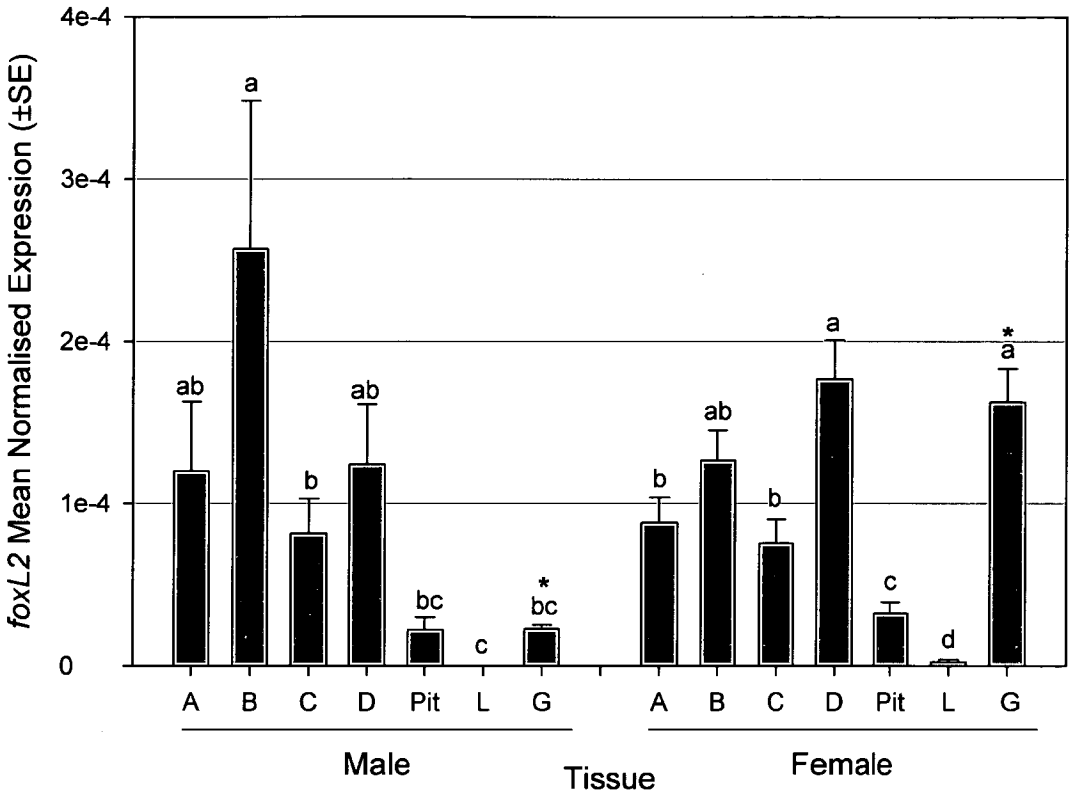


Figure 6.2 Tissue- and sex-specific differences in adult common carp *foxL2* expression.
Each bar represents the mean normalised expression (±SE; n = 3). X-axis labels: (A-D) brain sections (see Figure 2.1); Pit, pituitary; L, liver; G, gonad. One-way ANOVA was used to analyse data by tissue type within each sex, followed by SNK analysis to determine which means differed significantly ($P < 0.03$), as indicated by letters (a-c).

6.4.3 Ontogenic and larval development of common carp

6.4.3.1 Growth

Following hatch (20 °C- 70 ddpf, 25 °C- 62.5 ddpf) the TL of larva was measured at the time of sampling. In both temperatures TL was found to increase steadily over time (20 °C- $F=28.419$, $P<0.000$, 25 °C- $F=79.338$, $P<0.000$) (Figure 6.3). At the higher temperature (25 °C) the growth rate of larva was significantly higher ($F=16.813$, $P>0.000$) at 25 °C mean TL was 47.75 ± 3.43 mm after 118 dpf compared to 31.13 ± 2.47 mm at 20 °C after 138 dpf.

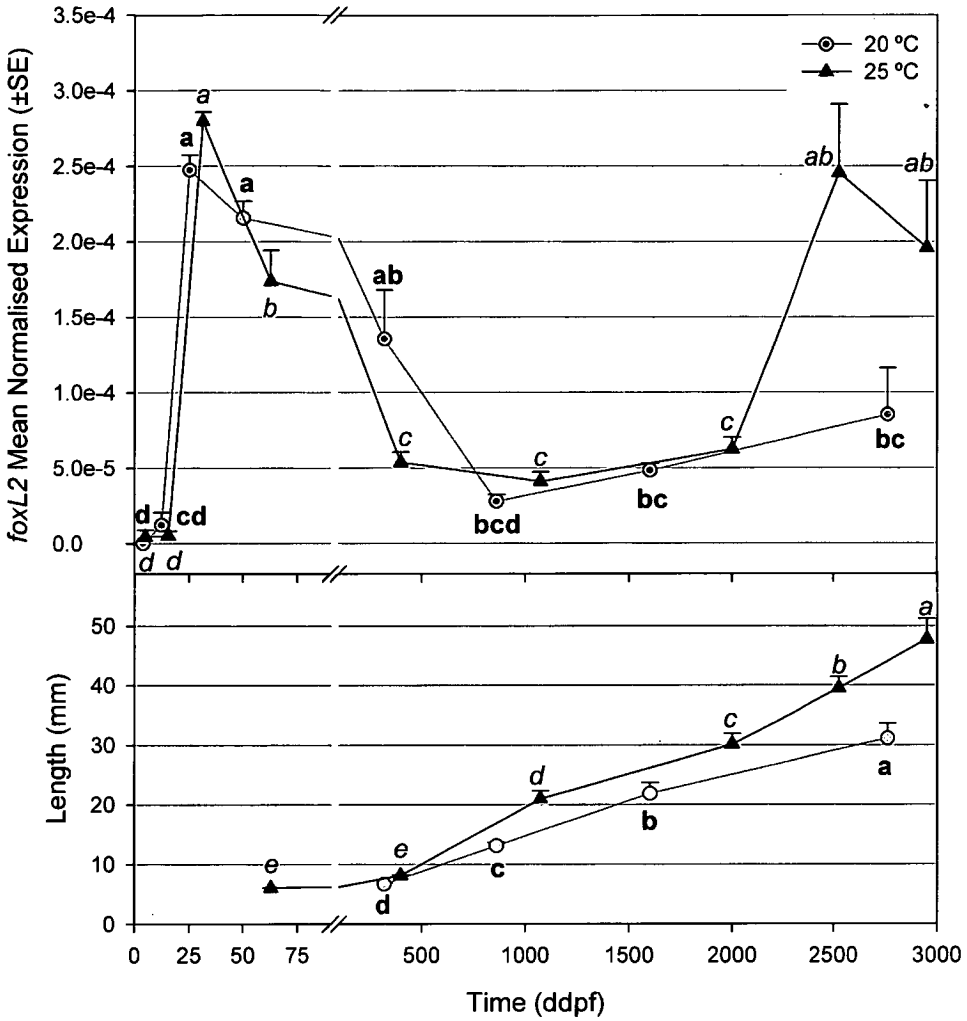


Figure 6.3 Temporal changes in length and *foxL2* expression of embryos and larvae reared at 20 or 25 °C.

One-way ANOVA used to analyse data for specific differences within each treatment, followed by SNK analysis to determine which means differed significantly ($P < 0.01$), as indicated by font style (20 °C- bold, 25 °C- italic). Note: hatch occurred at 70.8 and 62.5 ddpf in the 20 and 25 °C treatments respectively.

6.4.3.2 Embryonic and larval foxL2 expression

Temporal changes in *foxL2* expression during development were quantified by real-time PCR up to 2760 ddpf in the 20 °C treatment and 2950 ddpf at 25 °C (Figure 6.3). Expression of *foxL2* was not observed in unfertilised eggs, however significant changes in expression were observed over time in both treatments (20 °C- $F=12.250$, $P<0.000$, 25 °c- $F= 38.735$, $P<0.000$). In both temperatures expression of *foxL2* peaked before hatching at 25 ddpf (morula stage) at 20 °C and 31 ddpf (late blastula

stage) in the 25 °C treatments. In the 20 °C treatment, expression had dropped 8-fold by 860 ddpf and although it remained low for the remainder of the experimental period, an increasing trend appears to occur. In the 25 °C treatment expression dropped 5-fold by 400 ddpf and remained at this level until 200 ddph. A second peak was observed at 2525 ddpf in the warmer (25 °C) treatment with expression at similar level as the initial peak maintained through to completion of the experimental period.

6.5 Discussion

FoxL2 is one of the few ovarian differentiation genes known in vertebrates (Baron et al., 2005). This transcription factor is known to be involved in ovarian development, granulosa cell differentiation, and thus the proper maintenance of ovarian function (Cocquet et al., 2003; Loffler et al., 2003; Ottolenghi et al., 2005; Pisarska et al., 2004; Schmidt et al., 2004; Uda et al., 2004; Yao, 2005). The forkhead domain present in FOXL2 is highly conserved among vertebrates and hence its role in the sex-determining cascade is likely conserved. The presence of two genetically independent paralogue genes, *foxL2a* and *b* were previously reported in rainbow trout, fugu and tetraodon (Baron et al., 2004; Vizziano-Cantonnet et al., 2008; Vizziano et al., 2007). This study also found that a predicted zebrafish paralog of *foxl2b* formed a clade with the rainbow trout *foxL2b* isoform, while partial sequences were isolated from the medaka and three-spined stickleback genomes but not included in the phylogenetic analysis. This duplication across a range of fish species is further evidence of a whole genome duplication in the teleost linage (Taylor et al., 2003). While *foxL2a* appears to have maintained the main functional role, *foxL2b* has taken on specialised function in some species and may also have lost function in other species.

Spatial expression of *foxL2* was examined in different sections of the brain with a view to elucidate sex specific differences and potential roles in regulation of downstream molecules. In general, high levels of *foxL2* expression observed in this study correspond to consistantly high levels of *cyp19b* expression in brain sections observed in an earlier study (Chapter 3 / Barney et al., 2008). Similarly a positive correlation between the expression of *foxL2* and *cyp19b* has been reported in Southern catfish (Liu et al., 2007) and Nile tilapia (Kwon et al., 2001; Wang et al., 2004). Different levels of both *foxL2* and *cyp19b* are expressed within different sections of both male and female brain in the common carp, suggesting that it plays a role in differentiation and maintenance of brain sex. The expression of aromatase is concentrated in reproductive control centres of basal forebrain, especially in the telencephalic lobes, and in the hypothalamus/preoptic area, with lower levels of activity present in virtually all other regions of the brain, including basal midbrain, optic lobes, cerebellum, medulla and spinal cord in common carp (Chapter 3 / Barney et al., 2008) and goldfish (Callard et al., 1993). Aromatase activity has been shown to be restricted to radial glial cells in the anterior brain quarter in three fish species (Pellegrini et al., 2005). Expression of *foxL2* in the brain is not reported in chicken (Govoroun et al., 2004) or mammals (Cocquet et al., 2003) suggesting a specialised role in teleost fish, likely associated with the divergence of two aromatase isoforms. To further understand the role and location of *foxL2* in the teleost brain it would be interesting to map its expression in relation to aromatase expressing cells using *in situ* hybridisation.

Expression levels of *foxL2* in the gonad also mirrored earlier reported *cyp19a* expression (Chapter 3 / Barney et al., 2008) with sexually dimorphic and preferential expression of both genes (*cyp19a* and *foxL2*) in the ovary suggesting a ovarian

specific role for the *foxL2* in regulation of *cyp19a*. Regulation of *foxL2* on *cyp19a* gene transcription in a female-specific manner has been previously reported in tilapia (Wang et al., 2007). *FoxL2* has been shown to up-regulate ovarian expression of aromatase in both mammals and fish (Pannetier et al., 2006; Wang et al., 2007; Yamaguchi et al., 2007), and in turn estrogens, the biosynthetic product of *cyp19* activity, up-regulates *foxL2* in fish (Baron et al., 2004; Wang et al., 2007), suggesting regulation through a positive feedback loop between the two genes. However, it appears that both *foxL2* and SF-1 activate *cyp19* expression by binding directly to the promoter, with *foxL2* enhancing SF-1 activated transcription by forming a heterodimer (Nagahama, 2005). Although FOX binding sites are found in both *cyp19* promoters, SF-1 binding motif is present only in the promoter region of the *cyp19a* gene in common carp (Chapter 5), Nile tilapia (Wang et al., 2007), European seabass (Galay-Burgos et al., 2006) and Japanese flounder (Yamaguchi et al., 2007).

Sex reversal studies in tilapia show that *in vivo* expression of a an engineered mutation in the *foxL2* protein (that abolishes its function and also inhibits the function of simultaneously expressed wild-type protein) entitled 'transgenic dominant-negative-mutant-*foxL2* construct' reduced aromatase expression in females causing ovaries to degenerate and testicular tissue to develop with complete sex-reversal resulting in viable males (Nagahama, 2005). Baron et al (2004) examined the expression of *foxL2* in the ovaries of estrogen-induced sex-reversed female (genetic male) rainbow trout with and without aromatase inhibitor treatment. They observed two key patterns; 1) the feminising estrogen treatment (of males) induced expression of *foxL2* but did not up-regulate aromatase expression, suggesting exogenous estrogens are enough to drive the feminisation process, and 2) the

masculinising treatment (of females) with aromatase inhibitors quickly down regulated aromatase and *foxL2* expression.

The current study indicates that *foxL2* plays a role in early sex development, with a peak in expression observed in the morula and late blastula stages at 20 and 25 °C respectively. This is before peaks in larval expression of aromatase (*cyp19*) which occur after hatch, around 350 ddpf. The early larval onset of *foxL2* compared to *cyp19* suggest that *foxL2* is involved in the initiation of aromatase transcription during early ovarian differentiation in common carp as shown in medaka (Nakamoto et al., 2006) and chicken (Govoroun et al., 2004). However, unlike *foxL2*, *cyp19a* transcripts are maternally inherited in common carp (Chapter 3 / Barney et al., 2008), therefore it is possible that *foxL2* expression may itself be initiated by endogenously produced estrogen resulting from the activity of maternally inherited *cyp19a* aromatase transcript. In rainbow trout, estrogens strongly and quickly up-regulate both *foxL2a* and its divergent paralog, *foxL2b* (Baron et al., 2004; Vizziano-Cantonnet et al., 2008). However, it was found in medaka that not all *foxL2*-positive cells eventually expressed aromatase, and as in the current study, there was a significant delay from onset of expression of *foxL2* to that of aromatase (Chapter 4 and Nakamoto et al., 2006). This may indicate that expression of *foxL2* alone is insufficient to initiate aromatase expression, and other specific factor(s) might be required.

Nonetheless, *foxL2* is temporally co-expressed with *cyp19a* during the initial steps of ovarian differentiation in rainbow trout and Nile tilapia, (Ijiri et al., 2008; Vizziano et al., 2007; Wang et al., 2007). Moreover its expression is co-localized with *cyp19a* in some somatic cells of the differentiating ovaries in Japanese medaka, Japanese flounder and Nile tilapia (Nakamoto et al., 2006; Wang et al., 2007; Yamaguchi et

al., 2007). Therefore, the positive feedback loop between *foxL2* and *cyp19a* could account for the sexually dimorphic expression of both these isoforms in many teleosts. Although the expression of *cyp19a* was found to show sexually dimorphic expression in larval development in common carp (Chapter 4), the current study was unable to identify sex related differences in *foxL2* expression during larval development. This is made difficult as the sex of individuals can only be determined by observation of the differentiated gonad. The increase trend in *foxL2* expression in the 20 °C treatment and the second peak of expression late in the 25 °C treatment correlate with increase *cyp19a* levels in putative females (Chapter 4) and may be an indication of this feedback loop starting up to support ovarian development and granulosa cell differentiation within differentiating ovaries.

Interestingly, expression of *foxL2* does not appear to be affected by temperature in the common carp. This suggests that the suppression of *cyp19a* under warmer (25 °C) conditions previously found in the species (Chapter 3 / Barney et al., 2008 and Chapter 4) is regulated via another transcription factor, such as SF-1.

6.6 Conclusion

In summary, the *foxL2* gene was identified in the common carp and as in other species showed gonadal sexually dimorphic expression predominately in the ovary associated with high levels of ovarian aromatase. High levels of *foxL2* were also found to correspond to high levels of brain aromatase in the four brain sections examined. Analysis of embryonic and larval development demonstrated that this gene is not maternally inherited, with expression initiating early in development before the expression of aromatase. Subsequent expression was found to dissipate with no sex-specific expression observed in the experimental period. Temperature

does not appear to affect *foxL2* expression, suggesting that other transcription factors are responsible for the thermosensitive expression of ovarian aromatase.

6.7 Acknowledgments

Support and assistance provided by CSIRO, Invasive Animal Cooperative Research Centre, Inland Fisheries Service Tasmania and K&C Fisheries. This project received funding support from the Fisheries Research and Development Corporation.

6.8 References

Barney, M.L., Patil, J.G., Gunasekera, R.M., Carter, C.G., 2008. Distinct cytochrome P450 aromatase isoforms in the common carp (*Cyprinus carpio*): Sexual dimorphism and onset of ontogenic expression. Gen Comp Endocrinol, 156, 499-508.

Baron, D., Batista, F., Chaffaux, S., Cocquet, J., Cotinot, C., Cribiu, E., De Baeree, E., Guiguen, Y., Jaubert, F., Pailhoux, E., Pannetier, M., Vaiman, D., Vigier, B., Veitia, R., Fellous, M., 2005. *Foxl2* gene and the development of the ovary: a story about goat, mouse, fish and woman. Reprod, Nutr, Dev, 45, 377-382.

Baron, D., Cocquet, J., Xia, X., Fellous, M., Guiguen, Y., Veitia, R.A., 2004. An evolutionary and functional analysis of *FoxL2* in rainbow trout gonad differentiation. J Mol Endocrinol, 33, 705-715.

Callard, G.V., Drygas, M., Gelinas, D., 1993. Molecular and cellular physiology of aromatase in the brain and retina. J Steroid Biochem Mol Biol, 44, 541-547.

Cocquet, J., De Baere, E., Gareil, M., Pannetier, M., Xia, X., Fellous, M., Veitia, R.A., 2003. Structure, evolution and expression of the FOXL2 transcription unit. Cytogen. Genome Res., 101, 206-211.

Galay-Burgos, M., Gealy, C., Navarro-Martín, L., Piferrer, F., Zanuy, S., Sweeney, G.E., 2006. Cloning of the promoter from the gonadal aromatase gene of the European sea bass and identification of single nucleotide polymorphisms. Comp. Biochem. Physiol., A, 145, 47-53.

Govoroun, M.S., Pannetier, M., Pailhoux, E., Cocquet, J., Brillard, J.-P., Couty, I., Batellier, F., Cotinot, C., 2004. Isolation of chicken homolog of the *FOXL2* gene and comparison of its expression patterns with those of aromatase during ovarian development. Dev. Dynam., 231, 859-870.

Ijiri, S., Kaneko, H., Kobayashi, T., Wang, D.-S., Sakai, F., Paul-Prasanth, B., Nakamura, M., Nagahama, Y., 2008. Sexual dimorphic expression of genes in gonads during early differentiation of a teleost fish, the Nile tilapia *Oreochromis niloticus*. Biol Reprod, 78, 333-341.

Kwon, J.Y., McAndrew, B.J., Penman, D.J., 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*). Mol Reprod Dev, 59, 359-370.

Liu, Z., Wu, F., Jiao, B., Zhang, X., Hu, C., Huang, B., Zhou, L., Huang, X., Wang, Z., Zhang, Y., Nagahama, Y., Cheng, C.H.K., Wang, D., 2007. Molecular cloning of doublesex and mab-3-related transcription factor 1, forkhead transcription factor gene 2, and two types of cytochrome P450 aromatase in Southern catfish and their possible roles in sex differentiation. J Endocrinol, 194, 223-241.

Loffler, K.A., Zarkower, D., Koopman, P., 2003. Etiology of ovarian failure in blepharophimosis ptosis epicanthus inversus syndrome: FOXL2 is a conserved, early-acting gene in vertebrate ovarian development. Endocrinology, 144, 3237-3243.

Nagahama, Y., 2005. Molecular mechanisms of sex determination and gonadal sex differentiation in fish. Fish Physiol Biochem, 31, 105-109.

Nakamoto, M., Matsuda, M., Wang, D.-S., Nagahama, Y., Shibata, N., 2006. Molecular cloning and analysis of gonadal expression of Foxl2 in the medaka, *Oryzias latipes*. Biochem Biophys Res Commun, 344, 353-361.

Oshima, Y., Uno, Y., Matsuda, Y., Kobayashi, T., Nakamura, M., 2008. Molecular cloning and gene expression of *Foxl2* in the frog *Rana rugosa*. Gen Comp Endocrinol, 159, 170-177.

Ottolenghi, C., Omari, S., Garcia-Ortiz, J.E., Uda, M., Crisponi, L., Forabosco, A., Pilia, G., Schlessinger, D., 2005. *Foxl2* is required for commitment to ovary differentiation. Hum Mol Genet, 14, 2053-2062.

Pannetier, M., Fabre, S., Batista, F., Kocer, A., Renault, L., Jolivet, G., Mandon-Pepin, B., Cotinot, C., Veitia, R., Pailhoux, E., 2006. FOXL2 activates P450 aromatase gene transcription: towards a better characterization of the early steps of mammalian ovarian development. J Mol Endocrinol, 36, 399-413.

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

Pisarska, M.D., Bae, J., Klein, C., Hsueh, A.J.W., 2004. Forkhead L2 is expressed in the ovary and represses the promoter activity of the steroidogenic acute regulatory gene. Endocrinology, 145, 3424-3433.

Ramsey, M., Shoemaker, C., Crews, D., 2007. Gonadal expression of *Sf1* and aromatase during sex determination in the red-eared slider turtle (*Trachemys scripta*), a reptile with temperature-dependent sex determination. Differentiation, 75, 978-991.

Schmidt, D., Ovitt, C.E., Anlag, K., Fehsenfeld, S., Gredsted, L., Treier, A.-C., Treier, M., 2004. The murine winged-helix transcription factor Foxl2 is required for granulosa cell differentiation and ovary maintenance. Development, 131, 933-942.

Taylor, J.S., Braasch, I., Frickey, T., Meyer, A., Van de Peer, Y., 2003. Genome duplication, a trait shared by 22,000 species of ray-finned fish. Genome Res, 13, 382-390.

Uda, M., Ottolenghi, C., Crisponi, L., Garcia, J.E., Deiana, M., Kimber, W., Forabosco, A., Cao, A., Schlessinger, D., Pilia, G., 2004. *Foxl2* disruption causes mouse ovarian failure by pervasive blockage of follicle development. Hum Mol Genet, 13, 1171-1181.

Vizziano-Cantonnet, D., Baron, D., Mahe, S., Cauty, C., Fostier, A., Guiguen, Y., 2008. Estrogen treatment up-regulates female genes but does not suppress all early testicular markers during rainbow trout male-to-female gonadal trans-differentiation. J Mol Endocrinol, 41, 277-288.

Vizziano, D., Randuineau, G., Baron, D., Cauty, C., Guiguen, Y., 2007. Characterization of early molecular sex differentiation in rainbow trout, *Oncorhynchus mykiss*. Dev. Dynam., 236, 2198-2206.

Wang, D.-S., Kobayashi, T., Zhou, L.-Y., Paul-Prasanth, B., Ijiri, S., Sakai, F., Okubo, K., Morohashi, K.-I., Nagahama, Y., 2007. Foxl2 up-regulates aromatase gene transcription in a female-specific manner by binding to the promoter as well as interacting with Ad4 binding brotein/Steroidogenic Factor 1. Mol Endocrinol, 21, 712-725.

Wang, D.-S., Kobayashi, T., Zhou, L.-Y., Nagahama, Y., 2004. Molecular cloning and gene expression of Foxl2 in the Nile tilapia, *Oreochromis niloticus*. Biochem Biophys Res Commun, 320, 83-89.

Yamaguchi, T., Yamaguchi, S., Hirai, T., Kitano, T., 2007. Follicle-stimulating hormone signaling and Foxl2 are involved in transcriptional regulation of aromatase gene during gonadal sex differentiation in Japanese flounder, *Paralichthys olivaceus*. Biochem Biophys Res Commun, 359, 935-940.

Yao, H.H.-C., 2005. The pathway to femaleness: current knowledge on embryonic development of the ovary. Mol Cell Endocrinol, 230, 87-93.

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 interaction1. Biol Reprod, 68, 1545-1553.

CHAPTER 7

Cloning and expression of *dmrt1* in the common carp (*Cyprinus carpio*): Sexual dimorphism and expression during development

Barney, M.L. and Patil, J.G. Expression of *dmrt1* in the common carp, (*Cyprinus carpio*): Sexual dimorphism and expression during development. In preparation.

Chapter 7 – Cloning and expression of *dmrt1* in the common carp, (*Cyprinus carpio*): Sexual dimorphism and expression during development.

7.1 Abstract

Using RT-PCR and subsequent RACE a *dmrt1* cDNA was cloned from common carp testis. Alignment of this sequence with known *dmrt1* sequences from vertebrates confirmed conservation of the DM-domain among protein sequences. Real-time PCR revealed gonad-specific expression of *dmrt1* in the common carp, with 31-fold higher expression in the testis than the ovary. Examination of expression during development found that *dmrt1* expression peaked prior to hatch with higher expression in the warmer (25 °C) treatment, and subsequent expression in the 20 °C treatment appeared to be suppressed. During larval development sex-specific differences were not evident, suggesting that sexually dimorphic expression, as seen in mature gonads, must occur subsequent to the period examined in this study.

7.2 Introduction

The *doublesex* and *mab-3* (DM)-related transcription factor 1 (*dmrt1*) belongs to the DM domain gene family of novel zinc finger transcription factors. This family of genes were the first identified that show sexually dimorphic expression across both vertebrate and invertebrate phyla (Sinclair et al., 2002; Smith et al., 1999). Graves and Shetty (2001) suggested that *dmrt1* has been associated with sex determination for as long as a billion years, making it a likely candidate for the bird/reptile sex-determining gene.

The *dmrt1* gene was recently cloned from a critical region at the end of the short arm of human chromosome 9p, pinpointed by a sex reversing syndrome associated with deletions (Raymond et al., 1998). Since this human chromosome is equivalent to the bird Z chromosome, it is not surprising to find that *dmrt1* maps to the chicken Z chromosome (Nanda et al., 1999). With no copy on the W, there is a dosage difference- two copies in males to one in females. The gene is expressed specifically in the testis at about the same time as *sox9* is in both mice and chicken, so it is obviously an ancient component of the vertebrate sex-determining pathway (Smith et al., 1999). The name reflects this and is derived from its relatedness to two transcriptional factors involved in sex determination in invertebrates, the fly, *Drosophila melanogaster (Drosophila) doublesex* and worm, *Caenorhabditis elegans, mab-3* (Coschigano and Wensink, 1993; Shen and Hodgkin, 1988). It is the only gene found to have conserved structure and function in male sex determination and differentiation among the different phyla of the animal kingdom including mammals, (De Grandi et al., 2000; Moniot et al., 2000; Pask et al., 2003; Raymond et al., 1999a; Smith et al., 1999), birds (Nanda et al., 1999; Shan et al., 2000; Shetty et al., 2002; Smith et al., 1999), reptiles (Kettlewell et al., 2000; Smith et al., 1999) and fish (Brunner et al., 2001; Huang et al., 2005; Marchand et al., 2000). The correlation of *dmrt1* expression with testicular differentiation strongly suggests that *dmrt1* homologs are involved in testicular differentiation in the process of normal sex development and sex reversal in non-mammalian vertebrates (Kobayashi et al., 2008).

In teleost fish, *dmrt1* is an autosomal gene thought to function downstream in the pathway of genes regulating testis differentiation due to its expression before and during testis differentiation and development (Brunner et al., 2001; Marchand et al.,

2000; Yamaguchi et al., 2006), rather than determination. *Dmrt1* may also play a role in female gonadal development as low expression was observed in differentiating ovaries of zebrafish, *Danio rerio* (Guo et al., 2005), rainbow trout, *Oncorhynchus mykiss* (Marchand et al., 2000) and rice field eel, *Monopterus albus,* and spiny eel, *Mastacembelus aculeatus* (Huang et al., 2005). Expression of *dmrt1* has also been linked to both natural sex change from female to male, and in sex hormone-dependant sex reversal (Guan et al., 2000; He et al., 2003; Marchand et al., 2000). *Dmrt1* expression in Nile tilapia, *Oreochromis niloticus* suggests a role in early testis differentiation (Guan et al., 2000) and also an involvement in regulating *foxL2* and aromatase expression (Ijiri et al., 2008; Nagahama, 2005). A transgenic study demonstrated that over-expression of DMRT1 in the gonads of XX Nile tilapia fry caused a reduction in aromatase expression resulting in degeneration of ovaries and development of testicular tissue, with complete sex reversal in some individuals (D.S. Wang et al., unpublished in Nagahama, 2005). The mechanism of sex reversal was explored using luciferase assays, suggesting that DMRT1 bound to SF-1 itself, repressed SF-1 activated transcription of aromatase (Nagahama, 2005).

To date research has focused on the testis inducing characteristics of *dmrt1*. However the discovery of a female-specific DM domain gene (DMO) directing ovarian differentiation in tilapia (Guan et al., 2000) suggests strong evolutionary parallels between invertebrate and vertebrate sex-determining mechanisms as the *Drosophila doublesex* gene regulates somatic sexual differentiation in both sexes (Burtis and Baker, 1989). Interestingly, a SRY (sex-determining region Y)-consensus site is present in the 5' upstream region of *dmrt1,* but not in the 5' flanking region of *DMO* in tilapia. This suggests one of the upstream regulatory genes of *dmrt1* could be an SRY-like gene from the Y chromosome which probably upregulates *dmrt1*

expression to promote testis formation. In females, without the Y chromosome, *dmrt1* is either not upregulated or possibly repressed by an unknown ovary determining factor, thus blocking testis formation. In XX males, Guan et al. (2000) found environmental or hormonal influences compensate for the absence of a signal from the Y chromosome which results in XX-sex reversal and upregulation of *dmrt1* expression.

In medaka , *Oryzias latipes* a duplicate of *dmrt1* known as *DMY or dmrt1bY* has been identified as a master sex-determining gene (Matsuda et al., 2002). *DMY* is located on the Y chromosome and is expressed exclusively in XY embryos (Matsuda et al., 2002; Nanda et al., 2002). This gene is therefore thought to be the initiating switch in male sex determination in medaka (Mank et al., 2006). Although this gene has not been isolated in other species, it is highly likely that similar sex-determining genes have evolved in different species. Such sex-determining genes may direct male or female development zygotically, and or sex of an individual may be determined by the strength of genetic factors received from parents (Devlin and Nagahama, 2002).

To address its role in sex differentiation in the common carp, *dmrt1* was cloned and the expression profile determined in key adult tissues. Expression was also analysed through embryonic and larval development at high and low temperatures to determine maternal inheritance, initial zygotic expression, expression through the period of sex differentiation and also examined the effect of temperature on its biosynthesis.

7.3 Material and Methods

7.3.1 Sample collection

Breeding, larval rearing and sampling procedures were as previously described (Chapter 2).

7.3.2 Molecular biology

General molecular biology techniques and sample preparation procedures were as previously described (Chapter 2).

7.3.2.1 Oligonucleotides

Oligonucleotide primers used for cloning and real-time assays are listed in Table 7.1. Cloning primers, DmDom.1F and R1, were designed based on gene-specific conserved DM domain. Real-time primers, cDMRT1.F4 and cDMRT1.R3, were designed according to the requirements of SYBR Green Chemistry (Applied Biosystems) and also to be gene specific. The common carp *ß-actin* specific primers cBActin4.F and cBActinD.R (Chapter 3 / Barney et al., 2008) were used as an endogenous control in the relative quantitative PCR assay.

Table 7.1 Primers for cloning and real-time PCR expression analysis of *dmrt1* in common carp

Primer Name	Sequence 5' - 3'	Amplicon
DmDomR1	CKSAGSGCSACCTGSGCTGCCAT	5' RACE
DmDom1F	TGCGCVMGRTGCMGRAAYCACGG	3' RACE
cDMRT1.F1	GTCACCGCTGAAGGGCCACAAACG	3' nested RACE
cDMRT1.F4	GTCTGGAACCTTCTCTGTCGACTC	RT PCR
cDMRT1.R3	AATTAGATGGGAGACTGGTGCAG	RT PCR
cBActin4.F	CCCATCGAGCACGGTATTG	RT PCR
cBActinD.R	AAGGTGTGATGCCAGATCTTCTC	RT PCR

7.3.2.2 Real-time assay

Real-time assay procedures were as previously described (Chapter 2). At the time of this study no *dmrt* sequences were available for common carp. Therefore, to check

the specificity of real-time *dmrt1* primers, all available zebrafish, *dmrt* nucleotide gene sequences were aligned with the common carp sequence and compared at the primer binding sites.

The efficiency value for *dmrt1* (0.99) and the average efficiency value for *β-actin* (1.80) (described in Chapter 3 / Barney et al., 2008) were used in subsequent analysis. Correlation coefficients were 0.99 and 0.98 for *dmrt1* and *β-actin* respectively.

7.3.3 Data analysis

General data analysis procedures were previously described (Chapter 2). To determine an effect of two temperatures on expression the linear sections of larval expression (25 ddpf to 3000 ddpf) were analysed by ANCOVA with temperature as a fixed factor and time as a covariate. Significance was set at $P < 0.05$.

7.4 Results

7.4.1 Cloning and phylogenic analysis

The common carp *dmrt1* gene was cloned using RACE ready cDNA from testis tissue. As seen in Figure 7.1 a main amplicon of ~0.2 kb (lane 1, arrow 'a') and only a single amplicon of ~2 kb (lane 2, arrow 'b') were obtained in the 5' and 3' RACE respectively. The larger lighter band (~1.2 kb) in the 5' RACE (Figure 7.1), lane 1, dashed arrow) represents a non specific amplification. The consensus cDNA was 2151 bp in length with an open reading frame (ORF) of 271 AA (schematic diagram in Appendix), with 99% identity to a 402 bp partial sequence of *dmrt1* from common carp (DQ241767) and 98% identity to the 134 AA sequence (ABB72995).

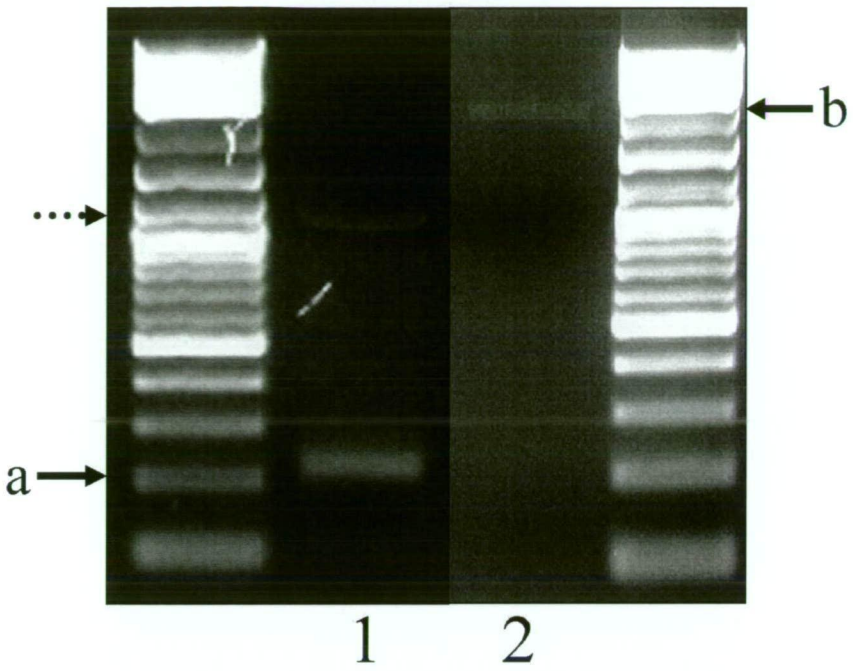


Figure 7.1 Gel picture of *dmrt1* reaction fragments of 5' (lane 1) and 3' (lane 2) nested RACE showing ~0.2 kb (a), ~2 kb (b) and a ~1.2 kb (dashed arrow) bands.

Examination of the common carp DMRT1 protein found that it contained a conserved DM DNA binding domain of 37 AA. When this conserved section of DMRT1 was compared to other species it showed 86-97% identity with other teleosts, 83% with human and 80% homology to several *Drosophila* species. The resulting tree of the deduced amino acid sequences from the ORF of teleosts and other vertebrates demonstrates close homology of the common carp sequence to zebrafish (Figure 7.2).

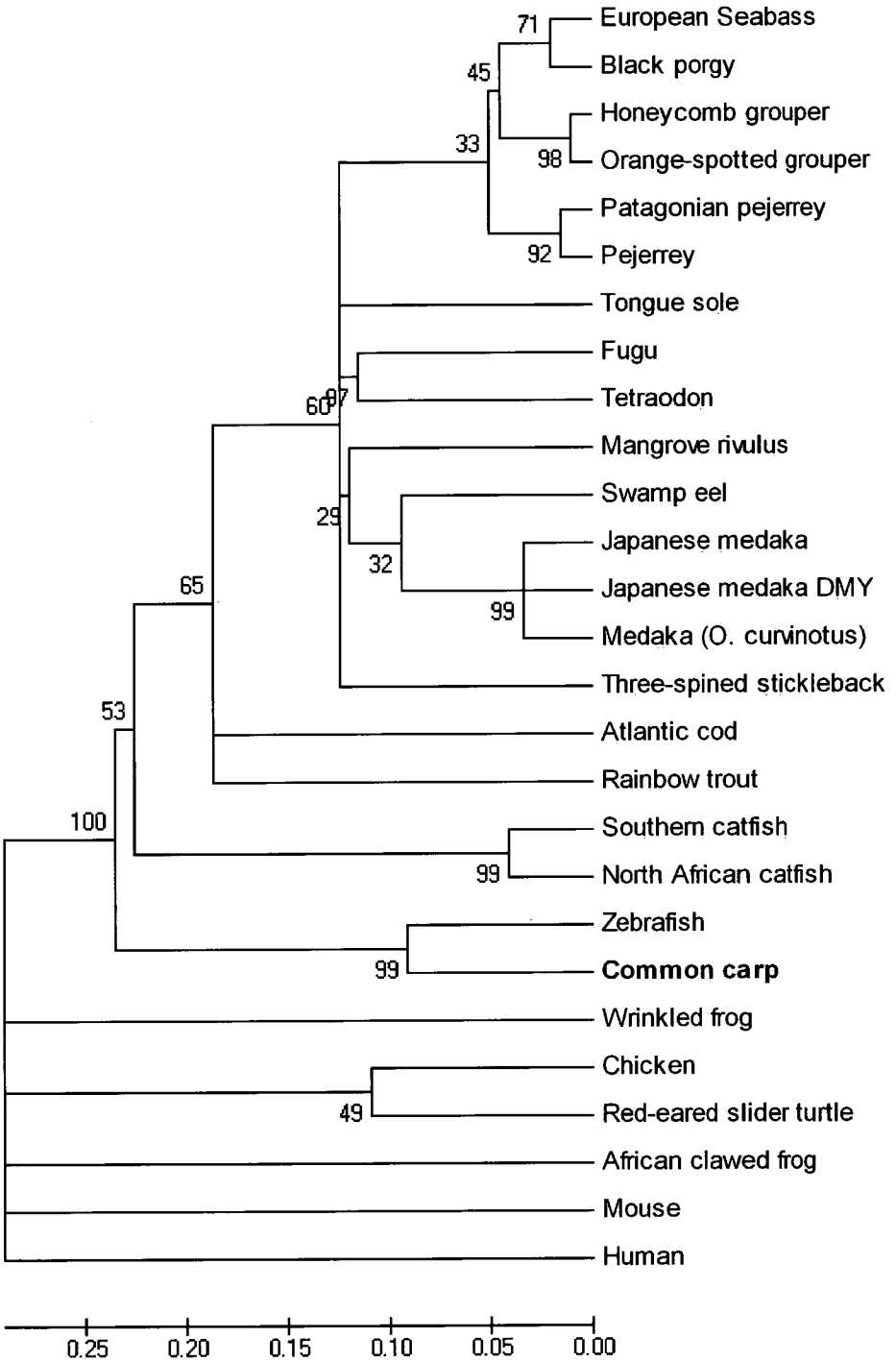


Figure 7.2 Phylogenetic tree of available DMRT1 proteins.

Deduced amino acids aligned from Tongue sole, *Cynoglossus semilaevis* (EU007655), Three-spined stickleback, *Gasterosteus aculeatus* (AY867870), European Seabass, *Dicentrarchus labrax* (AM993095), Black porgy, *Acanthopagrus schlegelii* (AY323953) zebrafish, *Danio rerio* (AF439562), Honeycomb grouper, *Epinephelus merra* (EU555179), Patagonian pejerrey, *Odontesthes hatcheri* (EU864152), Pejerrey, *Odontesthes bonariensis* (AY319416), Rainbow trout, *Oncorhynchus mykiss* (NP_001124269), Atlantic cod, *Gadus morhua* (EU561663), Southern catfish *Silurus meridionalis* (EF015487), fugu, *Takifugu rubripes* (NM_001037949), Orange-spotter grouper, *Epinephelus coioides* (EF017802), Mangrove rivulus, *Kryptolebias marmoratus* (DQ683742), Rice feild eel, *Monopterus albus* (AF421347), Japanese medaka, *Oryzias latipes* (AY524417) and DMY

(AY448017), Medaka, *Oryzias curvinotus* (AB091696), North African catfish, *Clarias gariepinus* (AF439561), Tetraodon, *Tetraodon nigroviridis* (AY152820), Wrinkled frog, *Rana rugosa* (AB272609), Red-eared slider turtle, *Trachemys scripta* (AF201387), Mouse (NM_015826), Chicken (AF123456), Human (NM_021951).

7.4.2 Tissue and sex-specific expression of dmrt1 mRNA in adult common carp

Examination of the *dmrt1* expression was carried out on the brain, pituitary, liver and gonad of male and female common carp (Figure 7.3). *Dmrt1* was found to be gonad specific showing sexually dimorphic expression with testis expressing at a level 31-fold higher (F=99.39, P>0.001) than ovary.

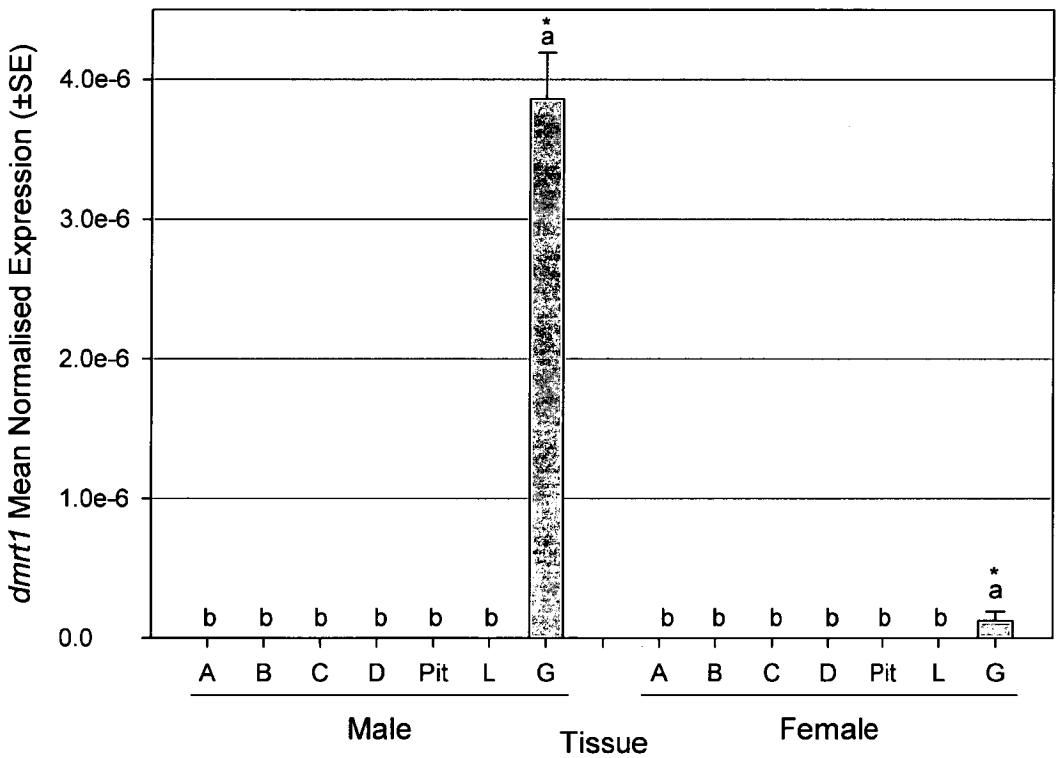


Figure 7.3 Tissue- and sex-specific differences in adult common carp *dmrt1* expression.
Each bar represents the mean normalised expression (±SE; n = 3). X-axis labels: (A-D) brain sections (see Figure 2.1); Pit, pituitary; L, liver; G, gonad. One-way ANOVA was used to analyse data by tissue type within each sex, followed by SNK analysis to determine which means differed significantly, as indicated by letters (a-b). Student's t test was used for pairwise comparison of gonad tissue between sex, and significant differences ($P < 0.05$) are labelled with asterisks (*).

7.4.3 Ontogenic and larval development of common carp

7.4.3.1 Growth

Following hatch (20 °C- 70 ddpf, 25 °C- 62.5 ddpf) the total length (TL) of larva was measured at the time of sampling. In both temperatures length was found to increase steadily over time (20 °C- F=28.419, P<0.000, 25 °C- F=79.338, P<0.000) (Figure 7.4). Higher temperature (25 °C) was found to cause the growth rate of larva to be significantly higher (F=16.813, P>0.000) with individuals reared at 25 °C reaching a mean TL of 47.75 ± 3.43 mm after 118 dpf compared to 31.13 ± 2.47 mm at 20 °C after 138 dpf.

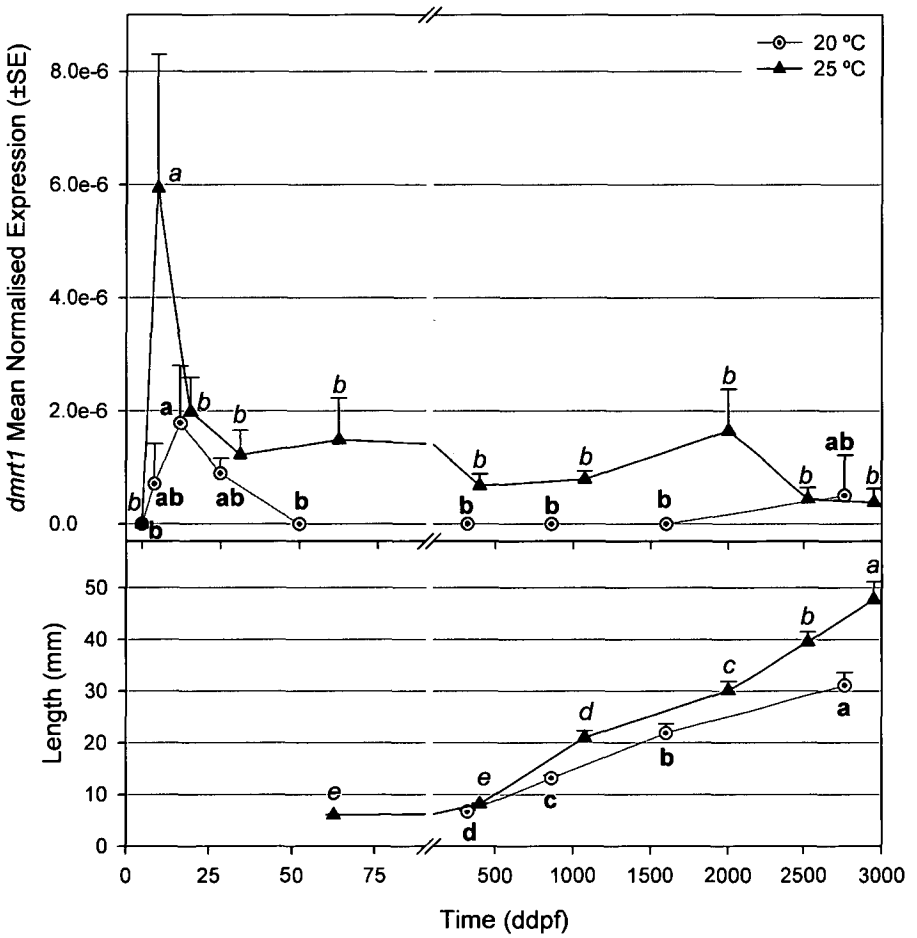


Figure 7.4 Temporal changes in TL and *dmrt1* expression of embryos and larvae reared at 20 or 25 °C.

One-way ANOVA used to analyse data for temperature specific differences, followed by SNK analysis to determine which means differed significantly (P < 0.01), as indicated by font style (20 °C- bold, 25 °C- italic). Note: Hatching occurs at 70.8 and 62.5 ddpf at 20 and 25 °C respectively.

7.4.3.2 Embryonic and larval dmrt1 expression

Temporal changes in *dmrt1* expression during development were quantified by real-time PCR up to 2760 ddpf in the 20 °C treatment and 2950 ddpf at 25 °C. Expression of *dmrt1* was not observed in unfertilised eggs, however significant changes in expression were observed over time in both treatments (20 °C- $F=3.170$, $P<0.01$, 25 °c- $F= 2.995$, $P<0.01$) (Figure 7.4). Expression peaked soon after fertilisation at about 4 and 5 ddpf coinciding with the morula and late blastula developmental stage at 20 °C and 25 °C respectively. At the point of peak expression, the level of *dmrt1* transcript was 3-fold higher in larvae reared at 25 °C than those at 20 °C. Following this peak, the expression levels in both treatment groups declined and remained at low levels, with no detectable expression between 50 and 1600 ddpf in the 20 °C treatment group. However at 2760 ddpf expression was again detected in larvae reared at 20 °C and was at levels comparable to those observed in 25 °C treatment group. After reaching a size of 20 mm TL (at 1600 and 1000 ddpf in the 20 and 25 °C treatments respectively) small SE for the data points of each temperature indicated little individual difference in expression at a sample point.

Analysis of data from 25 ddpf onwards using ANCOVA found expression of *dmrt1* to be suppressed at the lower temperature (20 °C).

7.5 Discussion

Of the genes known to be involved in sex determination/differentiation the *DMRT* family of genes are of special interest because of their presence in both invertebrates and vertebrates and highly conserved DNA-binding DM domain. In particular, the sexually dimorphic expression of *dmrt1* in both mammalian and other vertebrates indicates a role in male sex differentiation (Sinclair et al., 2002). However, to date,

no upstream regulators or downstream targets of *dmrt1* have been identified, making it difficult to place *dmrt1* in any gene regulatory pathway.

In the present study, common carp *dmrt1* cDNA was cloned and the encoded polypeptide was shown to contain the characteristic DM-domain shared by all DMRT proteins. The conserved nature of the common carp *dmrt1*, DM domain with those of other vertebrates and invertebrates indicates that the DM-domain has remained highly conserved during evolution suggesting a conserved function for the gene in sex differentiation. Despite targeting the conserved region of *dmrt1* gene a single transcript was cloned from the testicular tissue of common carp, suggesting that it is not alternatively spliced in the tissue. In contrast multiple alternative splice variants of *dmrt1* have been identified in different classes of animals. Especially, studies in teleosts, such as European sea bass, *Dicentrarchus labrax* (Deloffre et al., 2009), zebrafish (Guo et al., 2005) and rice field eel (Huang et al., 2005) have reported alternative spliced variants of *dmrt1*. Similar observations in human (Cheng et al., 2006), mouse (Lu et al., 2007) and chicken (Zhao et al., 2007) have revealed differentially expressed multiple transcribed isoforms of *dmrt1* in testis, suggesting that *dmrt1* is regulated by alternative splicing during male sexual development. In medaka, the *dmrt1* is known to have undergone whole gene duplication resulting in two copies of the gene- *dmrt1a* and *DMY/dmrt1bY*, of which the DMY is master regulator for male sex determination (Matsuda et al., 2002).

As well as gene structure, it appears the expression pattern and role of *dmrt1* are conserved across taxa. Tissue distribution studies in common carp indicate that *dmrt1* is expressed exclusively in the gonads, being higher in the testis than in the ovary supporting its role as a potential testis differentiation factor. This is consistent

with the results reported in other teleosts such as medaka (Kobayashi et al., 2004), southern catfish, *Silurus meridionalis* (Liu et al., 2007), Nile tilapia (Kobayashi et al., 2008) rainbow trout (Marchand et al., 2000) and higher vertebrates such as humans (Raymond et al., 1999b), chicken (Nanda et al., 2000), turtles (Kettlewell et al., 2000) and amphibians (Shibata et al., 2002). Even in the rice field eel, which naturally changes sex from female to male in its lifespan, *dmrt1* expression is upregulated when the testis development starts from its precursor ovotestis (Huang et al., 2005). In the zebrafish however, *dmrt1* expression was similar in the testis and ovary and also present in the liver (Guo et al., 2005). This non-sexually dimorphic expression was also seen during development of zebrafish, with *dmrt1* expression clearly observed in developing germ cells of both ovary and testis, especially in developing oocytes indicating expression is not only associated with testis development, but also, important in ovary differentiation of zebrafish (Gao et al., 2005). In the present study, expression of *dmrt1* in common carp was seen to peak early in development, coinciding with the morula and late blastula stages in the 20 and 25 °C treatments respectively. Early expression of *dmrt1*, well before the onset of sexual differentiation indicates a position early in the sex differentiation cascade and suggests other factors are also necessary to initiate testis formation (Sinclair et al., 2002).

In medaka and European sea bass, high temperature incubation during early development increases the percentage of males in the population (Hattori et al., 2007; Piferrer et al., 2005), our previous study indicates this may also be so for common carp (Chapter 4). In the current study, peak expression was higher in the warmer (25 °C) treatment, and subsequent expression in the 20 °C treatment appeared to be suppressed. It is therefore possible that repression of *dmrt1* in common carp could

result in phenotypic sex reversal in the species. However, sex related differences were not discernable (low SE between individuals) during early larval development in either temperature within the experimental period suggesting a role for *dmrt1* in general early larval development rather than sex differentiation. It is possible that the sex-specific differences in expression levels occur some time later than the period examined in the current study. Alternatively the sex specific dose differences are too minute to be distinguished technically but may yet be biologically significant to trigger sexual differentiation into male or the female pathway in a dose dependent manner. In general expression of *dmrt1* is considerably upregulated in developing testes compared to ovaries of mammals, with the timing of this up-regulation varying between species, but generally occurring in the late sex-determining or early testis-differentiation period (Koopman and Loffler, 2003). At present common carp can only be sexed by examination of the gonads since a master sex-determining gene has not yet been discovered in this species. In the present study little variation of *dmrt1* expression between individuals was detected in larva exceeding 20 mm, the size at which morphological sex differentiation is known to occur in common carp (Chapter 4). Suggesting that sexual dimorphism occurs later in the testis-differentiation period of common carp than covered in this study. In European sea bass the onset of *dmrt1* expression coincides with the period when the gonads start to differentiate and expression increases in a similar manner in both male and female gonads up until the gonads are developed, after which the two transcripts (*dmrt1a* and *b*) are down-regulated in the ovary and become specific of the developing testis. In contrast, *dmrt1* was found to be expressed exclusively during the early stages of male gonad differentiation, but not in the female gonad in Nile tilapia (Guan et al., 2000) and rainbow trout (Marchand et al., 2000). It is possible that other genes, even sex-determining genes, may be expressed earlier than *dmrt1* in the common carp sex

determination pathway. In medaka there is a significant delay between the first appearance of *dmy* and that of *dmrt1* in XY gonads during differentiation of the testes, showing that *dmrt1* expression occurs much later than *dmy* expression (Kobayashi et al., 2004).

Interestingly, in rainbow trout treatment with exogenous estrogen caused down-regulation of *dmrt1* expression (Marchand et al., 2000). In Nile Tilapia *dmrt1* may be required for the down-regulation of *cyp19a* during testicular differentiation (Wang and Nagahama, 2008) indicating that estrogens may actually be needed to repress male differentiation. This inhibition of the synthesis of the inhibitor (estrogen) would then allow testicular differentiation to proceed (Guiguen et al., 2009). However, there is little information on the influence of aromatase as an inhibitor on *dmrt1* expression in fish and further investigations are necessary to establish their causal relationship.

In vertebrates *dmrt1* is highly conserved having in general male-specific expression in the developing male gonads and that it plays a very important role from being a master regulator to a downstream gene required for the male gonad development. Although not identified in this study, it is likely that, like other teleosts, alternate transcripts of *dmrt1* exist in common carp. Identification of these transcripts and their regulation during gonadogenesis require further investigation. Further studies on the transcriptional regulation of *dmrt1* and the identification of target(s) of *dmrt1* will be required to determine the roles and actions of *dmrt1* in testicular differentiation in common carp.

7.6 Acknowledgements

Support and assistance provided by CSIRO, Invasive Animal Cooperative Research Centre, Inland Fisheries Service Tasmania and K&C Fisheries. This project received funding support from the Fisheries Research and Development Corporation.

7.7 References

Barney, M.L., Patil, J.G., Gunasekera, R.M., Carter, C.G., 2008. Distinct cytochrome P450 aromatase isoforms in the common carp (*Cyprinus carpio*): Sexual dimorphism and onset of ontogenic expression. Gen Comp Endocrinol, 156, 499-508.

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

Burtis, K.C. & Baker, B.S., 1989. Drosophila *doublesex* gene controls somatic sexual differentiation by producing alternatively spliced mRNAs encoding related sex-specific polypeptides. Cell, 56, 997-1010.

Cheng, H.H., Ying, M., Tian, Y.H., Guo, Y., McElreavey, K., Zhou, R.J., 2006. Transcriptional diversity of DMRT1 (dsx- and mab3-related transcription factor 1) in human testis. Cell Res, 16, 389-393.

Coschigano, K.T. & Wensink, P.C., 1993. Sex-specific transcriptional regulation by male and female doublesex proteins of *Drosophila*.Genes & Dev. 7:42–54. Genes Dev, 7, 42-54.

De Grandi, A., Calvari, V., Bertini, V., Bulfone, A., Peverali, G., Camerino, G., Borsani, G., Guioli, S., 2000. The expression pattern of a mouse doublesex-related gene is consistent with a role in gonadal differentiation. Mech Dev, 90, 323-326.

Deloffre, L.A.M., Martins, R.S.T., Mylonas, C.C., Canario, A.V.M., 2009. Alternative transcripts of DMRT1 in the European sea bass: Expression during gonadal differentiation. Aquaculture, 293, 89-99.

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.

Gao, S., Zhang, T., Zhou, X., Zhao, Y., Li, Q., Guo, Y., Cheng, H., Zhou, R., 2005. Molecular cloning, expression of *Sox5* and its down-regulation of *Dmrt1* transcription in Zebrafish. Journal of Experimental Zoology Part B: Molecular and Developmental Evolution, 304B, 476-483.

Graves, J.A.M. & Shetty, S., 2001. Sex from W to Z: Evolution of vertebrate sex chromosomes and sex determining genes. J Exp Zool, 290, 449-462.

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*). Biochem Biophys Res Commun, 272, 662-666.

Guiguen, Y., Fostier, A., Piferrer, F., Chang, C.F., 2009. Ovarian aromatase and estrogens: a pivotal role for gonadal sex differentiation and sex change in fish. Gen Comp Endocrinol, In press, corrected proof.

Guo, Y., Cheng, H., Huang, X., Gao, S., Yu, H., Zhou, R., 2005. Gene structure, multiple alternative splicing, and expression in gonads of zebrafish *Dmrt1*. Biochem Biophys Res Commun, 330, 950-957.

Hattori, R.S., Gould, R.J., Fujioka, T., Saito, T., Kurita, J., Strüssmann, C.A., Yokota, M., Watanabe, S., 2007. Temperature-dependent sex determination in Hd-rR medaka *Oryzias latipes*: Gender sensitivity, thermal threshold, critical period, and DMRT1 expression profile. Sexual Development, 1, 138-146.

He, C.-L., Du, J.-L., Wu, G.-C., Lee, Y.-H., Sun, L.-T., Chang, C.-F., 2003. Differential Dmrt1 transcripts in gonads of the protandrous black porgy, *Acanthopagrus schlegeli*. Cytogenetic and Genome Research, 101, 309-313.

Huang, X., Guo, Y., Shui, Y., Gao, S., Yu, H., Cheng, H., Zhou, R., 2005. Multiple alternative splicing and differential expression of *dmrt1* during gonad transformation of the rice field eel. Biol Reprod, 73, 1017-1024.

Ijiri, S., Kaneko, H., Kobayashi, T., Wang, D.-S., Sakai, F., Paul-Prasanth, B., Nakamura, M., Nagahama, Y., 2008. Sexual dimorphic expression of genes in gonads during early differentiation of a teleost fish, the Nile tilapia *Oreochromis niloticus*. Biol Reprod, 78, 333-341.

Kettlewell, J.R., Raymond, C.S., Zarkower, D., 2000. Temperature-dependent expression of turtle *Dmrt1* prior to sexual differentiation. Genesis, 26, 174-178.

Kobayashi, T., Kajiura-Kobayashi, H., Guan, G., Nagahama, Y., 2008. Sexual dimorphic expression of *DMRT1* and *Sox9a* during gonadal differentiation and hormone-induced sex reversal in the teleost fish Nile tilapia (*Oreochromis niloticus*). Dev. Dynam., 237, 297-306.

Kobayashi, T., Matsuda, M., Kajiura-Kobayashi, H., Suzuki, A., Saito, N., Nakamoto, M., Shibata, N., Nagahama, Y., 2004. Two DM domain genes, *DMY and DMRT1*, involved in testicular differentiation and development in the Medaka, *Oryias latipes*. Dev. Dynam., 231, 518-526.

Koopman, P. & Loffler, K.A., 2003. Sex determination: The fishy tale of Dmrt1. Curr Biol, 13, R177-R179.

Liu, Z., Wu, F., Jiao, B., Zhang, X., Hu, C., Huang, B., Zhou, L., Huang, X., Wang, Z., Zhang, Y., Nagahama, Y., Cheng, C.H.K., Wang, D., 2007. Molecular cloning of doublesex and mab-3-related transcription factor 1, forkhead transcription factor gene 2, and two types of cytochrome P450 aromatase in Southern catfish and their possible roles in sex differentiation. J Endocrinol, 194, 223-241.

Lu, H., Huang, X., Zhang, L., Guo, Y., Cheng, H., Zhou, R., 2007. Multiple alternative splicing of mouse Dmrt1 during gonadal differentiation. Biochem Biophys Res Commun, 352, 630-634.

Mank, J.E., Promislow, D.E.L., Avise, J.C., 2006. Evolution of alternative sex-determining mechanisms in teleost fishes. Biol. J. Linn. Soc., 87, 83-93.

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*. BBA-Gene Struct. Expr., 1493, 180-187.

Matsuda, M., Nagahama, Y., Shinomiya, A., Sato, T., Matsuda, C., Kobayashi, T., Morrey, C.E., Shibata, N., Asakawa, S., Shimuzu, 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.

Moniot, B., Berta, P., Scherer, G., Sudbeck, P., Poulat, F., 2000. Male specific expression suggests role of DMRT1 in human sex determination. Mech Dev, 91, 323-325.

Nagahama, Y., 2005. Molecular mechanisms of sex determination and gonadal sex differentiation in fish. Fish Physiol Biochem, 31, 105-109.

Nanda, I., Kondo, M., Hornung, U., Asakawa, S., Winkler, C., Shimizu, A., Shan, Z., Haaf, T., Shimizu, N., Shima, A., Schmid, M., Schartl, M., 2002. A duplicated copy of DMRT1 in the sex-determining region of the Y chromosome of the medaka, *Oryzias latipes*. P. Natl. Acad. Sci. USA, 99, 11778-11783.

Nanda, I., Shan, Z., Schartl, M., Burt, D.W., Koehler, M., Nothwang, H., Grutzner, F., Paton, I.R., Windsor, D., Dunn, I., Engel, W., Staeheli, P., Mizuno, S., Haaf, T., Schmid, M., 1999. 300 million years of conserved synteny between chicken Z and human chromosome 9. Nat Genet, 21, 258–259.

Nanda, I., Zend-Ajusch, E., Shan, Z., Grützner, F., Schartl, M., Burt, D.W., Koehler, M., Fowler, V.M., Goodwin, G., Schneider, W.J., Mizuno, S., Dechant, G., Haaf, T., Schmid, M., 2000. Conserved synteny between the chicken Z sex chromosome and human chromosome 9 includes the male regulatory gene DMRT1: a comparative (re)view on avian sex determination. Cytogenetic and Genome Research, 89, 67-78.

Pask, A.J., Behringer, R.R., Renfree, M.B., 2003. Expression of DMRT1 in the mammalian ovary and testis -- from marsupials to mice. Cytogenetic and Genome Research, 101, 229-236.

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.). Gen Comp Endocrinol, 142, 102-110.

Raymond, C.S., Kettlewell, J.R., Hirsch, B., Bardwell, V.J., Zarkower, D., 1999a. Expression of Dmrt1 in the Genital Ridge of Mouse and Chicken Embryos Suggests a Role in Vertebrate Sexual Development. Dev Biol, 215, 208-220.

Raymond, C.S., Parker, E.D., Kettlewell, J.R., Brown, L.G., Page, D.C., Kusz, K., Jaruzelska, J., Reinberg, Y., Flejter, W.L., Bardwell, V.J., Hirsch, B., Zarkower, D., 1999b. A region of human chromosome 9p required for testis development contains two genes related to known sexual regulators. Hum Mol Genet, 8, 989-996.

Raymond, C.S., Shamu, C.E., Shen, M.M., Seifert, K.J., Hirsch, B., Hodgkin, J., Zarkower, D., 1998. Evidence for evolutionary conservation of sex-determining genes. Nature, 391, 691-695.

Shan, Z., Nanda, I., Wang, Y., Schmid, M., Vortkamp, A., Haaf, T., 2000. Sex-specific expression of an evolutionarily conserved male regulatory gene, DMRT1 , in birds. Cytogenetic and Genome Research, 89, 252-257.

Shen, M.M. & Hodgkin, J., 1988. *mab-3*, a gene required for sex-specific yolk protein expression and a male-specific lineage in *C. elegans*. Cell, 54, 1019–1031.

Shetty, S., Kirby, P., Zarkower, D., Graves, J.A.M., 2002. DMRT1 in a ratite bird: evidence for a role in sex determination and discovery of a putative regulatory element. Cytogenetic and Genome Research, 99, 245-251.

Shibata, K., Takase, M., Nakamura, M., 2002. The *Dmrt1* expression in sex-reversed gonads of amphibians. Gen Comp Endocrinol, 127, 232-241.

Sinclair, A., Smith, C., Western, P., McClive, P., 2002. A comparative analysis of vertebrate sex determination. In: The Genetics and Biology of Sex Determination (eds D. Chadwick, Goode, J.), Vol. 244, pp. 102-114. Novartis Foundation.

Smith, C.A., Smith, M.J., Sinclair, A.H., 1999. Expression of chicken Steroidogenic Factor-1 during gonadal sex differentiation. Gen Comp Endocrinol, 113, 187-196.

Wang, D.S. & Nagahama, Y., 2008. DMRT1 represses AD4BP/SF1/FOXL2 activated aromatase gene transcription: a possible mechanism favoring the male pathway in tilapia. In Proc. Sixth Int. Symp. Fish Endocrinol., pp. 62, Calgary.

Yamaguchi, A., Lee, K.H., Fujimoto, H., Kadomura, K., Yasumoto, S., Matsuyama, M., 2006. Expression of the DMRT gene and its roles in early gonadal development of the Japanese pufferfish *Takifugu rubripes*. Comp. Biochem. Phys. D, 1, 59-68.

Zhao, Y., Lu, H., Yu, H., Cheng, H., Zhou, R., 2007. Multiple alternative splicing in gonads of chicken DMRT1. Development Genes and Evolution, 217, 119-126.

CHAPTER 8

Sox9 genes in the common carp (*Cyprinus carpio)*: Cloning and expression in adults and during development.

Barney, M.L. and Patil, J.G. *Sox9* genes in the common carp (*Cyprinus carpio)*: Cloning and expression in adults and during development. In preparation.

Chapter 8 - *Sox9* genes in the common carp (*Cyprinus carpio*): Cloning and expression in adults and during development.

8.1 Abstract

Two *sox9* genes (*sox9a* and *sox9b*) were cloned from common carp testis by RT-PCR and subsequent RACE. Alignment of the deduced protein sequences with other known s*ox9* genes found that they each segregate into separate clades corresponding to either s*ox9a* or s*ox9b* of other teleosts. Real-time PCR revealed that both isoforms were predominately expressed in the brain of both sexes, with s*ox9a* contributing to the bulk of the expression. Both genes were also expressed in the pituitary, liver and gonad; however no sex-specific differences in expression were observed. Ontogenic expression showed that only the *sox9a* transcript is inherited maternally, with both genes peaking early during embryonic development before dissipating to a basal level. At this basal level expression of *sox9a* was predominate, with no potential sex-specific differences observed between individuals. The early expression of both *sox9* genes with no subsequent sex-related differences in the experimental period suggests these two genes play a role in early development in common carp but not in sex determination or differentiation in the species.

8.2 Introduction

Failed attempts to isolate a teleostean equivalent to the mammalian testis determining factor SRY (sex-determining region Y) identified a *SRY*-related gene, *sox9* (*SRY*-like HMG-box 9) in fish, which is involved in mammalian sex determination down-steam of SRY (Morrish and Sinclair, 2002). To date, *sox9* has been identified in many different species, namely human (Foster et al., 1994), mouse (Kanai and Koopman, 1999; Wright et al., 1995), chicken (da Silva et al., 1996), lizard, *Calotes versicolor*

(Choudhary et al., 2000), frog, *Rana rugosa* (Takase et al., 2000), turtle, *Trachemys scripta* (Spotila et al., 1998), alligator, *Alligator mississippiensis* (Western et al., 1999) and several fish species, thereby demonstrating its conserved role in evolution.

Two *sox9* genes (*sox9a* and *sox9b*) have been found in several teleost fish; zebrafish, *Danio rerio* (Chiang et al., 2001), three-spined stickleback, *Gasterosteus aculeatus* (Klüver et al., 2005), fugu, *Takifugu rubripes* (Koopman et al., 2004), medaka, *Oryzias latipes* (Yokoi et al., 2002) and rice field eel, *Monopterus albus* (Zhou et al., 2003). Investigations on these fish showed that not only have the genes been duplicated, but also their functions have been partitioned in lineage-specific ways. The combined expression pattern of the two *sox9* genes in zebrafish and three-spined stickleback (Chiang et al., 2001; Cresko et al., 2003; Yan et al., 2002) correspond approximately to that of the single *sox9* in mouse (Kent et al., 1996; Pompolo and Harley, 2001), indicative of a partitioning of ancestral sub-functions. This split expression pattern of *sox9a/b* in fish may merely mimic an ancestral role of a single SOX9 which functioned in both testis and developing ovary in other species, including human and frog (Hanley et al., 2000; Takase et al., 2000). Interestingly, in zebrafish, *sox9a* is a putative regulator of the gonadal expression of an SF-1 homologue (von Hofsten and Olsson, 2005). However expression pattern studies in medaka and rice field eel indicate that the function of *sox9* genes are conserved in cartilage development (chondrogenesis) of teleosts, but may not be conserved for male sex determination and differentiation (Yokoi et al., 2002; Zhou et al., 2003). Interestingly in adult medaka, *sox9a* was expressed predominantly in the ovary and at much lower levels in the testis than *sox9b* (Klüver et al., 2005). This predominant expression of *sox9* genes in the ovary so far remains to be specific to some fish species such as medaka. In contrast, zebrafish *sox9a* is expressed in adult testis while

sox9b is expressed in adult ovary (Chiang et al., 2001). Previous work in common carp isolated the *sox9b* isoform and using semi-quantitative RT-PCR found highest expression in the brain and testis, with expression also in the liver, heart and quite low expression in the ovary and kidney (Du et al., 2007). This previous paper also reported a '*sox9a*' isoform, however this was identified by the authors as a splice variant of *sox9b*.'

To explore the role of *sox9* in sex differentiation in the common carp, the full *sox9a* isoform and a partial *sox9b* fragment were cloned and their expression profile determined in key adult tissues. Expression was also analysed through embryonic and larval development at high and low temperatures to determine maternal inheritance, initial zygotic expression, expression through the period of sex differentiation and also examined the effect of temperature on its biosynthesis.

8.3 Material and Methods

8.3.1 Sample collection

Breeding, larval rearing and sampling procedures were as previously described (Chapter 2).

8.3.2 Molecular biology

General molecular biology techniques and sample preparation procedures were as previously described (Chapter 2).

8.3.2.1 Oligonucleotides

Oligonucleotide primers used for cloning and real-time assays are listed in Table 8.1. Cloning primers, cSox9.F1 and R1, were designed based on the gene-specific conserved HMG box domain with nested *sox9a* primers, cSox9.R2 and F3, designed

from amplified targets. Cloning and real-time primers for *sox9b* were based on the previously reported nucleotide sequence (Du et al., 2007).

Real-time primer pairs, cSox9a.F2 / cSox9a.R2 and cSox9b.F4 / cSox9b.R4, were designed according to the requirements of SYBR Green Chemistry (Applied Biosystems) and also to be isoform specific. The common carp *ß-actin* specific primers cBActin4.F and cBActinD.R (Chapter 3 / Barney et al., 2008) were used as an endogenous control in the relative quantitative PCR assay.

Table 8.1 Primers used for cloning and real-time PCR expression analysis of *sox9* in common carp

Primer Name	Sequence 5' - 3'	Amplicon
cSox9.R1	TCAGACGCTCGGCCTCCTCCACRA	5' RACE
cSox9.F2	GCAGGAARCTSGCGGAYCAATA	3' RACE
cSox9.R2	BGGACGCTTYTCRCCCTCRTTCA	5' nested RACE
cSox9.F3	CTTTGGAGATTGCTGAACGAGGGTGA	3' nested RACE
cSox9b.F2	ACAACGCCGAGCTCAGCAAGACCCT	3' RACE
cSox9b.R2	GAAGATGGCGTTGGGTGAGATGTGGGT	5' RACE
cSox9b.F3	GAACGAGGGCGAGAAGCGTCCGTTC	3' nested RACE
cSox9b.R3	CCCTGGGACTGACCTGAGTGGTCTCC	5' nested RACE
cSox9a.F2	GACGCTCGGCGAGTTCA	RT PCR
cSox9a.R2	ACCCTTTCAGCACCTGAGACA	RT PCR
cSox9b.F4	GAGAGCAGCCGCCTCACA	RT PCR
cSox9b.R4	CCGCGTCCCTGATGCA	RT PCR
cBActin4.F	CCCATCGAGCACGGTATTG	RT PCR
cBActinD.R	AAGGTGTGATGCCAGATCTTCTC	RT PCR

8.3.3 Sequencing and phylogenetic analysis

Sequencing procedure and phylogenetic analysis was as previously described (Chapter 2). In species where only one *sox9* isoform is described to date, the classification of the isoform as either *sox9a* or *b* was not identified by the authors. Only sections of the aligned sequence that were conserved among species were used to construct the phylogenetic tree.

8.3.4 Real-time analysis

Real-time assay procedures were as previously described (Chapter 2). Specificity of *sox9* primers was tested by both standard- and real-time PCR by pairing both primer combinations with cloned *sox9a* and *sox9b* cDNA as templates. Specificity was also demonstrated through dissociation/melt-curve analysis.

The efficiency values for each *sox9* real-time primer pair and the average efficiency value for *β-actin* (described in Chapter 3 / Barney et al., 2008) were used in subsequent analysis. These values were 1.95, 1.98 and 1.80, with correlation coefficients of 0.99, 0.99 and 0.98 for *sox9a*, *sox9b* and *β-actin* respectively.

8.3.5 Data analysis

General data analysis procedures were previously described (Chapter 2).

8.4 Results

8.4.1 Phylogenic analysis

The full length cDNA for *sox9a* was isolated and cloned using the RACE ready cDNA prepared from testis as template. This was 1425 bp in length, with an open reading frame (ORF) of 394 AA (schematic diagram in Appendix). The conserved HMG box DNA binding domain had 89% identity with *sox9a* of triploid crucian carp (DQ201318) and 87% identity with zebrafish *sox9a* (NM_131643). As *sox9b* in the species has been previously published (Du et al., 2007) only a partial cloning and sequencing of the *sox9b* cDNA was undertaken to serve as template for testing specificity of the real-time primers. Of the partial fragments sequenced, the 5'-end had 86% identity over 794 bp and the 3'-end had 98% identity over 544 bp to the

common carp *sox9b* sequence (AY956415) (subsequently used for phylogenetic analysis).

A phylogenetic tree was generated from available deduced proteins of *sox9* genes from teleosts and higher vertebrates (Figure 8.1). The teleostean Sox9a and b proteins formed two distinct clades. The Sox9a protein determined for common carp in the current study shows highest identity (96%) to zebrafish Sox9a (NP_571718) and also 90% identity with Sox9a of triploid crucian carp (Accession ABA41588) (Guo et al., 2008). These carp Sox9a proteins also clade closely with the zebrafish Sox9a protein described by Yan (2002). The previously described common carp Sox9b protein lies in the Sox9b clade close to zebrafish Sox9b.

Interestingly, the unspecified fugu Sox9 isoform (AF329945) clusters with the teleostean Sox9b clade, whilst the one designated Sox9b (AY277965) clusters with teleostean Sox9a clade. Furthermore, blast-p analysis (NCBI), showed that the unspecified fugu Sox9 protein has highest protein identity (89%) with three-spined stickleback Sox9b (AAQ62979)(Cresko et al., 2003) and 87% with medaka Sox9b (NP_001098556)(Nakamoto et al., 2005), while the fugu Sox9b (AAQ18508) was most similar (82% identity) to three-spine stickleback Sox9a (AAQ62978) and also had 79% identity with medaka Sox9a (AAX62152) and 74% identity with medaka SOX9b (AAX62151)(Klüver et al., 2005). Two unnamed proteins from the Tetraodon, *Tetraodon nigroviridis*, were found to have high identity with each of the fugu Sox9 proteins in the Sox9a clade (93%)(Accession CAF97001) and the Sox9b clade (95%)(Accession CAG00200). The deduced amino acid for swamp eel Sox9 isoforms (*sox9a1* and *a2*) cluster with each of the Sox9a and b clades respectively. Based on homology and the phylogenetic tree the previously unspecified *sox9* gene of Hong Kong grouper, *Epinephelus akaara* (AY676309), orange-spotted grouper,

Epinephelus coioides (GQ232762), pejerrey, *Odontesthes bonariensis* (AY319415), guppy, *Poecilia reticulata* (DQ683727), rainbow trout, *Orcorhynchus mykiss* (NM_001124179) and Atlantic salmon, *Salmo salar* (BT059262) are potentially *sox9b* while the blue tilapia, *Oreochromis aureus* (EU373500) is potentially *sox9a*.

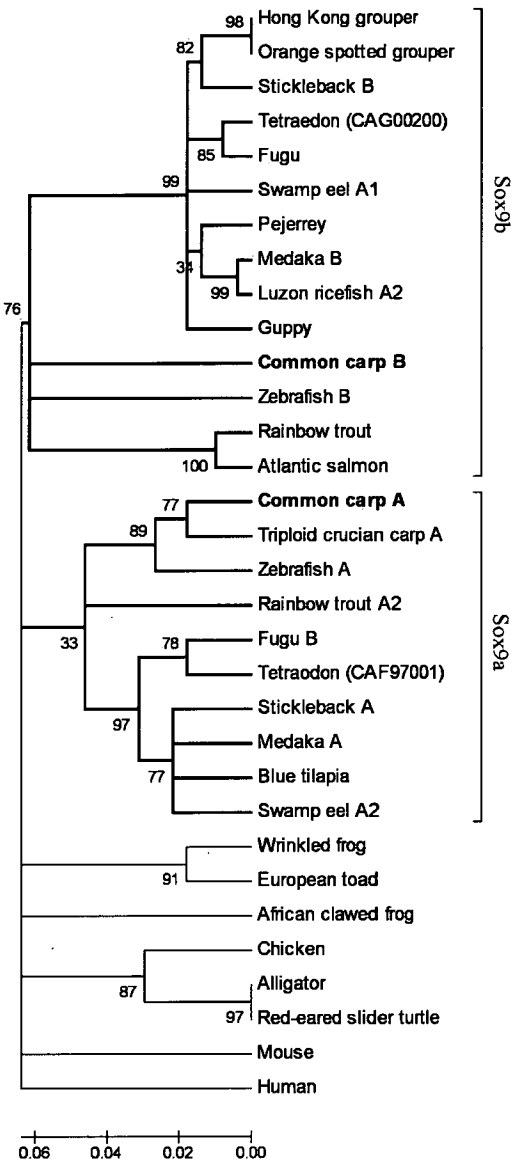


Figure 8.1 Phylogenetic tree of Sox9 proteins.

Deduced amino acid sequences were used from; common carp, *Cyprinus carpio* B (AY956415), Triploid crucian carp, *Carassius auratus* x *Cyprinus carpio* x *Carassius cuvieri* (triploid hybrids of tetraploid and Japanese crucian carp) A (DQ191773), Guppy, *Poecilia reticulata* (DQ683727), Blue tilapia, *Oreochromis aureus* (EU373500), Rainbow trout, *Orcorhynchus mykiss* (NM_001124179), A2 (NM_001124270), Atlantic salmon, *Salmo salar* (BT059262), Hong Kong grouper, *Epinephelus akaara* (AY676309), Orange-spotted grouper, *Epinephelus coioides* (GQ232762), Pejerrey, *Odontesthes bonariensis* (AY319415), Swamp eel, *Monopterus albus* A1 (AF378150), A2 (AF378151), Luzon ricefish, *Oryzias luzonensis* (AB428664), Zebrafish, *Danio rerio* A

(NM_131643), B (NM_131644), fugu, *Takifugu rubripes* (AF329945), B (AY277965), Japanese medaka, *Oryzias latipes* A (AY870394), B (AY870393), Three-spine stickleback, *Gasterosteus aculeatus* A (AY351914), B (AY351915), Unnamed proteins Tetraodon, *Tetraodon nigroviridis* (CAF97001 and CAG00200), Wrinkled frog, *Rana rugosa* (AB035887), African clawed frog, *Xenopus laevis* (NM_00190807), European toad, *Bufo bufo* (AJ786387), Alligator, *Alligator mississippiensis* (AF106572), Red-eared slider turtle, *Trachemys scripta* (EU914820), Chicken (GGU12533), Mouse (NM_011448), Human (NM_000346).The tree was rooted by human SOX9.

8.4.2 Expression of sox9 genes in adult common carp

8.4.3 Comparative expression of sox9 isoforms within tissues

Expression of both *sox9* genes was examined in the brain (4 sections), pituitary, liver and gonad of common carp by real-time PCR. Expression of *sox9a* was significantly higher than that of *sox9b* in all tissues tested including the gonads (Figure 8.2). The highest fold difference was observed in the gonad, where *sox9a* was expressed 29-fold higher than *sox9b* in the ovary ($t(4)= 5.763$, $P<0.004$) and 17-fold higher in the testis ($t(4)= 3.480$, $P<0.025$). The expression of *sox9a* was also much higher than *sox9b* in all the brain sections of male fish studied with 8-fold higher expression in both sections A ($t(4)= 5.326$, $P<0.006$) and B ($t(4)= 8.038$, $P<0.001$), followed by 7- and 6-fold higher in section C ($t(4)= 8.267$, $P<0.001$) and in section D ($t(4)=26.048$, $P<0.000$) respectively. Expression of these genes in the female pituitary was comparable ($t(4)= 1.381$, $P<0.239$) while differential expression was observed in the pituitary of males with 10-fold higher expression of *sox9a* than *sox9b* ($t(4)= 4.208$, $P<0.014$). Similarly in female brains, 9-fold higher *sox9a* expression was seen in both brain sections B ($t(4)= 9.363$, $P<0.001$) and D ($t(4)= 6.561$, $P<0.003$). In brain sections A ($t(4)= 12.247$, $P<0.000$) and C ($t(4)= 12.149$, $P<0.000$) of females, the expression of *sox9a* was 8-fold higher than *sox9b* expression.

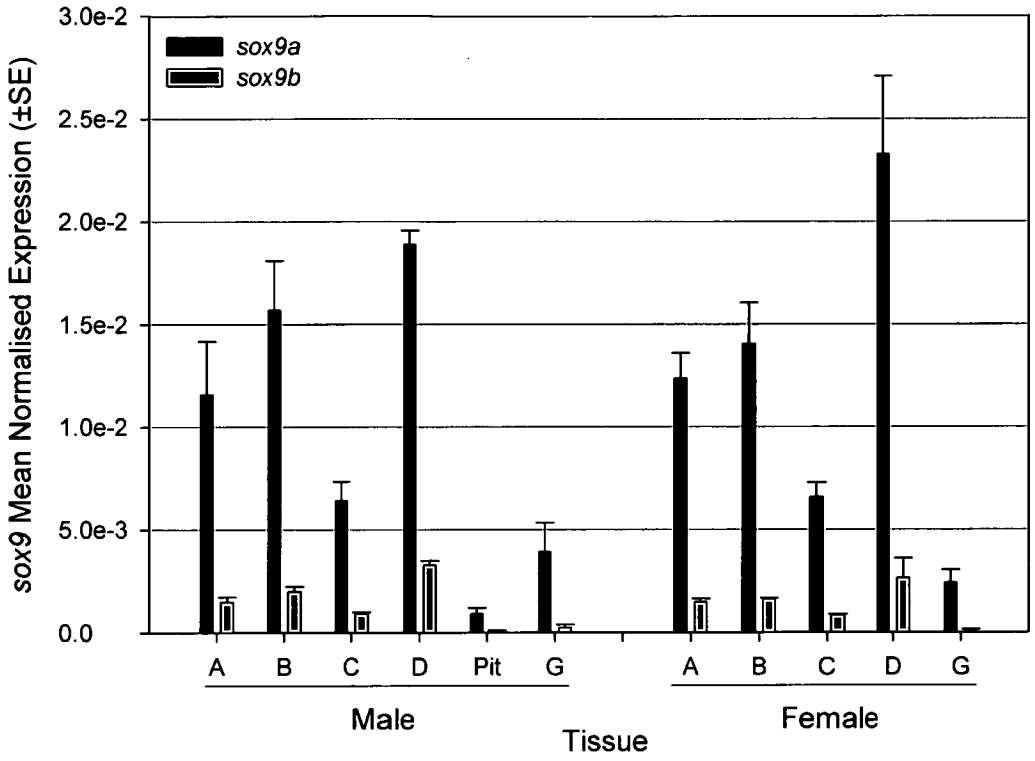


Figure 8.2 Comparative expression of common carp *sox9* genes within tissues showing significantly higher expression of *sox9a* than *sox9b*.

Each bar represents the mean normalised expression (±SE; n = 3). X-axis labels: (A–D) brain sections (see Figure 2.1); Pit, pituitary; G, gonad. Student's t test was used for pairwise comparisons of a both *sox9* genes within a given tissue ($P < 0.025$).

8.4.4 Spatial expression of sox9 genes in adult tissues

Tissue-specific expression of both *sox9* genes is summarised in Figure 8.3. Highest expression of *sox9a* was in brain sections B (optic lobe) and D (medulla) in males ($F= 32.106$, $P<0.000$), with 2-fold lower expression in section A (telencephalon) and 3-fold lower in section C (cerebellum). Expression of *sox9a* in the testis was 5-fold lower than in brain section D and 21- and 87-fold lower in the pituitary and liver in brain section D.

In females highest expression of *sox9a* was observed in brain section D ($F= 18.802$, $P<0.000$) with 2-fold lower expression in brain sections A and B and 3-fold lower in section C. In the pituitary and gonad, *sox9a* expression was 6- and 10-fold lower than

in brain section D. Lowest expression was observed in the liver (105-fold lower than in brain section D).

Expression of *sox9b* followed a similar trend to *sox9a* albeit at a much lower level. In males, brain section D had the highest *sox9b* expression ($F=29.934$, $P<0.000$) with 2-fold lower expression in brain sections A and B and 4-fold lower in section C. Expression in the pituitary was 35- fold lower than the highest expression (brain section D) and 9-fold lower in the liver. In the testis, expression of *sox9b* was 14-fold lower than in brain section D.

Female brain section D also had the highest *sox9b* expression ($F=9.002$, $P<0.000$) with 2-fold lower expression in brain sections A and B and 3-fold lower in section C. In the female pituitary *sox9b* expression was 5-fold lower and in the liver 10-fold lower than the highest female *sox9b* expression in the brain section D. The ovary had the lowest *sox9b* expression with 32-fold lower expression than in female brain section D.

Student's independent T-test between corresponding tissues of both sexes showed no sex-specific differences in expression of either gene.

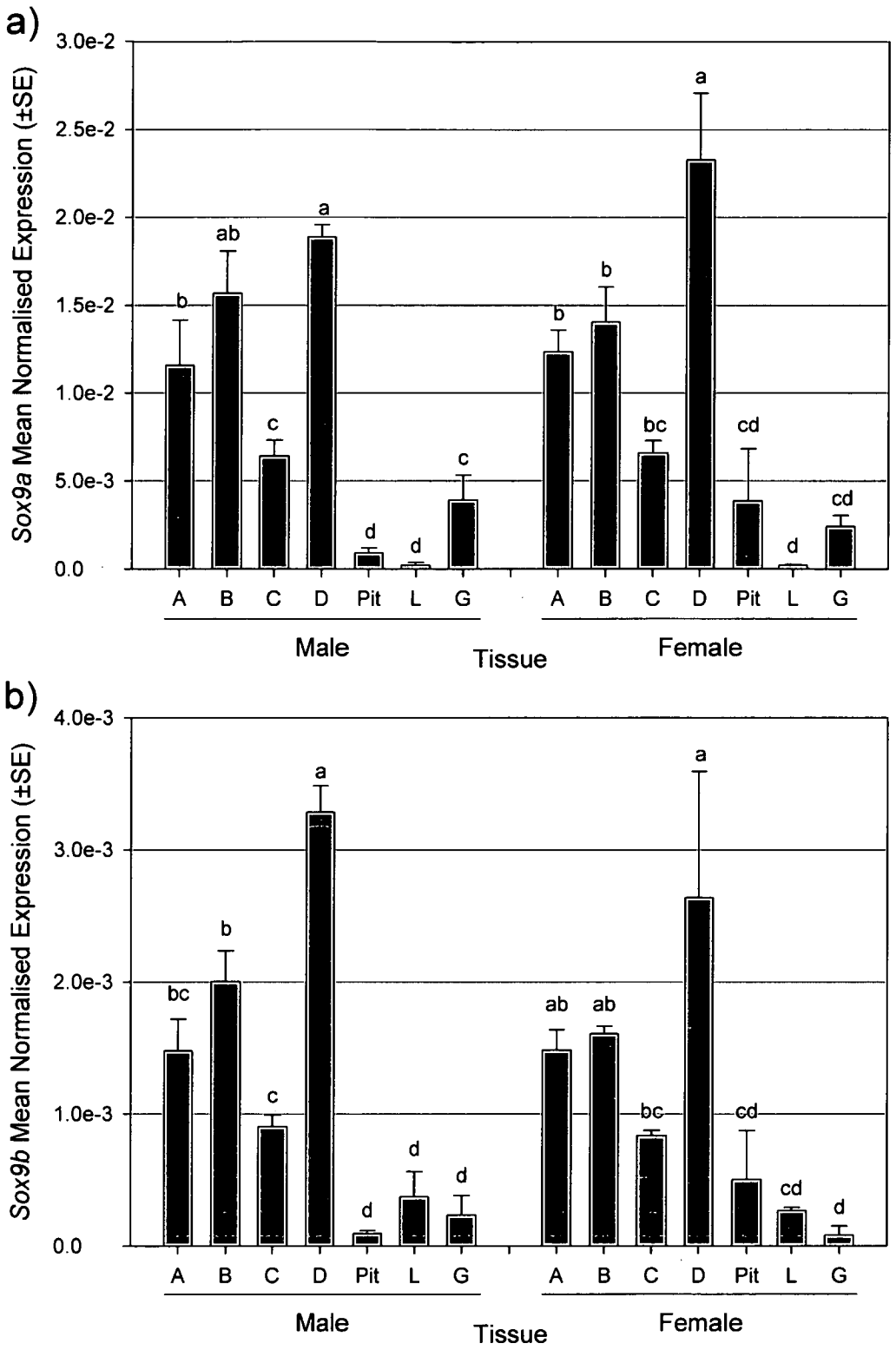


Figure 8.3 Expression of (a) *sox9a* and (b) *sox9b* in select adult common carp of tissues.

Each bar represents the mean normalised expression (±SE; n = 3). X-axis labels: (A-D) brain sections (see Figure 2.1); Pit, pituitary; L, liver; G, gonad. One-way ANOVA was used to analyse data by tissue type within each sex, followed by SNK analysis to determine which means differed significantly ($P<0.03$), as indicated by letters (a-d). Note scale difference in (a) and (b).

8.5 Embryonic and larval development

8.5.1 Growth

Following hatch (20 °C- 70 ddpf, 25 °C- 62.5 ddpf) the TL of larva was measured at the time of sampling. In both temperatures TL was found to increase steadily over time (20 °C- $F=28.419$, $P<0.000$, 25 °C- $F=79.338$, $P<0.000$) (Figure 8.4). At the higher temperature (25 °C) the growth rate of larva was significantly higher ($F=16.813$, $P>0.000$) at 25 °C mean TL was 47.75 ± 3.43 mm after 118 dpf compared to 31.13 ± 2.47 mm at 20 °C after 138 dpf.

8.5.2 Embryonic and larval expression of sox9 genes

Temporal changes in *sox9a* and *sox9b* expression during larval development were quantified by real-time PCR in unfertilised eggs up to 138 and 118 dpf in both 20 °C and 25 °C treatment groups respectively (Figure 8.4).

In unfertilised eggs expression of *sox9a* was similar to that found in the mature ovary ($F=2.609$, $P<0.182$). Over time there were significant changes in *sox9a* expression in both the 20 °C ($F= 110.524$, $P<0.000$) and 25 °C ($F=77.031$, $P<0.000$). Following fertilisation levels of *sox9a* declined by 2-fold, corresponding with the morula (~4 ddpf) and late blastula stages (~5 ddpf) in 20 and 25 °C respectively. At 20 °C this reduced level of expression was maintained until 12.5 ddpf, followed by a peak expression at 25 ddpf (mid-somite) ~3-fold higher than the maternally inherited level. Following this peak, expression fell 2-fold and continued to dissipate steadily through to 860 ddpf (13.13 ± 0.58 cm) where expression was maintained at a basal level to the end of the experimental period.

At 25 °C, *sox9a* expression increased 3-fold (from the previous measurement) at 15.6 ddpf corresponding to early somite stage. This was followed by a peak at 31.25 ddpf measuring 2-fold higher than the maternal level. This peak therefore was lower than that observed in the 20 °C treatment. This level of expression was maintained through hatching (62.5 ddpf) and subsequently declined following a similar trend observed at 20 °C, with low basal levels of expression maintained between 1075 ddpf (21.0 ± 1.31 mm TL) until the termination of the experiment (2950 ddpf : 47.75 ± 3.43 mm TL).

Expression of *sox9b* was not detected in unfertilised eggs, however temporal changes in expression were observed in both temperatures (20 °C- $F= 394.673$, $P<0.000$ and 25 °C- $F= 186.850$, $P<0.000$). Expression of *sox9b* remained low following fertilisation, but increased 172-fold and peaked to $5.5e^{-3}$ at about 15.6 ddpf in the 25 °C treatment (early somite stage). Expression of *sox9b* in the 20°C treatment increased gradually (105-fold over 21 degree days) before peaking at $3.2e^{-3}$ at 25 ddpf (mid-somite), albeit at a lower level than observed at 25 °C. Following the peak in both temperatures expression gradually dissipated, reaching a basal level at 320 ddpf (6.67 ± 0.22 mm TL) at 20 °C and 400 ddpf (8.15 ± 0.08 mm TL) at 25 °C. This basal level of *sox9b* expression was significantly lower than that of *sox9a* ($F=12.643$, $P<0.003$).

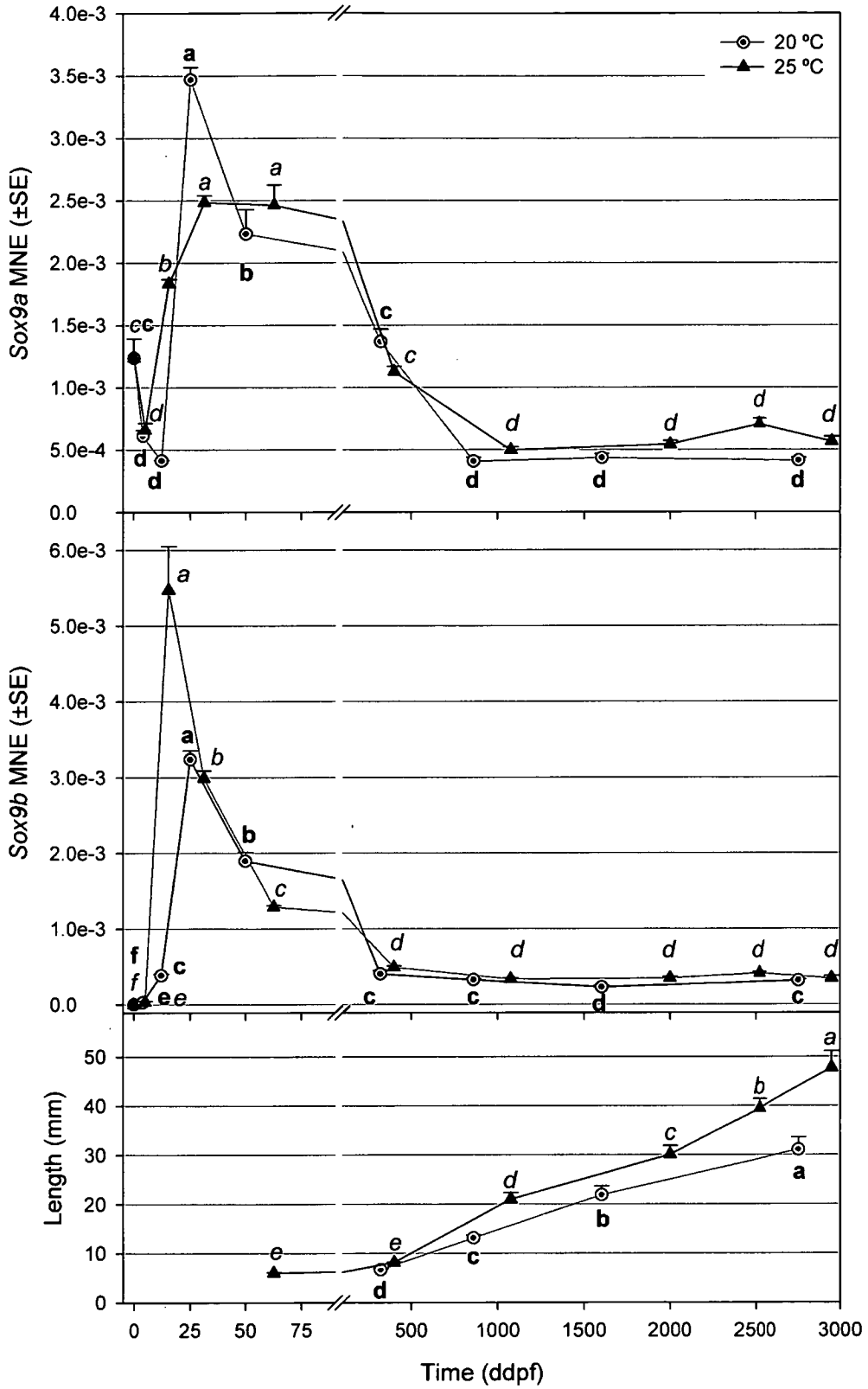


Figure 8.4 Temporal changes in length and *Sox9a* and *b* expression of embryos and larvae reared at 20 or 25 °C.

One-way ANOVA used to analyse data for temperature specific differences, followed by SNK analysis to determine which means differed significantly ($P < 0.01$), as indicated by font style (20 °C- bold, 25 °C- italic). Note: hatch occurred at 70.8 and 62.5 ddpf in the 20 and 25 °C treatments respectively.

8.6 Discussion

Sox9 is known to act down stream of SRY in mammalian sex determination and a single isoform of *sox9* is present in higher vertebrates such as in human (Foster et al., 1994), mouse (Kanai and Koopman, 1999; Wright et al., 1995), chicken (da Silva et al., 1996) and alligator (Western et al., 1999). In teleosts, however, two isoforms (*sox9a* and *b*) have been reported with their functions partitioned in lineage-specific ways (Cresko et al., 2003; Klüver et al., 2005; Postlethwait et al., 2004). Expression pattern studies indicate that the function of *sox9* genes in cartilage development (chondrogenesis) is conserved in fish, but its role in male sex determination and differentiation may not be (Yokoi et al., 2002; Zhou et al., 2003).

8.6.1 Phylogenetics

In common carp, the *sox9* gene was previously explored by Du et al. (2007) who amplified two versions from genomic DNA which they termed *sox9a* and *sox9b*, however these only differed in having an intron of different length, but coding for the same transcript, that is homologous to other teleostean *sox9b*. Again, as evidenced from the phylogenetic analysis carried out in this study, the deduced common carp Sox9b shared greater homology with other teleost Sox9b proteins than a distinct *sox9* transcript isolated in this study. The deduced protein from this distinct *sox9* transcript has much higher homology to Sox9a proteins described in other teleosts and therefore it is proposed that this be named the common carp *sox9a* gene. The presence of two *sox9* genes in common carp is consistent with the hypothesis that they are duplicated as shown in other teleost fishes such as zebrafish (Chiang et al., 2001) and three-spined stickleback (Cresko et al., 2003). It has been suggested that this duplication occurred before the divergence of these lineages, as did the partitioning of most sub-functions assayed by embryonic expression analysis (Cresko

et al., 2003). This supports a shared ancestry of the two separated genes and the existence of two paralogous genes suggested to be due to the occurrence of genome duplication early in the teleost linage (Taylor et al., 2003).

Interestingly the unassigned fugu Sox9 deduced protein (AAL32172) groups with Sox9b clade, whilst the assigned fugu Sox9b (AAQ18508) clades with the teleostean Sox9a clade, suggesting that these genes have been incorrectly assigned. It is therefore recommend that the fugu *sox9b* (AY277965) be renamed *sox9a* and the unassigned protein (AAL32172) be assigned as Sox9b. However the high identity of both of the fugu *sox9* genes with two different medaka *sox9b* isoforms reported by different authors (Klüver et al., 2005; Nakamoto et al., 2005) suggests that more research is required to tease this out. So far only one isoform has been reported for Hong Kong grouper, orange-spotted grouper, pejerrey, guppy, rainbow trout, Atlantic salmon and blue tilapia. Based on their homology and phylogenetic relationship it is tempting to assign orthology to either *sox9a* or as *sox9b*, but in the absence of the data on the second gene it is difficult to be sure if the gene is indeed duplicated in these species. However, as and when this information becomes available it will help place isoforms of these and other species, as has occurred with the two rice field eel *sox9* genes (*a1* and *a2*). When first analysed by Zhou et al. (2003) these genes were only compared with higher vertebrates and a single rainbow trout *sox9* and zebrafish *sox9a* and *b*. Analysis in the current study suggests that the swamp eel *sox9a2* ortholog is in fact more similar to other teleostean *sox9b* homologues.

8.6.2 Expression in adults

Although the structure of *sox9* genes have been conserved, the tissue in which they are expressed and hence their function differs widely among teleost species (Table 8.2).

Table 8.2 *Sox9* gene expression in adult tissues (Adapted and amended from Klüver et al., 2005).

	Brain		Testis		Ovary		Eye		Gills		Heart		Kidney		Liver		Spleen		Skin		Muscle		Blood		Fin	
	a	*b*	*a*	*b*	*a*	*b*	*a*		*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*
Common carp	+++	++	++	+	++	+	N.R.	N.R.	N.R.	N.R.	N.R.	N.R.	N.R.	N.R.	+	+	N.R.	N.R.	N.R.	N.R.	N.R.	N.R.	N.R.	N.R.	N.R.	N.R.
Common carp[1]		+++		+++		+		N.R.		N.R.		++		+		++		N.R.		N.R.		N.R.		N.R.		N.R.
Medaka	++	++	+	+++	+++	-	+	+	+	+	-	+	-	-	-	-	-	+	-	-	-	-	N.R.	N.R.	N.R.	N.R.
Zebrafish	++	+	++	-	-	+++	+	-	+	+	+	-	+	+	-	-	-	+	+	-	+	-	N.R.	N.R.	N.R.	N.R.
Swamp Eel	+	-	+	+	+	+	N.R.	N.R.	N.R.	N.R.	+	-	N.R.	N.R.	N.R.	N.R.	N.R.	N.R.	N.R.	N.R.	N.R.	N.R.	N.R.	N.R.	N.R.	N.R.
Rainbow Trout[2]		+		+		+		N.R.		N.R.		+		N.R.		+		+		N.R.		N.R.		N.R.		N.R.
Fugu	N.R.	N.R.	++	-	+	++	N.R.	N.R.	N.R.	N.R.	N.R.	N.R.	N.R.	N.R.	N.R.	N.R.	N.R.	N.R.	N.R.	N.R.	+	-	-	-	+	-
Pejerrey[3]		+++		+++		+		N.R.		N.R.		N.R.		N.R.		N.R.		N.R.		N.R.		N.R.		N.R.		

+++ High Expression ++ Medium Expression + Low Expression N.R., not reported [1]*Du et al, 2007* [2]*Presence/absence data only* [3]*Pejerrey isoform unknown*

Analysis of *sox9* genes in common carp in the current study found predominant expression of both isoforms in the brain, with the bulk of expression contributed by *sox9a*. The expression and importance of *sox9* in the brain development of higher vertebrates has been reported in rodents (Pompolo and Harley, 2001) and mammals (Andrew et al., 2002; Wagner et al., 1994). In contrast to the present study, Du et al (2007) found *sox9b* expression at similar levels in both the brain and testis employing a semi-quantitative technique. In contrast the real-time PCR assay used in the current study is more sensitive in both detection and quantitation. In rainbow trout *sox9* was found to be expressed in both the brain and testis (Takamatsu et al., 1997), however the isoform was not classified as either *sox9a* or *b*. Phylogenetic analysis in this study (Figure 8.1) places the described rainbow trout *sox9* gene in the *sox9a* clade. In zebrafish only *sox9a* was expressed in the brain, again at a high level (Chiang et al., 2001).

In chicken, mouse and turtle, both *sox9* genes are expressed in the testis but not the ovary (Kent et al., 1996; Moreno-Mendoza et al., 1999; Spotila et al., 1998; Wright et al., 1995) suggesting functional importance of *sox9* for male differentiation in tetrapods is conserved. However, the present study found *sox9* expression in the testis and ovary, with predominant expression of *sox9a* and no sex specific differences of either gene. In a previous study on the same species, Du et al. (2007) found high expression of *sox9b* in the testis while expression in the ovary was lower, sex specific expression of either *sox9a* or *b* is also found in other species of teleosts (Table 8.2). Discrepancies in expression domains of *sox9* observed between species of teleosts is likely due to species specific specialisation of function or could simply be a reflection of physiological condition of the tested animals. For example in zebrafish *sox9a* is expressed in the testis while *sox9b* was only expressed in the ovary (Chiang et al., 2001) and in fugu *sox9a* was detected in gonad of both sexes while *sox9b* was only detected in ovary (Shen et al., 2007). This indicates that the role of *sox9* function may not be entirely conserved between teleost, but may have undergone lineage specific functional specialisation, following genome duplication. Also, the non sex-specific expression of either isoform in common carp, suggests that *sox9* may not play a significant role in sex determination or sex-specific maintenance in the common carp.

The expression domains and role of *sox9* genes in non-gonadal tissues also appears to be species specific (Table 8.2). The present study found lower levels of *sox9b* expression as previously reported in the common carp liver (Du et al., 2007). Neither *sox9* gene were detected in medaka or zebrafish liver (Chiang et al., 2001; Yokoi et al., 2002).

8.6.3 Maternal inheritance and expression during development

Expression of both *sox9* genes was examined in unfertilised eggs and through early and larval development at two temperatures, 20 and 25 °C. Unfertilised eggs were found to express only the *sox9a* isoform, with levels of expression decreasing post-fertilisation. The maternal transfer of the *sox9a* transcript suggests that this isoform plays an important role in the very early stages of embryonic development. To our knowledge this is the first time that maternal inheritance of *sox9* genes has been investigated in teleosts.

Zygotic expression of both genes was observed as a peak in expression at ~25 ddpf, corresponding to mid-somite stage with the notochord distinct in both temperature treatments. This peak correlates well with observations of Pashine and Marathe (1977) where at 60 hpf (TL of 3.73 mm) the foundation of the cartilaginous skull had been laid down in common carp embryos, indicating chondrogenesis is well underway. Similarly expression of both *sox9* genes in zebrafish was detected in the head region (forebrain and midbrain–hindbrain boundary) and somites of embryos during the late somite stage with persisting expression through chondrogenesis (Chiang et al., 2001). As in zebrafish (Chiang et al., 2001), the current study found the expression of both *sox9* genes in common carp were progressively down-regulated around the time of hatching (70.8 and 62.5 ddpf in the 20 and 25 °C treatments respectively) likely coinciding with completion of early embryonic chondrogenesis. The role of *sox9* as the master regulator of chondrogenesis is conserved in higher vertebrates (Akiyama, 2008; Bi et al., 1999; Healy et al., 1996, 1999). Although this role is also conserved in teleosts, species specific patterns of embryonic expression of the two isoforms have been reported. For example, *sox9a* is not expressed in the forebrain, somites and midbrain–hindbrain boundary of medaka

(Klüver et al., 2005) but is only expressed in the forebrain and weakly in somites of three-spined stickleback (Cresko et al., 2003). In contrast, *sox9b* is expressed in generally similar patterns in zebrafish, medaka and three-spined stickleback, with medaka having additional lineage-specific expression domains (Klüver et al., 2005). Species specific expression patterns reported for *sox9* genes during embryonic development of teleosts indicates that spatio-temporal regulation of these genes has altered during divergence of the corresponding lineages.

The role of *sox9* genes in sex determination have also altered with divergence. Through the period of gonadal differentiation in common carp ongoing expression of both *sox9* genes was maintained at a low level without any sex-specific differences. The size at which sex is able to be determined by histology in common carp is 20 mm TL (Chapter 4 and Beyer, 2004) reached at ~1000 and ~1500 ddpf in the 25 and 20 °C treatments respectively. As no sex-specific differences for either gene are seen in adult gonads, perhaps it is not surprising that differences are also not seen during gonadal development. Rather, the lack of sexually dimorphic expression during development supports the idea that *sox9* genes in common carp do not play a role in sex determination. In contrast, sexually dimorphic expression and localisation of *sox9a* were seen in medaka after 25 dph when sex difference in histological architecture appear (Kobayashi et al., 2008).

8.7 Conclusions

Despite distinct expression patterns of teleost *sox9* co-orthologs, the combined expression patterns are similar to that of *sox9* in other vertebrates. However, the expression of *sox9* genes in the developed ovary appears to be unique to the teleost lineage. The combined expression of *sox9a* and *sox9b* in the gonad of common carp, with predominant expression of *sox9a*, suggests that *sox9b* may have acquired an as

yet unknown new role following duplication, while *sox9a* mimics most functions observed in tetrapods and in addition has acquired a functional role in the ovary of teleosts. Similar expression levels of both isoforms in tissues of both sexes in addition to no significant sex-specific expression during gonadal development suggest that *sox9* does not play a role in sex differentiation of common carp. Peak expression of both genes during embryogenesis suggests that the role of *sox9* in chondrogenesis is also conserved in common carp.

8.8 Acknowledgments

Support and assistance provided by CSIRO, Invasive Animal Cooperative Research Centre, Inland Fisheries Service Tasmania and K&C Fisheries. This project received funding support from the Fisheries Research and Development Corporation.

8.9 References

Akiyama, H., 2008. Control of chondrogenesis by the transcription factor Sox9. Modern Rheumatology, 18, 213-219.

Andrew, J.P., Jenny, L.H., Jennifer, A.M.G., Rachel, J.W.O.N., Sharon, L.L., Geoffrey, S., Marilyn, B.R., 2002. SOX9 has both conserved and novel roles in marsupial sexual differentiation. genesis, 33, 131-139.

Barney, M.L., Patil, J.G., Gunasekera, R.M., Carter, C.G., 2008. Distinct cytochrome P450 aromatase isoforms in the common carp (*Cyprinus carpio*): Sexual dimorphism and onset of ontogenic expression. Gen Comp Endocrinol, 156, 499-508.

Beyer, J., 2004. Early gonad development in the common carp, *Cyprinus carpio (L.)* at 20 °C and 25 °C. Honours Thesis, University of Tasmania, Launceston.

Bi, W., Deng, J.M., Zhang, Z., Behringer, R.R., de Crombrugghe, B., 1999. Sox9 is required for cartilage formation. Nat Genet, 22, 85-89.

Chiang, E.F.L., Pai, C.-I., Wyatt, M., Yan, Y.-L., Postlethwait, J., Chung, B.-c., 2001. Two Sox9 genes on duplicated Zebrafish chromosomes: Expression of similar transcription activators in distinct sites. Dev Biol, 231, 149-163.

Choudhary, B., Ganesh, S., Raman, R., 2000. Evolutionary conservation of the gene *Cvsox9* in the lizard, *Calotes versicolor*, and its expression during gonadal differentiation. Dev. Genes Evol., 210, 250-257.

Cresko, W.A., Yan, Y.-L., Baltrus, D.A., Amores, A., Singer, A., Rodríguez-Marí, A., Postlethwait, J.H., 2003. Genome duplication, subfunction partitioning, and lineage divergence: *Sox9* in stickleback and zebrafish. Dev. Dynam., 228, 480-489.

da Silva, S.M., Hacker, A., Harley, V., Goodfellow, P., Swain, A., Lovell-Badge, R., 1996. Sox9 expression during gonadal development implies a conserved role for the gene in testis differentiation in mammals and birds. Nat Genet, 14, 62-68.

Du, Q.Y., Wang, F.Y., Hua, H.Y., Chang, Z.J., 2007. Cloning and study of adult-tissue-specific expression of Sox9 in Cyprinus carpio. J Genet, 86, 85-91.

Foster, J.W., Dominguez-Steglich, M.A., Guioli, S., Kwok, C., Weller, P.A., Stevanovic, M., Weissenbach, J., Mansour, S., Young, I.D., Goodfellow, P.N., Brook, J.D., Schafer, A.J., 1994. Campomelic dysplasia and autosomal sex reversal caused by mutations in an SRY-related gene. Nature, 372.

Guo, X., Yan, J., Liu, S., Xiang, B., Liu, Y., 2008. Isolation and expression analyses of the Sox9a gene in triploid crucian carp. Fish Physiol Biochem.

Hanley, N.A., Hagan, D.M., Clement-Jones, M., Ball, S.G., Strachan, T., Salas-Cortes, L., McElreavey, K., Lindsay, S., Robson, S., Bullen, P., Ostrer, H., Wilson, D.I., 2000. SRY, SOX9, and DAX1 expression patterns during human sex determination and gonadal development. Mech Dev, 91, 403–407.

Healy, C., Uwanogho, D., Sharpe, P.T., 1996. Expression of the chicken Sox9 gene marks the onset of cartilage differentiation. Ann N Y Acad Sci, 785, 261-262.

Healy, C., Uwanogho, D., Sharpe, P.T., 1999. Regulation and role of Sox9 in cartilage formation. Developmental Dynamics, 215, 69-78.

Kanai, Y.A. & Koopman, P., 1999. Structural and functional characterization of the mouse Sox9 promoter, implications for campomelic dysplasia. Hum. Mol. Genet., 8, 691–696.

Kent, J., Wheatley, S.C., Andrews, J.E., Sinclair, A.H., Koopman, P., 1996. A male-specific role for SOX9 in vertebrate sex determination. Development, 122, 2813-2822.

Klüver, N., Kondo, M., Herpin, A., Mitani, H., Schartl, M., 2005. Divergent expression patterns of Sox9 duplicates in teleosts indicate a lineage specific subfunctionalization. Dev. Genes Evol., 215, 297-305.

Kobayashi, T., Kajiura-Kobayashi, H., Guan, G., Nagahama, Y., 2008. Sexual dimorphic expression of *DMRT1* and *Sox9a* during gonadal differentiation and hormone-induced sex reversal in the teleost fish Nile tilapia (*Oreochromis niloticus*). Dev. Dynam., 237, 297-306.

Koopman, P., Schepers, G., Brenner, S., Venkatesh, B., 2004. Origin and diversity of the *Sox* transcription factor gene family: genome-wide analysis in *Fugu rubripes*. Gene, 328, 177-186.

Moreno-Mendoza, N., Harley, V.R., Merchant-Larios, H., 1999. Differential expression of SOX9 in gonads of the sea turtle *Lepidochelys olivacea* at male- or female-promoting temperatures. The Journal of Experimental Zoology, 284, 705-710.

Morrish, B.C. & Sinclair, A.H., 2002. Vertebrate sex determination: many means to an end. Reproduction, 124, 447-457.

Nakamoto, M., Suzuki, A., Matsuda, M., Nagahama, Y., Shibata, N., 2005. Testicular type Sox9 is not involved in sex determination but might be in the development of testicular structures in the medaka, *Oryzias latipes*. Biochem Biophys Res Commun, 333, 729-736.

Pashine, R.G. & Marathe, V.B., 1977. The development of the chondrocranium of *Cyprinus carpio* Linn. Proc. Indian Acad. Sci., 85, 351-363.

Pompolo, S. & Harley, V.R., 2001. Localisation of the SRY-related HMG box protein, SOX9, in rodent brain. Brain Res, 906, 143-148.

Postlethwait, J., Amores, A., Cresko, W., Singer, A., Yan, Y.-L., 2004. Subfunction partitioning, the teleost radiation and the annotation of the human genome. Trends Genet, 20, 481-490.

Shen, X., Cui, J., Yang, G., Gong, Q., Gu, Q., 2007. Expression detection of DMRTs and two sox 9 genes in *Takifugu rubripes* (Tetraodontidae, Vertebrata). Journal of Ocean University of China (English Edition), 6, 182-186.

Spotila, L., D. , Spotila, J., R. , Hall, S., E. , 1998a. Sequence and expression analysis of WT1 and Sox9 in the red-eared slider turtle, *Trachemys scripta*. The Journal of Experimental Zoology, 281, 417-427.

Spotila, L.D., Spotila, J.R., Hall, S.E., 1998b. Sequence and expression analysis of WT1 and Sox9 in the red-eared slider turtle, *Trachemys scripta*. J Exp Zool, 281, 417-427.

Takamatsu, N., Kanda, H., Ito, M., Yamashita, A., Yamashita, S., Shiba, T., 1997. Rainbow trout SOX9: cDNA cloning, gene structure and expression. Gene, 202, 167-170.

Takase, M., Noguchi, S., Nakamura, M., 2000. Two *Sox9* messenger RNA isoforms: Isolation of cDNAs and their expression during gonadal development in the frog *Rana rugosa*. FEBS Lett, 466.

Taylor, J.S., Braasch, I., Frickey, T., Meyer, A., Van de Peer, Y., 2003. Genome duplication, a trait shared by 22,000 species of ray-finned fish. Genome Res, 13, 382-390.

von Hofsten, J. & Olsson, P.-E., 2005. Zebrafish sex determination and differentiation: Involvement of FTZ-F1 genes. Reprod. Biol. Endocrin., 3, 63.

Wagner, T., Wirth, J., Meyer, J., Zabel, B., Held, M., Zimmer, J., Pasantes, J., Bricarelli, F.D., Keutel, J., Hustert, E., Wolf, U., Tommerup, N., Schempp, W., Scherer, G., 1994. Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the SRY-related gene SOX9. Cell, 79, 1111–1120.

Western, P.S., Harry, J.L., Graves, J.A.M., Sinclair, A.H., 1999. Temperature-dependent sex determination: Upregulation of *SOX9* expression after commitment to male development. Dev. Dynam., 214, 171-177.

Wright, E., Hargrave, M.R., Christiansen, J., Cooper, L., Kun, J., Evans, T., Gangadharan, U., Greenfield, A., Koopman, P., 1995a. The *Sry*-related gene *Sox9* is expressed during chondrogenesis in mouse embryos. Nat Genet, 9, 15-20.

Yan, Y.L., Miller, C.T., Nissen, R.M., Singer, A., Liu, D., Kirn, A., Draper, B., Willoughby, J., Morcos, P.A., Amsterdam, A., Chung, B.C., Westerfield, M., Haffter, P., Hopkins, N., Kimmel, C., Postlethwait, J.H., Nissen, R., 2002. A zebrafish *Sox9* gene required for cartilage morphogenesis. Development, 129, 5065–5079.

Yokoi, H., Kobayashi, T., Tanaka, M., Nagahama, Y., Wakamatsu, Y., Takeda, H., Araki, K., Morohashi, K., Ozato, K., 2002. *Sox9* in a teleost fish, medaka

(*Oryzias latipes*): Evidence for diversified function of *Sox9* in gonad differentiation. Mol Reprod Dev, 63, 5-16.

Zhou, R., Liu, L., Guo, Y., Yu, H., Cheng, H., Huang, X., Tiersch, T.R., Berta, P., 2003. Similar gene structure of two *Sox9a* genes and their expression patterns during gonadal differentiation in a teleost fish, rice field eel (*Monopterus albus*). Mol Reprod Dev, 66, 211-217.

CHAPTER 9

General Discussion

Chapter 9 - General Discussion

9.1 General discussion

This thesis investigated the molecular mechanisms of sex determination and differentiation in common carp, *Cyprinus carpio*, with a focus on candidate genes known to be involved in these processes in higher vertebrates and invertebrates. More specifically, this thesis indentified and described several genes for the first time in this species and examined expression profiles in adult tissues as well as during embryonic and larval developmental under two temperature regimes (20 and 25°C). The following chapter will discuss the potential interactions between these genes and how the information produced in this thesis contributes to our understanding of the processes of sex determination and differentiation in common carp with a wider view to teleost fish as well as higher vertebrates and invertebrates. This chapter will also discuss the potential impacts of this knowledge to aquaculture, fish biology research and pest control methods and will highlight the commercial utility and future research directions.

9.1.1 Interactions of candidate genes in the sex determination and differentiation pathway

Hormones and environmental factors have very important roles in the genetic sex determination mechanisms of non-mammalian vertebrates. Among these factors, estrogens are involved in various aspects of sexual differentiation, gonadal growth and development of vertebrates (Lange et al., 2003; Simpson et al., 2002) including fish (reviewed by Guiguen et al., 2009), amphibians (Yu et al., 1993), and birds (Elbrecht and Smith, 1992; Smith and Sinclair, 2004). The production of endogenous estrogen relies on the availability and activity of steroid synthesizing enzymes, and in

particular cytochrome P450 aromatase complex expressed by the *cyp19* genes (Fenske and Segner, 2004).

This thesis examined the expression of ovarian (*cyp19a*) and brain (*cyp19b*) aromatase and analysed the promoter regions of both aromatase genes *in silico* to predict potential binding sites. This analysis indicated that both isoforms share binding sites for fork head box (FH) and combined SRY/Sox binding factors. These sites facilitate binding of the conserved domains of *foxL2* and *sox9*, genes which are investigated in Chapters 6 and 8, respectively. Unique sites were also identified in each aromatase promoter; full estrogen responsive elements (EREs) were unique to *cyp19b*, whilst steroidogenic factor 1 (SF-1) binding sites were unique to *cyp19a* promoter. The presence of full EREs in the *cyp19b* isoforms indicates that only this isoform is directly responsive to estrogen, suggesting that exogenous estrogens may affect gonadal development, at least partially, through up-regulation of *cyp19b*.

In contrast, the presence of SF-1, which is known to be thermolabile (Fleming et al., 1999), only in the *cyp19a* promoter, may explain why only this isoform (*cyp19a)* is suppressed at warmer (25 °C) temperature (Chapter 3 / Barney et al., 2008). SF-1 is also reported to be involved in aromatase regulation and sex differentiation of vertebrates through interaction with *foxL2* and *dmrt1*. Interestingly *foxL2* was not found to be affected by temperature (Chapter 6) while *dmrt1* does appear to be thermosensitive in common carp (Chapter 7). Further functional analysis of the promoters is essential to determine which of these potential binding sites identified in this thesis are in fact active.

The involvement of *foxL2* in the transcriptional regulation of *cyp19* has been reported in chicken (Govoroun et al., 2004; Hudson et al., 2005) and goat (Pannetier

et al., 2006). In an alternate system in mice, where estrogen is not involved in ovarian differentiation (Toda et al., 2001), knock-out of *foxL2* did not affect early sex differentiation or cause female to male sex reversal, but blocked differentiation of granulosa cells followed by follicle degeneration (Schmidt et al., 2004; Uda et al., 2004). Interestingly in these knock-out mice, genes involved in testis differentiation, such as *sox9* are upregulated after birth (Ottolenghi et al., 2005), suggesting that *foxL2* has a critical role in granulosa cell differentiation, and its function does not depend on estrogen.

However, in Nile tilapia, *Oreochromis niloticus*, during early stages of ovarian differentiation *foxl2* and SF-1 were found to be co-localized with *cyp19a* (Wang et al., 2007). Here FoxL2 protein interacted with SF-1 to enhance SF-1-activated *cyp19a* transcription in a female-specific manner. Although SF-1 was not examined in the current study, expression of *foxL2* and *cyp19a* were significantly higher in the ovary than the testis. Interestingly, when treated with aromatase inhibitors (AI) (Fadrozole and/or Tamoxifen) *foxL2/cyp19a* expression in the gonad and *foxL2/cyp19b* expression in the brain of southern catfish decreased significantly and was highly correlated with the female-to-male sex reversal (Liu et al., 2007). These results strongly suggest that *foxL2* regulates expression of *cyp19b* in brain and *cyip19a* in the ovary. Furthermore, this co-down-regulation of *foxL2/cyp19b* and *foxL2/cyp19a* in brain and ovary may contribute to the brain and ovarian sex differentiation in that species following AI treatment.

As has been observed in species including Paralichthyd flounders (Kitano et al., 1999; Luckenbach et al., 2003), Atlantic halibut (van Nes and Andersen, 2006), European sea bass (Mylonas et al., 2005), and Nile tilapia (D'Cotta et al., 2001;

Kwon et al., 2000) the expression of larval *cyp19a* at warmer (25 °C) conditions was suppressed (Chapter 4). Interestingly, *dmrt1 (doublesex* and *mab*-3 related transcription factor 1) was also found to be thermosensitive, but unlike *cyp19a* was found to be upregulated under warmer conditions. Therefore it is possible that *dmrt1* may interact with *cyp19a* as a negative regulator. Based on the knowledge of sex differentiation in mammals, it is now hypothesised that counterparts of mammalian testis-determining factors (e.g. *dmrt1*) in teleosts suppress the expression of *cyp19a* gene. This is supported by the inverse expression profile of *dmrt1* and *cyp19a* in gonads of common carp (Chapter 7 and 3/4 respectively). *Dmrt1* was found to be exclusively expressed in the gonad of common carp with predominant expression in the testis. This conserved testis-determining factor has been shown to repress basal, as well as SF-1 activated and *foxL2*-enhanced *cyp19a* transcription in Nile tilapia (Wang and Nagahama, 2008). It is suggested that *dmrt1* is required during testicular differentiation to down-regulate aromatase gene expression, possibly shifting the entire steroidogenic pathway towards androgen production (Wang and Nagahama, 2008). Investigation into the promoter region and transcriptional factor of *dmrt1* may allow insights into the mechanisms by which temperature is able to alter the expression of this gene. Further research is required to tease out the mechanism of temperature on the steroidogenic pathway and subsequent changes in sex ratio. It is also possible that either i) *dmrt1* acts upstream of *cyp19a* potentially down-regulating its expression during testicular differentiation, or ii) *cyp19a* may act as a testicular repressors with early testicular differentiation requiring active repression of *cyp19a* expression.

S*ox9* was not found to be expressed in a sex-specific manner in common carp, therefore not playing a significant role in sex determination or differentiation in the

species. Despite this the many SRY/SOX binding sites found in the promoter regions of both aromatase genes suggests that it may be involved on a transcriptional level in the pathway.

9.1.2 Insights into sex determination and differentiation

9.1.2.1 Common carp

Based on the expression profile observed in this study, *cyp19a* plays an important role in ovarian differentiation and maintenance in the common carp as in other teleosts. This is demonstrated through the sexually dimorphic expression of *cyp19a* during larval development (Chapter 4) and in the adult gonad, with predominant expression in the ovary, (Chapter 3). Interestingly *cyp19a* was also expressed in the testis, this may either be required for maintenance of testicular function (reviewed by Carreau et al., 2003) or this may be a vestige from an ancestral role. However, expression of only one aromatase gene (*cyp19a)* in unfertilised eggs indicates that it has a critical role in early growth and neural differentiation in both genetic males and females.

An apparent male skew in the sex ratio of larva reared in warmer (25 °C) rearing conditions suggests that sex determination in common carp may be influenced by temperature. This is likely through the suppression of *cyp19a*, as observed in this thesis, and hence suppression of ovarian development. This effect of temperature on sex determination in common carp was suggested by Nagy et al. (1981) and to the best of our knowledge this is the first time that it has been examined in relation to *cyp19a* expression. However, this preliminary data requires further investigation encompassing a larger sample size.

As in other teleost fish, the *dmrt1* gene was found to be expressed specifically in the common carp gonad, with predominant expression in the testis. This indicates a conserved function in testis differentiation, however sex-specific differences in larval expression were not found in the current study. This suggests that activation or sex-specific regulation occurs later in development.

FoxL2 in common carp was suggested to have a role in the regulation of aromatase, specifically in the ovary where expression was significantly higher than in the testis. High levels of *foxL2* expressed within different sections of both male and female brain in the common carp corresponded to the high levels of *cyp19b*, suggesting that *foxL2* plays a role in regulating both ovarian and brain aromatase, but in a tissue localised context.

A peak in expression of both *foxL2* and *dmrt1* prior to hatch, with subsequent sex specific expression in the adult gonad indicates a role in early sex differentiation in common carp. In contrast, expression of both *sox9* genes also peaked prior to hatch but failed to show sex-specific differences in adult tissues. This indicates that the role of *sox9* in sex determination may have been lost in common carp, but its role in chondrogenesis has been conserved.

This thesis was able to confirm previous findings that gonadal differentiation in common carp occurs at a total length of 20 mm and is visually distinguishable upon dissection (Beyer, 2004) and also appears to correspond with up-regulation of *cyp19a* in females and its down-regulation in males. Further studies are required to demonstrate this fully as whole individuals were used for expression analysis of

developmental stages meaning that the gonad was not identified to allow confirmation of sex.

9.1.2.2 Other teleosts

Duplication of aromatase and *sox9* genes found in the current study gives further evidence of a whole genome duplication in this species and the teleost linage (Taylor et al., 2003). Although the sequence structure of these genes is conserved, they appear to have undergone sub-functionalisation and either i) each isoform has taken on different functions of the single ancestral isoform, or ii) one isoform has taken on the majority of the role and the other isoform may have lost or taken on new species-specific functions. The former scenario is true for aromatase genes where the neural roles of aromatase are predominantly carried out by *cyp19b* and the gonadal roles by *cyp19a*. The degree to which ancestral roles are separated appears species specific. The second scenario of loss of function is demonstrated in the role of *sox9* in common carp, where unlike higher vertebrates and some fish species these genes do not appear to be involved in sex determination or differentiation despite a conserved role in chondrogenesis.

This thesis also demonstrated maternal transfer of both *cyp19a* and *sox9a* genes. In many animal species, maternally inherited mRNAs and proteins are used to program the earliest stages of development but are degraded by the mid-blastula transition, allowing genetic control of development to be regulated by zygotically synthesised transcripts (Pedersen, 1998). Expression of *cyp19a* in unfertilised common carp eggs was lower than expression observed in the whole ovary, consistent with findings in zebrafish (Sawyer et al., 2006). Expression of *cyp19b* in unfertilised eggs and through early embryonic development was undetectable at both temperatures of the

current study, suggesting that this transcript in not inherited maternally, unlike *cyp19a*. This conflicts with findings in zebrafish where a 10-fold enrichment of *cyp19b* was seen from whole ovary to unfertilised egg (Sawyer et al., 2006). However, these results are contradicted by another study on zebrafish using more sensitive Taqman real-time PCR, where neither aromatase isoform were detected until after hatching (Trant et al., 2001). It is therefore apparent that different species, and perhaps strains, have a different array of maternally inherited genes that contributes to both early development and sex determination. The current study demonstrates a unique view of aromatase maternal inheritance in a teleost species, however further study is required to determine the function of aromatase early during embryonic development

9.1.2.3 Higher vertebrates and invertebrates

A greater understanding of sex determination and its evolution in fish will inturn shed light on factors involved in the sex determination of invertebrates and higher vertebrates. The different roles of two aromatase isoforms in common carp and other teleost fishes may give insights into the regulation of their single ancestral gene. The expression of these two isoforms in separate or overlapping domains may demonstrate important functions of aromatase that otherwise might not be discernable. Also the impact of steroidogenic disruptors of enzymes, such as aromatase, in fishes helps us to understand the potential impacts on humans and other species. Aromatase is also known to be involved in human conditions and diseases, such as the development of breast cancer (Brodie et al., 2006), therefore fish would serve as model species for understanding disease development and the testing of different treatments.

The male-determining factor, *dmrt1*, is also known to be involved in human disorders when deletion of this gene occurs (Vinci et al., 2007). By exploring the expression of *dmrt1* in other species it is possible to develop a better understanding of its role and its evolution. In common carp and other teleost fish, the expression of *dmrt1* in both the male and female gonads is evidence of a shared history with the *Drosophila melanogaster (Drosophila) doublesex* gene which is expressed in the developing gonad of both sexes. Although *dmrt1* would be one of the downstream effectors of *Sry* in most mammals, Herpin and Schartl (2009) found the timing of their expression during the respective sex determination windows in mice and medaka is compatible with a similar conceptualised action as a molecular switch for pre-Sertoli cell induction.

Expression of *foxL2* in the brain is not reported in chicken (Govoroun et al., 2004) or mammals (Cocquet et al., 2003) suggesting a specialised role in teleost fish, likely associated with the divergence of two aromatase isoforms. To further understand the role and localisation of *foxL2* in the teleost brain it would be interesting to map its expression in relation to aromatase.

In common carp the role of *sox9* genes in chondrogenesis in conserved but the function in sex determination appears to have diminished. This may suggest that genes that are perhaps more functional in higher vertebrates may have developed a specialised function in teleosts. These genes may have subtle but important roles in other developmental processes in some teleost species.

Despite the many interacting factors in the pathways governing sex determination, the general model is valuable for understanding these highly diverse processes.

Ongoing analysis for novel differentially expressed genes specific to fish, avian, reptile or amphibian systems, as well as the development of methods to test the functional involvement of these genes, will be important in making progress toward further understanding the molecular basis of sex determination in non-mammalian species.

9.1.3 Applied application of outcomes for research, aquaculture and pest control

Although common carp is highly important in aquaculture and has been highly researched surprisingly little is known about mechanisms of sex determination and differentiation in the species. Although morphologically distinct sex chromosomes cannot be identified in common carp (Kirpichnikov, 1981), sex determination is thought to be of the XX/XY system with conventional diploid offspring yielding 1:1 sex ratios (Cherfas et al., 1994; Komen et al., 1992; Manzoor Ali and Satyanarayana Rao, 1989).

Fish, including common carp are also becoming increasingly important indicators of environmental health, with respect to pollution, habitat restriction and degradation. In cases where the reproductive capacities of fish populations appear to be compromised, it is important to understand the natural reproductive parameters, population dynamics and life history characteristics of the species involved. Part of this understanding involves knowledge of the strategies and mechanisms involved in reproduction and sex determination to better facilitate investigation into the points of impact on fauna.

Understanding the mechanisms involved in the development of ovary or testis is important for manipulating phenotypic sex and reproductive activity in fishes. This

may be required in aquaculture where one sex may be more desirable than the other. For example, the suppression of reproduction is fundamental to increase productivity, as unrestrained reproduction during grow-out often leads to wastage of energy in the form of gamete production and reproductive behaviour that could otherwise be channelled to somatic growth. Second, in a number of species, one sex grows faster or has better survival than the other, or has certain other characteristics that appeal to particular markets. In carp species, 7 month old females can be 10-40% bigger than their male counterparts (Hollebecq and Haffray, 1999).

The ability to control phenotypic sex is also important for the control of pest species. For example, in Australia an eradication program, the Daughterless Carp Project (DCP), focuses on genetically manipulating the population sex ratio of common carp by introducing exclusively male-bearing fish. Through the use of gene technology, it is hoped to introduce multiple copies of a 'daughterless' gene which will be carried by male common carp, and introgressed though the pest population. This is anticipated to result in male bias population sex ratios and ultimately result in a drastic reduction of female offspring in the population and potentially leading to localised extinction of pest populations. The primary target for this approach is aromatase, a critical gene conserved in the steroidogenic pathway which catalyses androgens to estrogens which are essential steroids required for both female development and maintenance of the ovary. Despite being the initial target for this eradication method, the aromatase genes had not been fully described in this species. A primary objective of this thesis was to characterise, profile their expression in adults and during development and explore the effect of temperature on its biosynthesis (Chapters 3 and 4).

A key finding of the current research was the maternal inheritance of the *cyp19a* gene suggesting it plays a key role in the early development in both male and female individuals in common carp. In addition, adults showed expression of *cyp19a* in the testis as well as the ovary, albeit at lower levels. The role of *cyp19a* in the testis of common carp therefore requires more research, especially in light of the DCP where the repression of *cyp19a* to produce exclusively male-bearing offspring needs to be heritable and therefore allow production of functional males. Predominant expression of *cyp19a* in ovary suggests that this remains a viable candidate for development of daughterless technology in common carp, albeit low levels of expression were observed in other tissues, particularly brain. Therefore inadvertent blocking of its expression in domains other than ovary needs further investigation, before it can be deployed in the generation of large scale production of 'daughterless carp' lines for pest control.

Interestingly, *dmrt1* was found to be a critical male differentiating factor in common carp as it is in other teleost fishes. Further, the common carp *dmrt1* was found to be exclusively expressed in the gonad of common carp and expression of this gene was initiated at mid-blastula transition, indicating an early role in sex-determining pathway. Taken together, this gene lends itself as a strong candidate for development of daughterless technology in common carp- by directing ectopic expression of the gene in putative gonads of the genetic females, resulting in their sex reversal (to males). This thesis has therefore identified a potential new candidate for manipulation of sex ratios by ectopic but targeted expression of this gene with unlikely non-target affects in other tissues. There is large potential for this approach in both aquaculture and pest management which warrants urgent investigation.

9.2 Future Directions

To understand the interactions of genes involved in sex determination in teleosts, and more specifically common carp, further research is required including protein studies and cell culture work to determine which of the potential aromatase promoter sites are active.

The evidence presented in this thesis suggests that *cyp19a* and *dmrt1* are good molecular markers of female and male differentiation respectively. However, the development of a sex-specific marker using techniques such as RAPD (random amplified polymorphic DNA) or AFLP (amplified fragment length polymorphism) will have great benefit to both aquaculture, to allow early sexing of individuals, and also for pest management to test the effectiveness of the 'daughterless' technology by determining the genetic sex of offspring of common carp carrying the genetic construct.

The development of a gonad specific common carp microarray would also allow greater understanding of concurrent changes in sex-determining factors through early larval development or sex-reversal using exogenous steroids.

Other candidate genes

Due to the conserved nature of genes involved in sex determination and differentiation it is possible to look at the increasing number of fully described genomes to determine factors that may be involved in these processes in different teleost fish. Genes that are expressed exclusively in the gonads or that are known to be involved in the transcription of sex steroid are primary targets.

One such gene originally described in *Drosophila* is *FtzF1* (fushi tarazu factor1) which in mammals encodes SF-1 (von Hofsten and Olsson, 2005). As SF-1 plays a pivotal role in the steroidogenic pathway and hence differentiation of sex in many species of teleosts, examining this gene in common carp may give insight into its role in the transcription of aromatase and other genes involved in sex differentiation of common carp.

Another potential regulator of *cyp19a* is the transcription factor *Dax1*, through its repressive action on the SF-1-mediated transactivation of the *cyp19a* promoter (Wang et al., 2001). In fish, *Dax1* has also been found to down-regulate both SF-1 and *foxL2*-mediated *cyp19a* expression in Japanese medaka ovarian follicles (Nakamoto et al., 2007). In rainbow trout, the up-regulation of *Dax1*, either by estrogens or androgens, is always associated with a simultaneous down-regulation of both SF-1 and *cyp19a* (Vizziano-Cantonnet et al., 2008; Vizziano et al., 2008), suggesting a similar down-regulation of *cyp19a* by a SF-1-mediated transactivation of the *cyp19a* promoter (Wang et al., 2001).

The process of sex determination and differentiation in common carp and other teleosts remains to be fully understood. Through the investigation of key candidate genes in species of aquaculture, pest or evolutionary interest, these pathways and their evolutionary background will be further understood. Information gathered in this thesis has developed a greater knowledge of gene expression domains and of normal expression levels in common carp that will not only allow greater ability to manipulate reproduction for aquaculture production but will aid in environmental studies that use common carp as a sentinel species. This thesis is expected to assist with the development of 'daughterless' technology in common carp, which could

lead to further use of this pest control method in other invasive fish species, such as tilapia, and also in the cane toad. More broadly, understanding the role of genes involved in the determination and differentiation of sex in teleosts, which display a wide diversity of species, will give insights into the functions of these genes in higher vertebrates, such as humans.

9.3 References

Barney, M.L., Patil, J.G., Gunasekera, R.M., Carter, C.G., 2008. Distinct cytochrome P450 aromatase isoforms in the common carp (*Cyprinus carpio*): Sexual dimorphism and onset of ontogenic expression. Gen Comp Endocrinol, 156, 499-508.

Beyer, J., 2004. Early gonad development in the common carp, *Cyprinus carpio (L.)* at 20 °C and 25 °C. Honours Thesis, University of Tasmania, Launceston.

Brodie, A., Sabnis, G., Jelovac, D., 2006. Aromatase and breast cancer. J Steroid Biochem Mol Biol, 102, 97-102.

Carreau, S., Lambard, S., Delalande, C., Denis-Galeraud, I., Bilinska, B., Bourguiba, S., 2003. Aromatase expression and role of estrogens in male gonad: a review. Reprod Biol Endocrinol, 1, 35.

Cherfas, N.B., Gomelsky, B.I., BenDom, N., Peretz, Y., Hulata, G., 1994. Assessment of triploid common carp (*Cyprinus carpio* L.) for culture. Aquaculture, 127.

Cocquet, J., De Baere, E., Gareil, M., Pannetier, M., Xia, X., Fellous, M., Veitia, R.A., 2003. Structure, evolution and expression of the FOXL2 transcription unit. Cytogen. Genome Res., 101, 206-211.

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*. Mol Reprod Dev, 59, 265-276.

Elbrecht, A. & Smith, R.G., 1992. Aromatase enzyme activity and sex determination in chickens. Science, 255, 467-470.

Fenske, M. & Segner, H., 2004. Aromatase modulation alters gonadal differentiation in developing zebrafish (*Danio rerio*). Aquat Toxicol, 67, 105-126.

Fleming, A., Wibbels, T., Skipper, J.K., Crews, D., 1999. Developmental expression of Steroidogenic Factor 1 in a turtle with temperature-dependent sex determination. Gen Comp Endocrinol, 116, 336-346.

Govoroun, M.S., Pannetier, M., Pailhoux, E., Cocquet, J., Brillard, J.-P., Couty, I., Batellier, F., Cotinot, C., 2004. Isolation of chicken homolog of the *FOXL2* gene and comparison of its expression patterns with those of aromatase during ovarian development. Dev. Dynam., 231, 859-870.

Guiguen, Y., Fostier, A., Piferrer, F., Chang, C.F., 2009. Ovarian aromatase and estrogens: a pivotal role for gonadal sex differentiation and sex change in fish. Gen Comp Endocrinol, In press, corrected proof.

Herpin, A. & Schartl, M., 2009. Molecular mechanisms of sex determination and evolution of the Y-chromosome: Insights from the medakafish (*Oryzias latipes*). Mol Cell Endocrinol, 306, 51-58.

Hollebecq, M.G. & Haffray, P., 1999. Genetic improvements. In: R. Billard (Ed.), Fisheries Research: Carp: Biology and Culture, Praxis Publishers Ltd., Chichester, UK. pp. 342.

Hudson, Q.J., Smith, C.A., Sinclair, A.H., 2005. Aromatase inhibition reduces expression of *FOXL2* in the embryonic chicken ovary. Developmental Dynamics, 233, 1052-1055.

Kirpichnikov, V.S., 1981. Genetic bases of fish selection. Springer-Verlag, Berlin, pp. 410.

Kitano, T., Takamune, K., Kobayashi, T., Nagahama, Y., Abe, S., 1999. Suppression of P450 aromatase gene expression in sex-reversed males produced by rearing genetically female larvae at a high water temperature during a period of sex differentiation in the Japanese flounder (*Paralichthys olivaceus*). J Mol Endocrinol, 23, 167-176.

Komen, J., de Boer, P., Richter, C.J.J., 1992. Male sex reversal in gynogenetic XX females of common carp (*Cyprinus carpio* L.) by a recessive mutation in a sex-determining gene. J. Hered., 83, 431-434.

Kwon, J.Y., Haghpanah, V., Kogson-Hurtado, L.M., McAndrew, B.J., Penman, D.J., 2000. Masculinization of genetic female Nile tilapia (*Oreochromis niloticus*) by dietary administration of an aromatase inhibitor during sexual differentiation. J Exp Zool, 287, 46-53.

Lange, I.G., Hartel, A., Meyer, H.H.D., 2003. Review: Evolution of oestrogen functions in vertebrates. J Steroid Biochem Mol Biol, 83, 219-226.

Liu, Z., Wu, F., Jiao, B., Zhang, X., Hu, C., Huang, B., Zhou, L., Huang, X., Wang, Z., Zhang, Y., Nagahama, Y., Cheng, C.H.K., Wang, D., 2007. Molecular cloning of doublesex and mab-3-related transcription factor 1, forkhead transcription factor gene 2, and two types of cytochrome P450 aromatase in Southern catfish and their possible roles in sex differentiation. J Endocrinol, 194, 223-241.

Luckenbach, J.A., Godwin, J., Daniels, H.V., Borski, R.J., 2003. Gonadal differentiation and effects of temperature on sex determination in southern flounder (*Paralichthys lethostigma).* Aquaculture, 216, 315-327.

Manzoor Ali, P.K.M. & Satyanarayana Rao, G.P., 1989. Growth improvement in carp, *Cyprinus carpio* (Linnaeus), sterilized with 17alpha-methyltestosterone. Aquaculture, 76, 157-167.

Mylonas, C.C., Anezaki, L., Divanach, P., Zanuy, S., Piferrer, F., Ron, B., Peduel, A., Atia, I.B., Gorshkov, S., Tandler, A., 2005. Influence of rearing temperature during the larval and nursery periods on growth and sex differentiation in two Mediterranean strains of *Dicentrarchus labrax*. J. Fish Biol., 67, 652-668.

Nagy, A., Bercsenyi, M., Csani, V., 1981. Sex reversal in carp (*Cyprinus carpio*) by oral administration of methyltestosterone. Can. J. Fish. Aquat. Sci., 38, 725-728.

Nakamoto, M., Wang, D.-S., Suzuki, A., Matsuda, M., Nagahama, Y., Shibata, N., 2007. *Dax1* suppresses *P450arom* expression in medaka ovarian follicles. Mol Reprod Dev, 74, 1239-1246.

Ottolenghi, C., Omari, S., Garcia-Ortiz, J.E., Uda, M., Crisponi, L., Forabosco, A., Pilia, G., Schlessinger, D., 2005. *Foxl2* is required for commitment to ovary differentiation. Hum Mol Genet, 14, 2053-2062.

Pannetier, M., Fabre, S., Batista, F., Kocer, A., Renault, L., Jolivet, G., Mandon-Pepin, B., Cotinot, C., Veitia, R., Pailhoux, E., 2006. FOXL2 activates P450 aromatase gene transcription: towards a better characterization of the early steps of mammalian ovarian development. J Mol Endocrinol, 36, 399-413.

Pedersen, R.A., 1998. Early mammalian embryogenesis. In: E. Knobil, J.D. Neill, L.L. Ewing, G.S. Greenwald, C.L. Markert & D.W. Pfaff (Eds.), The Physiology of Reproduction, Vol. 1, Raven Press, New York. pp. 187-230.

Sawyer, S., 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. Gen Comp Endocrinol, 147, 108-117.

Schmidt, D., Ovitt, C.E., Anlag, K., Fehsenfeld, S., Gredsted, L., Treier, A.-C., Treier, M., 2004. The murine winged-helix transcription factor Foxl2 is required for granulosa cell differentiation and ovary maintenance. Development, 131, 933-942.

Simpson, E.R., Clyne, C., Rubin, G., Boon, W.C., Robertson, K., Britt, K., Speed, C., Jones, M., 2002. Aromatase - A brief overview. Annu Rev Physiol, 64, 93-127.

Smith, C.A. & Sinclair, A., 2004. Sex determination: insights from the chicken. BioEssays, 26, 120-132.

Taylor, J.S., Braasch, I., Frickey, T., Meyer, A., Van de Peer, Y., 2003. Genome duplication, a trait shared by 22,000 species of ray-finned fish. Genome Res, 13, 382-390.

Toda, K., Takeda, K., Okada, T., Akira, S., Saibara, T., Kaname, T., Yamamura, K., Onishi, S., Shizuta, Y., 2001. Targeted disruption of the aromatase P450 gene (Cyp19) in mice and their ovarian and uterine responses to 17β-oestradiol. J Endocrinol, 170, 99-111.

Trant, J.M., Gavassa, S., Ackers, J., Chung, B.-C., Place, A.R., 2001. Developmental expression of cytochrome P450 aromatase genes (CYP19a and CYP19b) in zebrafish fry (*Danio rerio*). J Exp Zool, 290, 475-483.

Uda, M., Ottolenghi, C., Crisponi, L., Garcia, J.E., Deiana, M., Kimber, W., Forabosco, A., Cao, A., Schlessinger, D., Pilia, G., 2004. *Foxl2* disruption causes mouse ovarian failure by pervasive blockage of follicle development. Hum Mol Genet, 13, 1171-1181.

van Nes, S. & Andersen, O., 2006. Temperature Effects on Sex Determination and Ontogenetic Gene Expression of the Aromatases *cyp19a* and *cyp19b*, and the Estrogen Receptors *esr1* and *esr2* in Atlantic Halibut (*Hippoglossus Hippoglossus)*. Mol Reprod Dev, 73, 1481-1490.

Vinci, G., Chantot-Bastaraud, S., El Houate, B., Lortat-Jacob, S., Brauner, R., McElreavey, K., 2007. Association of deletion 9p, 46,XY gonadal dysgenesis and autistic spectrum disorder. Mol. Hum. Reprod., 13, 685-689.

Vizziano-Cantonnet, D., Baron, D., Mahe, S., Cauty, C., Fostier, A., Guiguen, Y., 2008. Estrogen treatment up-regulates female genes but does not suppress all

early testicular markers during rainbow trout male-to-female gonadal trans-differentiation. J Mol Endocrinol, 41, 277-288.

Vizziano, D., Baron, D., Randuineau, G., Mahe, S., Cauty, C., Guiguen, Y., 2008. Rainbow trout gonadal masculinization induced by inhibition of estrogen synthesis is more physiological than masculinization induced by androgen supplementation. Biol Reprod, 78, 939-946.

von Hofsten, J. & Olsson, P.-E., 2005. Zebrafish sex determination and differentiation: Involvement of FTZ-F1 genes. Reprod. Biol. Endocrin., 3, 63.

Wang, D.-S., Kobayashi, T., Zhou, L.-Y., Paul-Prasanth, B., Ijiri, S., Sakai, F., Okubo, K., Morohashi, K.-i., Nagahama, Y., 2007. Foxl2 up-regulates aromatase gene transcription in a female-specific manner by binding to the promoter as well as interacting with Ad4 binding brotein/Steroidogenic Factor 1. Mol Endocrinol, 21, 712-725.

Wang, D.S. & Nagahama, Y. (2008) DMRT1 represses AD4BP/SF1/FOXL2 activated aromatase gene transcription: a possible mechanism favoring the male pathway in tilapia. In Proc. Sixth Int. Symp. Fish Endocrinol., pp. 62, Calgary.

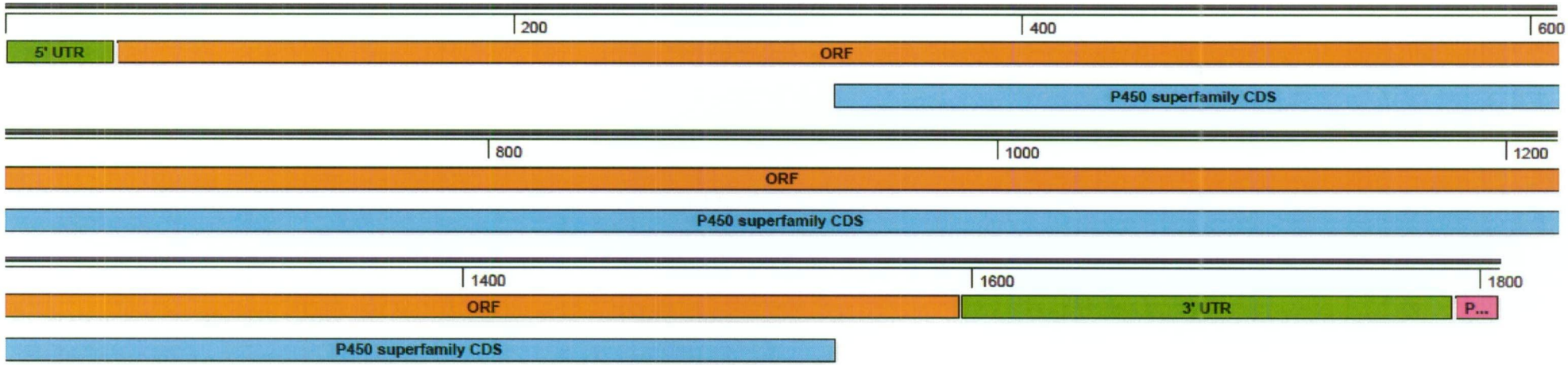
Wang, Z.J., Jeffs, B., Ito, M., Achermann, J.C., Yu, R.N., Hales, D.B., Jameson, J.L., 2001. Aromatase (*Cyp19*) expression is up-regulated by targeted disruption of *Dax1*. PNAS, 98, 7988-7993.

Yu, N.-W., Hsu, C.-Y., Ku, H.-H., Chang, L.-T., Liu, H.-W., 1993. Gonadal differentiation and secretions of estradiol and testosterone of the ovaries of *Rana catesbeiana* tadpoles treated with 4-hydroxyandrostenedione. J Exp Zool, 265, 252-257.

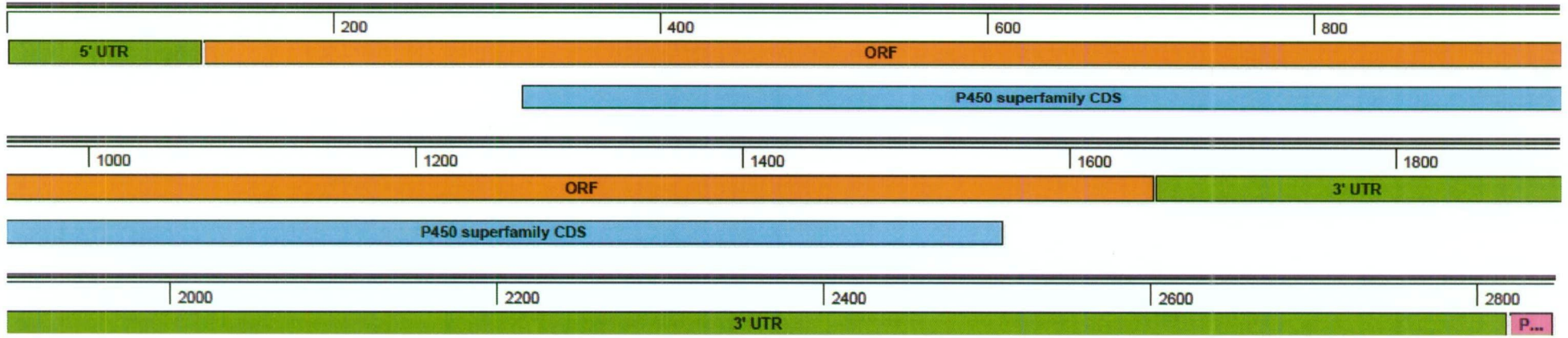
Appendix

Schematic diagrams of gene structures, showing; in green- 5' and 3' Untranslated Regions (UTR), in orange- Open Reading Frame (ORF), in pink- Poly A and in blue- Conserved Domain (CDS).

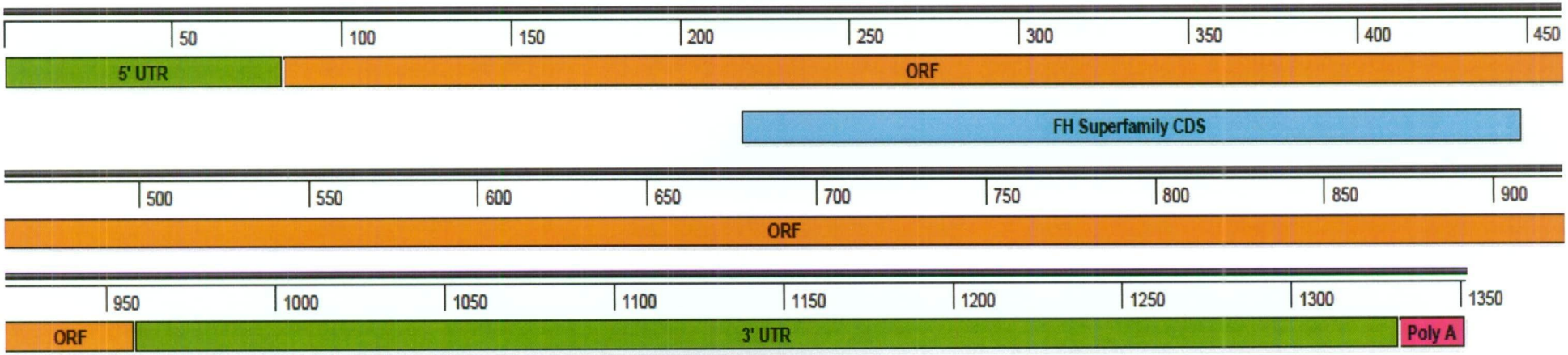
cyp19a



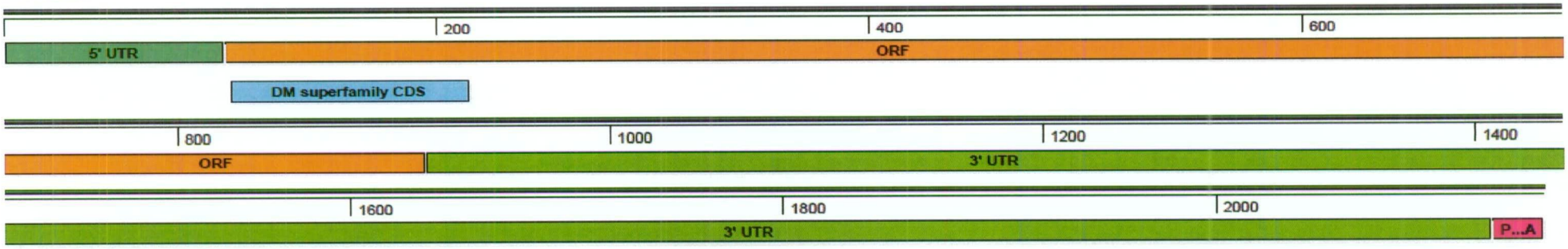
cyp19b



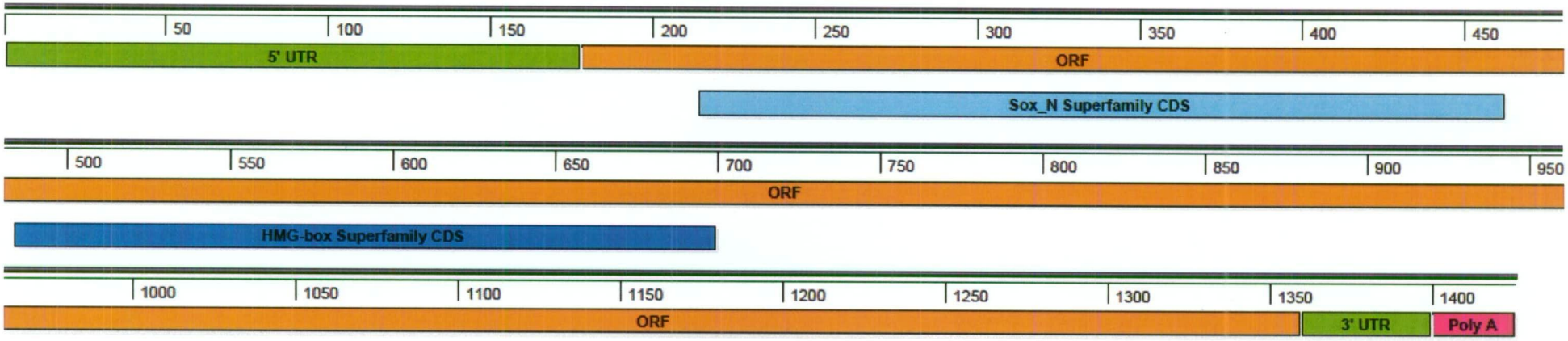
foxL2


50
100
150
200
250
300
350
400
450
5' UTR
ORF
FH Superfamily CDS
500
550
600
650
700
750
800
850
900
ORF
950
1000
1050
1100
1150
1200
1250
1300
1350
ORF
3' UTR
Poly A

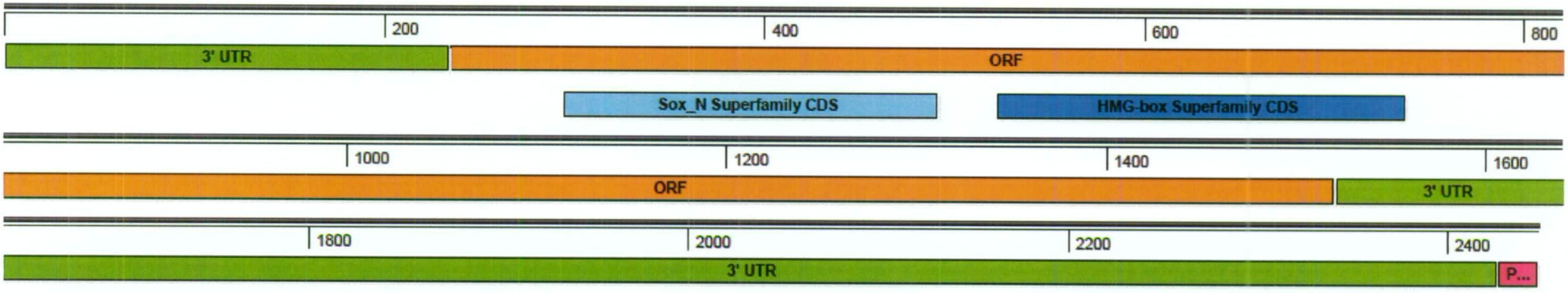
DMRT1


200
400
600
5' UTR
ORF
DM superfamily CDS
800
1000
1200
1400
ORF
3' UTR
1600
1800
2000
3' UTR
P...A

Sox9a


50
100
150
200
250
300
350
400
450
5' UTR
ORF
Sox_N Superfamily CDS
500
550
600
650
700
750
800
850
900
950
ORF
HMG-box Superfamily CDS
1000
1050
1100
1150
1200
1250
1300
1350
1400
ORF
3' UTR
Poly A

Sox9b


200
400
600
800
3' UTR
ORF
Sox_N Superfamily CDS
HMG-box Superfamily CDS
1000
1200
1400
1600
ORF
3' UTR
1800
2000
2200
2400
3' UTR
P...