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Cytochrome P450 Aromatase (CYP19) and Sex Differentiation in the Nile Tilapia *Oreochromis niloticus*

A Thesis Submitted for the Degree of Doctor of Philosophy

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DECLARATION

I declare that this thesis has been composed by myself based on my own research. It has neither been accepted nor submitted for any other degrees. All information from other sources has been properly acknowledged.

Signature of Candidate

Signature of Supervisor

Signature of Supervisor

Date 2nd June 2000

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ABSTRACT

Sex steroids are generally considered as natural sex inducers in fish. Aromatase (cytochrome P450 aromatase) catalyses androgens into oestrogens in the steroidogenic pathway. Three different approaches were taken to elucidate the action of aromatase in relation to sex differentiation in the Nile tilapia *Oreochromis niloticus.*

The first was treatment with Fadrozole™, a non-steroidal aromatase inhibitor (AI), by incorporating it in the diet or by immersing fish in a solution containing AI during the sex differentiation period. The AI treatment masculinised genetic females, indicating the importance of aromatase in sex differentiation. The result revealed that the most sensitive time to AI lies between 11-18 dpf (days post fertilisation).

A partial brain type aromatase cDNA (1707bp) was identified from a brain cDNA library of *O. niloticus*. The amino acid sequence (that corresponds to exon 2-9) derived from this showed 63.7% identity to a previously reported ovarian aromatase gene of this species, and 96.7% identity to the brain type aromatase gene of a closely related species *O. mossambicus*. A semi-quantitative RT-PCR method was established to investigate expression of brain and ovarian aromatase genes during ontogeny. No sexually dimorphic expression of brain aromatase mRNA was detected. However, expression of ovarian aromatase was down-regulated from 15 to 23 dpf in genetic males but upregulated in genetic females. This period overlaps closely with the most sensitive period to AI.

The pattern of temperature-dependent sex determination (TSD) was examined using three different genotypes (XX, XY, YY) at two temperatures (28 and 36°C). The results showed a bidirectional pattern of TSD. YY groups showed a significant percentage of feminisation at the higher temperature, which was suppressed by the AI treatment, implying that aromatisation is mechanistically associated with TSD in this species.

All of these data consistently suggest that aromatase plays a crucial role in sex differentiation, and that the decisive aromatisation takes place between 13-25 dpf in this species. Considering the timing (26-30 dpf) of the first appearance of steroid producing cells in the gonadal area, the decisive aromatisation is not likely to take place there. The brain could be the primary aromatisation site in fish sex differentiation.

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LIST OF FISH SPECIES

Note: Some fish species do not have widely recognised common names. These species are indicated by a dash (—) in place of the common name.

Scientific name	Common name
Ambloplites rupestris	Rock bass
Anguilla anguilla	European eel
Anthias squamipinnis	Wreckfish
Apistogramma sp.	— (a neotropical Cichlid)
Barbus conchonius	Rosy barb
Betta splendens	Fighting fish
Carassius auratus	Goldfish
Colisa lalia	Dwarf gourami
Coregonus clupeaformis	Whitefish
Coryphaenoides rupestris	Roundnose grenadier
Cyprinus carpio	Common carp
Danio rerio	Zebrafish
Dicentrarchus labrax	See bass
Etheostoma whipplei	Redfin darter
Glossolepis incisus	Red Australian rainbow
Hippoglossus hippoglossus	Atlantic halibut
Hoplosternum littorale	Atipa
Ictalurus punctatus	Catfish
Menidia menidia	Atlantic silverside
Merluccius productus	Pacific whiting
Misgurnus anguillicaudatus	Loach
Monopterus albus	Ricefield eel
Myoxocephalus	— (a marine teleost)
Odonthesis argentinensis	Sea pejerrey
Odonthestes bonariensis	Pejerrey
Oncorhynchu keta	Pacific salmon

Scientific name	Common name
Oncorhynchus kisutch	Coho salmon
Oncorhynchus mykiss	Rainbow trout
Oncorhynchus tshawytscha	Chinook salmon
Oreochromis aureus	Blue tilapia
Oreochromis macrochir	—
Oreochromis mossambicus	Mozambique tilapia
Oreochromis niloticus	Nile tilapia
Orochromis urolepsis hornorum	—
Oryzias latipes	Medaka
Paralichthys olivaceus	Olive flounder, hirame
Patagonina hatcheri	Patagonian freshwater pejerrey
Perca fluviatilis	Perch
Platichthys stellatus	Starry flounder
Pleuronectes quadrituberculatus	Alaska plaice
Poecilia latipinna	Balloon molly
Poecilia melanogaster	— (a Jamaican live-bearing Poecilid)
Poecilia reticulata	Guppy
Poecilia velifera	Sailfin molly
Poeciliopsis lucida	—
Puntius gonionotus	Silver barb
Salmo salar	Atlantic salmon
Scophthalmus maximus	Turbot
Stizostedion vitreum	—
Synbranchus marmoratus	Diandric fish
Thalassoma bifasciatum	Bluehead wrasse
Xiphophorus variatus	Sunset platy

CHAPTER 1

GENERAL INTRODUCTION

1.1. Introduction

Differences of size, colour and reproductive organs etc. between the males and females make the sexes easily identifiable from each other in many animals. However, the differences between the sexes are sometimes not at all easily distinguishable especially when we attempt to identify the sex of the animals at the very early stages. This is mainly because, with few exceptions, most gonochoristic vertebrates in their early life stages go through undifferentiated and potentially ambisexual stages.

This interesting phenomenon brings about many questions to scientists: How does one distinctive sex emerge dramatically from the indifferent stages? What governs the sex of an organism? Can that be simply answered by sex chromosomes? These questions have partly been answered. However, the entire mechanism of sex determination and differentiation in animals is still far from our present knowledge despite the centuries' efforts by many biologists. What makes it more difficult to understand is that the mechanism appears to vary from class to class and even species to species.

Fish, one of the biggest groups in vertebrates, comprise more than 25,000 species and exemplify the great variety of reproductive strategies and sex determining systems found in animals. Within fish, the variety of different sex determining systems is even dazzling. At a glance, the effort to uncover sex determining systems in fish seems to get no comprehensible answer. Nevertheless, unveiling the mechanisms of sex determination and differentiation have been of particular importance because of the great demands from aquaculture for economic as well as biological interests.

Fish breeders tends to prefer to breed males and females separately or to achieve a monosex culture depending on biological or economic traits, because there are differences in growth rate, behaviour pattern, breeding time, body colour, shape, or size between the

sexes in each cultured species (Yamazaki 1983). For instance, in the salmonids, females grow better than males; in cichlids, males are the preferred gender for culture, and monosex populations are indispensable for any effective culture. Sterile fish appear to be of an even greater advantage for ongrowing, as the energy required for gonadal recrudescence and reproductive activity is invested in somatic growth (Yaron and Zohar 1993). For this reason, various techniques have been developed to control the sex and have been employed in the field of aquaculture to produce economic benefits, although it still has lots of problems and barriers to overcome. To improve these techniques and utilise them properly, understanding of the mechanisms of sex determination and differentiation in fish is essential.

Many efforts have been made to understand the mechanisms of sex determination and differentiation in fish over the last century. Heteromorphic sex chromosomes have been identified in some species (Chen and Ebeling 1966; Chen 1969b; Uyeno and Miller 1971; Thorgaard 1977; Nanda *et al.* 1990; Foresti *et al.* 1993; Bertollo *et al.* 1998; Carrasco *et al.* 1999) and sex steroid hormones have been found to be possible natural sex inducers (Yamamoto 1969). In addition, recent developments in molecular biology have revolutionised our understanding of the way that the sexually undifferentiated young fish eventually develop into either male or female fish. By this technique, many steroidogenic enzymes were found to play some important roles differentiation (Chang *et al.* 1996). Among these enzymes, particular attention has been paid to cytochrome P450 aromatase CYP19 (aromatase) that converts androgens into oestrogens (Chang *et al.* 1997; Guiguen *et al.* 1999; Kitano *et al.* 1999).

The ultimate goal of this thesis is to improve our understanding of sex differentiation in fish by focusing on the role of aromatase during the sex differentiation period, using the Nile tilapia *Oreochromis niloticus* as a model animal. In this chapter, recent information

on sex determination and differentiation in animals, especially in tilapia will be reviewed to clarify what needs to be studied. With special regards to tilapia, the role of sex steroids, environmental factors that affect sex differentiation, aromatase, sex differentiation period and initial aromatisation sites will be reviewed. Finally, a summary of questions to be answered and several postulations to be tested will be given together with the entire experimental scheme and objectives of experiments. However, before going on to study sex differentiation, the classification of sex in fish and sexual dimorphism and the significance of sex control in aquaculture will first be introduced.

1.2. Sexual dimorphism and the significance of sex control in aquaculture

1.2.1. Sexual dimorphism in fish

The differences between the sexes can be categorised into three different levels such as physiological factors (gonads and sex steroid hormones), genetic factors (sex chromosomes and sex specific genes or differential gene expression) and external factors (body colour, shape or size, growth performance). The differences of physiological and genetic factors facilitate the classification of fish sex (Table 1.1) while the differences of external factors contribute to economical interests in aquaculture.

Table 1.1. Classification of physiological and genetic sex in fish (from Yamazaki 1983)

Physiological sex	Gonadal sex	Gonochorism	undifferentiated differentiated
		Hermaphroditism	synchronous protogynous protandrous
	External sex	Sex accessories Secondary sex characteristics	
	Ethological sex	Sexual behavioural patterns	
Genetic sex (chromosomal)	Monomorphic chromosomal sex	male heterogamety female heterogamety genetic homogamety	
	Heteromorphic chromosomal sex	male heteromorphic XO, XY, XXY female heteromorphic ZW	

1.2.1.1. Physiological factors

Sex steroid profiles during sexual maturation reflect the sex of an animal. Androgens, characterised as male hormones, are mainly produced in the testis whereas oestrogens, female hormones, are produced in ovary although some minor production in other tissues has been reported. However, sex steroids alone can not identify the sex of an individual

fish because the level of these hormones fluctuates depending on reproductive stages and environmental conditions. Besides, these hormones exist both in male and female. Thus physiological fish sex is classified at the level of gonadal status, external reproductive organ(s) and sexual behavioural patterns by which sexual dimorphism is visualised. This physiological sex is believed to be the result of the biochemical process of ontogenesis under the control of genetic factors (Yamazaki 1983).

Gonadal sex in fish shows two different phenomena, namely gonochorism and hermaphroditism. Gonochorism is the condition of having sexes separate with either only functional testes or ovaries in one individual. Two types of gonochorism in fish have been reported (Yamamoto 1969). One is undifferentiated species where the gonads first develop into ovary-like gonads and then one-half of individuals become males and the other half females. Unlike these species, differentiated species directly develop into either male or female without going through an intermediate stage. Many fish species have been considered to be undifferentiated (Yamamoto 1969) while Pacific salmon *Oncorhynchus keta* (Robertson 1953) and medaka *Oryzias latipes* (Yamamoto 1969) have been found as differentiated species. However, whether a fish species is undifferentiated or differentiated remains yet to be clarified in many species.

Hermaphroditism is the condition of having both functional ovarian and testicular tissues within the same individual at the same time (synchronous hermaphrodites) or at different times (protogynous and protandrous hermaphrodites) in the life cycle of the same individual. Protogynous hermaphrodite fish function first as females then as males by sex reversal at a certain stage of their growth, and in protandrous hermaphrodites ovaries replace the testes by natural sex reversal through a transitory intersexual stage (Yamazaki 1983).

External reproductive organs such as the gonoduct and copulatory organs also result in sexual dimorphism and provide external sex identity. Spawning behaviour or sexual behavioural patterns are classified as ethological sex.

1.2.1.2. Genetic factors

Morphologically differentiated sex chromosomes are not common in fish, although several occurrences have been reported (Chen 1969b; Thorgaard 1977; Foresti *et al.* 1993; Bertollo *et al.* 1998; Carrasco *et al.* 1999). In the absence of distinguishable gonosomes, genetic crosses using phenotypic markers revealed male heterogamety, female heterogamety and genetic homogamety (functional hermaphrodites) in fish. Medaka *Oryzias latipes* (Yamamoto, 1953), tilapia *Oreochromis mossambicus* (Clemens and Inslee, 1968), goldfish *Carassius auratus* (Yamamoto and Kajishima, 1968), guppy *Poecilia reticulata* (Takahashi, 1975), and rainbow trout *Oncorhynchus mykiss* (Okada *et al.*, 1979) have been proposed as having monomorphic male heterogamety (XY), and *Oreochromis aureus* (Guerrero 1975; reviewed in Trombka and Avtalion 1993) as having female heterogamety (ZW).

However, sex chromosomes in fish that were classified once as monomorphic are by no means absolutely identical. For example, Okada *et al.* (1979) classified the sex chromosome of rainbow trout as monomorphic, but contradictorily Thorgaard (1977) found that a pair of subtelocentric chromosomes differs in the size of the short arm in males but not in females of one population of rainbow trout. He suggested that the sex chromosomes are at an early stage of differentiation in this species. Moreover, improved techniques have allowed us to find morphological differences at the level of the synaptonemal complex between sex chromosome sets that were once considered as monomorphic (e.g., tilapia *Oreochromis niloticus* Foresti *et al.* 1993 and Carrasco *et al.*

1999). Also, sex chromosomes of guppy *P. reticulata* that were considered to be monomorphic have been claimed as heteromorphic after identifying telomeric heterochromatin on the male Y chromosome and a male-specific simple tandem repeat locus by hybridizing genomic guppy DNA with the oligonucleotide probe $(GAGA)_4$ (Nanda *et al.* 1990). Further studies are likely to reveal more heteromorphic chromosomes in fish species and change the classification of genetic sex in fish.

In the same sense, sex-specific genes and differential gene expression could be found soon, although all attempts that have been made to find sex-specific genes in fish, based on bird and mammalian sex-specific genes have identified only the existence of some of those genes but not sex-specificity (Wachel *et al.* 1991; Tiersch *et al.* 1992; Ito *et al.* 1995). There have been several candidates of testis-determining gene in higher vertebrates. Banded krait minor (Bkm) minisatellite DNA isolated from the W chromosome of the banded krait, a snake, was found to be concentrated in the sex-determining region of the mouse Y chromosome (Singh *et al.* 1984). This Bkm-rich area of the Y was considered to be very closely linked or identical to testis-determining gene (Wachtel and Tiersch 1994). Zincfinger Y (ZFY) that resembles the proteins with multiple finger domains, was also thought to be the testis-determining gene (Page *et al.* 1987). However, male-specific appearances of Bkm (Kiel-Metzger *et al.* 1985) and ZFY (Palmer *et al.* 1989) were not universal. Another male-specific gene, sex determining region Y (SRY), has been proposed (Sinclair *et al.* 1990) and so far is still considered to be the true testis-determinant in mammals. Nonetheless, none of them was found to be sex-specific in catfish (Tiersch *et al.* 1992). An SRY-type gene that is expressed in pituitary glands of rainbow trout *Oncorhynchus mykiss* was not sex-specific either (Ito *et al.* 1995). More attempts to study these genes in many different fish species are required.

Genetic differences between males and females in fish are more likely to be identifiable at the transcriptional levels. A testis-specific 3-Kb long transcript was detected by Northern blot analysis and designated as SOX-LZ in rainbow trout (Takamatsu *et al.* 1995). This gene encodes a protein with a high mobility group (HMG) box and a leucine zipper motif. Sexual dimorphism has also been noticed from different expression levels of some genes. The expression level of Sox24 (SRY-related HMG box gene 24) in rainbow trout ovary was recognisably stronger than in testes (Kanda *et al.* 1998). Stage-specific differential expression of ovarian aromatase gene between males and females during ontogeny was detected in rainbow trout (Guiguen *et al.* 1999). All these sexual differences detected so far were confined to one species, rainbow trout. More studies for sexual differences at the level of aromatase gene expression in different fish species would provide useful information.

1.2.1.3. External factors (body colour, shape or size, growth performance)

Sexual dimorphism in body colour, shape or size and growth performance has been observed in many fish species (Table 1.2). Some of them have drawn great attention from fish culturists and prompted the development of various sex control techniques. As mentioned earlier, in some species, females grow faster than males; in others, males grow better. Body colour and shape are concerned in ornamental fish culture where males are the preferred gender because of its more attractive appearance (Piferrer and Lim 1997).

1.2.2. Significance of sex control in aquaculture

In the light of biology, males and females could be considered as different animals. As listed in Table 1.2, external differences between the sexes are obvious in fish. Differences can be extreme and may mean that the sexes may have very different biological requirements.

Table 1.2. Examples of external sexual dimorphism in fish

Species	Observed differences	Literatures
Ambloplites rupestris	Size[d]	Noltie 1988
Anguilla anguilla	Size[c]	Garcia-Gallego and Akharbach 1998
Barbus conchonius	Colour and shape[e]	Piferrer and Lim 1997
Betta splendens	Colour and shape[e]	Piferrer and Lim 1997
Bothidae	Size[c]	Déniel 1990
Colisa lalia	Colour and shape[e]	Piferrer and Lim 1997
Coryphaenoides rupestris	Size[c]	Bergstad 1990
Dicentrarchus labrax	Growth performance[a]	Carrillo *et al.* 1995
Etheostoma whipplei	Size[d]	Heins and Machao 1993
Glossolepis incisus	Colour and shape[e]	Piferrer and Lim 1997
Hippoglossus hippoglossus	Growth performance[a]	Björnsson 1995
Ictalurus punctatus	Growth performance[b]	Simco *et al.* 1989
Merluccius productus	Size[c]	Dorn 1992
Misgurnus anguillicaudatus	Size[c]	Kubota *et al.* 1988
Oncorhynchus mykiss	Growth performance[a]	Bye and Lincoln 1986
Oncorhynchus tshawytscha	Size[d]	Peterson *et al.* 1992
Oreochromis niloticus	Size[d]	Lowe-McConnell 1987
Paralichthys olivaceus	Growth performance[a]	Tabata 1991
Perca fluviatilis	Growth performance[a]	Tamazouzt 1995
Platichthys stellatus	Size[c]	Tokranov 1996
Pleuronectes quadrituberculatus	Size[c]	Tokranov and Zavarina 1992
Pleuronectidae	Size[c]	Berner and Sager 1985
Pleuronectiformes	Size[c]	De Veen 1976
Poecilia latipinna	Colour and shape[e]	Piferrer and Lim 1997
Poecilia reticulata	Colour and shape[e]	Piferrer and Lim 1997
Poecilia velifera	Colour and shape[e]	Piferrer and Lim 1997

Psettodidae	Size[c]	Druzhinin and Petrova 1980
Puntius gonionotus	Growth performance[a]	Pongthana *et al.* 1999
Salmo salar	Size[d]	Nævdal *et al.* 1983
Scophthalmus maximus	Growth performance[a]	Imsland *et al.* 1997
Sebastes sp.	Shape	Lenarz and Wyllie Echeverria 1991
Stizostedion vitreum	Growth performance[a]	Scott and Crossman 1973
Xiphophorus variatus	Colour and shape[e]	Piferrer and Lim 1997

[a]: Females grow faster than males; [b]: Males grow faster than females; [c]: Females are larger in size than males; [d]: Males are larger than females; [e]; Males are more colourful and attractive as ornamental fish.

Hence, they might require different diets, different environments, and different dosages of chemicals for disease control depending on their biological conditions. In nature, they can select the proper diets and environments when required. However, under confinement like culture tanks or cages, no choices are given to fish. It is impossible to find out and satisfy these different requirements from different genders under mixed-sex culture condition. Therefore, development of monosex culture technique should be an important issue in aquaculture.

Several reasons have been proposed for monosex or sterile fish culture in different fish species. Identified monosex female culture of salmonids reduces the cost of broodstock maintenance (Hunter and Donaldson 1983). In rainbow trout, adult males have poor growth rates, poor food conversion efficiencies, and poor survival when grown in seawater (Bye and Lincoln 1986). The precocious males that are common in salmonid culture lower the market value (Hunter and Donaldson 1983). Thus, in general, female or sterile fish culture is desirable in these species, although males have been reported to be larger than females in some salmonids (Nævdal *et al.* 1983; Peterson *et al.* 1992).

In many cichlids including the Nile tilapia *Oreochromis niloticus* (Lowe-McConnell 1987), males are larger than females. In addition, early maturation and high fecundity of

these species result in overcrowding of the ponds when fish are too small to be marketed (Hunter and Donaldson 1983). Besides, since *Oreochromis sp.* are mouthbrooders, females do not take any food while holding eggs and embryos in their mouth, resulting in detrimental effects on their growth. Monosex male culture is prefered in these species.

In many flatfish, females grow faster than males (*Paralichthys olivaceus,* Tabata 1991; *Hippoglossus hippoglossus,* Björnsson 1995; *Platichthys stellatus,* Tokranov 1996; *Scophthalmus maximus,* Imsland *et al.* 1997). The olive flounder, *P. olivaceus* is one of the most popular marine species for aquaculture in Japan and Korea. Atlantic halibut, *H. hippoglossus* and turbot, *S. maximus* are considered to be promising species for marine fish culture in Europe. Thus development of reliable monosex culture technique would be fruitful in these fish species.

Sexual dimorphism in colouration and fin structure of some fish provides another motive for sex control. Piferrer and Lim (1997) reported that the actual market value of males is as high as fourfold over females because of their more colourful skins and attractive fin structure in many ornamental fish (e.g., guppy, *Poecilia reticulata*; balloon molly, *P. latipinna*; sailfin molly, *P. velifera*; sunset platy, *Xiphophorus variatus*; dwarf gourami, *Colisa lalia*; fighting fish, *Betta splendens*; rosy barb, *Barbus conchonius*; red Australian rainbow, *Glossolepis insisus*). Therefore, application of sex reversal techniques to ornamental fish culture would be beneficial.

Due to the significance of sex control in aquaculture as mentioned above, many techniques are currently being used or are under development to control fish sex: e.g., steroid hormone treatments, hybridisation, triploidy, gynogenesis, androgenesis, and recently aromatase inhibitor treatment and temperature treatment. However, most of them have not yet been proved to be both completely reliable on fish farm and acceptable to

consumers. Moreover, none of them are based on a complete understanding of the sex determination/differentiation mechanism in the fish involved.

When monosex culture becomes possible in many aquaculture species, a wide range of investigation for the sexual differences of many biological aspects such as food selection, essential diets, preference to environments, susceptibility to chemical compounds and disease resistance would follow. Undoubtedly, reliable monosex culture will open a new era to advanced aquaculture.

To open this new era, however, improving our knowledge on sex determination and differentiation for more commercially important species is a prerequisite.

1.3. The mechanism of sex determination and differentiation

Sex determination designates the mechanisms that direct sex differentiation, and the development of testes or ovaries from the undifferentiated or bipotential gonad is termed as sex differentiation (Hayes 1998). In fish, however, sex determination and differentiation cannot be clearly separated, since the timing of sex determination and differentiation has yet to be established, unlike mammals that show clear genetic sex determination (GSD) in which the sex is determined at the time of fertilisation. Many lower vertebrates, including some fish, show environmental sex determination (ESD) either entirely or in combination with GSD. When a genetic female differentiates into a phenotypic male by the influence of temperature, strictly speaking, it should be termed as "determination of phenotypic sex" rather than "sex differentiation". Therefore, in this thesis, the term sex determination and differentiation will be used together without strict distinction. However, where they need to be clearly separated in meaning, either "determination of phenotypic sex" or "gonadal differentiation" will be used instead of "sex differentiation" against "determination of genetic sex".

In this section, several mechanisms of sex determination (GSD: mammals, birds and fish, ESD - mostly temperature-dependent sex determination (TSD): reptiles and fish) that have been found in vertebrates including fish, are briefly summarised.

1.3.1. Genetic sex determination

1.3.1.1. Sex chromosomes

Like all the other traits that are inherited by information on chromosomes, determination of genetic sex is controlled by chromosomal information. Up to now, female homogamety

(XX female, XY male: mammals) and male homogamety (ZW female, ZZ male: birds) are known as common sex chromosome models.

Females produce gametes (eggs) that carry an X or, Z or W chromosome and males produce gametes (sperm) that carry an X or Y, or Z chromosome. Hence, genetic sex is determined at the time of fertilisation when an X bearing egg accommodates either a Y bearing sperm or an X bearing sperm (or in the case of a ZW system: a Z bearing sperm and a Z or W bearing egg). In humans, the X chromosome is considerably longer than the Y chromosome so that identification of heteromorphic sex chromosomes can be done easily, like in many other mammals.

However, phenotypic sex does not always comply with genetic sex identified by heteromorphic sex chromosomes in mammals. XX males and XX hermaphrodites (Ferguson-Smith 1966; Singh and Jones 1982; Wachtel and Simpson 1994), and XY females and XY hermaphrodites are not extremely rare (Wachtel and Simpson 1994) in humans and mice. For example, the XX male syndrome occurs in about 1 in 20,000 male births (Wachtel 1994), which means about 1 in 10,000 human births. Considering the XY female syndrome, the discrepancy of phenotypic sex against genetic sex would be more frequent in humans. The XX male syndrome is explicable by X-Y translocation (Evans *et al.* 1979) and mutation of a downstream sex determining gene that is either autosomal or X-linked gene (Tommerup *et al.* 1993) although more evidence is required. The XY female syndrome is more complicated to explain. The most obvious explanation for the XY female syndrome is deletion or mutation within the Y-linked testis determining gene. However, the existence of a gene or group of genes on the X chromosome and autosomes, the mutation of which can block organogenesis of the normal testis, was also suggested (reviewed by Wachtel and Simpson 1994).

Aneuploidies of sex chromosomes, caused by non-disjunction during meiosis or mitosis, also drive a zygote into abnormal sex differentiation with much higher frequencies. For instance, Turner Syndrome (XO with the frequency of 1 in 5000 births) and metafemale (XXX with the frequency of 1 in 700 births) result in sterile females, while Klinefelter Syndrome (XXY with the frequency of 1 in 2000 births) causes sterile males (Weaver and Hedrick 1997).

Despite all these complicated phenomena of abnormal phenotypic sex, a "simple" XY sex chromosome system is generally accepted as the mechanism of sex determination in most mammals. Clearly heteromorphic sex chromosomes and many data from cytological studies put this simple sex chromosome system into a solid position as the main mammalian sex determining mechanism.

In fish, however, studies on the mechanism of sex determination have generally been hampered by the absence of easily identifiable heteromorphic sex chromosomes (Trombka and Avtalion 1993). In many cases, the proposed sex chromosome systems (XX/XY, ZW/ZZ) could not entirely explain the phenotypic sex ratio produced from genetic crosses. The complexity of sex determining systems in fishes will be reviewed in more detail using the tilapia as a model fish in section 1.6.

1.3.1.2. TDF (testis-determining factor) and its implication for fish

All explanations for the abnormal sex differentiation under an XY sex chromosome system are premised by the existence of a testis determining factor (TDF). As previously mentioned, among several candidates for testis-determining gene in higher vertebrates such as HYA (histocompatibility antigen), Bkm, ZFY and SRY, through a half century's intensive study, only one gene, SRY, was eventually proved to be a true testis determining gene (Sinclair *et al.* 1990) in mammals (Fig. 1.1).

The SRY gene encodes a testis-specific transcript that has a conserved DNA-binding motif present in the nuclear high-mobility-group protein, HMG (Sinclair *et al.* 1990). The evidence that this SRY is the true TDF is quite well established. The role of Sry (SRY equivalent in mouse) in testis development was directly confirmed in transgenic mouse experiments, in which XX mice bearing Sry developed into males (Koopman *et al.* 1991). Indirectly, mutations, including single-base substitutions and a four-nucleotide deletion, were found within the conserved DNA-binding motif of SRY in three human XY females (Berta *et al.* 1990; Jäger *et al.* 1990), and deletion of Sry was also found from the Y chromosome in certain XY female mice (Gubbay *et al.* 1990). These observations strongly support a role of SRY (or Sry in mouse) in primary sex determination.

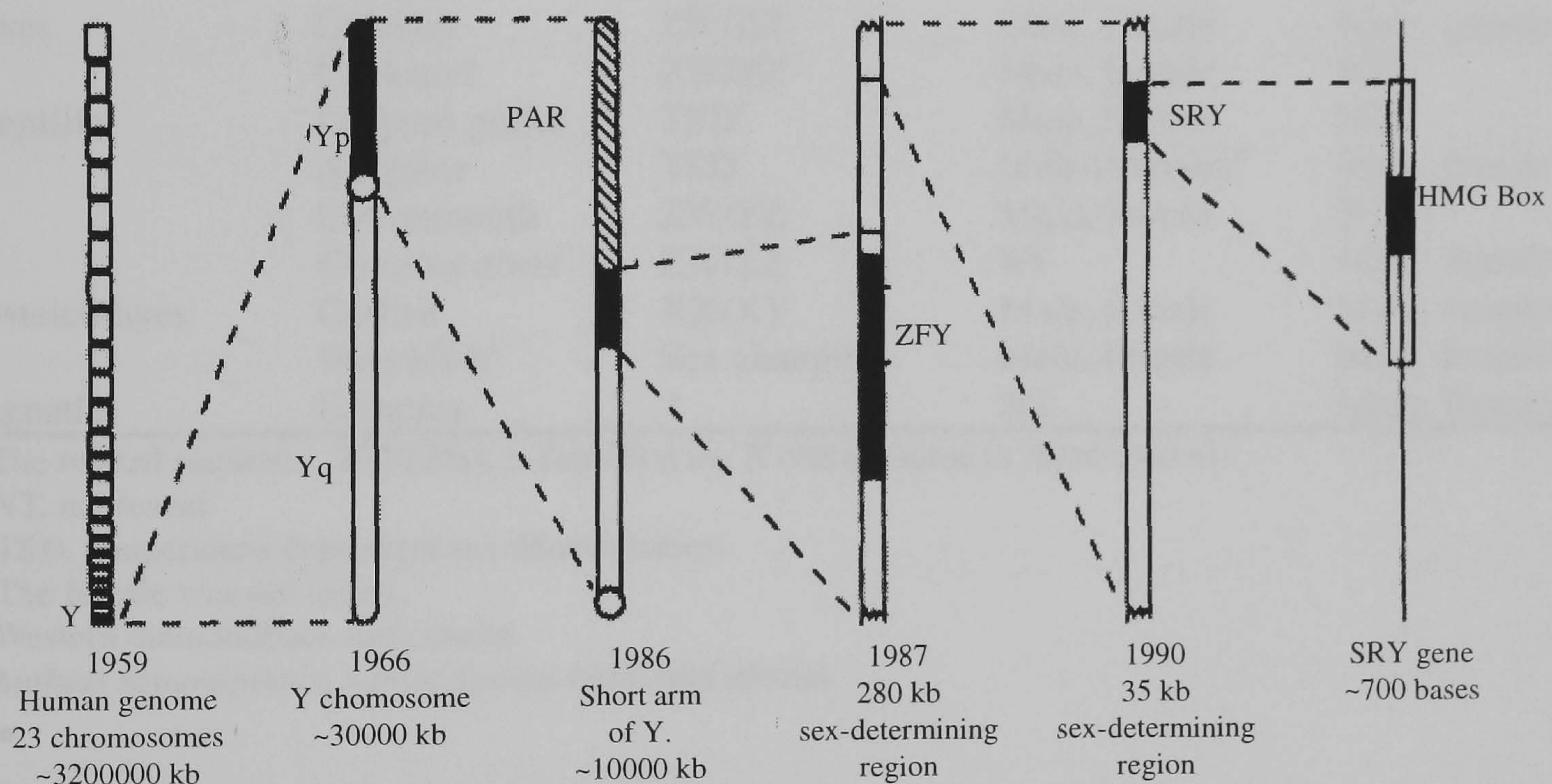


Fig. 1.1. Progressive narrowing down of the region of the human genome which must contain the testis-determining factor, from the demonstration that the Y chromosome is male determining to the isolation and characterisation of the SRY gene in 1990 (from McLaren 1991).

SRY belongs to a group of HMG box proteins that are expressed in a tissue-specific or developmentally regulated manner and that preferentially bind to specific DNA sequences (Laudet *et al.* 1993). Other HMG box proteins of this group have been isolated by virtue of their homology with SRY, and those with greater than 60% homology with the SRY HMG box are termed SOX genes. SOX9, one of these genes, is also involved in sex determination (reviewed by Parker *et al.* 1999).

Table 1.3. Conservation of ZFY and SRY (from Wachtel and Tiersch 1994)

Class	Species	Mechanism of sex determination	ZFY hybridisation in	SRY hybridisation in
Mammalia	Human	XX/XY	Male (female)[a]	Male
	Mouse	XX/XY	Male (female)[a]	Male
	Kangaroo	XX/XY	Male, female	Male
	Wallaby	XX/XY	Male, female	Male
Aves	Chicken	ZW/ZZ	Male, female	Male, female
	Cockatiel	ZW/ZZ	Male, female	NT[b]
Reptilia	Leopard gecko	TSD[c]	Male, female	NT
	Alligator	TSD	Male (female)[d]	Male, female
	Cottonmouth	ZW/ZZ	Male, female	NT
	Crotalus atrox[e]	ZW/ZZ	NT	Male, female
Osteichthyes	Catfish	XX/XY	Male, female	Male, female
	Wreckfish[f]	Sex changing	Male, female	Male, female
Agnatha	Lamprey	?	NT	Male, female

[a] The related sequence, ZFX(Zfx), is found on the X chromosome in these species.
[b] NT, not tested.
[c] TSD, temperature-dependent sex determination.
[d] The female was not tested.
[e] Western diamondback rattlesnake.
[f] *Anthias squamipinnis*, a protogynous coral reef species.

The SRY is now being considered as a master switch of sex determination in mammals. However, SRY does not explain the whole story of sex determination in vertebrates. Birds, reptiles and bony fishes shared a common ancestor about 400 million years ago with mammals (Nei 1987), so that their sex determining system must have derived from a common system. There are genes with homology to SRY in birds, reptiles and fish, but no sign that any of them are sex specific (Table 1.3). For example, in the catfish, multiple

cross-hybridising fragments were detected by Southern blotting with a probe including the conserved DNA binding motif of SRY, and in PCR, multiple fragments were amplified by use of primers specifying and flanking the conserved motif. Moreover, sequences corresponding to the conserved motif of SRY were observed in 23 other non-mammalian species representing four vertebrate classes (Tiersch *et al.* 1991). Sex specificity was not associated with the fragments identified in the catfish, and it remains to be determined whether sequences cross-hybridising to SRY are involved in sex determination in fish or other non-mammals (Tiersch *et al.* 1992). Fairly recently, an SRY-type gene was detected in pituitary glands of rainbow trout *Oncorhynchus mykiss* but again it was not sex-specific (Ito *et al.*1995).

From these observations, SRY appears to have evolved from a non-sex determining gene located on both members of a chromosome pair and obtained the sex determining function after differentiation of the chromosome pair into X and Y. Then, what would have been the common sex determining system? First, dosage dependency that occurs both in nematodes and mammals may provide a clue. In nematodes, sex is determined by the numbers of X chromosomes (i.e., XX/XO system). Mammals achieve dosage compensation by X inactivation in females (the number of active X chromosomes is always one both in male and female) (Lyon 1994). Thus, it can be expected that fish, amphibians and reptiles might have such dosage dependency in their sex determining systems. Second, some upstream or downstream genes of SRY in mammals might have been the primitive sex determinants in lower vertebrates. Apart from SRY, many more genes (Table 1.4) are known to participate in gonadal development, although it is not clear whether they are downstream or upstream of SRY.

Steroidogenic Factor 1 (SF-1) is an essential mediator of endocrine development and known to be involved in sex differentiation in mammals (Parker *et al.* 1999). Ad4 binding

protein (Ad4BP) is another name for this protein that shows high homology to *Drosophila fushi tarazu* factor 1 (FTZ-F1) (Lala *et al.* 1992). SF-1 is expressed in both male and female mouse embryos from the very earliest stages, but with the onset of testicular differentiation, the levels of SF-1 transcripts increase in testes and decrease in ovaries (Ingraham *et al.* 1994). More directly, SF-1 knockout mice exhibit male-to-female sex reversal of the internal and external urogenital tracts (Luo *et al.* 1994). Promisingly, FTZ-F1 in medaka fish has been found to regulate the transcription of P450 aromatase in ovarian follicles (Watanabe *et al.* 1999). SF-1 will be discussed in more detail with regard to gonadal differentiation together with aromatase gene expression in section 4 in this Chapter.

Table 1.4. Genes involved in the initial steps of sexual development: Their chromosomal localisation, their gene family, and presumed function are indicated (from Vilain and McCabe 1998)

Gene	Localisation	Family	Function	Phenotype of mutations
SF1	9q33	Orphan nuclear receptor	Transcription factor	Gonadal and adrenal agenesis in mouse
WT1	11p13	Zinc finger protein	Transcription factor	Denys-Drash and Frasier syndromes
SRY	Yp11	HMG protein	Transcription factor	XY gonadal dysgenesis
DAX1	Xp21.3	Orphan nuclear receptor	Transcription factor	XY gonadal dysgenesis
SOX9	17q24	HMG protein	Transcription factor	Campomelic dysplasia with XY gonadal dygenesis
MIS	19q13	TGFβ	Growth factor	Persistent Müllerian Duct syndrome

The Wilms tumor gene, WT1, was initially identified as an oncogene responsible for a pediatric kidney tumor and shown to be involved in the maintenance of gonadal development (reviewed by Vilain and McCabe 1998).

Duplication of a 160 kb region in Xp21.3 results in feminisation or ambiguous genitalia in XY humans. This region was named DSS for dosage-sensitive sex reversal. The locus for adrenal hypoplasia congenita (AHC) also maps to Xp21.3 and is contained within the 160 kb DSS critical region. This gene was identified and named DAX1 (DSS-AHC on the X chromosome, gene 1) (reviewed by Vilain and McCabe 1998). It is important to notice that DNA binding and transcriptional repression by DAX1 blocks steroidogenesis (Zazopoulos *et al.* 1997) although no physiological target for DAX1 is known yet. DAX-1 was also found to be involved in gonadal development (Muscatelli *et al.*, 1994; Zanaria *et al.*, 1994). It shows a similar distribution to SF-1 and is transcribed in foetal gonads and in adult testis, ovary, and adrenal gland (Zanaria *et al.* 1994). It is very likely that this transcription factor gene is also important in gonad differentiation of fish.

SOX9 is also involved in sex determination (Parker *et al.* 1999) and the role of SOX9 in sex determination is not limited to mammals. Male-specific expression of Sox9 (SOX9 in human) has been reported in chicken and turtle genital ridges, coincident with testis determination in those species (Kent *et al.*, 1996; da Silva *et al.*, 1996; Spotila *et al.*, 1998). The prominent expression of Sox9 in testis was also reported in rainbow trout (Takamatsu *et al.* 1997). SOX9 seems to be far closer to the origin of the common sex determining system than SRY. The way that SOX9 works is not clear, but potential SOX9 binding sites are found upstream of SF1. Thus, it is postulated that a possible target of SOX9 is SF1 (Nomura *et al.* 1995) that was suggested to be involved in the regulation of P450 aromatase transcription in fish (Watanabe *et al.* 1999).

Müllerian inhibiting substance (MIS), also called Anti-Müllerian hormone (AMH) or Müllerian inhibiting factor (MIF), is a glycoprotein dimer, produced by gonadal somatic cells, and responsible for the regression of Müllerian ducts in male foetuses (Jost 1953). Sexually dimorphic patterns of MIS production by the developing testis and ovary have

been reported in the rat (Hirobe *et al.* 1992), implying a role of MIS in sex differentiation. Furthermore, MIS inhibited the normal oogonial proliferation in the rat and this inhibition appeared to be associated with inhibition of aromatase biosynthesis (Vigier *et al.* 1989).

Apart from the genes mentioned above, DMT1, a human homologue of dsx (doublesex), a major regulatory gene involved in *Caenorhabditis elegans* sex determination, has recently been identified and is considered as a human sex determination candidate gene (Raymond *et al.* 1998).

Interestingly, most genes involved in mammalian sex determination relate to steroidogenesis via P450 aromatase directly or indirectly (SF1, SRY, DAX1, SOX9 and MIS). Hence, studying P450 aromatase gene in lower vertebrate such as reptiles, amphibians or fish is likely to make a good start for searching for the common sex determining system between vertebrate classes.

1.3.2. Environmental sex determination

Environmental sex determination (ESD) is mainly observed in lower vertebrates including reptiles, amphibians and fish. ESD can be defined into several categories by the factor that influences sex determination. External environments encompass TSD, pH-dependent sex determination and social environment-dependent sex determination. Endogenous sex steroid hormones might be considered as part of the internal environment that governs sex differentiation. Although there were several reports on pH-dependent sex determination (Heiligenberg 1965; Rubin 1985; Römer and Beisenherz 1996) and social environment-dependent sex determination (Shapiro 1979; Fricke and Fricke 1977; Francis and Barlow 1993), only TSD will be summarised here because it is more prevalent in gonochoristic (also in some hermaphroditic) species, especially fish. The influence of internal environment namely sex steroid hormones will be mentioned throughout the whole thesis

since it appears to be involved in many fundamental events during sex differentiation in vertebrates.

In terms of evolutionary history, reptiles, amphibians and fish are more closely related to each other than to mammals . Fish diverged from the mammalian line 400 million years ago, 50 million years later amphibian and another 50 million years later reptiles became an independent group (Nei 1987). Since then, it has been a long time-300 million years. Therefore, it is more likely that these three classes share the common sex determining system with each other than with mammals. It would be worth paying attention to TSD that occurs in these three classes of animals.

1.3.2.1. The pattern of temperature effect on sex determination

TSD is widespread in reptiles including all crocodiles, all lizards, some turtles and some snakes (reviewed by Pieau *et al.* 1994a), and the mechanism of TSD in reptiles has been extensively studied.

The period during which sex determination is sensitive to temperature has been determined in many species. On the basis of this thermosensitive period, male and female determining temperatures have been studied either by incubating fertilised eggs at different temperature (in the laboratory) or by recording nest temperatures and sex ratios of hatchlings (in the wild) (reviewed by Solari 1994). From these observations, Pieau *et al.* (1994a) proposed three patterns of temperature effect on sex determination (Fig. 1.2). Generally, both sexes occur within a narrow range of temperature, the transitional range of temperature (TRT). Within this range, 1:1 sex ratio is expected. Above and below the TRT, 100% phenotypic males or 100% phenotypic females are obtained. In some species, temperatures below TRT are masculinising whereas temperature above TRT are feminising (Fig. 1.2B: Pattern 1). In other species, the opposite occurs (Fig. 1.2C: Pattern 2).

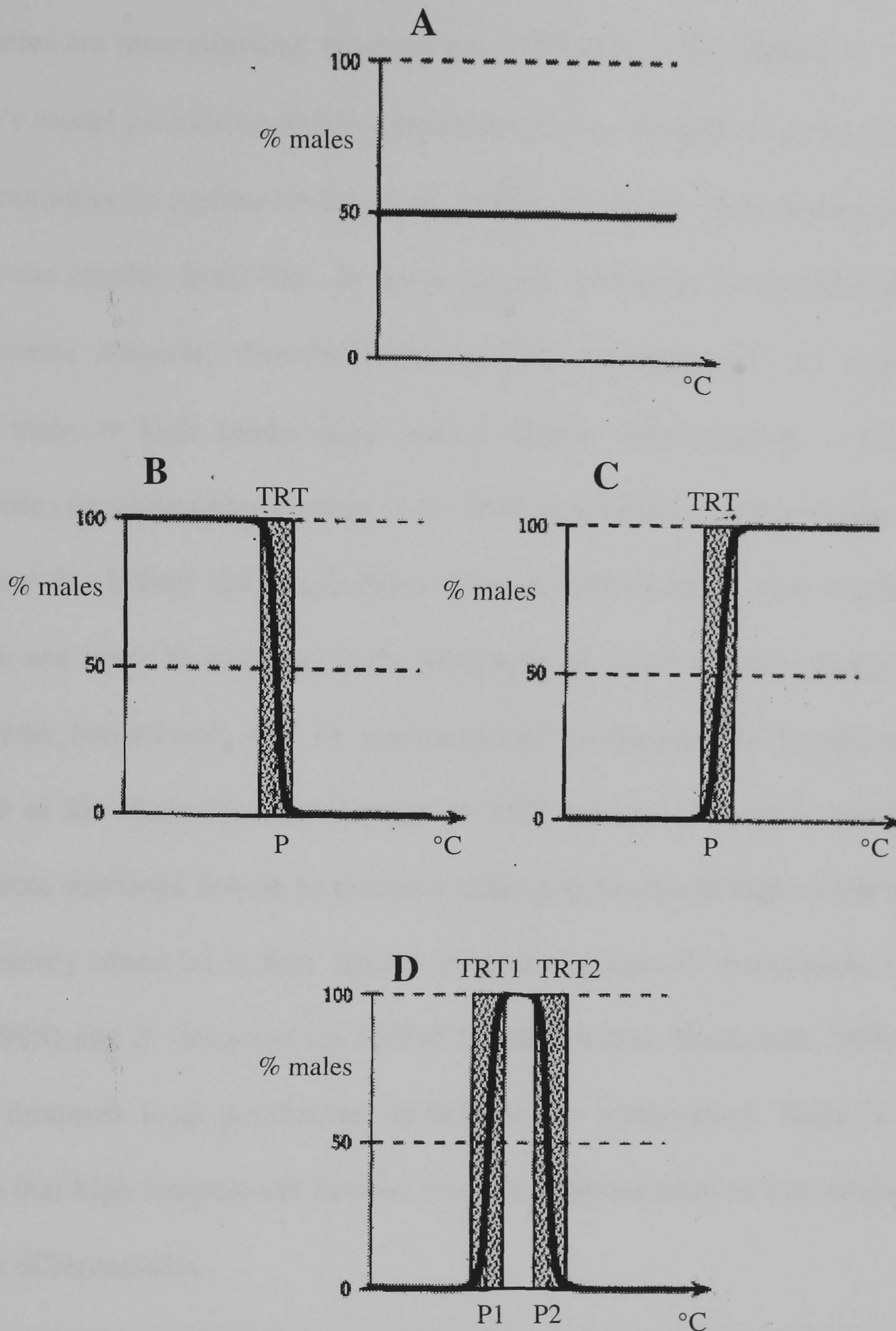


Fig. 1.2. Sexual differentiation as a function of the incubation temperature of eggs in reptiles: (A) no effect of temperature; (B) low temperatures are masculinising, high temperatures are feminising; (C) low temperatures are feminising, high temperatures are masculinising; (D) low and high temperatures are feminising, intermediate temperatures are masculinising. (TRT, TRT1, TRT2: transitional ranges of temperature; P, P1, P2: pivotal temperatures.) (from Pieau *et al.* 1994a).

In the third pattern, high and low temperatures are feminising and intermediate temperatures are masculinising: there are two TRTs (Fig. 1.2D: Pattern 3).

Pieau's model provide excellent characterisation of the pattern of temperature effect on sex determination in reptiles (Pieau *et al.*, 1994a). However, none of these patterns entirely explains the results from fish. In most species identified as thermosensitive, such as *Hoplosternum littorale*, *Poecilia lucida* and *P. melanogaster*, sex ratios are skewed towards male at high temperature and/or female differentiation is favoured by low temperatures (reviewed by Baroiller *et al.* 1999: also below). In the tilapia *O. niloticus*, *O. aureus* and the hybrid red tilapia from the Red Florida strain, low temperatures do not affect the sex ratio. In contrast, in the atherinids *Menidia menidia*, *Patagonina hatcheri*, *Odonthestes bonariensis and O. argentinensis*, predominantly female populations are produced at low temperatures. Contary to TSD patterns in reptile species, a complete change from monosex female to monosex male populations at high or low temperatures is not frequently observed in fish. So far, only two species *O. bonariensis* (Strüssman and Patiño 1995) and *P. olivaceus* (in half of families tested, Yamamoto 1999) are known to produce monosex male populations at high or low temperature. There is no convincing evidence that high temperature favours ovarian differentiation or low temperature induces testicular differentiation.

In most thermosensitive fish species, interactions between environmental factors and genotype have been strongly suggested. The Atlantic silverside *M. menidia*, a common estuarine fish in North American coast, that completes its life cycle in 1 year and breeds over 2-3 months period during the spring, shows skewed sex ratios towards female at low temperature (earlier groups of recruitment) and males at high temperature (later groups of recruitment) (Conover and Kynard 1981). Hence, females in this species are afforded a longer growing season and tend to be larger than males. The authors assumed large body

size is more important to the reproductive success of female than male, and concluded TSD in this species is adaptive event to the environment. Conover and Heins (1987) confirmed this idea by investigating several different populations of *M. menidia* that occur in several different environments. In their results, sex ratios from southern populations that have long growing season were more sensitive to temperature than that from northern populations. In an extreme northern population, temperature did not influence sex ratios. The authers explained that it was because the length of growing season in the region is so short (3 months) that the adaptive advantage of TSD has diminished. However, the authors' explanation does not cover the highest thermosensitivity of sex ratio in the southern most population where the length of growing season is 10 months which covers most of their life cycles. It can be better explained by the length of breeding season (1 month in Northern most; 3 months in southern most) rather than the length of growing season. Nonetheless, the work from Conover and his collegues provides a good model of TSD in fish and demonstrates evidences of temperature and genotype interactions by showing a highly variable thermosensitivity both within (Conover and Kynard 1981) and between populations (Conover and Heins 1987). Different thermosensitivity is also observed within the same strain in tilapia (Baroiller *et al.* 1995b; 1996). This will be discussed later (1.6).

In amphibians, high temperature (27 to 36°C) results in 100% males in all frogs studied (reviewed by Hayes 1998), whereas either 100% females (Dournon *et al.* 1984) or 100% males (Dournon and Houillon 1984) were produced depending on species in salamanders. Despite these clear temperature effects, it is not likely that temperature is important in normal sex determination in amphibians, because all these effects were obtained by exposure to temperatures that are not normally experienced by the species (Hayes 1998).

1.3.2.2. TSD and sex steroids

In some reptiles, amphibians and fish, the influence of temperature on sex determination is rather obvious. The next question to ask is how temperature influences the process of sex determination? Sex steroid hormones are likely to provide a close answer, since the effects of exogenous sex steroid hormones on sex differentiation have been proven in reptiles (Crews *et al.*, 1989; Jeyasuria *et al.* 1994; Pieau *et al* 1994b; Richard-Mercier *et al* 1995) and fish (Yamamoto 1969; Hunter and Donaldson 1983). In amphibians, both TSD and the role of sex steroids on sex differentiation are less clear and in doubt (Hayes 1998).

Sex steroids appear to be a final sex determinant, while genetic factors and/or environmental factors act as an initial sex determinant in reptiles (Crews *et al.* 1989) and fish (Yamamoto 1969). Many androgens and oestrogens have quite successfully been used as sex converters in some fish including salmonids, cichlids and carp for both commercial or experimental purposes (Yamamoto 1969; Hunter and Donaldson 1983). Hence, it is not necessary to discuss the effects here. Instead, attention should be paid to further upstream processes that govern the synthesis of sex steroid hormones. In the next section, the role of cytochrome P450 aromatase in the process of sex differentation in reptiles and fish is introduced, following summaries of basic steroid biosynthesis.

1.4. Steroid biosynthesis and cytochrome P450 aromatase

1.4.1. Steroid biosynthesis

Steroids are a group of lipids based on a skeleton of four fused rings and derivatives of cholesterol. The primary organs involved in their synthesis are known to be the gonads and the adrenal cortex, plus the placenta in pregnant female mammals. However, more organs are considered to be putative steroidogenic in recent biology (see 1.4.2 for details). In general, steroid hormones control metabolism at the gene level. They react with intracellular protein receptors and hormone-receptor complexes that bind to specific sites on the genome and affect transcription of neighboring genes.

They are grouped into five major classes: (1) the progestagens (e.g., progesterone), which regulate events during pregnancy and are the precursors to all other steroid hormones; (2) the glucocorticoids (e.g., cortisol and corticosterone), which promote gluconeogenesis and, in pharmacological doses, suppress inflammation reactions; (3) the mineralocorticoids (e.g., aldosterone), which regulate ion balance by promoting reabsorption of Na^+, Cl^-, and $HCO3^-$ in the kidney; (4) the androgens (e.g., androstenedione and testosterone), which promote male sexual development and maintain male sex characteristics; and (5) the oestrogens (e.g., oestrone and oestradiol), or female sex hormones, which produce female characteristics.

A general feature of steroid hormones is that they are not stored for release after synthesis. Therefore, the level of a circulating hormone is controlled primarily by its rate of synthesis, which is often controlled ultimately by signals from the brain.

The conversion of cholesterol to steroid hormones is a series of enzymatic reactions (Fig. 1.3). An enzyme complex called cholestrol desmolase (=cholesterol side chain cleavage cytochrome P450: P450scc) hydroxylates the side chain at C-20 and C-22 and

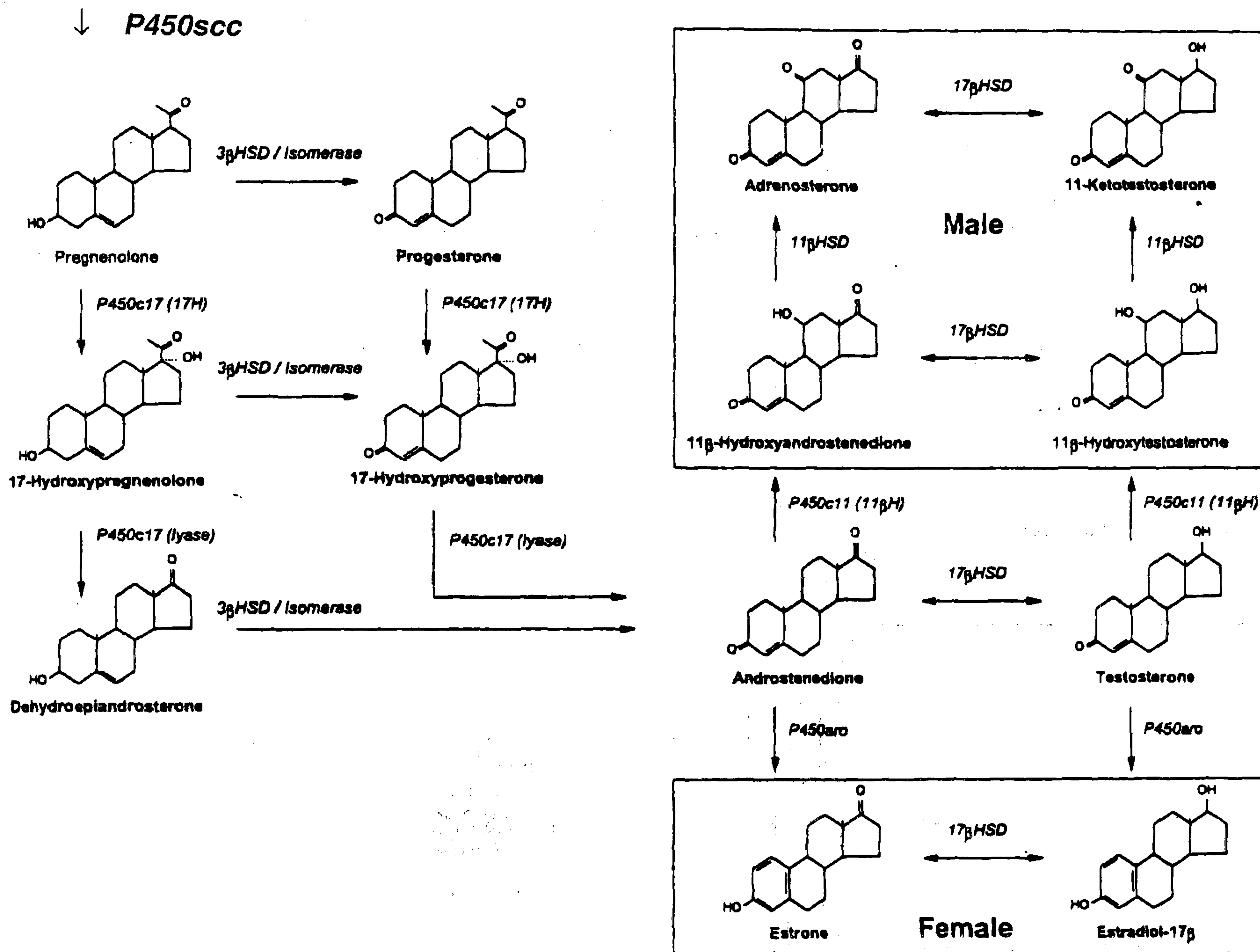


Fig. 1.3. Schematic representation of some gonadal steroidogenic pathways in fish and identified steroids (in bold type) following *in vitro* steroid metabolism with pregnenolone and androstenedione precursors in rainbow trout and/or tilapia differentiating gonads (from Baroiller at al. 1999).

cleaves it, to yield pregnenolone. Pregnenolone is converted to the steroid hormone progesterone by dehydrogenation and double-bond isomeration (3β-hydroxysteroid dehydrogenase-isomerase (3β-HSD). Hydroxylation of progesterone at C-17 by an enzyme 17α-hydroxylase/17,20-lyase cytochrome P450 (P450c17) gives 17α-hydroxyprogesterone. A gonadal enzyme 17α-hydroxylase/17,20-lyase cytochrome P450 (P450c17) cleaves the side chain of 17-hydroxyprogesterone at C-17, giving androstenedione, a precursor to other androgens and oestrogens. Androstenedione is further converted into testosterone (by 17β-hydroxysteroid dehydrogenase: 17β-HSD, this reaction is reversible). Both androstenedione and testosterone are catalysed by cytochrome P450 aromatase (aromatase or P450aro), giving estrone and estradiol, respectively.

1.4.2. Cytochrome P450 aromatase

Cytochrome P450 aromatase (aromatase) is the enzyme responsible for the aromatisation of androstenedione into estrone and testosterone into estradiol-17ß. The aromatisation of androgens to oestrogens takes place in the agranular endoplasmic reticulum in several steroidogenic cells and is classified as a mixed-function oxidase reaction. The aromatase enzyme complex (CYP19) is the sole mediator for the conversion of androgens to oestrogens in vertebrates. The "aromatase" complex consists of two membrane-bound P450 enzymes, which are aromatase (which binds the androgen substrate and inserts oxygen into the molecule) and a ubiquitous flavoprotein, NADPH-cytochrome P450 reductase. Reducing equivalents from NADPH are transferred via the flavoprotein to aromatase. The overall reaction involves a three step hydroxylation, release of formic acid, and a resulting spontaneous aromatisation of the A ring.

Due to its function of synthesis of sex steroids, aromatase has been of interest with regards to the mechanism of sex differentiation. Many recent applications of aromatase

inhibitors have proved that it is possible to sex reverse female embryos of fish (Piferrer *et al.* 1994), amphibians (Yu *et al.* 1993), reptiles (Jeyasuria *et al.* 1994) and birds (Elbrecht and Smith 1992; Wartenburg *et al.* 1992) simply by blocking oestrogen synthesis in the undifferentiated gonad. Conversely, application of oestrogen to male embryos results in development of an ovary. All of these data suggest that the enzyme necessary for oestrogen synthesis (aromatase) in the developing gonad plays a critical role in sex differentiation in these vertebrates.

In fish, the role of aromatase in sex differentiation has been proposed in three different species. Piferrer *et al.* (1994) showed that immersion in an aromatase inhibitor (Fadrozole CGS16949A) solution for only 2 hours caused genetic females to develop into normal males in chinook salmon (*O. tshawytscha*), suggesting that aromatase plays a pivotal role in the process of gonadal differentiation. Nakamura *et al.* (1997) demonstrated that the initiation of the critical aromatisation during sex differentiation appears to take place just before 23-26 days post-hatch (dph) in tilapia *O. niloticus* using a polyclonal antibody against tilapia aromatase and subsequent histological observations. In rainbow trout *O. mykiss*, aromatase mRNA was specifically detected in female gonads 2 weeks before the first sign of a histological sex differentiation (Guiguen *et al.* 1997). However, so many questions for the role of aromatase in fish sex differentiation still remain unanswered. These questions include the specific time and place of aromatisation, and the activators of aromatase gene expression during the sex differentiation period.

The specific time of aromatisation might be different by species because of the different developmental periods. Thus, it seems to be very difficult to generalise the aromatisation period for all species. It should be investigated separately in each species.

The genes for aromatase have been cloned from several mammalian species (Simpson *et al.* 1994), chicken (McPhaul *et al.* 1988), zebra finch (Shen *et al.* 1994), alligator and

turtle (Jeyasuria *et al.* 1995), and six species of teleost fish (rainbow trout: Tanaka *et al.* 1992; catfish: Trant 1994; medaka: Tanaka *et al.* 1995; tilapia *O. niloticus*: Chang *et al.* 1997; goldfish brain: Gelinas *et al.* 1998; goldfish ovary: Tchoudakova and Callard 1998; a flounder *P. olivaceus*: Kitano *et al.* 1999), and the regulation of its tissue-specific expression in mammals has been the subject of considerable research (Simpson *et al.* 1994). In mammals the full sequence of the aromatase gene is not known, but it spans a distance of at least 75 kb and may be larger (Simpson *et al.* 1994). In the rainbow trout, medaka and tilapia, the aromatase genes only span 2.6 kb (Tanaka *et al.* 1992; Tanaka *et al.* 1995; Chang *et al.* 1997) whereas in the catfish it spans 3.4 kb (Trant 1994) and 3.0 kb in the goldfish brain (Gelinas *et al.* 1998). Aromatase genes that have been reported so far contain 9 exons (McPhaul *et al.* 1993; Tanaka *et al.* 1995).

Aromatase is expressed in a variety of cells and tissues including the ovary, testis (Silberzahn *et al.* 1988), placenta (Kellis and Vickery 1987), adipose tissue (Lueprasitsakul and Longcope 1991), central nervous system (Pasmanik and Callard 1989; Gelinas and Callard 1993) and skin fibroblasts (Stillman *et al.* 1991). However, it is not clear yet where the aromatase is expressed during sex differentiation period in fish, although Nakamura *et al.* (1997) and Guiguen *et al.* (1997) focused on the undifferentiated gonads. Possibly the aromatase to be expressed in the undifferentiated gonads would act directly on the process of gonadal differentiation, but this needs to be clarified.

The regulation of aromatase gene expression during the sex differentiation period is of particular importance, because the regulation of expression of the aromatase gene has been suggested to play a key role in sexual differentiation of gonads in all vertebrates. There is no satisfactory answer yet, though various possible factors have been suggested.

Steroidogenic factor I (SF- 1), also known as Ad4BP, an orphan nuclear receptor with a zinc finger DNA-binding domain, the mammalian homologue of *fushi tarazu* factor I

(FTZ-FI) from the fruit fly (Lavorgna *et al.* 1991), is known to be a key regulator of steroidogenic enzyme gene expression (Rice *et al.* 1991; Lynch *et al.* 1993; Morohashi *et al.* 1993a,b). This nuclear protein of 52,000 daltons binds to the promoter region of all steroidogenic P450 genes including aromatase and has been shown to act as a transcriptional activator. SF-1 has also been shown to be important in AMH expression (Shen *et al.* 1994), and pituitary expression of LH, FSH, and the receptor for gonadotropin releasing hormone (Ingraham *et al.* 1994).

In rats, SF- I was first detected in the primordial adrenal glands and testes of the 13.5-day pc foetus, but only trace amounts were detected in the foetal ovaries (Hatano *et al.* 1994). When the gene for this factor was disrupted in mice, the embryos developed without gonads or adrenal glands and died shortly after birth (Luo *et al.* 1994). Clearly this factor is a key determinant of gonadal development. However, given the widespread tissue distribution of SF-1 and its multiple roles in steroidogenesis and pituitary function, it is obvious that other factors must also be involved. In rat granulosa cells, for example, aromatase activity increases in response to FSH (GTH I in fish) via an increase in cyclic AMP (cAMP). The promoter region of rat aromatase gene has a cAMP response element (CRE) that binds the transcription factor, CRE binding protein (CREB), and an SF-1 binding site (Richards 1994).

In fish, SF1/Ad4BP or FTZ-F1 also appear to regulate aromatase gene expression. The promoter region of medaka aromatase gene contains potential Ad4BP sites (Tanaka *et al.* 1995). Recently, FTZ-F1 has been suggested as a potential regulator of the transcription of aromatase gene in medaka ovarian follicles (Watanabe *et al.* 1999). In the study, the expression pattern of FTZ-F1 transcripts during oogenesis coincided with that of ovarian aromatase transcripts.

In some reptiles where the sex is mainly determined by the incubation temperature, the expression of the aromatase gene is temperature-dependent. However, whether temperature acts on the expression of the aromatase gene itself or on that of another upstream gene is not known.

Pieau *et al.* (1994a) postulated a cascade reaction for vertebrate sex differentiation focusing on aromatase action (Fig. 1.4). Anti-Müllerian hormone (AMH) inhibits aromatase activity in differentiated foetal ovaries, and it induces testis-like structures in undifferentiated gonads of rat genotypic females. This masculinisation could result from the decrease in the level of gonadal endogenous oestrogens. In adipose cells and ovarian granulosa cells of mammals, the regulation of expression of the aromatase gene appears to be multifactorial. Some factors such as cAMP and gonadotropins activate its transcription, other factors such as prolactin and epidermal growth factor repress it. For testicular differentiation in mammals, the testis-determining factor (SRY or homologs) could repress transcription of the aromatase gene itself, or could activate transcription of the AMH gene or that of another gene, the product of which represses the aromatase gene. It is well known that steroid hormones activate the transcription of some genes, whereas they repress the transcription of others. Structural genes for both ovarian and testicular differentiation are present in all individuals. When the level of oestrogens is high, ovarian differentiation genes are activated, whereas testicular differentiation genes are repressed. When the level of oestrogens remains low, testicular differentiation genes are not repressed whereas ovarian differentiation genes are inactivated.

Recent studies show that the mechanism of sex determination/differentiation in vertebrates could be more complicated than this postulated cascade reaction in undifferentiated gonads. There appears to be interactions between genes involved in sex determination/differentiation – e.g., between DAX1 and SF1, and between WT1 and SF1

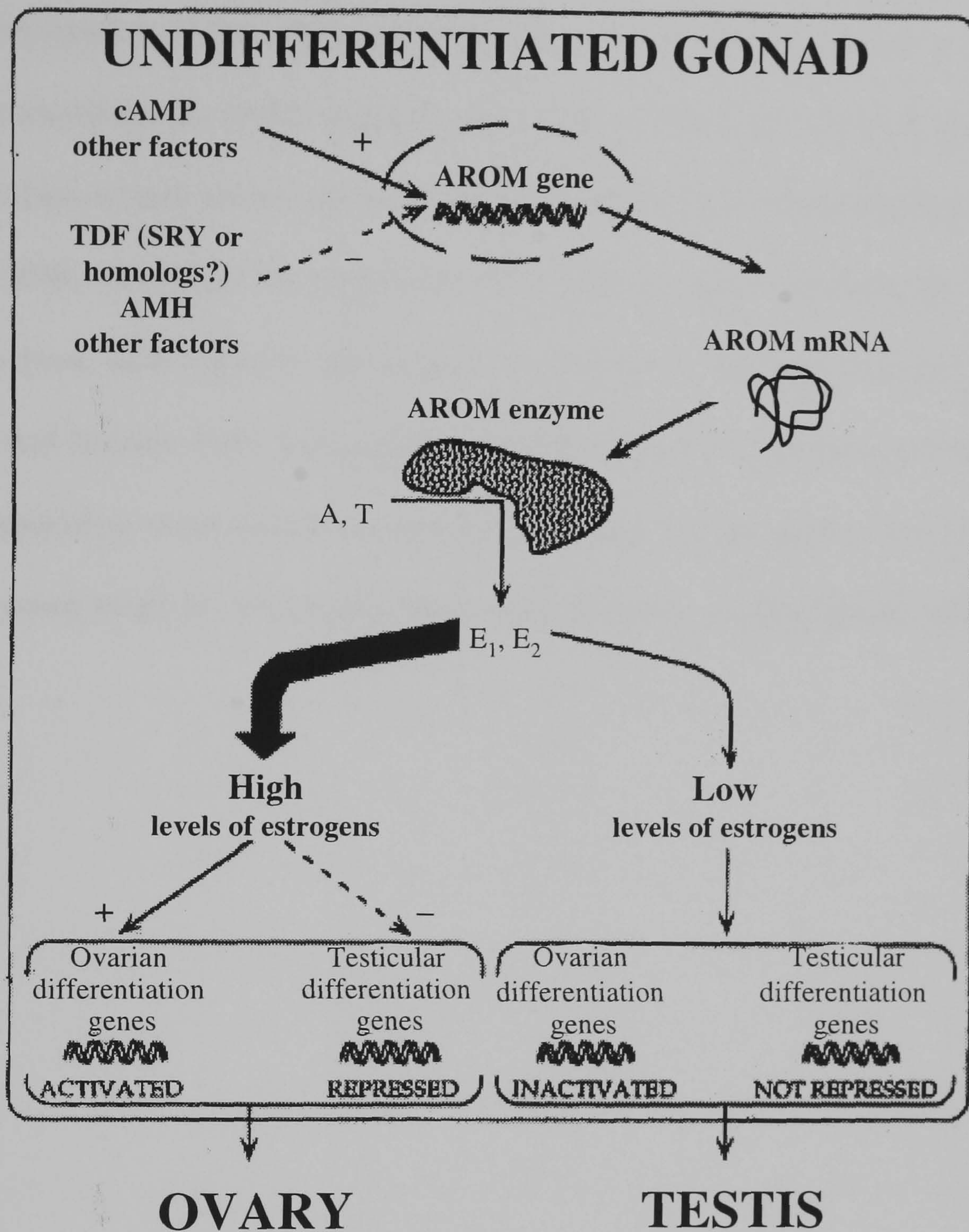


Fig. 1.4. Postulated involvement of cytochrome P450 aromatase (AROM) in the sexual differentiation of gonads in vertebrates. (+, Activation of transcription; –, repression of transcription; A, androstenedione; E1, estrone; E2, estradiol-17β; T, testosterone; AMH, anti-Müllerian hormone; cAMP, adenosine 3':5'-cyclic monophosphate; SRY, sex determining region of the Y chromosome; TDF, testis-determining factor.) (from Pieau *et al.* 1994a).

(reviewed by Parker *et al.* 1999). Moreover, Vilain and McCabe (1998) pointed out that normal sexual development requires not only normal gonadal differentiation but also normal development of the entire hypothalamic-pituitary-adrenal-gonadal axis, referred to as the reproductive axis. GnRH neurons play a role in the secretion of LH and FSH by the pituitary, which in turn induce the secretion of testicular and ovarian hormones (Vilain and McCabe 1998). It should be noted that SF-1, the possible transcriptional regulator of aromatase gene, also regulates the expression of LH and FSH in pituitary gonadotropes (Barnhart and Mellon 1994; Keri and Nilson 1996). Thus, it could be possible to speculate that the expression of aromatase is controlled by more than one factor, and that the central nervous system might be involved in the aromatase action during the sex differentiation.

1.5. The role of brain during sex differentiation

1.5.1. Ontogeny and functional anatomy of the brain

The brain is the enlargement of the central nervous system (CNS) of most bilaterally symmetrical animals with an antero-posterior axis. The CNS is one of the earliest groups of tissues to develop embryologically. In most cases the neural tube, formed by the closure of the neural groove in the dorsal mid-line, is developed fully by the time 10-15% of embryonic life (from fertilisation to hatching) has passed. The anterior thickening of this tube which occurs, to form the brain, indicates the cephalization of both sense organs and integrative centres which has occurred in vertebrates. Outgrowths of this early nervous system and connections with nervous tissue outside the neural tube form the spinal and cranial nerves. These are the routes by which the animal receives information from its own tissues and environment, and relays commands to muscles, glands and sense organs through which a response is mediated.

The anterior thickening of the neural tube develops into three distinguishable components: the prosencephalon (forebrain), mesencephalon (midbrain) and rhombencephalon (hindbrain). These three hollow chambers or vesicles are traditionally associated with the three primary senses of olfaction, vision and audition respectively, and together constitute the brainstem in the adult. Each develops a secondary outgrowth: the telencephalon (cerebrum), optic tectum and cerebellum respectively (Table 1.5, Fig. 1.5).

Localisation of function in the brain has not been well established for fish. By rough estimation, the vagal lobes of cyprinids are concerned with taste and general cutaneous sensation as well as vibration sense (reviewed by Laming 1981; also below). The optic tectum of teleosts in general is not only a region for the synthesis of visual information but is also concerned with the integration of visual and auditory sensory inputs. The

telencephalon of telosts is concerned with both olfactory reception and such complex functions as learning and appetitive behaviour (Laming 1981). The telencephalon also appears to function as an organiser and integrator of segments of innate behavior and maintains aggression, sexual activity, and parental behavior of fishes in the proper balance to insure successful reproduction of the species (Bernstein 1970).

Table 1.5. The major divisions of the vertebrate brain (from Laming 1981)

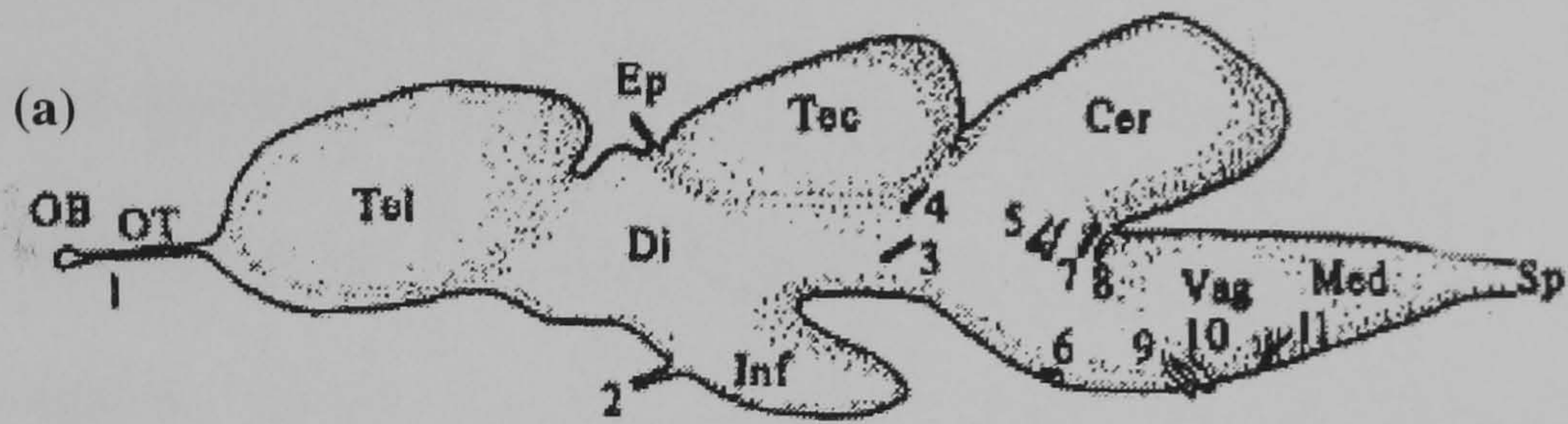
Primary vesicle	After formation of secondary vesicles	Major constituent regions
Prosencephalon (forebrain)	*Telencephalon* (cerebrum, 'forebrain')	Olfactory bulb Pallium (cortex) Corpus striatum
	Diencephalon	Epithalamus Thalamus Hypothalamus
Mesencephalon (midbrain)	Optic tectum (tectum)	
	Tegmentum	
Rhombencephalon (hindbrain)	Cerebellum or *Metencephalon* (anterior medulla and cerebellum)	
	Medulla oblongata (medulla) or *Myelencephalon* (posterior medulla)	

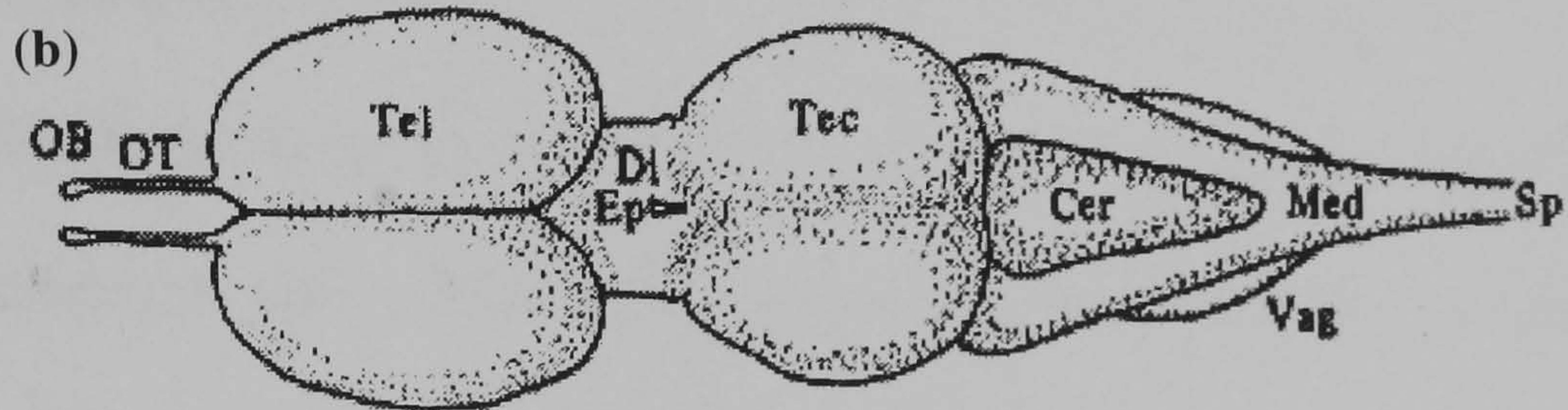
Embryological divisions are shown in *italic* type.

1.5.2. Sex differences in the brain

Males and females show different behaviours that are easily found in the realm of reproduction. It was hypothesised that these different behaviours between the sexes might originate from the fundamental differences of the CNS. Countless reports on sexual dimorphism in the nervous systems of vertebrate and invertebrate species have been published (reviewed by Gahr 1994; also below). These differences include: total brain size weight; the size of certain brain regions or sub-regions called brain nuclei; the numbers of

A





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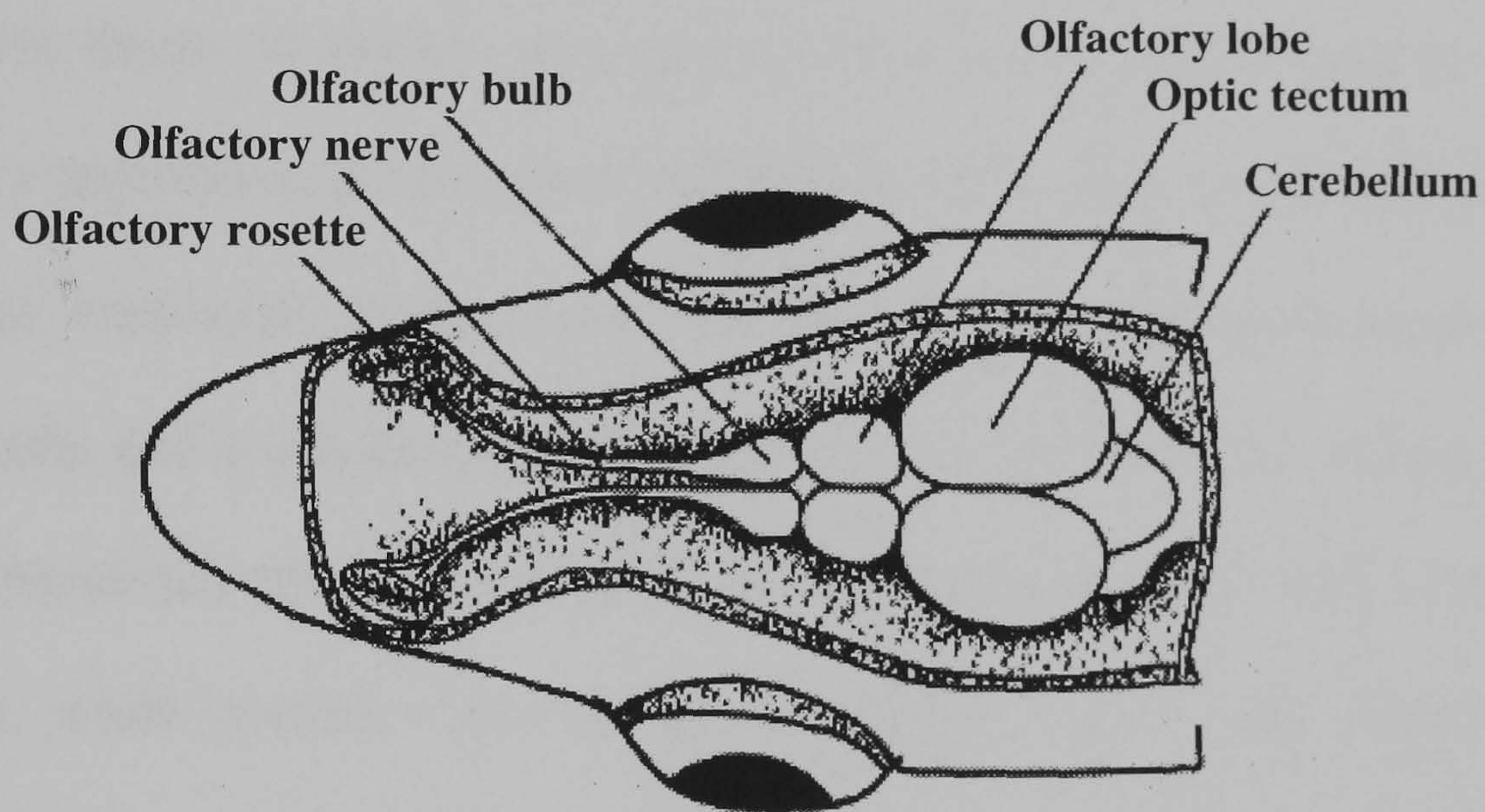


Fig. 1.5. A. Lateral (A-a) and dorsal (A-b) views of a generalised lower vertebrate brain to show the main superficial features. Cer, cerebellum; Di, diencephalon, Ep, epiphysis (pineal); Inf, inferior lobe of hypothalamus; Med, medulla oblongata; OB, olfactory bulb; OT, olfactory tract; Sp, spinal cord; Tec, optic tectum; Vag, vagal lobes; Tel, telencephalon; 1-11, cranial nerves. B. Dorsal view of the brain and the olfactory organ of lake whitefish (*Coregonus clupeaformis*). (A from Laming 1981; B from Hara 1981).

neurons; glial cells and axons in certain brain areas; the size of the cell bodies in the same brain region in males and females; dendritic branching patterns of the same neurons in males and females; patterns of synaptic organisation; the structure of neuronal plasma membrane; the distribution and relative concentration of enzyme activities; neuropeptides; neuropeptide receptors; steroid hormone receptors; and cytoskeletal proteins in male and female brains. How do male and female brains become different?

Sex steroid hormones provided the answer to this question. Since Phoenix *et al.* (1959) published a fundamental study on the effects of sex steroid hormone administration during prenatal development in adult guinea pigs, suggesting that the action of gonadal steroids may cause the differentiation or organisation of the CNS during a critical period of development, much experimental evidence has been reported. Exposure to testosterone (T) masculinises the brains of female neonatal rats as shown by an increase in intromission and ejaculatory behaviours (Christensen and Gorski 1978). The injection of as little as 10 μg testosterone propionate to the female rat shortly after birth permanently prevented ovulation (Gorski and Barraclough 1963). Castration of the male rat within a few days of postnatal life feminised the male's brain in that ovulation occurred after ovarian grafting (Harris 1965), while ovulation did not occur when ovarian grafts placed in a male gonadectomised as an adult became polyfollicular. On the basis of all these findings, it is now generally believed that sex steroids direct the differentiation of brain sex from lower vertebrates (although there is no evidence of this yet for fish) to mammals (reviewed by Hutchison 1997). Further studies revealed that masculinisation of the CNS in a critical period is not achieved by testosterone itself but by its metabolite, oestrogen that is locally aromatised in the brain (Naftolin 1994). However, the mechanism by which locally aromatised oestrogen masculinises the CNS is not clear.

1.5.3. Brain aromatase

It is known and well established that specific areas of the CNS convert androgens to oestrogens by an aromatisation mechanism (reviewed by Lephart 1996). The aromatisation of androgens to oestrogens also occurs in the ovary, testis (Silberzahn *et al.* 1988), placenta (Kellis and Vickery 1989), pituitary (Pasmanik and Callard 1989), adipose tissue (Lueprasitsakul and Longcope 1991) and skin fibroblasts (Stillman *et al.* 1991). Thus, a question arises: Is the aromatase that is expressed in different tissues the same?

Due to its biological significance, the gene encoding aromatase has been cloned and sequenced, and the pattern of aromatase gene expression has been studied in many animals including mammals, bird, reptiles and fish. Among these animals, some of them appear to have more than one type of aromatase gene transcripts or genes. Three types of tissue specific aromatase promoters that result in different transcripts have been proposed in humans (ovary, placenta and adipose: Simpson *et al.* 1994) and rats (hypothalamus, ovary, cerebral cortex: Kato *et al.* 1997). Tissue-specific isoforms of aromatase genes have also been reported in pigs (ovary, blastocyst and placenta: Choi *et al.* 1996) and goldfish (brain and ovary: Tchoudakova and Callard 1998). Further studies are likely to reveal more tissue-specific transcripts and isoforms in many animals, including fish.

Interestingly, teleost fish show exceptionally high levels of brain aromatase activity, that are 100 to 1000 fold higher than in mammalian brains (Callard *et al.* 1981; Pasmanik and Callard 1988a). Why should fish brains have such exceptionally high aromatase activity, which is more than 10 times higher than ovarian aromatase activity in the same species? Does this imply that in fish the major steroidogenic organ is not the gonads but the brain? No matter what the answers would be, it can be postulated that all the reproductive events including sex differentiation of the brain and gonads in fish might be controlled through different steroid mechanisms from those in mammals. Thus, it would

be required to identify both ovarian and brain type aromatase genes or transcripts and study gene expression patterns with regards to reproductive events in fish.

1.5.4. Sex determination and the brain in fish

In vertebrates, it is generally believed that sex is first differentiated in the gonads, and the sex of gonads directs sexual differentiation of all other tissues including the brain. However, this may not be true in fish. Francis (1992) suggested a new idea that unlike higher vertebrates, in teleosts, instead of the gonads determining the fate of the relevant brain structures, events in the brain may determine the fate of the gonads. Despite the fact that there is no direct experimental evidence at all for this revolutionary idea, it seems to be worth reviewing, considering the implication of ESD observed in fish.

As mentioned previously, in some fish, amphibians and reptiles sex is influenced by environmental factors (e.g., temperature and pH). Such environmental cues would be detected by peripheral sensors (e.g., thermoreceptors in the skin: Prosser 1991) and delivered to the brain. These input would be analysed and integrated, following the production of responses through the neuroendocrine system. It is also known that some cichlids change their sex by social environments (Francis and Barlow 1993). This is likely to be a response to the changing social environments via the CNS.

Francis (1992) made an assumption that the sex determining gene on a teleost sex chromosome would initiate events in the brain (perhaps in the GnRH cell populations in the preoptic area, POA, and nervous terminalis), and only through these events it will determine the fate of the gonads. For this assumption to be true, a couple of prerequisite should be found in sex determination of fish. First the CNS, especially the hypothalamo-pituitary axis, should be active around the time of sex differentiation and steroids can have a feedback effect on this axis. Many experimental studies reported that it is, indeed, active,

and has a feedback effect of steroids (reviewed by Kah *et al.* 1993). Second, treatment with GnRH or GTH I and II (equivalent to FSH and LH in mammals, respectively) could cause sex-reversal. In two hermaphrodite fish species, the possible sex reversal effect of GnRH has been suggested (ricefield eel, *Monopterus albus,* Tao *et al.* 1993; a protogynous diandric fish, *Synbranchus marmoratus*, Ravaglia *et al.* 1997). Human chorionic gonadotropin (hCG) and neuropeptide Y also induced sex reversal in hermaphrodite fish (reviewed by Baroiller *et al.* 1999). These results indirectly support Francis's idea, although there is, yet, no convincing evidence for this in gonochoristic fish species.

1.6. Sex determination in tilapia

The Cichlidae, which tilapia belong to, is one of the most mysterious fish families in terms of sex determination. Apparently most tilapia show GSD. However, many incidences of skewed sex ratios have been reported from normal crosses, and unexpected sex ratios from hormone treatments and gynogenesis (see below for details). The influence of temperature has been noticed in tilapia, and the influence of pH or social environment was also reported in some Cichlidae. Even within GSD, some tilapia show XX female homogamety while other show ZZ male homogamety. Autosomal influence is also considered in some tilapia (see below for details). Tilapia shows high sensitivity to exogenous sex steroids. Tilapia is, indeed, one of the most interesting animals in respect to its sex determination. This section summarises the proposed pattern of sex determination in tilapia.

1.6.1. Genetic sex determination in tilapia

1.6.1.1. Simple monofactorial model

Sex should be determined either by a male determining gene or a female determining gene on sex chromosomes in this model. Male heterogamety (XX female and XY male) and female heterogamety (WZ female and ZZ male) were proposed to occur in this family (Hickling 1960; Chen 1969a). Based on this model, *O. mossambicus* and *O. niloticus* have male heterogamety, and *O. macrochir*, *O. urolepsis hornorum* and *O. aureus* have female heterogamety.

This model has been challenged by many unexpected sex ratios that have been produced from genetic cross experiments. For example, in interspecific crosses, this model failed to explain many of the ratios obtained with hybrid combinations beyond the F1 generation (i.e., offspring of the cross between the WZ male of the all-male brood and XW

female of the reciprocal cross) and the subsequent backcrosses (Chen 1969a; Jalabert *et al.* 1971). Lack of information on heteromorphic sex chromosomes and lack of sex determining gene(s) such as SRY, also push this model into a corner. However, no other sex determining system was able to completely replace this model, so far.

1.6.1.2. Autosomal influence

While interspecific cross studies in tilapia provided useful information on tilapia sex determining system, at the same time it also brought about a great complexity to understanding sex determining system in this family. To explain many unexpected sex ratio from interspecific crosses, several ideas based on autosomal influences were put forward (Avtalion and Hammerman 1978; Hammerman and Avtalion 1979; Kallman 1984; Majumdar 1984; Mair *et al.* 1990; Mair *et al.* 1991a,b; Wohlfarth and Wedekind 1991).

An autosomal (AA or aa) plus gonosomal (W, X, Y, where Y=Z) sex determining system (autosomally influenced three gonosome model) was proposed through interspecific studies (Table 1.6) (Avtalion and Hammerman 1978; Hammerman and Avtalion 1979). Based on this model, *O. mossambicus* and *O. niloticus* are classified as AAXX females and AAXY males, and *O. macrochir*, *O. urolepsis hornorum* and *O. aureus* are classified as aaWY females and aaYY males. This is a gene balance model assuming that if the sum effects of the four sex determining chromosomes exceeds a certain threshold, the individual develops into a male; if less, a female (Hammerman and Avtalion 1979).

The model of Avtalion and Hammerman (1978) provided better prediction of sex ratio from interspecific crosses, but not from intraspecific crosses. For intraspecific data, Mair *et al.* (1991b) suggested the possibility of an autosomal recessive sex modifying gene in *O.*

aureus, epistatic to the major sex determining factors W and Z (Y). Hussain *et al.* (1994) also hypothesized the existence of an autosomal locus (with alleles SR and sr) epistatic to the gonosomal locus and which induces female to male sex reversal when sr is homozygous. However, it still does not explain all aberrant sex ratios observed: for example, occurrence of female in the progeny of YY males (Mair *et al.* 1997). Mair *et al.* (1997) proposed the action of several autosomal sex modifying genes. Additionally, Sarder *et al.* (1999) speculated the existence of an autosomal sex modifying gene with limited penetrance.

Wohlfarth and Wedekind (1991) suggested that in intraspecific studies, sex ratio behaves as a quantitative trait (i.e., polygenic, and susceptible to environments). However, their suggestion has not been experimentally proved as yet. Moreover, Mair *et al.* (1991a) commented that sex inversion and gynogenesis experiments refute the polygenic model of sex determination in tilapias.

Table 1.6. Influence of autosomal pairs on sex determination in interspecific crosses

Sex chromosomes	*Autosomal factors*		
	AA	Aa	aa
YY	m	m	m
WY	m	m	f
XY	m	m	f
WW	m	f	f
WX	f	f	f
XX	f	f	f

m: male, f: female (from Avtalion and Hammerman 1978).

1.6.1.3. Crossing-over

Recombination in the chromosomal region between the centromere and the sex determining genes in the prophase of the first meiotic division was hypothesised to explain a predominance of females in the F1 generation of gynogenetic *O. aureus* (Penman *et al.*

1987a). This hypothesis was further investigated in three generations of gynogenetic *O. aureus* (Avtalion and Don 1990). The sex ratio they obtained could be explained by the crossing-over occurring between the centromere and the sex determining genes.

In *O. niloticus*, Hussain *et al.* (1994) suggested an autosomal sex determining locus (SDL-2: see 1.6.1.2) and calculated a recombination rate of y=0.85 between the centromere and this SDL-2 based on the low proportion of males among the meiogynes derived from one female. Müller-Belecke and Hörstgen-Schwark (1995) assumed an even higher recombination rate of y=1.00 between the centromere and the SDL-2 on the basis of the absence of males among the 163 meiogynes produced by two females which had males among their mitogyne offspring in their study. However, this theory could not explain a low proportion of males in the offspring of a mitogyne male when mated to normal brood stock females in the study. The authors suggested the occurrence of two or more autosomal minor sex determining factors.

1.6.2. Environmental effects (temperature)

Temperature effects on sex ratio have been reported in several tilapia species including *O. niloticus* (Baroiller *et al.* 1995a,b; Baroiller *et al.* 1996; Abucay *et al.* 1999), *O. mossambicus* (Mair *et al.* 1990), *O. aureus* (Mair *et al.* 1990; Desprez and Mélard 1998), and a red Florida strain (Baroiller *et al.* 1995b). Most tilapia species studied so far respond significantly to high temperature (34-37°C), producing high percentages of males; the one exception is *O. mossambicus* which responded to low temperature.

The sensitivity to high temperature varied from species to species. Among the tilapia species, *O. aureus* was the most sensitive to high temperature, yielding 97.8% male (Desprez and Mélard 1998). Moreover, a high variability in thermosensitivity was observed within the same species (Abucay *et al.* 1999) and even within the same strain in

tilapia (Baroiller *et al.* 1995b; 1996). Abucay *et al.* (1999) also claimed that high temperature can influence sex ratio not only towards male but also towards female by providing evidence of feminisation in high temperature treated-YY male groups. These studies imply that a genotype-temperature interaction may exist in tilapia as proposed in a different species, *M. menidia* (Conover and Kynard 1981).

It seems obvious that temperature can participate in the process of sex determination in tilapia, although it is still questionable whether this is relevant to sex determination under "normal or natural" condition. What mechanisms are likely to be involved in this temperature effect? Does temperature turn on or off some specific genes that relate to sex determination? Or does it work through the endocrine regulation system such as hypothalamus-pituitary-axis and/or steroidal action? Temperature action through aromatisation of androgen to oestrogen has been suggested in a flounder, *P. olivaceus* (Kitano *et al.* 1999), and in many reptiles (reviewed by Jeyasuria and Place 1998). It will be worth testing whether aromatase plays an important role in the temperature mediated effects during sex determination in tilapia..

1.6.3. Parental effect

There seem to be parental effects on sex determination in tilapia (Shelton *et al.* 1983). Wohlfarth and Wedekind (1991) stated the evidence of the stability of sex ratio in repeat spawns of the same parental pair in intraspecific crosses. This also implies the possibility of parental influence on sex ratio of progenies. A strong parental influence on the response to high temperature was also noticed in *O. niloticus* (Baroiller *et al.* 1995b).

In contrast, Mair *et al.* (1991a) used five males and five females to produce progeny from 25 different parental combinations and found no evidence for paternal or maternal influences. However, it should be noted that, among the five females they used, one

female produced a consistently higher proportion of intersex (5.8 – 26%) with all five males compared to intersex percentages in the other four females (mostly 0%, apart from only two parental combinations of 3%). Thus, parental effects on sex ratios of progeny may still need to be postulated to explain some unusual sex ratios from tilapia.

Sex ratios of tilapia progenies deviate from 1:1 either significantly or not when produced by normal crosses (between XX females and XY males) (Mair *et al.* 1991a). Under this circumstance, it would be difficult to identify the parental effects on sex ratios from the mixed sex groups. Using clonal fish and sex reversed males (XX) as broodstock might magnify the differences of sex ratios from different parental combinations.

Overall, it seems likely that one will never reach an answer for sex determining system in tilapia as long as one just relies on genetic cross experiments, which produce complex sex ratio data. Advanced cytological studies and molecular studies would be helpful to clarify sex determining system(s) in tilapia. Conclusion(s) should be postponed until enough cytological and molecular evidence are collected. In this thesis, to get one step closer to the conclusion, a molecular approach is introduced, focusing on the role of aromatisation in routine sex differentiation and temperature-affected sex differentiation in the Nile tilapia, *O. niloticus*.

1.7. Postulations and questions to be tested

Several facts have become clear throughout this general introduction. Following the cascade of sex determination, fish sex is mainly determined by genetic information with possible environmental influence. Then, a series of biochemical reactions takes place to develop a sex, either male or female (or rarely intersex). Sex steroid hormones (androgens and oestrogens: see 1.3.2.2) and environmental factors (e.g., temperature: see 1.3.2.1 and 1.6.2) have been shown to be crucial in the determination of phenotypic sex in fishes and other vertebrates. The conversion of androgens into oestrogens is catalysed by aromatase (one of the P450 enzymes) and such enzymatic action can be also affected by the environment (see 1.4.2).

Initially, the genetic information to determine the sex (chromosomal and/or autosomal) can be related to a specific gene like Sry, though no sex-specific Sry gene has found in fish yet. The results of the biochemical reactions would reach, at a certain stage, the aromatase gene through a series of unknown processes (possibly in the CNS and/or presumptive gonadal area). Some of the results will activate the aromatase gene and some of them will depress it. Endogenous sex steroids will be produced in a different pattern as the results of the expression of the aromatase gene. The sex steroids profile in each individual would determine the sex of that individual (on the basis of exogenous steroids effects). A picture can be drawn for this story, but all the suggestions described here need to be proven in fish.

Tilapia is a good experimental animal to study the mechanism of sex determination. There are many benefits in using this species as well as the fact that this is an important species in aquaculture. (1) The sex of the embryos can be manipulated at will simply by fertilising eggs and sperm from XX, XY and YY male or female; (2) Extensive experience

with successful handling of the eggs and embryos of this fish is at hand; (3) The full-length cDNA for the tilapia ovarian aromatase gene has been obtained by Chang *et al.* (1997); (4) Females of this species spawn every 20-30 days when appropriate environmental conditions are provided, and males are always available. (5) This species shows TSD as well as GSD.

This study is based on two premises (Table 1.7): 1. The sex of the Nile tilapia *O. niloticus* is determined primarily by genetic factors (an XX/XY system with mild autosomal influence); 2. Sex steroid hormones are natural sex inducers in fish.

It has been postulated that XX/XY genetic sex determination is masked by parental effects. Thus, parental effects were studied using normal, clonal and sex reversed broodstocks (Chapter 2). In this study, it was also intended to identify the best brood stock lines for other studies that are described below.

Another postulation that emerges from the previous studies, is that aromatase gene may play an important role in sex differentiation in tilapia. Only a 2-hour treatment with aromatase inhibitor (AI) resulted in masculinisation in chromosomally female salmon (Piferrer *et al.* 1994) and two weeks treatment with AI in *O. niloticus* resulted in masculinisation (Kogson-Hurtado 1997). Nakamura *et al.* (1997) suggested the initial aromatisation might take place just before 23-26 days post-hatch (dph), but Kogson-Hurtado (1997) showed aromatase worked properly from 7 to 38 dph using a range of two or three week treatment regimes. The sex determination period suggested by Kogson-Hurtado (1997) includes 7-20 dph which is earlier than the period suggested by Nakamura *et al.* (1997). It should be possible to narrow down the period of crucial aromatisation during sex differentiation in tilapia.

Table 1.7. The list of hypotheses and questions to be tested in this thesis

Hypothesis and questions I

Premise : The sex of the Nile tilapia *Oreochromis niloticus* is determined primarily by genetic factors (an XX/XY system with mild autosomal influence).

Postulation : Genetic sex determination is masked by parental effect and temperature effect.

Questions to be tested :

- Are there parental effects on the sex ratio of progenies?
- Does temperature affect tilapia sex determination?
- Do different genotypes (XX, XY, YY) respond differently to temperature?
- Does the temperature effect go through the pathway of steroidogenesis?

Hypothesis and questions II

Premise : Sex steroid hormones are natural sex inducers in fish.

Postulation : Cytochrome P450 aromatase that catalyses androgens to oestrogens may play a crucial role in sex differentiation .

Questions to be tested :

- Does blocking aromatisation by a chemical inhibitor alter sex ratio?
- When does aromatisation take place with regards to sex differentiation?
- Does the brain aromatase gene differ from the ovarian aromatase gene?
- Does the expression of brain and ovarian aromatase genes coincide with sex differentiation?
- Where does the crucial aromatisation take place?

As the first step to uncover where and when aromatase activity has an effect on sex differentiation in tilapia, sexually undifferentiated tilapia were subjected to AI (Fadrozole CGS16949A) treatment by oral administration or immersion (Chapter 3). The ontogeny of aromatase gene expression was also studied. To explore the possibility of the involvement of the CNS in sex determination since the initial aromatisation site is not known, an attempt was made to clone and sequence tilapia brain aromatase gene and to study the expression pattern of this gene as well as the ovarian aromatase gene (Chapter 4).

In addition, temperature effects on different genotypes were studied in combination with treatment of aromatase inhibitor to examine the involvement of aromatase action in TSD. Based on the results, an explanation for the relationship between GSD and TSD in this species was proposed (Chapter 5).

Finally, findings from each chapters were discussed together to make some suggestions for the sex determination in this species (Chapter 6).

The findings and suggestions from these studies could provide better understanding of sex determination and differentiation in this species, and may lead to development of better techniques to manipulate fish sex for aquaculture.

CHAPTER 2

PARENTAL EFFECT

2.1. Introduction

It has been proposed that sex in the Nile tilapia *Oreochromis niloticus* is mainly determined by a monofactorial system with male heterogamety (XX/XY system) (Mair *et al.* 1991a; Mair *et al.* 1997). However, this simple sex determining system (XX/XY) often fails to explain some unexpected sex ratios in this species, as introduced in Chapter 1. To explain these unexpected sex ratios, environmental influence (Mair *et al.* 1990; Baroiller *et al.* 1995a,b; Baroiller *et al.* 1996; Abucay *et al.* 1999), autosomal influence (Mair *et al.* 1991b; Hussain *et al.* 1994), a polygenic system (Wohlfarth and Wedekind 1991) and parental influence (Shelton *et al.* 1983; Sarder *et al.* 1999; Tuan *et al.* 1999) have been suggested.

Amongst these explanations, the possibility of parental influence on progeny sex ratios have not received much attention from fish biologists and aquaculturists, although the possibility was first suggested more than a decade ago (Shelton *et al.* 1983). Recently, the possibility of parental influences on sex ratios have been proposed in other animals including insects (Hurst and Majerus 1993), crustaceans (Juchault *et al.* 1992), birds (Bradbury and Blakey 1998; Nager *et al.* 1999) and many mammals (Moses *et al.* 1995; Manning *et al.* 1996; James 1996; Monard *et al.* 1997; Grant 1996; Andersson and Bergstrom 1998; Hewison *et al.* 1999; Kruuk *et al.* 1999; Fisher 1999; Nonaka *et al.* 1999). Parental influence on sex ratios could possibly be a common phenomenon throughout the animal kingdom including fish.

However, the definition of "parental influence or effect" is not well established. This term has been used to describe a tendency to produce skewed sex ratios of offspring from a certain mother and/or father in many animals from invertebrates to mammals. There are many possible causes of this parental influence. These include genetic factors such as

"multiple sex determining/modifying gene(s)" and "maternal effect", and secondary sex control system such as "pre-fertilisation control" and "post-fertilisation control".

The existence of "multiple sex determining/modifying gene(s)" was proposed to explain highly variable sex ratios and parental influence in some tilapia species (Shelton *et al.* 1983; Wohlfarth and Wedekind 1991; Tuan *et al.* 1999).

"Maternal effect" (Russell 1996) is defined as a phenotype in an individual that is established by the maternal nuclear genome, as the result of mRNA and/or proteins that are deposited in the oocyte prior to fertilisation. These inclusions direct early development of the embryo.

In mammals that have male heterogametic sex determining system (XX/XY), all eggs produced by females are X-bearing while males produce two types of sperm, X and Y in equal numbers as a consequence of meiotic cell division. Offspring sex is determined by whether an X- or a Y-bearing sperm fertilise the egg. "Pre-fertilisation control" is that females either actively prevent or facilitate the passage of one type of sperm through the reproductive tract and select sperm at the site of fertilisation. "Post-fertilisation control" is that females selectively abort embryos of the undesired sex (reviewed by Hardy 1997).

However, none of these possible causes have been proven by direct evidence. The cause of parental influence on sex ratio is not clear in any animal. Therefore, it should be pointed out that establishing a polygenic sex determining system based on the tendency of parental influence is not relevant, at least too early at the moment. Parental influence on sex ratio in fish requires further attention and appropriate interpretation.

In tilapia, *O. niloticus,* Wohlfarth and Wedekind (1991) showed the evidence of the stability of sex ratio in repeated spawns of the same parental pair in intraspecific crosses. This implies the possibility of parental influences on sex ratio of progenies. A strong

parental influence on the response to high temperature was also noticed in *O. niloticus* (Baroiller *et al.* 1995b).

In contrast, Mair *et al.* (1991a) produced progeny from 25 different parental combinations and found no evidence for paternal or maternal influences. However, it should be noted that, among the five females that they used, one female produced consistently higher proportion of intersex (5.8 – 26%) with all five males compared to the proportion of intersexes from the four other females (mostly 0%, except two parental combinations giving 3% intersex). Parental effect on sex ratios of progeny in this species should still be tested through further studies.

The existence of parental influences on sex ratio of progenies is arguable. Furthermore, it is not clear whether the parental influences observed in some species was associated with genetic or non-genetic events. If there is non-genetic parental influences, it might not be easy to recognise the non-genetic parental influences under the presence of autosomal influence and temperature effect. As already mentioned, strong evidence of autosomal influences on tilapia sex determination has been reported, including an epistatic locus (SDL-2, two alleles, SR and sr: Hussain *et al.* 1994) and rare autosomal recessive sex-influencing genes (Mair *et al.* 1991a). Temperature also significantly altered sex ratios in this species (Abucay *et al.* 1999). Tuan *et al.* (1999) proposed the possibility of parental influence in *O. niloticus*, but the experimental temperature was not mentioned. Fish were raised in earthen ponds in their experiment. Water temperature would need to be maintained at a range of constant temperatures in which sex determination is not influenced.

Recently, Sarder *et al.* (1999) established clonal lines of *O. niloticus*, and propagated them by means of mitotic gynogenesis and subsequent meiotic gynogenesis. Such gynogenetic clonal fish that should produce all female progeny in theory, even under the

interference of an autosomal sex modifying gene such as SDL-2, could increase the chance to recognise non-genetic parental effects.

For the experiment presented in this chapter, a series of crosses were carried out using gynogenetic clonal fish, neomales (sex reversed XX males), normal males and females, and YY fish in recirculating breeding and rearing systems where temperature was controled. The main objective of this study was to investigate the possibility of parental effects on sex ratios of tilapia progenies. Additionally, through these crosses, it was intended to identify the best broodstock lines for other studies presented in this thesis.

2.2 Materials and Methods

All types of broodstock of *O. niloticus* (originated from Lake Manzala, Egypt) that were used in this experiment were produced in the Institute of Aquaculture, University of Stirling through a series of sex determination studies on this species. They were maintained individually in glass aquaria within a recirculating system at 28±1°C. They were fed at least twice a day with commercial trout feed (BOCM PAULS Fish Feed Group, Renfrew, UK).

To examine autosomal and parental influences, a total of 12 males of four different genetic types and 18 females of three different genetic types were tested (Table 2.1). XX neomales were produced by dietary treatment of all female fry with 17α-methyltestosterone (MT) (SIGMA) at a dose of 50 mg kg^{-1} in diet for 1 month and progeny-tested before use. YY males were produced by crossing YY males and YY females from the YY fish lines in the Institute of Aquaculture, and some YY male fry were sex reversed by the treatment of diethylstilbestrol (DES) (SIGMA) to produce YY females. Fully inbred clonal male and female broodstocks were produced by previous research using mitotic and meiotic gynogenesis (Sarder *et al.* 1999). The clonal males, CM1 and CM2, were siblings of the clonal females that had produced high percentages of males in meiotic gynogenesis. A clonal male, CM3, and three clonal females were derived from all-female producing lines in previous studies (Table 2.2). All clonal lines were confirmed as completely homozygous and without any paternal influence by multilocus fingerprinting (Sarder *et al.* 1999).

Eggs were manually stripped from ovulated females. Milt were collected into glass capillary tubes and either used directly for fertilisation or transferred to 1.5 ml plastic

Table 2.1. Lists of broodstock used in this experiment

Tag No.	Genetic identity	Phenotypic sex	Identity code
00-0135-EA1B	XY normal	male	NM1
00-013C-BOEC	XY normal	male	NM2
00-013C-AFA5	XX clone, MT treated	male	CM1
00-013E-OA23	XX clone, MT treated	male	CM2
009-783-894	XX clone, MT treated	male	CM3
005-117-817	XX MT treated	male	MTM1
006-023-629	XX MT treated	male	MTM2
014-556-527	YY super	male	SM1
014-571-512	YY super	male	SM2
014-555-315	YY super	male	SM3
010-554-342	YY super	male	SM4
013-554-595	YY super	male	SM5
00-013E-12E1	XX normal	female	NF1
00-013E-3245	XX normal	female	NF2
00-013E-42E4	XX normal	female	NF3
005-831-633	XX normal	female	NF4
013-110-064	XX normal	female	NF5
00-013E-3BFC	XX normal	female	NF6
011-779-353	XX normal	female	NF7
00-013E-3A6C	XX normal	female	NF8
00-012C-13B7	XX normal	female	NF9
147-982-273	XX normal	female	NF10
Tag missing 1	XX normal	female	NF11
Tag missing 2	XX normal	female	NF12
00-013E-2F19	XX clone	female	CF1
00-013E-3466	XX clone	female	CF2
00-013E-OEDE	XX clone	female	CF3
00-013C-AD32	YY E_2 treated	female	E2-F-1
00-013C-B207	YY E_2 treated	female	E2-F-2
011-571-283	YY E_2 treated	female	E2-F-3

Table 2.2. Identities of clonal fish used in this experiment

Clones	Maternal lines	Paternal lines	Characteristics of clonal lines
CM1	002-046-539		Inbred, Occurrence of males reported*
CM2	002-046-539		Inbred, Occurrence of males reported*
CM3	010-036-092		Inbred, All female line*
CF1	009-356-316	010-036-092	Outbred, All female line*
CF2	006-812-566	010-036-092	Outbred, All female line*
CF3	006-812-566	010-036-092	Outbred, All female line*

* Further details on clonal lines and their founders can be found in Sarder *et al.* (1999).

centrifuge tubes for short-term storage. After fertilisation, eggs were placed into a 1 litre incubation jar and the resultant fry were transferred to an aquarium (5 L volume) in a recirculating system. When fish were 1-2 months old, they were transferred to bigger tanks (25 L volume) to allow for further growth and sexing. Fish were sexed at around 3 months old by the aceto-carmine gonad-squashing method (Guerrero and Shelton 1974). Temperature was maintained at 28±1°C from the incubation until the end of experiments.

Sex ratio data from all crosses were first analysed by chi-square goodness-of-fit test to determine any significant differences from 1:1 sex ratios. Data were transformed to arcsine values when necessary. In order to determine whether parents are independent of progeny sex ratios, sex ratios from the crosses between clonal males and normal females, and from the crosses between normal females and normal males were subjected to three-dimensional contingency table (2×6×3) analysis followed by chi-square tests. Afterwards, the data from single pair crosses and pooled data for each male or female parent with other females or males were again tested by two-dimensional chi-square contingency analysis to further determine which gender of parents are independent of or associated with progeny

sex ratio. Statistical differences of sex ratios from repeated spawns of the same parental pairs were determined by t-test and/or chi-square analysis of 2×2 contingency tables when applicable (Zar 1984). Regression analysis was performed to determine whether parental influences were attributed to skewed sex ratios for some crosses, and to examine the relationship between survival rates and sex ratios in three different groups of progenies (all female groups with predicted genotype XX; mixed sex groups with predicted genotype XX or XY; all male groups with predicted genotype XY or YY).

2.3 Results

2.3.1. Crosses between an inbred clonal male and outbred clonal females

A clonal male (CM1) was crossed to three different outbred clonal females, where both maternal and paternal lines were identified as all female producing clonal lines in the previous studies. No male progeny were observed in any of the crosses (Table 2.3).

Table 2.3. Sex ratios from the crosses between a clonal male (XX) and clonal females (XX)

Parents		Progenies		
Male	Females	♂	♀	%♂
CM1	CF1	0	37	0.00
	CF2	0	40	0.00
	CF3	0	16	0.00
Overall		0	93	0.00

2.3.2. Crosses between clonal males and normal females

Three clonal males, CM1, CM2 and CM3 were crossed to eight normal females NF1-8 (Table 2.4). Under the assumption that CM1 and CM2 are homozygous for a recessive sex modifying allele (i.e., XX srsr), the crosses between CM1 or CM2 and normal females were expected to produce some male progenies depending on the genotype of normal females for the sex modifying locus. As expected, the crosses between CM1 or CM2 and normal females produced some male progenies with variations between females mated (0-33.3% males for CM1; 0-31.8% males for CM2). However, the assumption was undermined by the occurrence of some males (0-17.9% depending on female parents) in the progenies of normal female parents and the clonal male parent (CM3) that was

expected to produce 100% female progeny based on the assumption that this clonal line posseses a homozygous dominant genotype to the autosomal sex modifying gene (i.e., XX SRSR).

Table 2.4. Sex ratios from the crosses between clonal males (XX) and normal females (XX). Ratios expressed as male:female, with percentage of males in parentheses

Parents	*Clonal males*			Pooled for each female parent
	CM1	CM2	CM3	
Normal females				
NF1	0:15 (0)	4:26 (13.3)	2:57 (3.4)	6:98 (5.8)
NF2	0:12 (0)	1:10 (9.1)	0:8 (6.7)	1:30 (3.2)
NF3	1: 8 (11.1)	7:15 (31.8)	0:17 (0)	8:40 (16.7)
NF4	5:10 (33.3)	2:16 (11.1)	1:23 (4.2)	8:49 (14.0)
NF5	8:20 (28.6)	0:19 (0)	12:55 (17.9)	20:94 (17.5)
NF6	2:21 (8.7)	0:17 (0)	0:20 (0)	2:58 (3.3)
NF7	NT	0:21 (0)	5:41 (10.9)	5:62 (7.5)
NF8	0: 3 (0)	2:45 (4.3)	NT	2:48 (4.0)
Pooled for each male parent	16:89 (15.2)	16:169 (8.6)	20:221 (8.3)	52:479 (9.8)
	χ^2 for male parents = 4.407, 2 d.f.			χ^2 for female parents = 20.046, 7 d.f.*

NT: not tested.

* Significantly associated with progeny sex ratios (P < 0.01).

Chi-square analysis of three-dimensional contingency table (2×6×3) for sex ratio from three clonal males and six normal females, NF1-6 revealed that male and female parents, and progeny sex ratios are not mutually independent of each other (χ^2 = 90.075, 27 d.f., P<0.001). Further two-dimensional contingency table analyses using pooled data for 3 male parents (2×3) and for 8 female parents (2×8) were performed. Female parents were

significantly associated with the progeny sex ratios ($\chi^2 = 20.046$, 7 d.f., $0.005 < P < 0.01$), whereas male parents were not strongly associated with the progeny sex ratios ($\chi^2 = 4.407$, 2 d.f., $0.05 < P < 0.15$).

2.3.3. Crosses between neomales and normal females

Two neomales (MTM1 and MTM2) which were not related to the established clonal lines, were crossed to either six normal females or three normal females (Table 2.5). The proportion of males in the progenies varied with female parents (7.8-59.2% in MTM1; 0-60.9% in MTM2).

Progeny sex ratios from MTM1 ($\chi^2 = 60.491$, 5 d.f.) and MTM2 ($\chi^2 = 28.072$, 2 d.f.) showed significant association with female parents ($P<0.001$), but not with male parents when analysed by 2×2 contingency table using pooled data ($\chi^2 = 0.955$, 1 d.f., $P>0.25$). However, the result of this analysis seemed to be biased by an extreme outlier NF10. When sex ratios from 5 females were further analysed by 2×5 contingency table after omitting the data from NF10, the association of female parents with progeny sex ratio ($\chi^2 = 8.178$, 4 d.f., $0.05<P<0.10$) was not as strong as when NF10 was included.

2.3.4. Sex ratios from repeated spawns

The stability of sex ratios from repeated spawns was examined using XX to XX cross (MTM1 × NF2 and MTM1 × NF4: all female progenies are expected), XY to XX cross (NM1 × NF1 and NM2 × NF3: mixed sex progenies are expected) and YY to XX cross (SM1 × NF5 and SM3 × NF12: all male progenies are expected). None of these repeated crosses produced significantly heterogeneous sex ratios (Fig. 2.1). All sex ratios of two repeated spawns from MTM1 × NF2 and MTM1 × NF4 were not significantly different

from 100% females (t-test, P>0.05). Chi-square analyses of 2×2 contingency tables for the sex ratios of these crosses also did not show any significant association of different spawning with progeny sex ratios (χ^2 = 1.322, 1 d.f.; χ^2 = 1.695, 1 d.f., P>0.10 for both). Repeated crosses of NM1 × NF1 (χ^2 = 0.035, 1 d.f.) and NM2 × NF3 (χ^2 = 0.035, 1 d.f.) were not associated with progeny sex ratios (P>0.75 for both), apart from the significantly different sex ratios for NM2 × NF3 to 1:1 sex ratios (t-test, P<0.05). Sex ratios from repeated YY to XX crosses were not statistically tested since all data repeatedly showed 100% males. Parental influences were also noticed in XX × XX crosses (χ^2 = 4.036, 1 d.f., P<0.05) and XY × XX crosses (χ^2 = 4.856, 1 d.f., P<0.05) as demonstrated earlier and later in this chapter.

Table 2.5. Sex ratios from the crosses between neomales (XX: sex reversed genetic females by MT treatment) and normal females (XX)

		Progeny sex ratios			χ^2 values for
♂ parents	♀ parents	♂	♀	%♂	female parents
MTM1	NF2	6	71	7.8	= 60.491, 5 d.f.
	NF4	34	162	17.3	(from 2×6
	NF7	21	81	20.6	
	NF10	29	20	59.2	contingency table)*
	NF11	18	130	12.2	
	NF12	3	28	9.7	
Pooled		111	492	18.4	
MTM2	NF9	0	9	0.0	= 28.072, 2 d.f.
	NF10	14	9	60.9	(from 2×3
	NF12	1	31	3.1	
Pooled		15	49	23.4	

χ^2 values for male parents = 0.955, 1 d.f. (from 2×2 contingency table)

* Significantly associated with progeny sex ratios (P < 0.001).

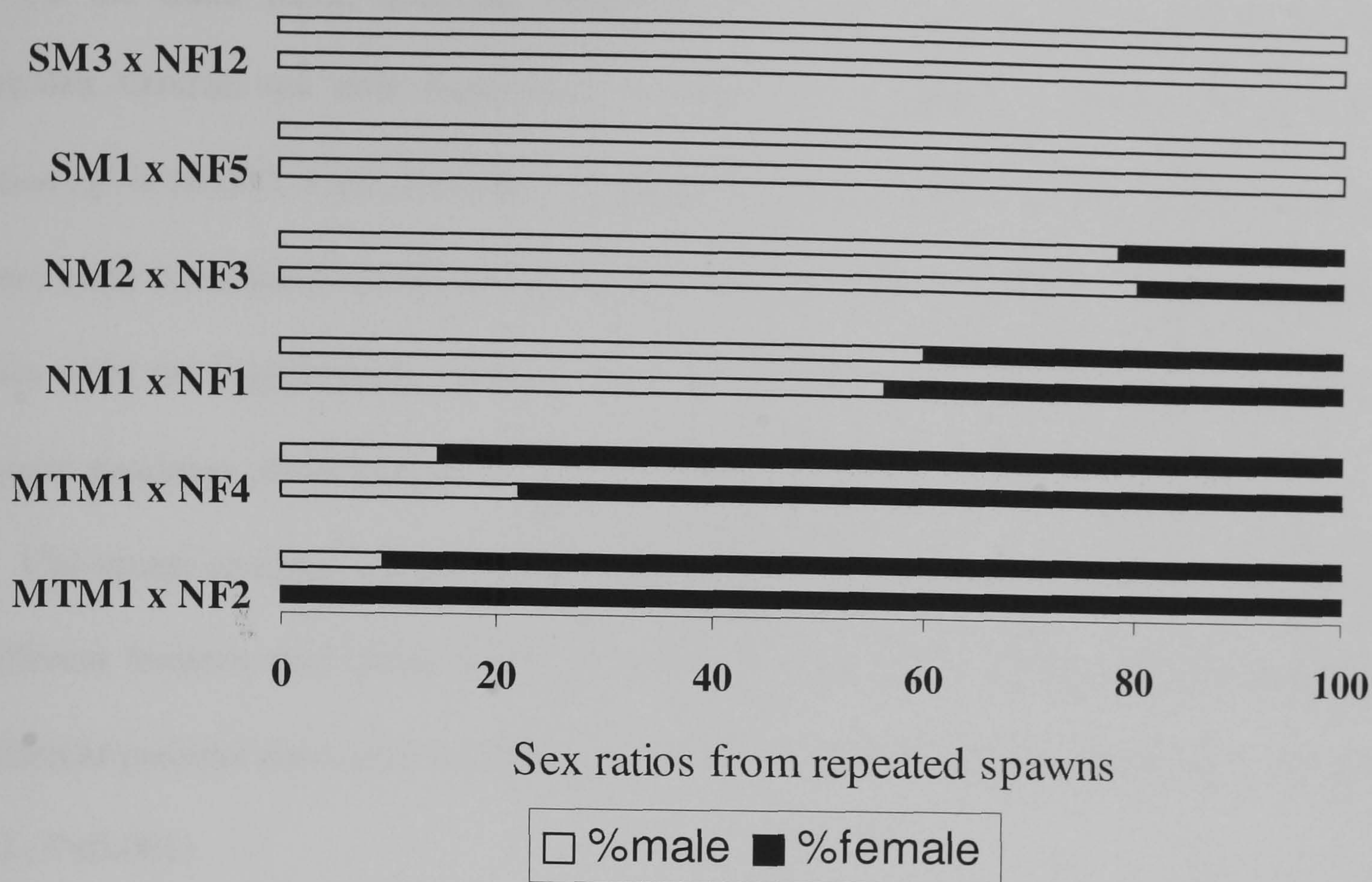


Fig. 2.1. Progeny sex ratios from repeated spawns. MTM1×NF2 and MTM1×NF4 (XX × XX): progenies are genetically all females; NM1×NF1 and NM2×NF3 (XY × XX): sex ratios of progenies are expected to be 1:1 (genetically mixed sex groups); SM1×NF5 and SM3×NF12 (YY × YY): progenies are genetically all males.

2.3.5. Crosses between normal males and normal females

Among 16 crosses between normal males and normal females, sex ratios of progenies from 6 crosses were significantly different to 1:1 sex ratio (P<0.05, 0.01 or 0.001, chi-square goodness-of-fit analysis, χ^2 values not shown) (Table 2.6). Sex ratios from NM1 with different normal females were homogeneous (χ^2 = 8.519, 7 d.f., P>0.25, heterogeneity chi-square analysis), while sex ratios from NM2 were heterogeneous (χ^2 = 30.133, 7 d.f., P<0.001, heterogeneity chi-square analysis), suggesting the possibility of paternal influences in sex determining process.

On the other hand, maternal influence was once again observed among different females. Overall sex ratio from these crosses were significantly different from 1:1 sex ratios (χ^2 = 18.000, 1 d.f., P<0.001, chi-square goodness-of-fit analysis) and NF3 and NF6 were main contributor to this skewness. Without sex ratios from NF3 and NF6, overall sex ratio were not significantly different from 1:1 sex ratio (χ^2 = 3.769, 1 d.f., P>0.05, chi-square goodness-of-fit analysis).

Chi-square analyses of 2×8 contingency tables for the sex ratios of NM1 and NM2 with different females also showed that progeny sex ratios were significantly associated with different parental pairs (not in NM1 χ^2 = 8.587, 7 d.f., P>0.25; but in NM2 χ^2 = 33.104, 7 d.f., P<0.001).

Table 2.6. Sex ratios from normal cross (XX female × XY male). Ratios expressed as male:female, with percentage of males in parentheses

Female parents (XX)	Male parents (XY)		
	NM1	NM2	Total
NF1	15 : 11 (57.7)	16 : 12 (57.1)	31 : 23 (57.4)
NF2	15 : 14 (51.7)	9 : 9 (50.0)	24 : 23 (51.1)
NF3	7 : 9 (43.6)	59 : 15 (79.7)***	66 : 24 (73.3)***
NF4	10 : 6 (62.5)	16 : 9 (64.0)	26 : 15 (63.4)
NF5	34 : 34 (50.0)	4 : 0 (100.0)[NT]	38 : 34 (52.8)
NF6	10 : 14 (41.7)	18 : 5 (78.3)**	28 : 19 (59.6)
NF7	13 : 4 (76.5)*	8 : 22 (26.7)*	21 : 26 (44.7)
NF8	NT	24 : 11 (68.6)*	24 : 11 (68.6)*
NF9	12 : 5 (70.6)	NT	12 : 5 (70.6)
Total	116 : 97 (54.5)	154 : 83 (65.0)***	270 : 180 (60.0)***

NT: not tested.

* Significantly different from 1:1 sex ratio (P<0.05, chi-square goodness-of-fit analysis).

** Significantly different from 1:1 sex ratio (P<0.01, chi-square goodness-of-fit analysis).

*** Significantly different from 1:1 sex ratio (P<0.001, chi-square goodness-of-fit analysis).

Maternal influence on progeny sex ratios was obvious in crosses between clonal males and normal females, and between neomales and normal females. However, no clear paternal influence was observed. Thus, the sex ratio data from the progeny of two normal males (NM1 and NM2) that were mated to 6 normal females (NF1, NF2, NF3, NF4, NF6 and NF7) were further analysed to determine whether the parental influences observed here were caused only by maternal side or also by paternal side (data from Table 2.6). Regression analysis revealed a negative correlation (r^2=0.7718, $P<0.05$, ANOVA) between the sex ratios of progenies from two males (NM1 and NM2), indicating a strong paternal influence on progeny sex ratios (Fig. 2.2).

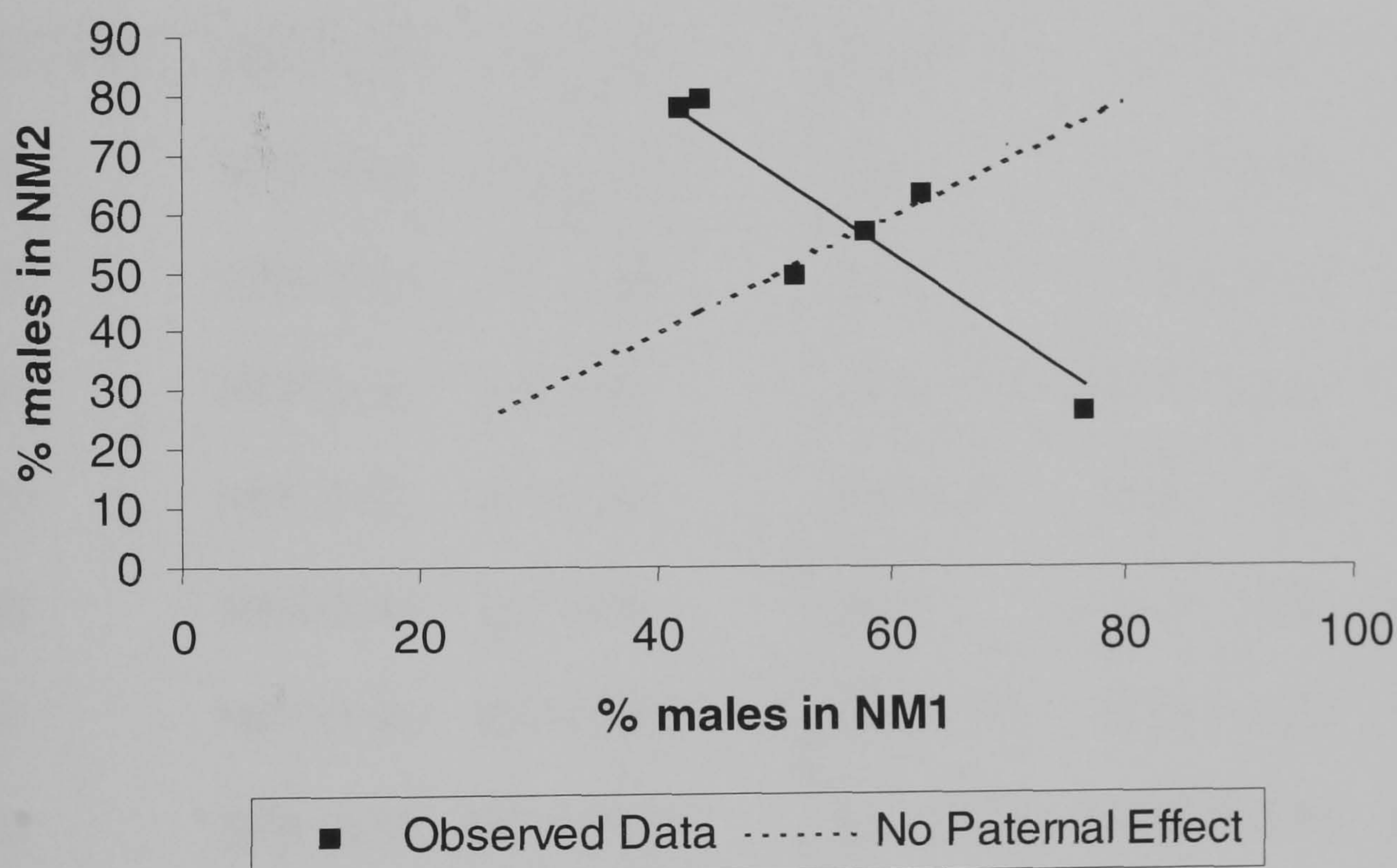


Fig. 2.2. Regression analysis of progeny sex ratios from two normal males that were mated to 6 normal females (data from Table 2.6). If there is no parental influence at all, theoretically sex ratios should be 1:1. If there is only maternal influence, the sex ratios between two males should show a positive correlation. This analysis indicates that the sex ratios observed are the consequence of an interaction between maternal factors and paternal factors. The broken line (-----) indicates a theoretical correlation between the sex ratios when there are no paternal influences.

2.3.6. Crosses between YY males and normal females or YY females

All crosses between YY parents or YY and XX resulted in 100% males with two exceptions of 95 and 96.7% (2 out of 18 crosses) (Table 2.7). Sex ratios were all significantly different from 1:1 (P<0.001, χ^2 values not shown). Among 393 progenies sexed, only 2 fish were identified as females. No parental influence was found in any of these crosses.

Table 2.7. Sex ratios from the crosses between YY males to normal females (XX) or YY female. Ratios expressed as male:female, with percentage of males in parentheses

Parents		Progeny	Parents		Progeny
♂	♀	Sex ratios	♂	♀	Sex ratios
SM1 (YY)	NF1 (XX)	13:0 (100)	SM1 (YY)	E2-F-1 (YY)	30:0 (100)
SM1	NF2 (XX)	20:0 (100)	SM1	E2-F-2 (YY)	126:0 (100)
SM1	NF3 (XX)	45:0 (100)	SM1	E2-F-3 (YY)	73:0 (100)
SM1	NF4 (XX)	6:0 (100)	SM2 (YY)	NF3 (XX)	15:0 (100)
SM1	NF5 (XX)	41:0 (100)	SM3 (YY)	NF10 (XX)	20:1 (95.2)
SM1	NF6 (XX)	3:0 (100)	SM3	NF12 (XX)	29:0 (100)
SM1	NF7 (XX)	40:0 (100)	SM4 (YY)	NF10 (XX)	14:0 (100)
SM1	NF8 (XX)	30:0 (100)	SM5 (YY)	NF12 (XX)	29:1 (96.7)
Total					391:2 (99.5)

2.3.7. Survival rates versus sex ratios

Survival rates of progenies from all crosses were 3.7-100.0% (42.1±28.9% in genetically all female groups), 6.7-81.3% (33.2±20.3% in genetically mixed sex groups) and 5.6-

100.0% (57.2±27.8% in genetically all male groups) (Fig. 2.3). No statistically significant relationship between survival rates and sex ratios of progenies was observed in any genotypic groups when the data were subjected to regression analysis ($r^2<0.100$ and $P>0.05$ for all).

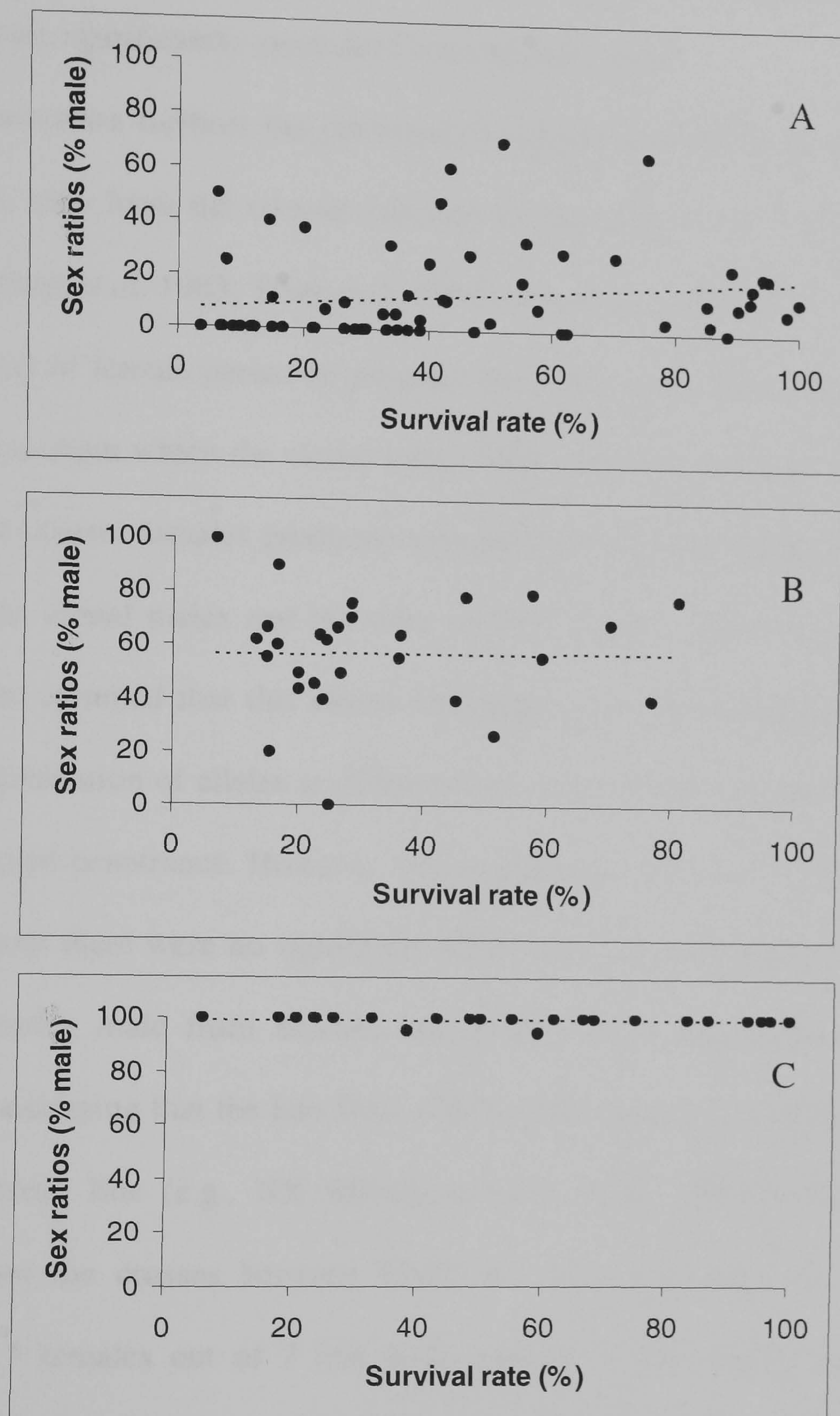


Fig. 2.3. Regression analysis of progeny sex ratios and survival rates. A: genetically all female progeny; B: mixed sex groups, C: genetically all male progeny.

2.4 Discussion

There appears to be strong parental influences on progeny sex ratios in this species. Not only in normal crosses but also in crosses using gynogenetic clonal males or neomales that should produce all genetically female progeny under the same temperature, sex ratios of progenies were significantly associated with parental pairs.

This observation furthers the previously proposed possibility of parental influence on progeny sex ratio from the crosses between normal males and normal females in tilapia species (Shelton *et al.* 1983; Tuan *et al.* 1999). Recently, Sarder *et al.* (1999) also observed a strong effect of female parent on progeny sex ratios from one clonal line (006 046 539) of *O. niloticus* from which the clonal males CM1 and CM2 belong. In their study, crosses to one of the control females produced only female offspring, while crosses to the mitotic mother of the clonal males and the other control female produced a high percentage of males. It was assumed that this clonal line might have been fixed genetically for some allele, or combination of alleles at different loci, which cause female to male sex reversal but with limited penetrance. However, this explanation does not exactly fit to the present results, because there were no significant differences between males from this line (CM1 and CM2) and a male from another line (CM3) when their progeny sex ratios were analysed. Considering that the line from which CM3 came was identified as an all female producing clonal line (e.g., XX SRSR) in their study, 100% female progenies were expected from the crosses between CM3 and normal females. Contradictory to this expectation, 5 females out of 7 that were crossed to this clonal male produced male progenies (3.4-17.9%). CM1 and CM2 showed the same pattern of progeny sex ratios as CM3 when crossed to the same 7 or 8 normal females. On the basis of these observations, it is assumed that CM1, CM2 and CM3 all have the same genotype (e.g., XX SRSR), and

that the unexpected males observed here may have been caused by other factors such as environments or genetic/non-genetic parental infuences.

In the work of Sarder *et al.* (1999), the founder of clonal line 006 046 539 and a normal female (11C) showed a high percentage of males and may have been the same cases of extreme maternal influence. NF10 in the present study also showed the extreme case of maternal influence. Excess male progenies, produced from two females, were also observed by Mair *et al.* (1991a), but these authors concluded these two females were 'naturally sex reversed' XY fish on the basis of progeny testing results. However, if we suppose that the sex ratio altering factor of the female is inherited, progeny testing results should also show the same skewed sex ratios. Cytological or molecular studies in future would have to judge whether these outlier females are 'naturally sex reversed' XY fish or true XX normal female with other sex ratio altering factors. It is not impossible that NF10 was one of the cases of 'naturally sex reversed XY female'. However, other normal females that produced high percentages of female progeny when crossed to clonal males also showed strong parental influence. Thus, it is not likely that the parental influences observed throughout this experiment was caused by 'naturally sex reversed' XY females.

Analysis of sex ratios from repeated crosses and relationship between survival rates and sex ratios also support the possibility of parental influence. Results from repeated crosses of the same parents in this study are consistent with the suggestion that progeny sex ratios in *O. niloticus* are stable and reproducible (Wohlfarth and Wedekind 1991). Sex ratios from repeated crosses of the same parents, however, were heterogeneous in another study of the same species (Tuan *et al.* 1999). This discrepancy may have been created by different experimental conditions. The study of Tuan *et al.* (1999) was carried out in fertilised earthen ponds where temperature influence is suspected while the present study was carried out in a recirculating system where temperature was maintained at 28°C, well

below the TSD threshold for this species. The stability of sex ratios from repeated crosses in this study implies that the varying sex ratios from different parents must have been derived by more than chance. In addition, survival rates did not appear to be the main cause of sex ratio variation in this study. None of the genotypic groups showed significant association of survival rate with progeny sex ratio.

Table 2.8 summaries the parental influences observed in this study. In the crosses between clonal males and normal females, it seems likely that the sex ratio altering factors were introduced from the normal females, since clonal male × clonal female crosses produced 100% female progenies. However, paternal influence was also noticed from normal male × normal female crosses. In these crosses, a strong effect of the interaction between maternal and paternal factors was observed (Fig. 2.2). No parental influence was found in the crosses between YY males and normal females (XX) or YY females. This supports an XX/XY sex determining system in this species.

Table 2.8. Summaries of parental influences on progeny sex ratios observed in this study

Types of Crosses	Results (progeny sex ratios)	Comments
Clonal males × Clonal females (XX × XX)	100% females	No parental influences
Clonal males × Normal females (XX × XX)	0 – 33.3% males depending on female parents	Maternal influence No paternal influence
Neomales × Normal females (XX × XX)	0 – 20.6% males (sex ratio from an extreme, NF10 not included)	Mild maternal influence No paternal influence
Normal males × Normal females (XY × XX)	26.7 – 79.7% males depending on both male and female parents	Maternal influence Paternal influence
YY males × Normal females (YY × XX)	98.8% males	No parental influence
YY females × YY females (YY × YY)	100% males	No parental influence

Parental influence appears to override the XX/XY sex determining system in some crosses. However, at the moment, in fish there is no evidence of what the parental influence might be. Parental influences on progeny sex ratios in fish have been noticed in several studies (Conover and Kynard 1981; Shelton *et al.* 1983; Wohlfarth and Wedekind 1991; Sarder *et al.* 1999; Tuan *et al.* 1999), and most of them were explained in favour of a polygenic sex determining theory. At least, all of them were discussed on the basis of genetics. However, it should be questioned that "sex ratios" do reflect only the consequences of genetic sex determination?

Assume that there are non-genetic parental influences, how could the parents alter progeny sex ratios against the genetic sex determination system? Can there be any sex filtering system in the maternal or paternal side? It is too early to make any firm hypothesis for fish, but several possibilities can be suggested based on an XX/XY sex determining system as suggested in mammals (Hardy 1997). First, the performance of sperm that carry X chromosomes could differ from that of sperm with Y chromosomes depending on the parents (paternal influence) ("Pre-fertilisation control"). Second, eggs may be able to discriminate X bearing sperm and Y bearing sperm by selectively accommodating sperm (maternal influence) ("Pre-fertilisation control"). Third, the composition of X and Y carrying sperms in semen may vary from male to male (paternal influence) ("Pre-fertilisation control"). Fourth, when females are in poor condition, they would produce poor quality eggs and the viability of male embryos might differ from female embryos, resulting in sex-biased mortality during very early developmental stages (maternal influence). Lastly, levels of maternal steroid hormones vary with its physiological condition. It would result in differential hormone deposition into eggs, causing hormonal sex-reversal when the hormone contents are extremely high or low (maternal influence).

Parental influences on sex ratios of offsprings have been observed in many mammals including humans (Moses *et al.* 1995; Grant 1996; James 1996; Manning *et al.* 1996; Monard *et al.* 1997; Andersson and Bergstrom 1998; Fisher 1999; Hewison *et al.* 1999; Kruuk *et al.* 1999; Nonaka *et al.* 1999). Does this imply that humans have a polygenic sex determining system or some strong sex modifying autosomal loci? The answer seems "No". In birds and mammals that have gonosomal sex determining systems, the nutritional status of female parents seemed to be the main contributor of parental influence. A bird, the zebra finch *Poephila guttata* showed significantly high percentages of males when female birds were in better body condition, while females in poorer condition hatched an equal sex ratio (Bradbury and Blakey 1998). In the experiment, female body condition was manipulated by feeding females with different diets for 3 months before allowing them to breed. A similar maternal influence was also suggested in wild birds (Nager *et al.* 1999). In woodrats, sex ratios were strongly correlated with prebreeding body weight in adult mothers, and authors suggested that adult woodrats adjust sex ratio at conception or during early gestation in relation to maternal condition (Moses *et al.* 1995). Maternal malnutrition was also associated with low male/female ratio of human children (Andersson and Bergstrom 1998). The nutritional status of female broodstock was not controlled in the present study. Thus, further studies on parental influence by manipulating nutritional status could produce useful information in this species. Seasonal variation and age of parents also need to be examined.

The absence of parental influences in crosses between clonal males and clonal females (Table 2.8) may infer that the parental influences observed in other crosses was the result of genetic differences between parental combinations. However, it could also be interpreted in favour of non-genetic parental influences. As summarised in Table 2.8, there were no paternal influences when males produce only one type of sperm (e.g., only

X-bearing sperm in gynogenetic clonal males and neomales or only Y-bearing sperm in YY males). In this case, selective accommodation of X- or Y bearing sperms by oocyte and competition between X- and Y-bearing sperms cannot be expected. Strong paternal effect observed in crosses between normal males and normal females support this interpretation. Maternal influences observed in some types of crosses may be the result of differential loading of steroids into eggs by female parents during oogenesis. However, it remains unknown whether the parental influences observed here was the consequences of genetic events or non-genetic events. The present findings attract further studies on parental influences on progeny sex ratios in fish.

Parental influence on progeny sex ratios are evident in this species although the cause of this influence is not clear. Thus, it is suggested that when sex ratio data from this species is interpreted, parental influence should be considered together with the influences of autosomal factors and temperature that could lead to misinterpretation. However, conclusions here once again remain with a monofactorial sex determination with autosomal, temperature and possible parental influence in this species since it is not yet clear whether the parental influence participates in the actual sex determining process or simply alters progeny sex ratios through secondary sex-filtering systems such as sperm selection by eggs.

Apart from identifying the parental influence, the best broodstock line was established for other studies in this thesis, based on progeny sex ratios, fecundity, and eggs and milt quality. NF1, NF2 and E2-F-3 were selected as female parents, and MTM1 and SM1 as male parents.

CHAPTER 3

EFFECT OF AROMATASE INHIBITOR

3.1. Introduction

Treating fish with exogenous sex steroid hormones during early developmental stages can cause sex-reversal against genotypic sex, or sterility. It has been proven in many fish species that exogenous androgens generally cause masculinising effects, while exogenous oestrogens cause feminising effects (Yamazaki 1983). However, the mechanism of action of these steroids on sex differentiation is, so far, not well understood. The exact nature of the role of the endogenous sex steroids in sex differentiation is not known (Rothbard *et al.* 1987; Feist and Schreck 1996), nor is it clear to what extent exogenous sex steroids mimic the role of endogenous sex steroids (Piferrer *et al.* 1994). A detailed understanding of steroid metabolism during sexual differentiation would be advantageous in furthering our knowledge of the role of steroids in this process.

Recently, the function of cytochrome P450 aromatase in sex determination has been examined, because it is the enzyme responsible for the aromatisation of androstenedione into estrone and testosterone into estradiol-17β (Jeyasuria *et al.* 1996). In some species, inhibiting the action of this enzyme caused masculinising effects similar to those caused by androgens (e.g. in the bullfrog *Rana catesbiana*, Yu *et al.* 1993; in the chicken *Gallus domesticus*, Elbrecht and Smith, 1992; Wartenburg *et al.* 1992; and in chinook salmon *Oncorhynchus tshawytscha*, Piferrer *et al.* 1994). Aromatisation in genetic females during early developmental stages appears to be a key step in ovarian ontogeny. Thus, the investigation of aromatase function in early developmental stages should increase our understanding of sex determination in fish.

The Nile tilapia, *Oreochromis niloticus,* is of major importance in aquaculture (FAO 1997). As the production of all male populations can eliminate unwanted reproduction during culture, the sex determination system of this species has been investigated and

various sex control techniques have been developed. These include treatment with exogenous sex steroids (McAndrew 1993; MacIntosh and Little 1995) and genetic manipulation of the sex determination system (Mair *et al.* 1997). *O. niloticus* has an XX/XY chromosomal sex determination mechanism (Jalabert *et al.*1974; Mair *et al.* 1991a; Carrasco *et al.* 1999; Chapter 2 in this thesis), although it has been shown that environmental factors (Baroiller *et al.* 1995a,b; 1996; Abucay *et al.* 1999), secondary (autosomal) genetic factors (Mair *et al.* 1991a; Hussain *et al.* 1994; Sarder *et al.* 1999) and parental factors (Chapter 2 in this thesis) can also influence sex determination.

Apart from preventing unwanted reproduction, sexual dimorphism in growth is also of interest in this species. Numerous studies have been conducted to investigate the difference of growth performance between male and female tilapia for several decades due to its economic importance (Marr *et al.* 1966; Fryer and Iles 1972; Lowe-McConnell 1987; McAndrew and Majumdar 1989; Palada-de Vera and Eknath 1993; Vera Cruz and Mair 1994; Marengoni *et al.* 1998; Schreiber *et al.* 1998). In tilapia culture, it is now generally believed that males grow faster than females. However, it is not clear when males start to grow faster than females since most studies conducted so far have focused on the juvenile stage to harvest size. Palada-de Vera and Eknath (1993) pointed out that the age or size at which divergence of growth performance between the sexes occurs has not been unequivocally established in tilapia.

O. niloticus is also a good model species in which to study the function of aromatase in relation to the mechanism of sex determination. It is possible to produce batches of XX fish, which should be all female, through gynogenesis (Penman *et al.* 1987) or crosses between neomales (hormonally masculinised genetic females, XX) and normal females (Jalabert *et al.* 1974), although such groups do not always conform exactly to the expected 100% female sex ratios (Mair *et al.* 1991a; Hussain *et al.* 1994; Baroiller 1996; Sarder *et*

al. 1999; Chapter 2 in this thesis). The profiles of sex steroids during early development have been studied (Rothbard *et al.* 1987; Hines *et al.* 1999) and the period of sex differentiation, on the basis of histological observation, has been proposed (Nakamura and Nagahama 1985 and 1989). Sex ratios can be assessed easily at the age of two or three months using a simple squash technique (Guerrero and Shelton 1974).

The first detectable signs of sexual differentiation in this species on the basis of histological observations is known to be around 26-30 dpf (Nakamura and Nagahama 1985 and 1989) and the sexually labile period lasts from first feeding to 35 dpf (Pandian and Sheela 1995) on the basis of the results from hormone treatments. However, Gale *et al.* (1999) successfully masculinized tilapia fry by immersion in 17α-methyldihydro-testosterone (MDHT) solution for 3 hours twice at 10 and 13 dpf, implying that the actual process of sex differentiation in this species could begin earlier. The timing of sex determination should be studied further. Further understanding of the labile period could improve current sex control techniques and also make a good start to study the exact mechanism of sex determination/differentiation in this species.

Aromatase inhibitors were developed as a human breast cancer therapy and have been successfully applied to study the action of steroids in lower vertebrates, including fish (Piferrer *et al.* 1994; Afonso *et al.* 1999). Various types of aromatase inhibitors have been developed and used, which include "competitive" and "suicide" types. Competitive inhibitors bind to the active site(s) of the enzymes and prevent product formation for as long as the inhibitor occupies the catalytic site. By contrast, suicide inhibitors initially compete with the natural substrate such as androstenedione or testosterone, and then induce the enzyme to yield reactive alkylating species that will irreversibly inactivate the enzyme (Perez and Borja 1992).

Competitive inhibitors are further divided into steroidal and non-steroidal inhibitors. Steroidal inhibitors are modified steroids that will compete with natural androgens and block aromatisation of them. Non-steroidal inhibitors are organic compounds that can bind to the heme iron of aromatase (Cole and Robinson 1990).

Fadrozole™ is a potent, non-steroidal, competitive inhibitor of aromatase which is relatively selective in its effects (Brodie 1991; Pérez and Borja 1992). It has previously been used in studies of aromatase activity in sex determination in the chinook salmon (Piferrer *et al.* 1994) and has been shown to inhibit oestrogen synthesis in maturing female coho salmon (*O. kisutch*) *in vivo* (Afonso *et al.* 1999).

In this study, a series of experiments was carried out in which batches of genetically female (XX) *O. niloticus* fry were treated with an aromatase inhibitor, Fadrozole™, either in the diet during the period when sexual differentiation is known to occur or by a short period of immersion. The objectives were to determine if inhibition of aromatase activity could cause genetic females to develop as phenotypic males and to narrow down the known sex determining period in this species. Additionally, growth performance between treatments were compared to see any possible effect of aromatase inhibitor on fish growth.

3.2. Materials and Methods

The broodstock used in this study were selected through the previous study and maintained as described in section 2.2 in Chapter 2 in this thesis.

Genetically female groups were produced by crosses between neomales and normal females. Eggs were manually stripped from ovulated females. Milt was collected into glass capillary tubes and either used directly for fertilisation or transferred to 1.5 ml plastic centrifuge tubes for short-term storage. After fertilisation, eggs were placed into 1 litre incubation jars and the resultant fry were used for each experiment following yolk sac resorption (11±1 days post-fertilisation, dpf = 7±1 days post-hatch, dph).

For the dietary treatments and controls, commercial trout pellets were mechanically ground and then passed through a 250 μm sieve. The appropriate amount of aromatase inhibitor (AI: Fadrozole™, CGS16949A, Novartis, Summit, NJ, USA) was dissolved in 99% ethanol and sprayed onto the powdered food (a total of 150 ml ethanol per kg of food). Food for control groups was prepared in the same way but using ethanol only. The food was then thoroughly mixed and left in a fume cupboard overnight to allow the ethanol to evaporate.

3.2.1. Experiment 1: Dietary treatments

Three trials were carried out involving AI treatments of one week duration within the first four weeks of exogenous feeding, using different concentrations and feeding regimes. The concentration of AI was determined in previous study (Haghpanah 1997; Kwon *et al.* 2000). Each treatment or control tank contained 50-57 fry at the start of the experiment. In trial-A, fish were fed four times per day, with AI at 500 $mgkg^{-1}$ in the diet. In trial-B, fish were fed twice per day, with AI at 500 $mgkg^{-1}$ in the diet. In trial-C, fish were fed twice

per day, with AI at 100 $mgkg^{-1}$ in the diet. Each trial contained three replicates of untreated controls, AI in week 1, AI in week 2, AI in week 3 and AI in week 4. Additionally, trials A and B had single positive controls (500 $mgkg^{-1}$ AI during weeks 1-4).

Water temperature was maintained at 26-27°C and feed was given *ad libitum*. Each group of fish was kept in an isolated aquarium (approximately 5 L volume). A partial water change (about 70% of water) was carried out every day to remove faeces and uneaten food and to maintain water quality. After feeding for thirty days with treated food, fish were transferred into tanks in a recirculating system (26-27°C) and grown until 10-12 weeks old when they were sexed by the aceto-carmine gonad-squashing method (Guerrero and Shelton 1974).

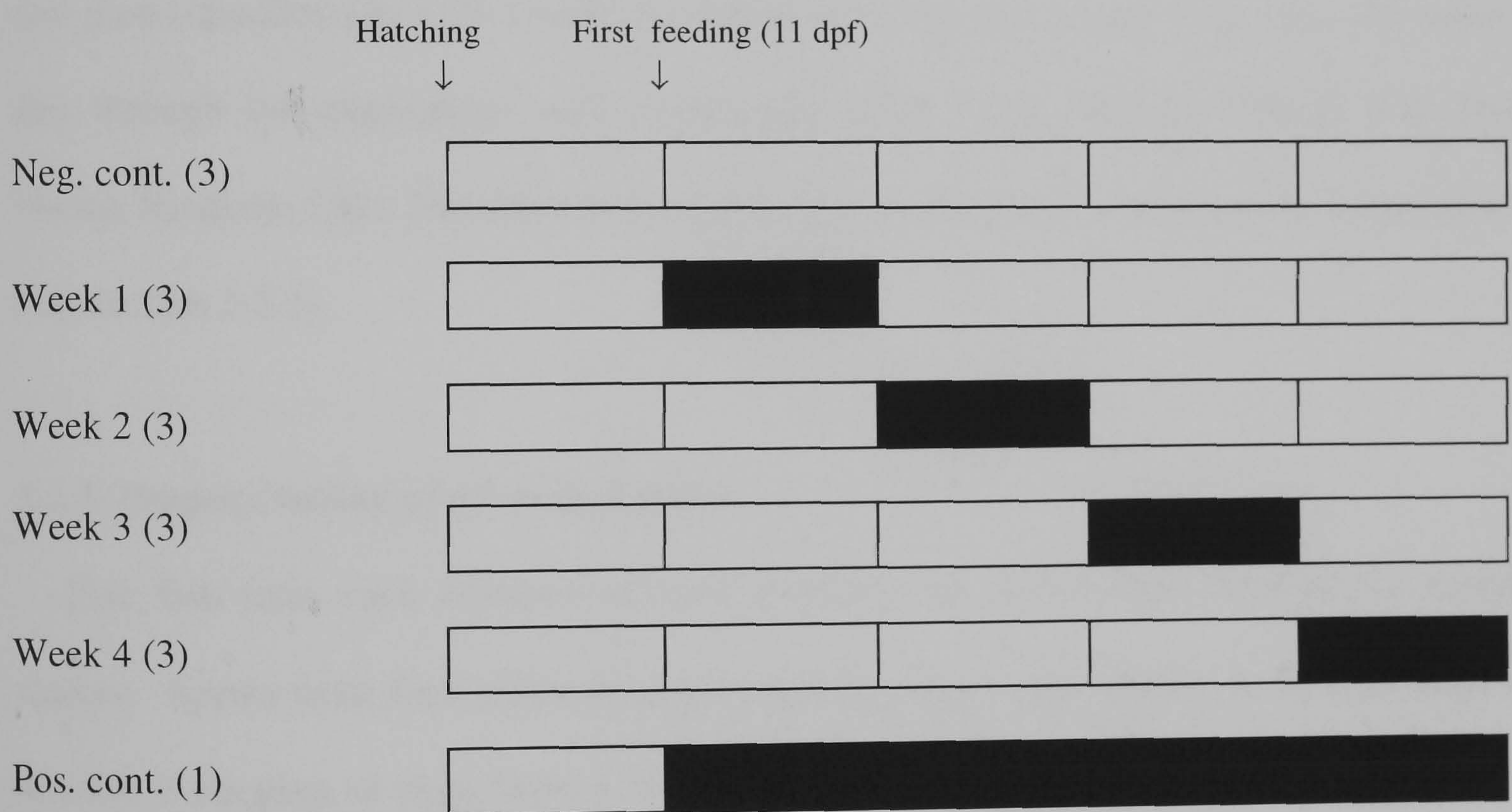


Fig. 3.1. Experimental design for dietary treatment with aromatase inhibitor (Experiment 1, Trial A). The numbers in parentheses indicate the number of replicates. Neg. cont.= negative control (no AI treatment); Pos. cont.= positive control (treatment for four weeks). Trial B and C were carried out identically to this design, but trial C did not have a positive control. (See the text for details).

3.2.2. Experiment 2: Immersion treatment

Genetically all female fry were immersed for 3 hours in a solution containing AI at 0, 50 or 100 mg L^{-1} once (at 11 or 13 dpf) or twice (at 11 and 13 dpf). AI stock solution was prepared in 99% ethanol and diluted to the experimental concentrations before use. All treatments including control groups contained equal volumes of ethanol. To facilitate the transfer of fish between treatments, each treatment group of fry was kept in a fine mesh fish carry net until they were housed in isolated aquaria in a recirculating system for ongrowing after immersion treatment. Fish were thoroughly washed three times after each immersion to prevent chemicals from being introduced into the rearing system. During immersion, the AI solutions were maintained at 26-27°C and aerated. Each replicate of the treated fish (n = 40) was grown in a 5 L plastic tank in a recirculating system for 2 months and then transferred to a 25 L tank for further growing until sexed. Fish were fed twice a day through the experiment with commercial trout foods (BOCM PAULS Fish Feed Group, Renfrew, UK). The other procedures were carried out as described in experiment 1 (see section 3.2.1).

3.2.3. Progeny testing of AI-treated males

Five fish from each replicate of each treatment in trial-A were retained for further studies. Sperm from four males from AI-treated groups and a control male were used to fertilise 5 aliquots of eggs from a single female. These batches of fertilised eggs were incubated separately and the fry were reared until they could be sexed.

3.2.4. Growth

To investigate possible effects of AI treatment on fish growth, total length (cm) and body weight (g) were measured at the end of trial-A when fish were sexed. Sexual dimorphism in growth was also assessed within each treatment.

3.2.5. Histology

Some of the retained fish from trial-A were dissected when they reached 6 month old, and gonads were removed for histology. Gonadal tissues were fixed in 10% phosphate-buffered formaldehyde solution for processing light microscopy. Sampled tissues were processed following the routine histological procedures in the Histopathology Laboratory, Institute of Aquaculture, University of Stirling. Briefly, fixed tissues were dehydrated in an automatic processor (Shandon Citadel Tissue Processor, Japan) and embedded in paraffin wax. Tissues were sectioned at 4-8 μm thickness and stained with haematoxylin and eosin for light microscopic observations.

3.2.6. Statistical analysis

All data are shown as mean ± S.E., although where appropriate they were subjected to arcsine transformation before being analysed by one-way ANOVA followed by Tukey's multiple range test. Sexual difference in growth within treatments in trial-A was analysed by t-test. A probability level of $P<0.05$ was used as the criterion for significance of differences between groups. Progeny sex ratios were tested by chi-square analysis against a 1:1 sex ratio (Zar 1984).

3.3. Results

3.3.1. Experiment 1: Dietary treatments

The survival rates (Table 3.1) were >80% in all but five groups: two replicates of the control (10.5, 38.6%) in trial-A; one replicate of the first week treatment (64.0%) in trial-B; two replicates of the first week treatment (71.7, 75.5%) and one replicate of the fourth week treatment (0.0%) in trial-C. There was no statistical association between mortality and AI treatment.

Table 3.1. Survival rates of tilapia *O. niloticus* fry treated with AI during the first, second, third and fourth week following yolk sac resorption (Experiment 1)

Trials	Treatments	Survival rates in each replicate (%)			Mean ± S.E.
Trial A	Neg. cont.	10.5	38.6	93.0	47.4 ± 24.2
	Week 1	91.2	98.2	100.0	96.5 ± 2.7
	Week 2	94.7	84.2	91.2	90.1 ± 3.1
	Week 3	94.7	80.7	93.0	89.5 ± 4.4
	Week 4	98.2	93.0	96.5	95.9 ± 1.5
	Pos. cont.	57.9			NA
Trial B	Neg. cont.	92.0	90.0	94.0	92.0 ± 1.2
	Week 1	64.0	96.0	94.0	84.7 ± 10.4
	Week 2	94.0	88.0	90.0	90.6 ± 1.8
	Week 3	94.0	90.0	94.0	92.7 ± 1.3
	Week 4	94.0	98.0	96.0	96.0 ± 1.2
	Pos. cont.	96.0			NA
Trial C	Neg. cont.	98.1	92.6	100.0	96.9 ± 2.3
	Week 1	71.7	92.6	75.5	79.9 ± 6.4
	Week 2	98.1	94.3	98.1	96.9 ± 1.3
	Week 3	94.3	90.6	90.6	91.8 ± 1.2
	Week 4	83.0	81.1		82.1 ± NA

Trial-A (500:4) = 500 $mgkg^{-1}$ AI, fed four times per day; Trial-B (500:2) = 500 $mgkg^{-1}$ AI, fed twice per day; Trial-C (100:2) = 100 $mgkg^{-1}$, fed twice per day. Neg. cont. = negative control (no AI treatment); Pos. cont. = positive control (500 $mgkg^{-1}$ AI treatment during weeks 1 – 4). Feeding frequencies for controls are the same as experimental groups in the same trial. NA: not applicable.

Final total length and body weight were analysed to assess the effect of AI on growth at the end of trial-A (Fig. 3.2A and B). Average total length and body weight were 6.00-6.29 cm and 3.88-4.46 g, and first week-treated groups were slightly larger both in length and weight than others, although there were no significant differences in growth between control groups and AI-treated groups in this experiment (P>0.25). However, further analysis revealed significant difference in growth between the sexes (Table 3.2). When pooled data were analysed, males were larger both in total length and body weight than females at around 100 dpf at the time of sexing (P<0.001). When analysed in each treatment, males were larger in all treatments with one exception of fourth week AI treated group where females were slightly larger than males, but differences were statistically different only in first week AI treated group and third week AI treated group. Size distribution of each sex were plotted in Fig. 3.3 and 3.4. The modal values of males in each treatment tended to be higher than that of females except third and fourth week treatments.

The sex ratios from this experiment are shown in Fig. 3.5. The negative control groups (no AI treatment) from trials A, B and C gave means of 14.5±6.1, 17.3±0.8 and 12.3±4.7% males respectively. The positive control groups (500 $mgkg^{-1}$ AI 1-4) contained 100% males. Treatment during week 1 produced sex ratios which were significantly different from the negative controls in all three trials. The only other one-week treatment which produced significantly more males than the negative control was week 2 in Trial-A (500 $mgkg^{-1}$).

Normal spermatogenesis was observed both in the group treated with AI in the first week and in the four week AI treated group (positive control) (Fig. 3.6B and C), while the negative control showed normal oogenesis (Fig. 3.6A). From histological study, no apparent abnormalities were observed in the spermatogenesis of the males sex-reversed by AI treatment.

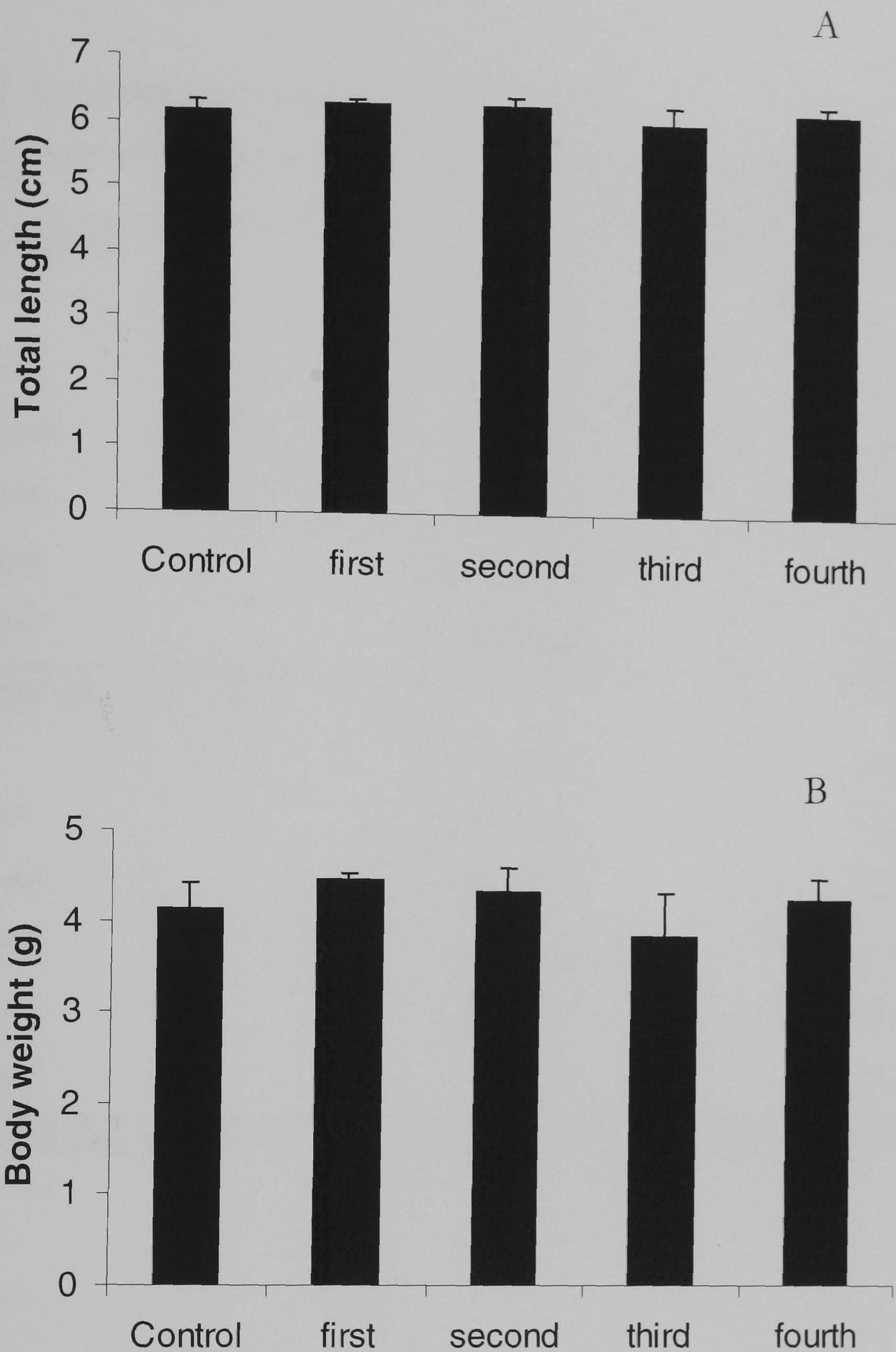


Fig. 3.2. Fish growth in groups of genetically female *O. niloticus* treated with 500 mg kg^{-1} AI during the first, second, third or fourth week following yolk sac resorption (Experiment 1, trial A). A: total length (cm), B: body weight (g). Each bar represents the mean ± S.E.

Table 3.2. Sexual difference in growth after AI treatment

Treatments	males	females
Total length (cm)		
Control	6.25±0.18	6.14±0.09
First week*	6.47±0.11	5.95±0.12
Second week	6.40±0.11	6.17±0.11
Third week*	6.48±0.16	5.93±0.09
Fourth week	6.20±0.16	6.27±0.08
Pooled data*	6.40±0.06	6.11±0.04
Body weight (g)		
Control	4.31±0.35	4.11±0.17
First week*	4.80±0.23	3.80±0.25
Second week	4.56±0.23	4.10±0.21
Third week*	4.69±0.34	3.66±0.16
Fourth week	4.04±0.29	4.33±0.17
Pooled data*	4.57±0.12	4.03±0.08

* Differences between the sexes are statistically significant (t-test, $P<0.05$). All data are shown as mean ± S.E.

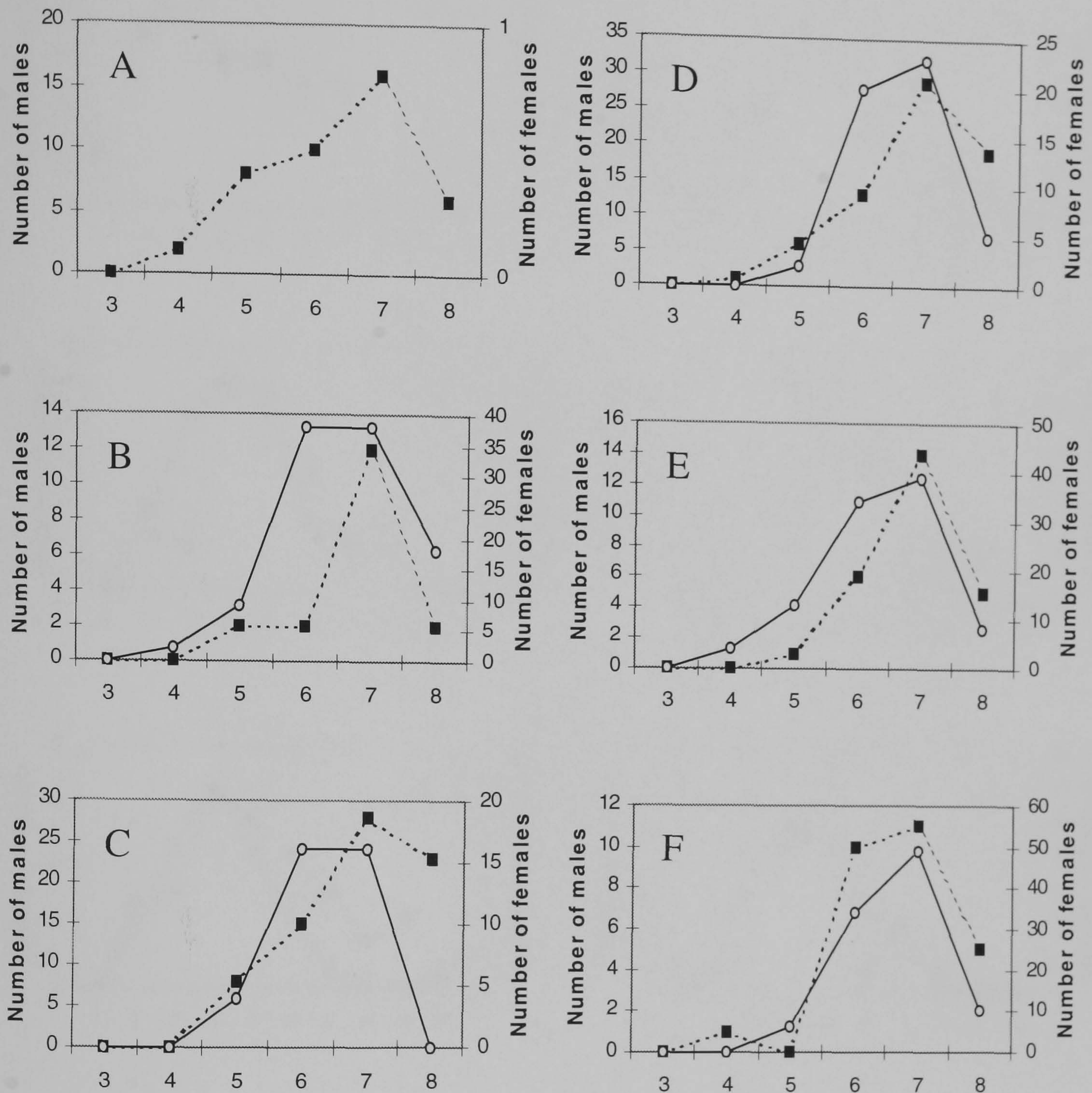


Fig. 3.3. Size distribution (X axis: total length, cm) of male (■) and female (O) fish in positive control (A), negative control (B), first week (C), second week (D), third week (E) and fourth week (F) in groups of genetically female *O. niloticus* treated with 500 mg kg^{-1} AI during the first, second, third or fourth week following yolk sac resorption (Experiment 1, trial-A). Fish were grouped by size. Each point represents the number of fish in each size group.

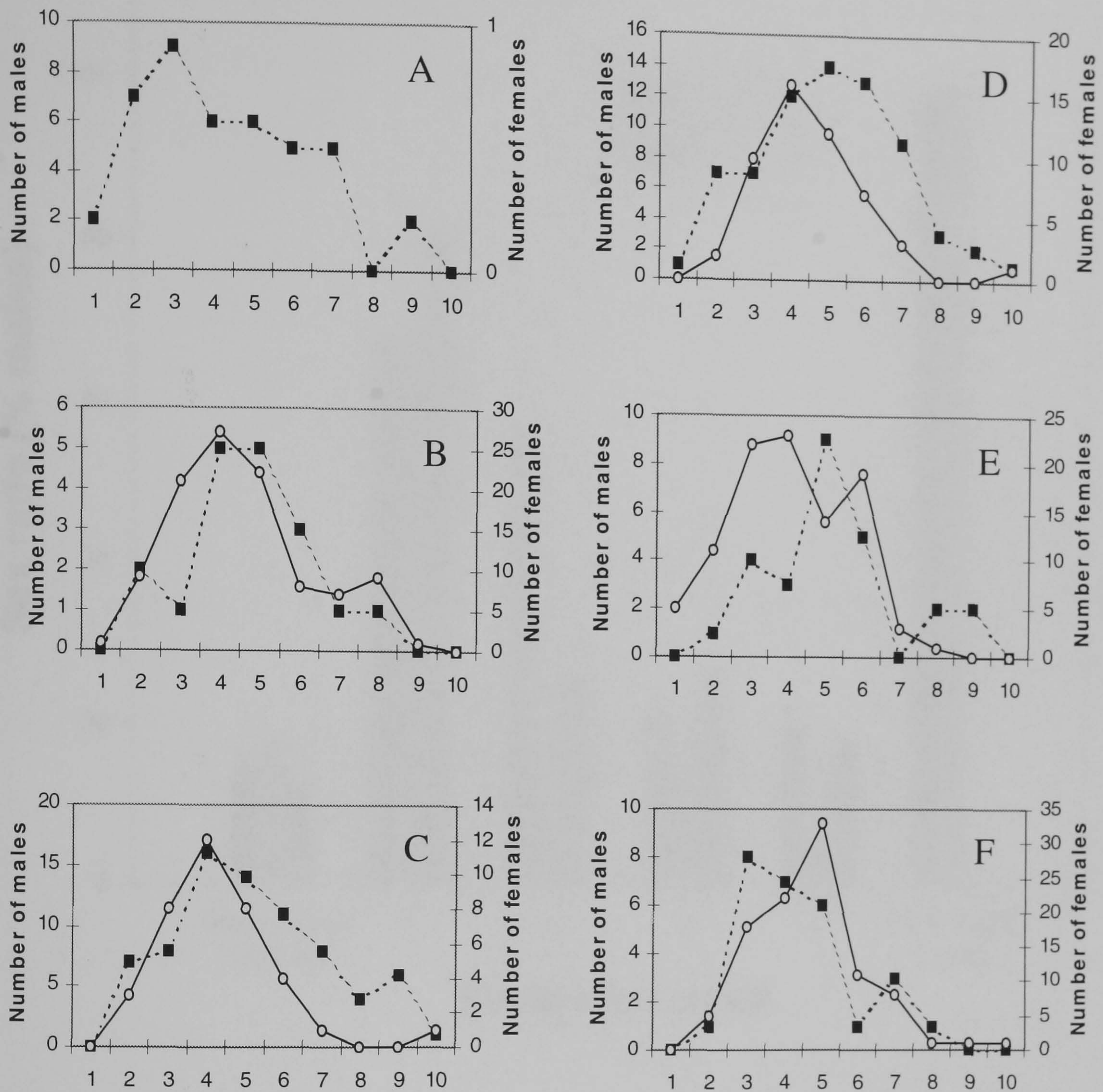


Fig. 3.4. Size distribution (X axis: body weight, g) of male (■) and female (O) fish in positive control (A), negative control (B), first week (C), second week (D), third week (E) and fourth week (F) in groups of genetically female *O. niloticus* treated with 500 mg kg^{-1} AI during the first, second, third or fourth week following yolk sac resorption (Experiment 1, trial-A). Fish were grouped by size. Each bar represents the number of fish in each size group.

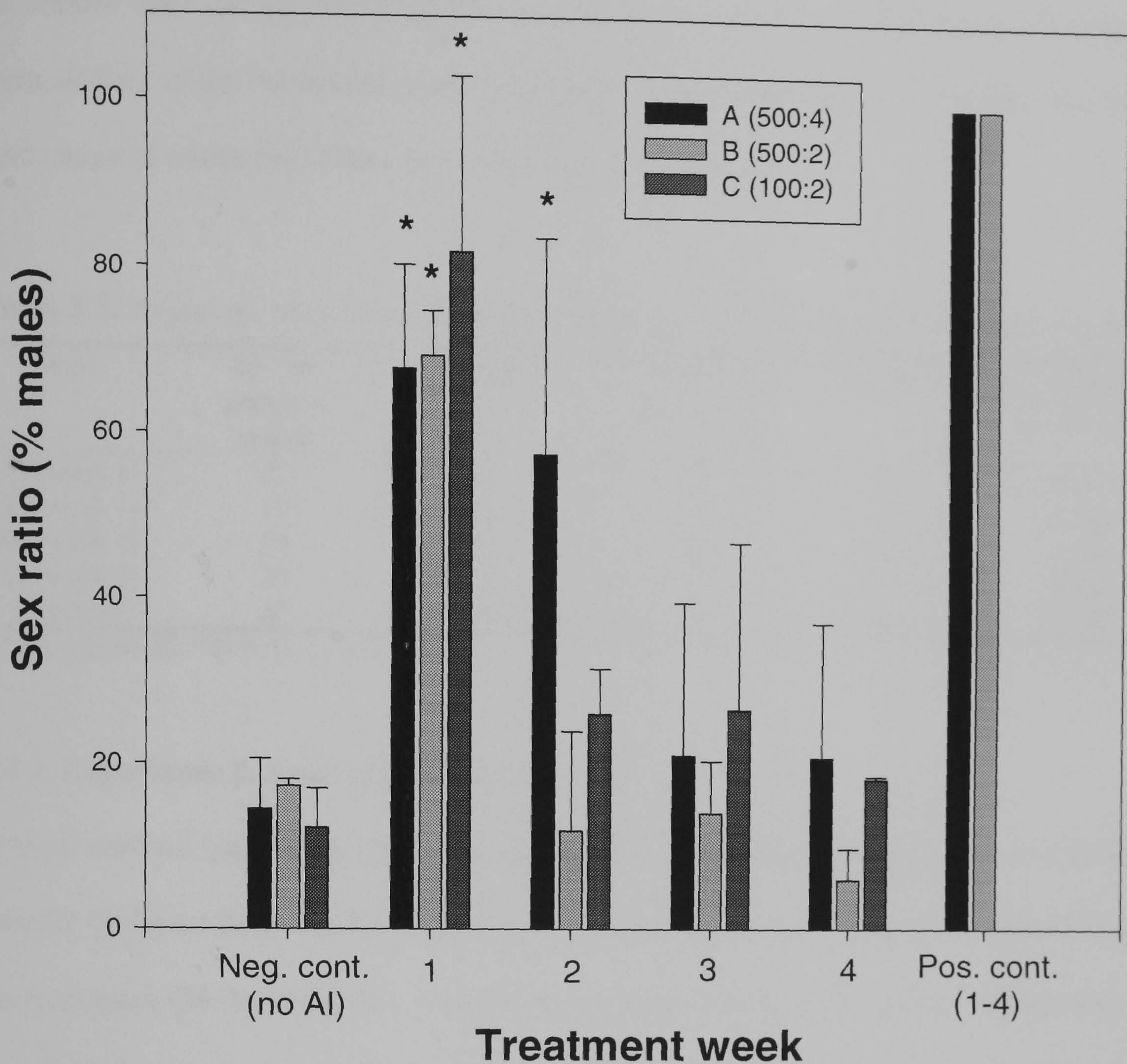


Fig. 3.5. Sex ratios in groups of genetically female *O. niloticus* treated with AI during the first, second, third or fourth week following yolk sac resorption (Experiment 1). Trial-A (500:4) = 500 mgkg^{-1} AI, fed four times per day; Trial-B (500:2) = 500 mgkg^{-1} AI, fed twice per day; Trial-C (100:2) = 100 mgkg^{-1}, fed twice per day. Neg. cont. = negative control (no AI treatment); Pos. cont. = positive control (500 mgkg^{-1} AI treatment during weeks 1 – 4). Feeding frequencies for controls are the same as experimental groups in the same trial. Treatments which gave sex ratios significantly different from negative control groups ($P<0.05$) are indicated by an asterisk (*). Each trial had three replicates except for the positive control. Each bar represents the mean ± S.E. except for the positive control.

Functional sex-reversal was further confirmed by progeny testing. Table 3.3 shows the progeny sex ratios from four AI-treated males and one control male. The progeny from the control male did not differ significantly from the expected 1:1 sex ratio, while progeny from all four of the AI-treated males contained a significant excess of females. The mean percentage of males from these four groups was 10.38±7.65.

Table 3.3. Progeny sex ratios from males from AI-treated groups (trial-A), crossed to a control female

Male	No. of progeny sexed	No. of males	No. of females	% males	$\chi 2$ (against 1:1 sex ratio)
AI (week 1)	22	2	20	9.1	14.73**
AI (week 1)	15	0	15	0.0	15.00**
AI (week 2)	34	11	23	32.4	4.24*
AI (week 2)	25	0	25	0.0	25.00**
Control	29	16	13	55.2	0.31^{ns}

ns: not significant; * P<0.05; ** P<0.001.

3.3.2. Experiment 2: Immersion treatment

Overall survival rates in the immersion experiment were poor (probably due to the delayed transfer of fry to larger tanks) (Table 3.4). Control groups (AI 0 mg L^{-1}) showed higher survival rates (53.3±5.8%) than treated groups (11dpf with AI 50 mg L^{-1}: 35.0±3.8%; 13 dpf with AI 50 mg L^{-1}: 30.0±4.3%; 11&13 dpf with AI 50 mg L^{-1}: 32.5±3.8%; 11 dpf with AI 100 mg L^{-1}: 39.2±5.8%; 13 dpf with 100 mg L^{-1}: 43.3±13.4%; 11&13 dpf with 100 mg L^{-1}: 40.0±14.2%). However, survival rate was not statistically associated with AI immersion treatment (P>0.25).

All treated groups responded positively to the AI treatment by immersion in terms of the percentage of males (Fig. 3.7). Immersion in AI solution once at 11 dpf did not significantly increase the percentage of males at the lower (50 mg L^{-1}) or higher (100 mg L^{-1}) AI concentrations (P>0.05), while immersion once at 13 dpf significantly increased the percentage of males at the higher concentration (P<0.05) but not at the lower

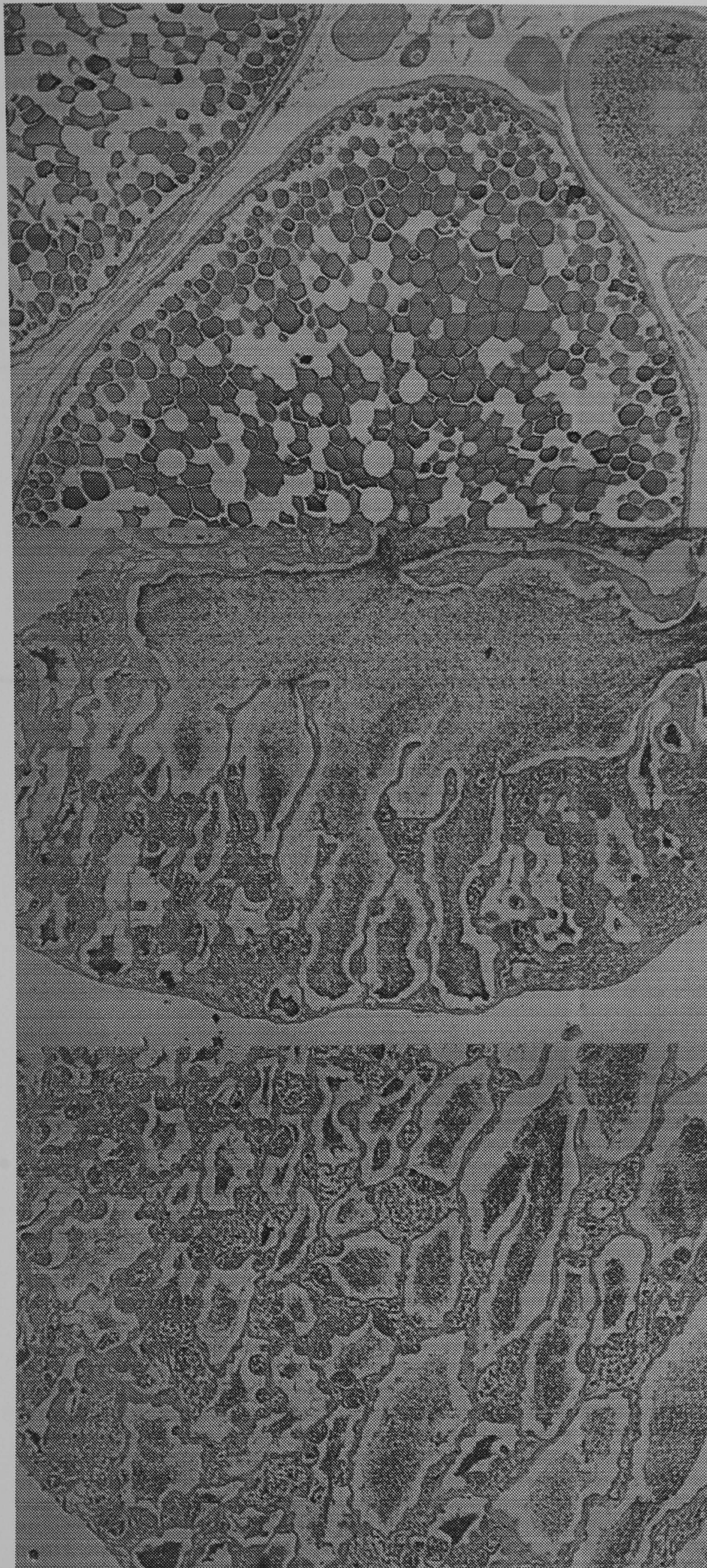


Fig. 3.6. Digital images of ovarian tissue section from negative control group (A), testis from group sex-reversed by AI treatment during the first week (B) and testis from group sex-reversed by AI treatment for four weeks - positive control (C). (Experiment 1, trial-A).

concentration. Immersion twice at 11 and 13 dpf significantly increased the percentage of males both at the lower and higher AI concentrations (P<0.05), demonstrating dose-dependency and cumulative effect of AI treatment.

Table 3.4. Survival rates of tilapia *O. niloticus* fry treated by immersion in 50 mg L^{-1} or 100 mg L^{-1} AI solution once at 11 or 13 dpf, or twice at 11 and 13 dpf (Experiment 2)

Treatments	Survival rates in each replicate (%)			Mean ± S.E.
Control	42.5	62.5	55.0	53.3 ± 5.8
11d - 50	40.0	37.5	27.5	35.0 ± 3.8
13d - 50	37.5	30.0	22.5	30.0 ± 4.3
11 & 13d - 50	25.0	37.5	35.0	32.5 ± 3.8
11d - 100	30.0	37.5	50.0	39.2 ± 5.8
13d - 100	27.5	32.5	70.0	43.3 ± 13.4
11 & 13d - 100	12.5	47.5	60.0	40.0 ± 14.2

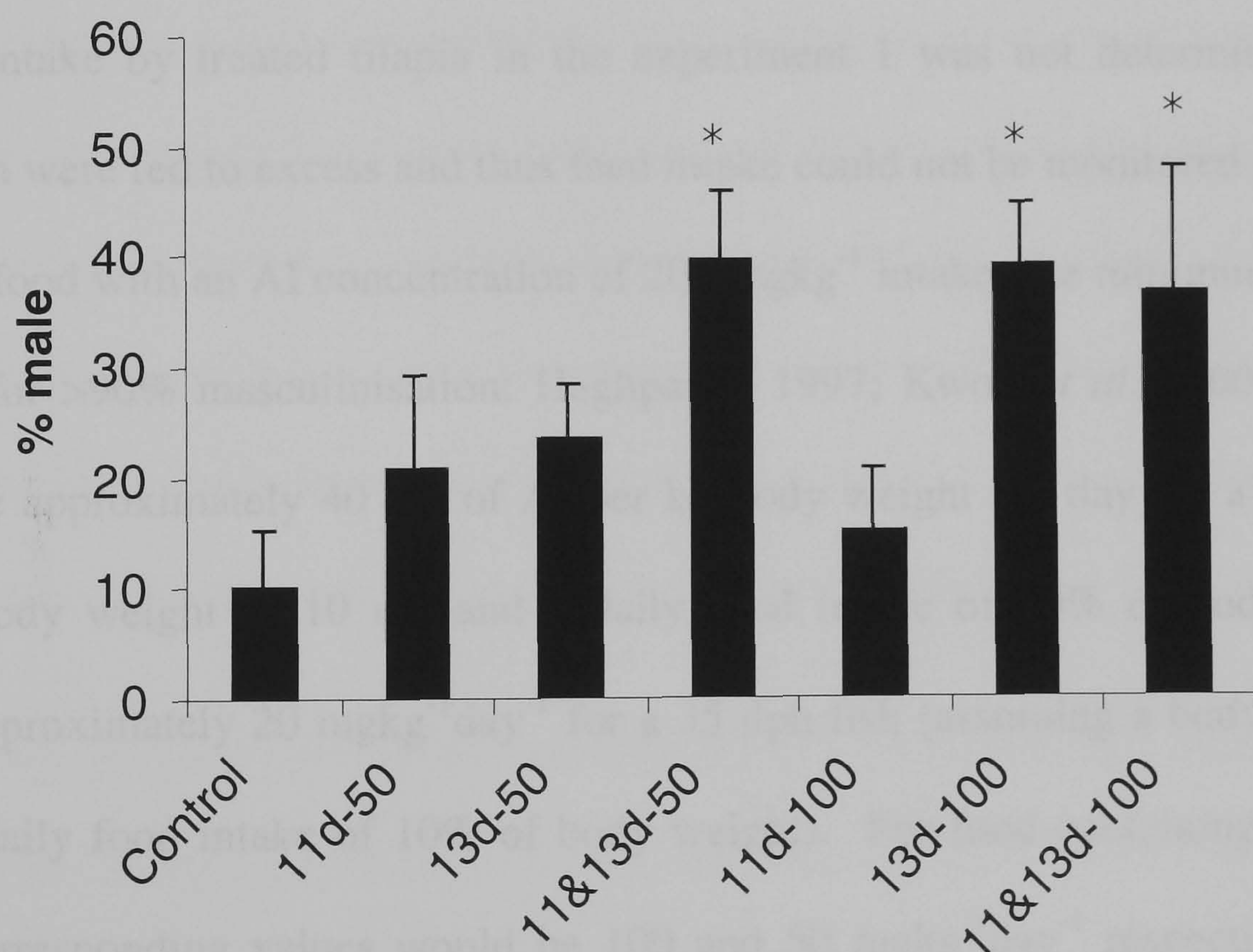


Fig. 3.7. Sex ratios in groups of genetically female *O. niloticus* treated by immersion in 50 mg L^{-1} or 100 mg L^{-1} AI solution once at 11 or 13 dpf, or twice at 11 and 13 dpf. * Significantly different from control (P<0.05). Each bar represents the mean ± S.E.

3.4. Discussion

Treatment with AI resulted in masculinisation of genetically female *O. niloticus* fry. The sensitivity of the sex determination system to AI declined during the first four weeks of exogenous feeding, with the first week being the most responsive. Furthermore, a single 3 hour immersion in AI solution or two immersions at 11 and/or 13 dpf significantly increased the percentage of males, strengthening the evidence for the importance of the first week in sex determination in this species. A cumulative effect and dose dependency were observed in immersion treatment, in which a single immersion at a lower dose did not significantly increase the percentage of males while higher dose did, and treatment once at 11 dpf or at 13 dpf at a lower dose did not increase the percentage of males but treatment twice at 11 and 13 dpf did.

Actual AI intake by treated tilapia in the experiment 1 was not determined, partly because the fish were fed to excess and thus feed intake could not be monitored accurately, but for treated food with an AI concentration of 200 $mgkg^{-1}$ intake (the minimum effective concentration for >90% masculinisation: Haghpanah 1997; Kwon *et al.* 2000) could be estimated to be approximately 40 mg of AI per kg body weight per day for a 7 dph fish (assuming a body weight of 10 mg and a daily food intake of 20% of body weight), declining to approximately 20 $mgkg^{-1}day^{-1}$ for a 35 dph fish (assuming a body weight of 75 mg and a daily food intake of 10% of body weight). For food containing AI at 500 $mgkg^{-1}$, the corresponding values would be 100 and 50 $mgkg^{-1}day^{-1}$ respectively (body weights from unpublished data and daily food intakes from Macintosh and Little 1995). These values are considerably higher than the amounts given to human patients, as described by Pérez and Borja (1992) (16 mg per day would be equivalent to 0.27 $mgkg^{-}$

^{1}day^{-1} assuming a body weight of 60 kg), but direct comparison may be relatively uninformative.

AI treatment did not cause any significant detrimental effects on fish growth and survival. The action of Fadrozole™ is reversible and 200 and 400 times more potent than another aromatase inhibitor of the same type, aminoglutethimide (Perez and Borja 1992), but Fadrozole™ is known to be less toxic than the latter. In humans, no severe, life-threatening or lethal toxicity was reported (Perez and Borja 1992). In this experiment, AI treatment at the first week seemed to even enhance fish growth though not significantly. Although there were no significant difference between control and treatments, sexual dimorphism for size was observed in one trial. Males are known to be larger than females in this species (Lowe-McConnell 1987), but it was not clear whether fry at early stages show sexual dimorphism in size (Palada-de Vera and Eknath 1993). The data shown here suggested that male tilapia may be growing faster than females at the age of 3 or 4 months. This idea could also explain the slightly larger size of the first week treated group since this group had a higher percentage of males than the other groups. However, it is not clear if inhibition of aromatisation is in someway related to fish growth. It may be worth noting that males were significantly larger than females in first week AI treated group while the trend was reversed in fourth week AI treated group where females were slightly larger.

Histology and progeny test results confirmed that functional and permanent sex reversal was achieved by AI treatments in this experiment. Four AI-treated males gave a mean of 10.38% males in crosses to a control female. Although one of these males gave a fairly high percentage of males (32.4%), this was significantly different from the sex ratio which would have been expected from an XY male and the sex ratios from these four males were similar to those obtained in comparable studies (e.g., Baroiller 1996).

Sexual differentiation appears to be fixed between 23 and 26 dph in *O. niloticus* at 25±1°C. Both ovarian differentiation, accompanied by the appearance of steroid producing cells (SPCs) (Nakamura and Nagahama 1985), and testicular differentiation, accompanied by the appearance of Leydig cells (Nakamura and Nagahama 1989) have been observed at this time. Although the present experiments were carried out at slightly higher temperatures (26-27°C), these are well below the minimum threshold (34-35°C) for temperature effects on sex determination in this species (Baroiller *et al.*1995a,b; 1996; Abucay *et al.* 1999).

Androgen treatments for 25 - 60 days duration (mostly around 30 days) starting at first feeding (usually 7 dph) are routinely used to produce all male populations (McAndrew 1993). Shorter treatments have, however, also been successful. MacIntosh and Little (1995) describe consistently high rates of sex reversal from MT treatments of 60 $mgkg^{-1}$ for 21 days from first feeding (i.e. 7-22 dph) and feminisation of YY male *O. niloticus* with diethylstilbestrol can be accomplished by dietary treatment for 20 days starting at 7 dph (Mair *et al.* 1997). For successful masculinisation of *O. niloticus* fry by dietary MT treatment, treatment must begin before 17 dph (MacIntosh and Little 1995). The timing of these steroid treatments is broadly in agreement with the histology-based studies quoted above. Fig. 3.8 summarises the timings derived from these histological and steroid studies.

The data presented here adds to the evidence for the role of sex steroids and aromatase in sex determination and differentiation in fish. Most previous studies on this in tilapia have focused on the effects of a variety of steroids on this process, as described in part above. Hines and Watts (1995) showed that an anti-oestrogen, Tamoxifen, could also cause masculinisation in hybrid *O. niloticus* x *O. aureus* fry. The effects of aromatase inhibitors have not been studied before in *O. niloticus*, but Fadrozole™ was shown to masculinise up

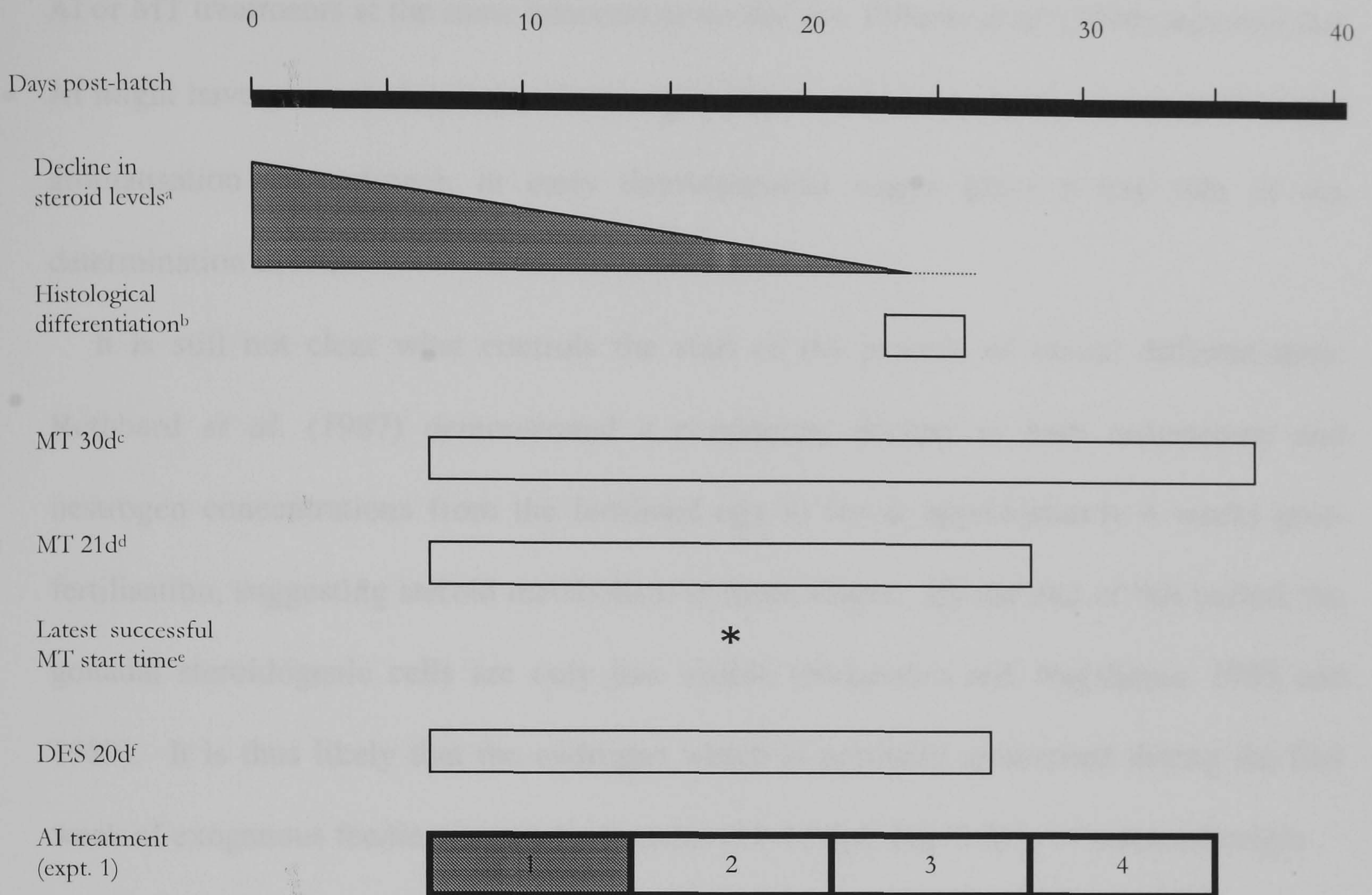


Fig. 3.8. Diagrammatic representation of chronology of steroid levels ([a] Rothbard *et al.*, 1987 – not to scale), visible gonadal differentiation at the histological level ([b] Nakamura and Nagahama, 1985 and 1989), effective periods for sex reversal using exogenous dietary steroids (MT = 17α-methyltestosterone; DES = diethylstilbestrol; [c] McAndrew, 1993; [d,e] Macintosh and Little,1995 ; [f] Mair *et al.*, 1997) and AI treatment periods in experiments 1. See Discussion for further details.

to 22% of genetically female chinook salmon, using an immersion treatment (Piferrer *et al.* 1994). Lower concentrations of AI were shown to interact with 17α-methyltestosterone (MT), in that a combined treatment resulted in high rates of masculinisation while separate AI or MT treatments at the same concentrations did not. Piferrer *et al.* (1994) assumed that AI might have prevented aromatisation of the 17α-methyltestosterone. It seems clear that aromatisation of androgen in early developmental stages plays a key role in sex determination in fish.

It is still not clear what controls the start of the process of sexual differentiation. Rothbard *et al.* (1987) demonstrated a continuous decline in both testosterone and oestrogen concentrations from the fertilized egg to fry at approximately 4 weeks post-fertilisation, suggesting steroid metabolism in these stages. By the end of this period, the gonadal steroidogenic cells are only just visible (Nakamura and Nagahama, 1985 and 1989). It is thus likely that the androgen which is normally aromatised during the first week of exogenous feeding in genetic females (11-17 dpf: Fig. 3.5) is of maternal origin.

The membrane of the endoplasmic reticulum in steroidogenic cells in several tissues including ovary, testis and brain are known to be major aromatisation sites (Silberzahn *et al.* 1988; Pasmanik and Callard 1989). As aromatase activity appears to be occurring in tilapia embryos before the development of gonadal steroidogenic cells, attention should be given to other tissues, such as the brain. In fact, it has been reported that the aromatase activity in the brain was 10 times higher than that of the ovary in adult goldfish, *Carassius auratus* (Pasmanik and Callard 1988a). Tchoudakova and Callard (1998) identified different aromatases in the brain and ovary of the goldfish. Francis (1992) suggested that, in fish, events in the brain may determine the fate of the gonads. Accordingly, it can be postulated that the brain of fry is the location of early aromatase activity, although there is no experimental evidence for this yet.

In conclusion, these results support the hypothesis that sex steroids are natural sex inducers, that aromatisation can take place before gonadal differentiation and that aromatisation can direct the fate of the gonad. In addition, the aromatase inhibitor used in this study, Fadrozole™, can be considered as a potent substance for further studies of sex determination and differentiation. Further studies on the timing and location of aromatase gene expression and activity, and on steroid profiles in both genetic sexes with and without steroid or AI treatments, would be beneficial.

Some answers became clear from this chapter as suggested above, but also many questions arose. Is the aromatase gene actually expressed before visible gonadal differentiation? If so, does a decisive aromatisation for sex determination take place during the first week after first feeding? Then, where is the decisive aromatisation site? Does aromatisation take place only in females? Is the ovarian aromatase the only aromatase in this species? And how does this aromatase work? In the following studies, these questions would be examined and discussed.

CHAPTER 4

CLONING AND DIFFERENTIAL EXPRESSION OF AROMATASE GENES

4.1. Introduction

Involvement of a steroidogenic enzyme, cytochrome P450 aromatse in sex determination and differentiation has been proposed in fish based on the masculinising effect of aromatase inhibitor (Piferrer *et al.* 1994; Kwon *et al.* 2000; Chapter 3) which is in agreement with the effect in other animals (Wartenburg *et al.* 1992; Yu *et al.* 1993). Up-regulation of ovarian aromatase gene expression in female juvenile fish was also reported in some species (a flounder *Paralichthys olivaceus*: Kitano *et al.* 1999; rainbow trout *Oncorhynchus mykiss*: Guiguen *et al.* 1999). However, a pharmacological effect of the aromatase inhibitors that have been used to inhibit aromatase action could be suspected. The up-regulation observed might also simply have reflected the result of the determined sex rather than the cause of sex differentiation since the up-regulation was detected from the gonads or histologically undifferentiated gonadal tissues. Moreover, when, where and how this aromatisation differentially occurs is not known.

Cytochrome (CYP19) P450 ovarian aromatase gene was the only known gene that codes for aromatase in fish until recently. However, a brain type isoform of aromatase gene was identified in goldfish *Carassius auratus* (Tchoudakova and Callard 1998) and tilapia *Oreochromis mossambicus* (Cruz and Canario 2000). The functional difference between brain type and ovarian type aromatase is not known, but it is possible that the brain type aromatase takes part in the sex determining process.

This chapter mainly consists of two parts: one is the identification of the brain type aromatase gene in *O. niloticus* and tissue specific expression of both brain type and ovarian type aromatase mRNA in adult fish; the other is differential expression of both brain and ovarian type aromatase mRNA between the sexes during ontogeny.

4.1.1. Identification of brain type aromatase gene and tissue specific gene expression

Teleost fish have 100-1000 fold higher levels of brain aromatase activity than mammals and the activity in the brain is around 10 fold higher than in the ovary (a marine teleost, *Myoxocepphalus*: Callard *et al.* 1981; goldfish, *C. auratus*: Pasmanik and Callard 1988a). Gelinas *et al.* (1998) showed that the brain type aromatase gene is the major contributor to the high enzyme activity in goldfish brain although ovarian type aromatase is also expressed in goldfish brain. Tchoudakova and Callard (1998) proved that eukaryotic cells (COS 7 African green monkey kidney cells) transfected with both brain and ovarian type aromatase gene constructs catalysed androgens to oestrogens, suggesting that there is no functional difference between the two isoforms in terms of aromatisation. However, it is not at all understood why the fish brain has to have such high levels of aromatase activity.

It is well understood that ovarian aromatase mRNA is involved in the reproductive cycle, especially in vitellogenesis (Nagahama *et al.* 1994). Brain aromatase mRNA in goldfish is also related to the reproductive cycle, showing seasonality and cyclicity in its expression (Gelinas *et al.* 1998), implying that both ovarian and brain type aromatase are related in the steroidal events for governing reproduction. In the same context, it could be postulated that these two aromatase genes might also be tightly related in the steroidal events in the sex determining process. The involvement of extragonadal tissue in the sex determining process in *O. niloticus* was suggested in the previous chapter (also Kwon *et al.* 2000) with the brain as the most promising candidate. Thus, it would be very informative to look at the level of brain type aromatase mRNA during ontogeny in studying the sex determining mechanism. However, no brain type aromatase gene has as yet been identified in this species.

First, an approach should be made to finding whether there is a possibility of the existence of a brain type aromatase gene in this species. A preliminary study revealed that

ovarian aromatase gene expression in the brain is much lower than in the ovary. This result does not conform to the previous finding that in fish, aromatase activity in the brain is 10 fold higher than that in the ovary (Pasmanik and Callard 1988a). Recently, a partial cDNA sequence of brain type aromatase gene was reported in the Mozambique tilapia *O. mossambicus* (unpublished, Genebank accession number: AF135850-1999). Considering that *O. niloticus* and *O. mossambicus* are very closely related species, it is natural to conclude that there would be a brain type aromatase gene in *O. niloticus* which differs from the ovarian aromatase gene.

The first objective of this study was to identify the brain type aromatase gene in *O. niloticus* and to develop a probe for this gene for further studies. The second objective was to investigate tissue specific expression of both brain and ovarian aromatase genes that would provide some clue to trace the crucial aromatisation site(s) for the sex determining process.

4.1.2. Differential expression of brain and ovarian aromatase genes during ontogeny

So far, no functional genetic differences between the sexes at the molecular level have been clearly demonstrated in fish (Baroiller *et al.* 1999) including this species where genetic sex determination was proposed (Mair *et al.* 1991a; Chapter 1 and 2 in this thesis). In mammals where sex is also determined genetically, a functional sex determining gene (SRY or Sry) has been identified (Sinclair *et al.* 1990) and many transcription factors (SF1, WT1, DAX1, SOX9 and MIS) that are involved in the cascade of the sex determining process have been proposed (see Chapter 1 in this thesis). Promisingly, most of these genes relate directly or indirectly to steroidogenesis via P450 aromatase (see also Chapter 1). Investigating aromatase gene expression at the transcriptional level during

ontogeny is likely to provide useful information for genetic differences between the sexes in fish.

The role of aromatase in the sex determining process has been extensively studied in reptiles (reviewed by Jeyasuria and Place 1998) where temperature-dependent sex determination has been widely demonstrated. In reptiles, aromatase activity is higher at female determining temperatures than at male determining temperatures, suggesting an important role of aromatase. However, the decisive aromatisation site and timing for sex determination is not clear. In the early embryogenesis of reptiles, steroidogenesis takes place in the adrenals, the mesonephros, and the liver, with some steroidogenic activity in the gut. However, there is little steroidogenesis in the genital ridge and the gonads of very early embryos (White and Thomas 1992). The brain was suspected as a primary aromatisation site for sex determination in reptiles (Jeyasuria and Place 1998). Aromatase mRNA was present in the brain at both male and female temperatures prior to the presence of aromatase mRNA in the presumptive ovary in their study. Additionally, the gonads of turtles that have been organ-cultured undergo differentiation *in vitro* but are not affected by "male" and "female" incubation temperatures, again implying that the signal for differentiation is under temperature control but comes from a source other than the gonad itself (Merchant-Larios and Villalpando 1990).

In fish, as pointed out earlier in this chapter, it is almost certain that sex steroid hormones are natural sex inducers (Yamamoto 1969). The involvement of aromatase in the sex differentiation process has been proposed (Piferrer *et al.* 1994; Guiguen *et al.* 1999; Kitano *et al.* 1999; Kwon *et al.* 2000; Chapter 3 of this thesis), but the initiation of aromatase gene expression in early embryogenesis is not known. Moreover, no strong hypotheses have been made yet for the timing and site of the decisive aromatisation.

The objective of this study was to determine the initiation of expression of both brain and ovarian type aromatase genes and to investigate the profile of expression of these genes during ontogeny, especially before and after the known gonadal differentiation period as defined by histological observation.

This study was facilitated by the well established broodstock lines that would produce all male and all female fry and the defined susceptible period to aromatase inhibitor from previous studies (Chapter 2 and Chapter 3 in this thesis).

4.2. Materials and Methods

4.2.1. Fish tissues

For cDNA library construction and Northern analysis, adult males and females (for each sex, n=10) were killed by an overdose of anaesthetic (ethyl p-amino-benzoate: Benzocaine). The brain and gonads were dissected out, immediately frozen in liquid nitrogen and kept until use. For tissue specific expression, a further three adult males and three adult females were killed in the same way. The brain, gonads, eyes, kidney, liver and spleen from each fish were dissected out and processed in the same way as above.

For the ontogeny of differential aromatase gene expression between the sexes, all male (XY) and female (XX) groups of tilapia fry were produced by crossing normal XX female to YY male and normal XX female to XX neomale, respectively. Fertilisation, incubation of embryos and maintenance of fry were carried out as described in section 2.2 in Chapter 2. Fish were sampled every day from fertilisation up to 10 days post fertilisation (dpf) to examine the initiation of both brain and ovarian aromatase mRNA expression, and every 4 days from 11 dpf up to 43 dpf to monitor the profile of the expression level of both brain and ovarian aromatase mRNA before and after gonadal differentiation. The sampling processes including killing, freezing and storing were the same as above. Fish were weighed when they were sampled. After the sampling period, the remaining fish were grown on until sexed.

4.2.2. RNA extraction

Total RNA was extracted using the RNAZOL method (Chomczynski and Sacchi 1987). Frozen tissue samples were homogenized using a mechanic tissue homogenizer in RNAZOL solution (2M guanidinium thiocyanate, 12.5 M sodium citrate pH 7.0, 0.5% N-

lauryl-sarcosinate, 0.72% β-mercaptoethanol, 50% water-saturated phenol, 0.2M sodium acetate pH 4.0) at tissue/RNAZOL ratio of 100 mg/2 ml. Approximately 0.1 volume of chloroform was added to each tissue homogenate, and vortexed vigourously for 15 seconds, subsequently kept on ice for 15 minutes, and then centrifuged at 4,000 g for 15 minutes at 4°C. The upper phase that contained RNA was transferred to a fresh tube and precipitated with an equal volume of isopropanol for more than 1 hour at –20°C. The supernatant was removed after centrifugation at 4,000 g for 10 minutes at 4°C, and the remaining white RNA pellet was washed twice with 1 ml of 75% ethanol (vortexed and spun for 8 minutes at room temperature between each wash). The pellet was dried down at room temperature for 15 minutes (no longer or the pellet becomes difficult to resuspend). The pellet was dissolved in an appropriate volume (depending on the size of the pellet) of nuclease free water. This solublisation was aided by heating up to 60°C for a few minutes as required.

Liver and egg samples were additionally treated with 0.5 ml of 4M lithium chloride to remove glycogen from the pellets after the isopropanol precipitation. Following vigorous vortexing, the insoluble RNA was pelleted at 4,000 g for 10 minutes in the microcentrifuge, the pellet was dissolved in 0.5 ml of RNAZOL solution, and then mixed with 50 μl of 2M sodium acetate (pH 4.0). The RNA was precipitated in 100% isopropanol for 30 minutes at –20°C and pelleted by centrifugation at 10,000g for 10 minutes at 4°C (Puissant and Houdebine 1990).

The concentration of extracted RNA was estimated from the OD 260 value measured by a spectrophotometer. The quality was checked by the OD 260/280 ratio and electrophoresis on an agarose gel (1%). Total RNA was used for Northern analysis, reverse transcription and further extraction of mRNA.

4.2.3. Identification of brain type aromatase gene and tissue specific gene expression

4.2.3.1. Construction of brain cDNA library

Poly(A)$^+$ RNA was purified from total RNA of brain tissue using an oligotex mRNA Mini Kit (QIAGEN). A brain cDNA library of *O. niloticus* was constructed using ZAP-cDNA Synthesis Kit (Stratagene).

Full details of the procedure can be found in the manufacturer's protocol. Briefly, first-strand cDNA was synthesized from 3 μg Poly(A)$^+$ RNA by Molony murine leukemia virus reverse transcriptase (MMLV-RT, Promega) (an aliquot of the first-strand reaction was carried out with α-^{32}P dNTP as a control). Second-strand cDNA was synthesized with second strand reaction buffer, dNTP mixture, α-^{32}P dNTP, Rnase H and DNA polymerase I. This second strand cDNA was made blunt end by cloned *Pfu* DNA polymerase and purified by phenol-chloroform extraction. The cDNA was then resuspended in a solution containing *Eco*R I adapters. Before proceeding, the first strand control reaction and 1μl of second strand reaction were run on an agarose gel with radio-labelled size marker to determine the size range of the cDNA. The size of both cDNA strands ranged approximately 500bp to 10000bp.

The *Eco*R I adapter was ligated to the cDNA using T4 DNA ligase and the *Eco*R I ends were then phosphorylated. The reaction mixture was then digested by *Xho* I and subjected to size fractionation. The cDNA reaction mixture was put through a sepharose CL-2B gel filtration column and collected in microcentrifuge tubes. The collected fractions of the desired sizes were pooled and purified by phenol-chloroform extraction, and then ligated into the Uni-Zap XR vector using T4 DNA ligase. The ligated cDNA was added to ZAP-cDNA Gigapack III Gold packaging extract (Stratagene) to enhance the efficiency of infection into *E. coli* cells. This cDNA was plated on the *E. coli* cell line XL1-Blue MRF'. About 100 000 plaques were obtained in the primary library. This cDNA library was

amplified and stored at 4°C for immediate use and -70°C for preservation.

4.2.3.2. PCR cloning of brain aromatase cDNA

DNA was purified from an aliquot of the cDNA library by proteinase K treatment and phenol-chloroform extraction, and used for PCR cloning. Two sets of primers (Table 4.1) were designed based on the brain aromatase cDNA sequence of the Mozambique tilapia *O. mossambicus* (Cruz and Canario 2000; Genebank accession number: AF135850). To determine the presence of brain aromatase transcripts in the constructed cDNA library, PCR was carried out using these primers and the product was sequenced. Ready-to-Go PCR beads (Amersham Pharmacia Biotech Inc.) were used for all following procedures in this study. The final reaction mixture contained 1.5 mM $MgCl_2$, Taq polymerase, reaction buffer, each primer, template, dNTP. PCR was initially performed using the following conditions: 1 cycle at 94°C for 2 minutes, 60°C for 1 minute, 72°C for 1 minute; 40 cycles at 94°C for 1 minute, 60°C for 30 seconds, 72°C for 1 minute; 1 cycle at 72°C for 6 minutes. However, the number of cycles and the annealing temperature were varied in different experiments (explained as required later).

The purified cDNA library was then amplified using these gene specific primers and universal sequencing primers (BF1→T7 and T3→BR2) by PCR. The PCR product of BF1→T7 and T3→BR2 were digested by *Xho* I and *Eco*R I, respectively. Both digested products were seperated on low melting point agarose gels. Target fragments were purified from the gel using glass fiber matrix column (Gel purification kit, Amersham Pharmacia Biotech, Inc.) and subcloned into T vector, modified from pBluescript II SK(+) by digestion with *Eco*R V to blunt the ends of the plasmid and adding dTTP to produce 3' overhanging ends (Mezei and Storts 1994). Transformation of these ligates was performed using Epicurian Coli XL-Gold Ultracompetent cells (Stratagene) following the

manufacturer's protocols. Transformed cells were plated on ampicillin agar medium with IPTG (isopropyl-1-thio-β-D-galactopyranoside) and X-gal (5-bromo-4-chloro-3-inodlyl-β-D-galactopyranoside) for blue/white color screening. White colonies were picked and screened by PCR with gene specific primers. Positive clones were used as templates for PCR with a gene specific primer + T3 or T7 primer.

Forward primer (BF1 or BF2) and T7 primer yielded a prominent specific band, but T3 primer and reverse primer (BR1 or BR2) did not yield the expected size of PCR product. More primers (Table 4.1) were designed based on *O. mossambicus* brain aromatase gene sequence to amplify further upstream 5'- sequence. PCR were carried out using the purified cDNA library as templates with primers, BF3 and BR1.

Table 4.1. Primers used for PCR cloning for brain aromatase gene of *O. niloticus*

Primers	Sequence (5'→3')
Forward primer 1 (BF1)	GACTTTGCAACAGAGCTCAT
Forward primer 2 (BF2)	CCCAGTCGTTACTTCCAGCC
Forward primer 3 (BF3)	TAGAGCGTCAGAAGTCACTG
Reverse primer 1 (BR1)	AGCATGAAGAAGAGGCTGAT
Reverse primer 2 (BR2)	TCGTGGGATGAAGCGCATGG
Reverse primer 3 (BR3)	ACACTTCAGAGGACCTGCTC
Reverse primer 4 (BR4)	ATGAGCTCTGTTGCAAAGTC

Size of PCR products: 123bp (BF1→BR1), 218bp (BF2→BR2), 657bp (BF1→BR2), 901bp (BF3→BR1).

4.2.3.3. Sequencing and sequence analysis

The PCR products were purified from the gel as described before and sequenced using an ABI automated sequencer based on the cycle sequencing method (Applied Biosystems).

The obtained nucleotide and deduced amino acid sequences were analysed using BCM search launcher (Baylor College of Medicine, Houston, TX) and NCBI blast search. A multiple sequence alignment program (ClustalW) was used to compare the deduced amino acid sequence from *O. niloticus* brain aromatase with those from brain and/or ovarian aromatase of other species.

4.2.3.4. Northern analysis

Both brain and ovarian total RNA (10 μg each) were resuspended in 10μl GFM buffer containing glyoxal, formamide and MOPS, and incubated at 55°C for 15 minutes. After mixing with 1μl of 10× loading buffer, these RNA samples were run on a 1% neutral gel in 1× MOPS buffer with a size marker alongside. The gel was then soaked for 30 minutes in 20× SSC. The RNA was transferred from the gel onto a positively charged nylon membrane (Hybond, Boehringer Mannheim) using the capillary transfer method in 20× SSC overnight. After transfer, the RNA on the membrane was immobilised by baking the membrane for 1 hour at 80°C.

The membrane was hybridised overnight at 68°C in 20 ml of Church-Gilbert solution (Church and Gilbert 1984) with the brain aromatase cDNA probe. The probe (657bp) was generated by PCR using primer BF1 and BR2, and labeled with α-^{32}P dCTP by nick translation (Nick Translation System, Promega). The membrane was washed three times over 2 hours at 68°C in 0.1× SSC/0.1% SDS and subjected to autoradiography to visualise hybridisation signals.

The size of the brain aromatase RNA transcript was estimated by comparison of the distance between the gel well and the hybridisation signal on the X-ray film with the RNA size markers on the original gel (SIGMA, 0.2-10kb).

4.2.3.5. RT-PCR analysis for tissue specific expression of aromatase mRNA

Total RNA isolated from the tissues of adult brain, kidney, eye, liver, spleen and gonads was treated with RNAse free DNAse I (Boehringer Mannheim). Each RNA (5μg) was reverse transcribed using MMLV-RT (Promega) following the standard procedure. Briefly, total RNA (5 μg) was transferred into a 0.2 ml microcentrifuge tube and 0.5 μg of either oligo $d(T)_{12-18}$ or oligo $d(T)_{15}$ (oligo $d(T)_{15}$ yielded better results) was added. Nuclease free water (DEPC treated water) was then added to final volume of 15 μl and incubated at 70°C for 5 minutes. After incubation, 5 μl of 5X MMRT reaction buffer and 4μl of 2.5mM of each dNTP mixture were added. The reaction mixture was incubated up at 42°C for 2 minutes, and 1 μl of MMRT (200 Units, Promega) was added. Reverse transcription was carried out at 42°C for 30 minutes, then at 65°C for 15 minutes. The resultant cDNA (1 μl) was used as template for subsequent PCR.

Several sets of ovarian aromatase gene primers were designed based on the *O. niloticus* ovarian aromatase cDNA sequence (Chang *et al.* 1997). After testing all these primers, one set of primers (forward primer, OF: 5'-CTGAGAATGTGACGCAGTGC-3'; reverse primer, OR: 5'-CAGCAGTGTCACCAAAATGG-3') was chosen for gene expression experiment. The PCR product from these primers was 490bp in length, and subsequent sequencing revealed that the product corresponded to the reported *O. niloticus* ovarian aromatase cDNA sequence. As a control, actin primers (forward primer, AF: 5'-AATCGTGCGTGACATCAAGG-3', reverse primer, AR: AGTATTTACGCTCAGGTGG G -3'; product size 392bp) were designed based on the actin cDNA sequence of a closely related species *O. mossambicus* (Genebank database accession number: Y18689). For brain aromatase gene primers, BF1 and BR2 were used (Table 4.1). PCR was carried out according to the conditions described above. PCR products were run on a 1% agarose gel

in 1× TBE buffer. Ethidium bromide (EtBr) was added both to the gel and running buffer at a concentration of 0.5 μg/ml. PCR products (3μl each) for the brain aromatase gene and the actin gene were loaded together into one lane for each tissue. In the same way, PCR products (3μl each) for the ovarian aromatase gene and the actin gene were also loaded together into one lane for each tissue. PCR products were visualised on a UV transilluminator (UVP) and photographed using a Polaroid camera (Genetic Research Instrumentation Ltd).

4.2.4. Differential expression of brain and ovarian aromatase genes during ontogeny

To estimate the differential expression level between the sexes and developmental stages, a semi-quantitative RT-PCR method was established (Fig. 4.1). Total RNA, isolated from the whole body of male and female groups collected throughout ontogeny, was treated with RNAse free DNAse I (Boehringer Mannheim). Each RNA aliquot (5μg) was reverse transcribed using oligo $d(T)_{15}$ following the standard procedure as described above. The resultant cDNA was purified using a DNA purification kit (Boehringer Mannheim) to remove enzymes, unincorporated dNTP and oligo d(T) primer.

The purified cDNA (0.5 μl of each sample) was mixed with 3 μl of 0.05 μg/ml EtBr and spotted on the surface of a transparent disposable petri dish to estimate the cDNA concentration of each sample. The petri dish was inverted and exposed to a UV transilluminator (hanging drop method). One cDNA sample was chosen as a standard. Using this cDNA, PCR was optimised to achieve a semi-quantitative response. The standard cDNA was subjected to serial dilution (1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024, 1:2048). Using these dilutions as templates, a series of PCRs was conducted with different number of PCR cycles (25, 30, 35 or 40 cycles) and with different annealing temperatures (55, 58, 60 or 63°C) for different gene specific primers.

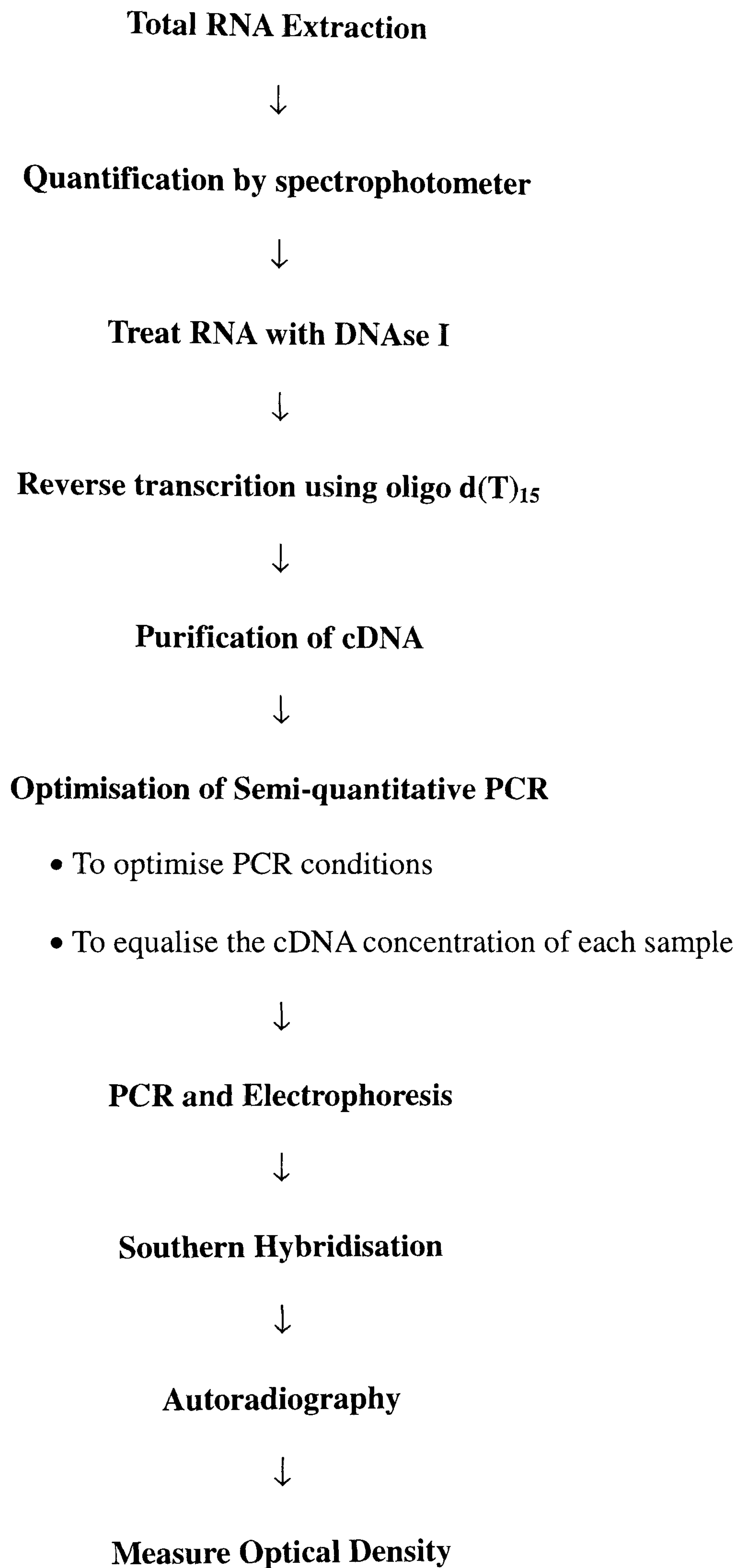


Fig. 4.1. Semi-quantitative RT-PCR process that used to study differential aromatase mRNA expression during ontogeny.

Through these PCRs, the range of cDNA concentration that responded quantitatively was determined. Since actin gene expression (or any other housekeeping gene) cannot cover 100% of the range between different samples, it was necessary to minimise the range of cDNA concentration between different samples. The concentrations of all cDNA samples were adjusted by diluting or concentrating (using mechanical concentrator) until the sample concentration fell into the detection range (Fig. 4.2). The optimal number of PCR cycles was determined as follows: 25 cycles for the actin gene, 35 cycles for the brain and ovarian aromatase genes. The optimal annealing temperature was determined as 60°C for all three genes.

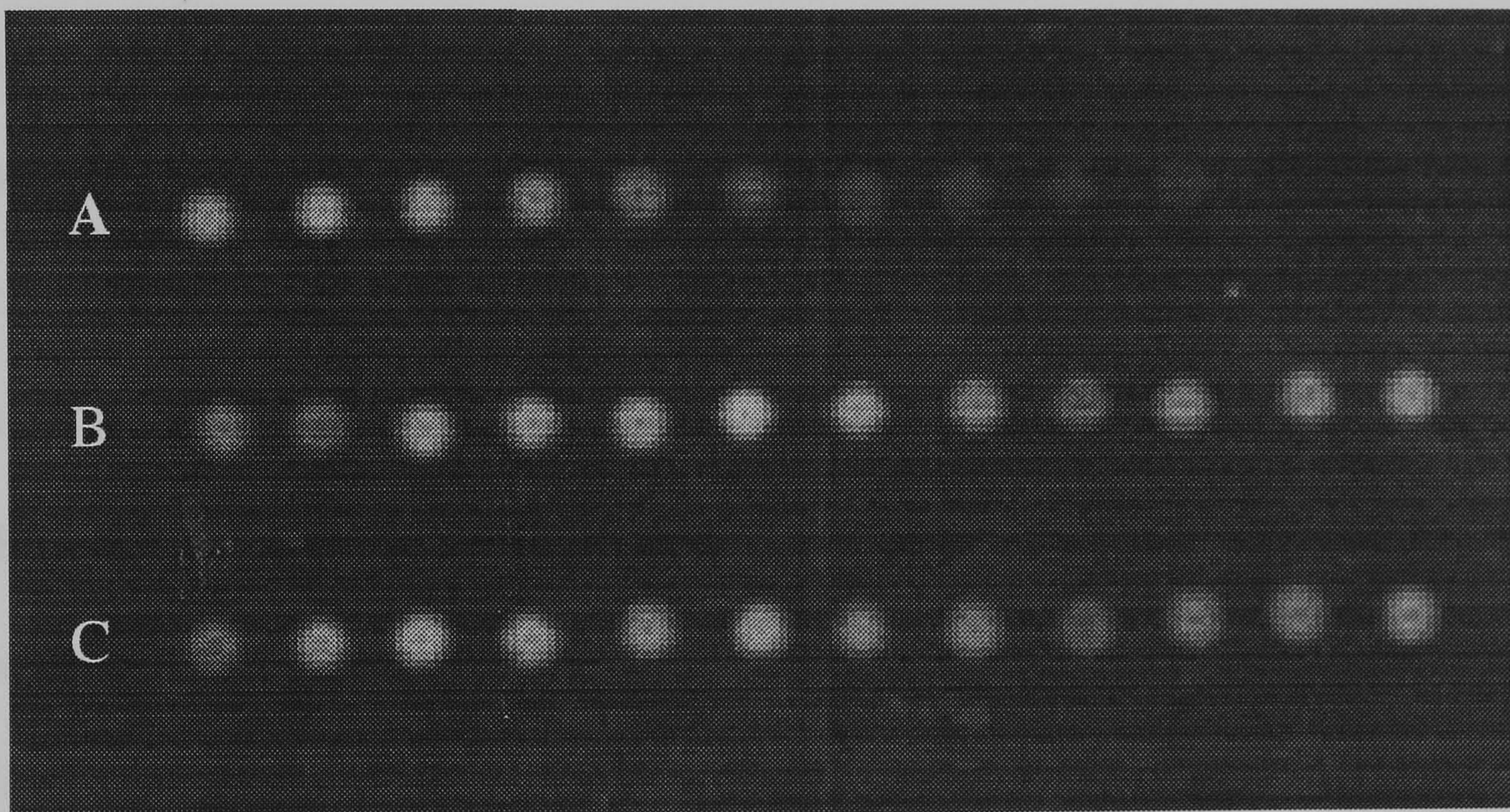


Fig. 4.2. A serial dilution of standard cDNA and optimised cDNA samples for semi-quantitative RT-PCR. The first row (A) shows standard serial dilution of cDNA concentrations (1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024 and 1:2048 in order); the second row (B) shows samples from an XX group; the third row (C) shows samples from an XY group. Samples from the XX and XY groups are (in order) 4, 6, 8, 11, 15, 19, 23, 27, 31, 33, 37, 39 and 43 dpf.

PCR was carried out for all samples (XX and XY groups, 4, 6, 8, 11, 15, 19, 23, 27, 31, 33, 37, 39 and 43 dpf) with the different gene primers (in triplicate). PCR products were run on 1% agarose gels in 1× TBE buffer with EtBr. The gel was briefly exposed to UV light to check the presence of PCR products and was then subjected to Southern hybridisation. The DNA on the gel was first denatured by soaking the gel for 45 minutes in 1.5M NaCl/0.5N NaOH with gentle agitation on a mechanical rotator and then neutralised by soaking for 45 min in 1M Tris (pH 7.4)/1.5M NaCl at room temperature. The DNA was transferred onto a nylon membrane in 10× SSC overnight using the capillary transfer method. The membrane was dried and baked for 1 hour at 80°C.

The membranes were hybridised at 68°C in 20 ml of Church-Gilbert solution with brain aromatase, ovarian aromatase, or actin probe in separate hybridisation chambers overnight. Each probe was generated by PCR using primer BF1 and BR2 for brain aromatase, OF and OR for ovarian aromatase and AF and AR for actin, and labelled with α-^{32}P dCTP by nick translation (Promega). The membrane was washed three times over 2 hours at 68°C in 0.1× SSC/0.1% SDS and subjected to autoradiography to visualise hybridisation signals.

Hybridisation signals were digitised by estimating the optical densities using an image analysis system (Image-Pro PLUS, Media Cybernetics). The relative expression level of aromatase genes to that of actin was calculated and used to compare the level between sexes and different developmental stages. The data were shown as mean±S.E. Statistical differences of relative aromatase mRNA expression to actin mRNA between the sexes were determined by t-test ($P<0.05$).

4.3. Results

4.3.1. Identification of brain type aromatase gene and tissue specific gene expression

4.3.1.1. Identification of brain aromatase gene

A partial brain aromatase cDNA (1707bp) was isolated from the brain cDNA library of tilapia *O. niloticus* using primers based on the *O. mossambicus* brain aromatase cDNA sequence. The multicloning site of the cloning vector (Uni-Zap XR) for the cDNA library contains T3 and T7 primer corresponding regions. An attempt to clone full length cDNA encoding brain aromatase was made by utilising these primers. PCR was carried out with BF1 (forward gene specific primer) + T7 and T3 + BR2 (reverse gene specific primer). Both PCR products were subcloned into pBluescript II and subjected to sequencing (Details in *Materials and Methods*). The BF1→T7 PCR product yielded a cloned DNA fragment of 929bp that had 97% identity to the 3'end sequence of the *O. mosambicus* brain aromatase cDNA sequence, and contained a poly adenylation signal. However, the sequence of T3→BR2 clone did not corresponded to any known aromatase cDNA sequences. To obtain further upstream sequence towards the 5' end, several upstream primers (Table 4.1) were designed and used for PCR. With the sequence obtained from these PCRs, a 1707bp partial brain aromatase cDNA sequence of *O. niloticus* was determined (Fig. 4.3). This cDNA sequence showed 97% identity to the *O. mosambicus* brain aromatase cDNA sequence and 67.9% identity to the *O. niloticus* ovarian aromatase cDNA sequence. However, the sequence towards the 5'end of the *O. niloticus* brain aromatase gene could not be determined in this experiment.

The identified sequence included a 1444bp open reading frame (ORF) (part of exon 1, and exon 2 - 9) and a 263bp 3'end untranslated region (3'UTR). The amino acid sequence

TAGAGCGTCAGAAGTCACTGCTGTCCTGCTTCTTCTGCTGCTGTTGTTGCTGCTT
TTCACCACCTGGAGACAAAGAAAACAGTCACACATACCAGGTCCTTTCTTCTTAG
CAGGACTCGGTCCAATTCTCTCCTACAGCAGATTCATCTGGTCTGGGATTGGAAC
AGCGTGTAACTACTACAACAACAAATATGGGAGCATTGTGCGGGTGTGGATAAAC
GGAGAGGAGACCCTGATTTTGAGCAGGTCCTCTGAAGTGTACCACGTTTTGAGGA
GTGCCCACTACACCTCCAGATTTGGCAGCAAAAAAGGACTCGAGTGCATCGGCAT
GTACGGAAATGGTATCATTTTCAACAGTGATGTCCTGCTTTGGAAAAAAGTGAGA
ACATACTTTTCTAAAGCTCTGACTGGACCCGGCCTGCAGAGGACCGTAGGAATCT
GTGTGAGCTCCACAGCCAAACACCTGGACAACTTACAGGACATGACTGACCCCTC
TGGACATGTAGATGCTCTCAATCTCCTGAGAGCCATCGTGTTGGACATCTCCAAC
CGGCTGTTCCTCAGAGTGCCGTTAAATGAGAAAGACTTCTTGACGAAAATTCACA
ACTACTTTGATACCTGGCAAACAGTTCTAATAAAACCAGATATATTCTTCAAGGT
TGGATGGCTGTACAACAAGCATAAGAGAGCAGCACAGGAGCTGCAAGATGCAATG
GAGAGCCTGCTTGAAGTTAAGAGAAAGATGATTCATGAAGCCGAGAAGCTGGACG
ACGAGCTCGACTTTGCAACAGAGCTCATCTTCGCCCAGAACCACGGAGAGCTATC
AGCAGATAACGTCAGGCAGTGTGTGCTAGAGATGGTGATCGCAGCCCCTGACACA
CTTTCCATCAGCCTCTTCTTCATGCCGATGCTGCTGAAACAGAACCCGGACATAG
AGCTGCAGCTAGTGGAGGAGATGAACACCATCTTGAATGAAAAAGACGTGGAAAA
TATCGATTACCAAAGCCTGAAGGTGATGGAGAGCTTCATCAACGAGTCTTTGAGG
TTTCATCCTGTGGTCGGTTTCACAATGAGGAAAGCTCTGGAGGACAACGACATCG
CAGGCACAAAAATCAAGAAGGGCACCAACATCATTCTCAACACTGGCCTCATGCA
CAAAACCGAATTCTTCCCCAAACCTGAAGAGTTCAACCACACGAACTTTGAAAAA
ACGGTACCCAATCGTTACTTCCAGCCCTTTGGCTGCGGGCCTCGTTCCTGTGTGG
GCAAACACATCGCCATGGTGATGATGAAGGCCATCCTGGTCACTCTCCTGTCTCG
GTACACTGTGTGTCCTCATCAAGGCTGCACACTCAGCAGCATCAAGCAGACCAAC
AACCTGTCACAGCAGCCGGTGGAAGACGAGCACAGCCTGGCCATGCGCTTCATCC
CACGAACGAAA**TAA**CCCCACGACAACAAGCTGCGAAACATAACAGCGCTTTTTAC
ACTTCAGATGAAAGCATCTTGAAATGGGTGTAAAGGCCTTAATGAAGTCCTGTAC
TAGGAAATACTAACAGTAAATTTGTATTAACAGCACATGTACGCCAAATTCAACT
TTACTAATCTTTAAAAAAATACACTTGTTAAGTAGAGTTTTCTGATTCATGTTTT
GACAATGTAAACAGCTCATACTTTT**AATAAA**GATCCATTTGCAAAAAAAAAAAAA
AA

Fig. 4.3. Partial nucleotide sequence of tilapia *O. niloticus* brain P450 aromatase cDNA. A stop codon and a polyadenylation signal are indicated with underlines.

for ORF derived from this partial *O. niloticus* brain aromatase cDNA sequence showed 96.7% identity with the amino acid sequence of the corresponding region of *O. mossambicus* and 69.9% with that of goldfish brain aromatase (Fig. 4.4). This partial sequence contains an I-helix region, an Ozol's peptide region, an aromatase specific conserved region, and a heme-binding region. These regions showed high homology between different animals or tissues. On the other hand, the termini of aromatase genes from different animals or tissues showed very low homology.

The identity of aromatase amino acid sequences between different animals is summarised in Table 4.2. Identities between brain type aromatases of different animals are higher than those between brain type and ovarian type aromatases within a species. Interestingly, the similarities of fish brain aromatase to chicken (McPhaul *et al.* 1988) and human (Corbin *et al.* 1988) aromatases are higher than those of fish ovarian aromatases to chicken and human aromatases. To illustrate the phylogenetic relationship of *O. niloticus* brain aromatase with other reported aromatases from different animals or different tissue, an evolutionary tree was constructed using the Neighbor-Joining method of Clustal X (Fig. 4.5). Fish brain aromatases clustered separately from fish ovarian aromatases and also from human and chicken aromatases. In the evolutionary context, the phylogenetic tree suggests that fish brain aromatase diverged from ovarian aromatase (or vice versa) after the divergence of bony fishes from the line of mammals and birds.

The identified cDNA sequence contains restriction sites for 121 enzymes (some of them appeared more than once) including *Afe* I, *Bal* I, *Bsa*B I, *Cla* I, *Dra* I, *Dra* II, *Eco*R I, *Hae* II, *Kpn* I, *Sac* I, *Xho* I, *Xho* II, etc. The overall restriction map derived from sequence data is shown in Appendix III.

Fig. 4.4. Alignment of tilapia *O. niloticus* brain P450 aromatase amino acid sequences (deduced from the partial cDNA sequence) with sequences of the brain and ovarian P450 aromatase from other animals. CF: catfish *I. punctatus*, FL: flounder *P. olivaceus*; GF: goldfish *C. auratus*; MEDA: medaka *O. latipes*; RT: rainbow trout *O. mykiss*; TOM: tilapia *O. mossabicus*, TON: tilapia *O. niloticus* (References for each animal are found in the text). The regions with homology more than 80% between different species or forms are shaded. The regions above underlines indicate a I-helix region (I), an Ozol's peptide region (II), an aromatase specific conserved region (III), and a heme-binding region (IV), respectively. Putative exon-intron boundaries are indicated by arrows.

➢ See next page for Fig 4.4

```
TON-BRAIN   ............................R.....ASEVTAVLL....LLLLLLLFTTWRQRKQSH.IPGPFFLAGLGP:41
TOM-BRAIN   ...............HEEELTAGPMVADR.....ASEVTAALL....LLLLLLLFTTWRQRKQSH.IPGPFFLAGLGP:54
GF-BRAIN    ................MEEVLKGTVNFAA.....AVQVTLMALTGTLLLILLHRIFTAKNWRNQSG.VPGPGWLLGLGP:57
TON-OVARY   .MDLIS.ACEQAMNPVGLDAVVARSLCDLK.....CHPIDGISMATRTLILLVCLLLVAWSHTDKKI.VPGPSFCLGLGP:72
TOM-OVARY   .MDLIS.ACEQAMNPVGLDAVVADLSVTSN.....AIQSHGISMATRTLILLVCLLLVAWSHTDKKI.VPGPSFCLGLGP:72
FL-OVARY    .MDRIP.ACDLAMTPVGLGAALGDLVSTSPN..ATAVRTPGISVASRTLILLVCVLLVAWSHTDRRT.VPGPPFCLGLGP:75
MEDA-OVARY  .MDLIP.ACDRTMSSSCLVAELVSIAPNTT.....VGLPSGIPMATRSLILLVCLLLMVWSHSEKKT.IPGPSFCLGLGP:72
RT-OVARY    .MDLLSPVCGRVMAVVCLDTVIADLLVSESRN.ATATRSEGISLATGSLLLLLCLLLAAWRHTDNNS.VPGPFFCLGVGP:77
GF-OVARY    MAGELLQPCG..MKQVHLGEAVLELLMQGAHNSSYGAQDNVCGAMATLLLLLLCLLLAIRHHWTEKDHVPGPCFLLGLGP:78
CF-OVARY    MAAHVFPMCERTRKPVHFSETVMEILLREARNGTDPRYENPRG.ITLLLLLCLVLLLTVWNRHEKKCSIPGPSFCLGLGP:79
CHICKEN     ...................MIPETLNPLNY..FTSLVPDLMPVATVPIIILICFLFLIWNHEETS..IPGPGYCMGIGP:56
HUMAN       ...................MVLEMLNPIHYN.ITSIVPEAMPAATMPVLLLTGLFLLVWNYEGTSS.IPGPGYCMGIGP:58

TON-BRAIN   ILSYSRFIWSGIGTACNYYNNKYGSIVRVWINGEETLILSRSSEVYHVLRSAHYTSRFGSKKGLECIGMYGNGIIFNSDV:121
TOM-BRAIN   ILSYSRFIWSGIGTACNYYNNKYGSIVRVWISGEETLILSRSSEVYHVLRSAHYTSRFGSKKGLECIGMYGNGIIFNSDV:134
GF-BRAIN    IMSYSRFLWMGIGSACNYYNEKYGSIARVWISGEETFILSKSSAVYHVLKSNNYTGRFASKKGLQCIGMFEQGIIFNSNM:137
TON-OVARY   LLSYLRFIWTGIGTASNYYNNKYGDIVRVWINGEETLILSRSSAVHHVLKNGNYTSRFGSIQGLSYLGMNERGIIFNNNV:152
TOM-OVARY   LLSYVRFIWTGIGTACNYYNKRYGDIVRVWINGEETLILSRSSAVHHVLKNGNYTSRFGSIQGLSCLGMNERGIIFNNNV:152
FL-OVARY    LMSYLRFIWTGIGTASNYYNNKYGDIVRVWIDGEETLILSRASAIYHVLKNGHYTSRFGSKQGLSCIGMYERGIIFNNNV:155
MEDA-OVARY  LLSYLRFIWTGIGTSANYYNSKYGDIVRVWINGEETLILSRASAVHHVLKNRKYTSRFGSKQGLSCIGMNEKGIIFNNNV:152
RT-OVARY    LLSYCRFIWSGIGTASNYYNSKYGDIVRVWINGEETFILSSSSAVHHVLRQGRYTSRFGSKQGLSCIGMDERGIIFNSNM:157
GF-OVARY    LMSYCRFIWMGIGTASNYYNEKYGDMVRVWISGEETLVLSRPSAVYHVLRKSLYTSRFGSKLGLQCIGMHEQGIIFNSNV:158
CF-OVARY    LISHGRFLWMGVGNACNYYNKTYGEFVRVWISGEETFIISKSSSVFHVMKHWNYVSRFGSKLGLQCIGMHEQGIIFNSNV:159
CHICKEN     LISHGRFLWMGIGSACNYYNRVYGEFMRVWISGEETLIISKSSSMFHIMKHNHYSSRFGSKLGLQCIGMYENGIIFNNNP:136
HUMAN       LISHGRFLWMGIGSACNYYNRVYGEFMRVWISGEETLIISKSSSMFHIMKHNHYSSRFGSKLGLQCIGMHEKGIIFNNNP:138

TON-BRAIN   LLWKKVRTYFSKALTGPGLQRTVGICVSSTAKHLDNLQD.MTDPSGHVDALNLLRAIVLDISNRLFLRVPLNEKDFLTKI:200
TOM-BRAIN   PLWKKVRTYFSKALTGPGLQRTVGICVSSTAKHLDNLQD.MTDPSGHVDALNLLRAIVLDISNRLFLRVPLNEKDLLTKI:213
GF-BRAIN    ALWKKVRTYFTKALTGPGLQKSVDVCVSATNKQLNVLQE.FTDHSGHVDVLNLLRCIVVDVSNRLFLRIPLNEKDLLIKI:216
TON-OVARY   TLWKKIRTYFAKALTGPNLQQTVDVCVSSIQAHLDHLDS.....LGHVDVLNLLRCTVLDISNRLFLNVPLNEKELMLKI:227
TOM-OVARY   TLWKKIRTYFAKALTGPNLQQTADVCVSSIQAHLDHLDS.....LGHVDVLNLLRCTVLDISNRLFLDVPLNEKELMLKI:227
FL-OVARY    SLWKKIRTHFTRALTGPGLQKTVEVCVSSTQTHLDDLDG.....LGHVDVLSLLRCTVVDISNRLFLDVPINEKELLVKI:230
MEDA-OVARY  ALWKKIRTYFTKALTGPNLQQTVEVCVTSTQTHLDNLSS.....LSYVDVLGFLRCTVVDISNRLFLGVPVDEKELLQKI:227
RT-OVARY    ALWKKTRTYFAKALTGPGLQKTVDVCVSSTQTHLDALQGPDGLMGGQVDVLSLLRCTVVDISNRLFLGVPLNEKELLQKI:237
GF-OVARY    ALWKKVRSFYAKALTGPGLQRTLEICITSTNTHLDNLSH.LMDARGQVDILNLLRCIVVDISNRLFLGVPLNEHDLLQKI:237
CF-OVARY    TLWRKVRTYFAKALTGPGLQRTLEICTMSTNTHLDGLSR.LTDAQGHVDVLNLLRCIVVDISNRLFLDVPLNEQNLLFKI:238
CHICKEN     AHWKEIRPFFTKALSGPGLVRMIAICVESTIVHLDKLEEVTTE.VGNVNVLNLMRRIMLDTSNKLFLGVPLDESAIVLKI:215
HUMAN       ELWKTTRPFFMKALSGPGLVRMVTVCAESLKTHLDRLEEVTNE.SGYVDVLTLLRRVMLDTSNTLFLRIPLDESAIVVKI:217

TON-BRAIN   HNYFDTWQTVLIKPDIFFKVG.WLYNKHKRAAQELQDAMESLLEVKRKMIHEAEKLDDELDFATELIFAQNHGELSADNV:279
TOM-BRAIN   HNYFETWQTVLIKPDIFFKVG.WLYNKHKRAAQELQDAMESLLEVKKKMIHEAEKLDDELDFATELIFAQNHGELSADNV:292
GF-BRAIN    HRYFSTWQAVLIQPDVFFRLN.FVYKKYHLAAKELQDEMGKLVEQKRQAINNMEKLD.ETDFATELIFAQNHDELSVDDV:294
TON-OVARY   QKYFHTWQDVLIKPDIYFKFR.WIHHRHKTATQELQDAIKRLVDQKRKNMEQADKLD.NINFTAELIFAQNHGELSAENV:305
TOM-OVARY   QKYFHTWQDVLIKPDIYFKFG.WIHHRHKTATQELQDAIKRLVDQKRKNMEQADTLD.NINFTAELIFAQNHGELSAENV:305
FL-OVARY    LKYFDTWQTVLIKPDIYFKFD.WIHQRHKAAVQELHDAIGDLVEQKRRDVEQADKLD.NINFTTGLIFAQNHGELSAENV:308
MEDA-OVARY  HKYFDTWQTVLIKPDIYFKFS.WIHQRHKTAAQELQDAIESLVERKRKEMEQAEKLD.NINFTAELIFAQGHGELSAENV:305
RT-OVARY    QKYFDTWQTVLIKPDVYFKLD.WIHEKHRRAAQELEDAIESLVDQKRRGLQEADKLD.HINFTADLIFAQSHGELSAENV:315
GF-OVARY    HKYFDTWQTVLIKPDVYFRLAWWLHRKHKRDAQELQDAIAALIEQKRVQLTRAEKFD.QLDFTAELIFAQSHGELSTENV:316
CF-OVARY    HRYFETWQTVLIKPDFYFRLK.WLHDKHRNAAQELHDAIEDLIEQKRTELQQAEKLD.NLNFTEELIFAQSHGELTAENV:316
CHICKEN     QNYFDAWQALLLKPDIFFKIS.WLCKKYEEAAKDLKGAMEILIEQKRQKLSTVEKLDEHMDFASQLIFAQNRGDLTAENV:294
HUMAN       QGYFDAWQALLIKPDIFFKIS.WLYKKYEKSVKDLKDAIEVLIAEKRCRISTEEKLEECMDFATELILAEKRGDLTRENV:296

TON-BRAIN   RQCVLEMVIAAPDTLSISLFFMPMLLKQNPDIELQLVEEMNTILNEKDVENIDYQSLKVMESFINESLRFHPVVGFTMRK:359
TOM-BRAIN   RQCVLEMVIAAPDTLSISLFFMLMLLKQNPDIELQLVEEMNNILNEKDVENIDYQSLKVMESFINESLRFHPVVDFTMRK:372
GF-BRAIN    RQCVLEMVIAAPDTLSISLFFMLLLLKQNSVVEEQIVQEIQSQIGERDVESADLQKLNVLERFIKESLRFHPVVDFIMRR:374
TON-OVARY   TQCVLEMVIAAPDTLSLSLFFMLLLLKQNPHVEPQLLQEIDAVVGERQLQNQDLHKLQVMESFIYECLSFHPVVDFTMRR:385
TOM-OVARY   TQCVLEMVIAAPDTLSLSLFFMLLLLKQNPHVEPQLLQEIDAVVGERQLQNQDLHKLQVMESFIYEMLRFHPVVDFTMRR:385
FL-OVARY    VQCVLEMVIAAPDTLSVSLFFMLLLLKQNPDVELQLLREIDTVVGERQLQNGDLQKLQVLESFINECLRFHPVVDFSMRR:388
MEDA-OVARY  RQCVLEMVIAAPDTLSISLFFMLLLLKQNPHVELQLLQEIDTIVGDSQLQNQDLQKLQVLESFINECLRFHPVVDFTMRR:385
RT-OVARY    RQCVLEMVIAAPDTLSISLEFMLLLLKQNPDVELQLLEEIDTAIGDRELHNSDLQNLRVLESFINESLRFHPVVDFTMRR:395
GF-OVARY    RQCVLEMVIAAPDTLSISVFFMLLLLKQNPDVELKILQEMNAVLAGRSLQHSHLSGFHILESFINESLRFHPVVDFTMRR:396
CF-OVARY    RQCVLEMVIAAPDTLSISVFFMLLLLKQNAEVERRILTEIHTVLGDTELQHSHLSQLHVLECFINEALRFHPVVDFSYRR:396
CHICKEN     NQCVLEMMIAAPDTLSVTLFFMLILIADDPTVEEKMMREIETVMGDREVQSDDMPNLKIVENFIYESMRYQPVVDLIMRK:374
HUMAN       NQCILEMLIAAPDTMSVSLFFMLFLIAKHPNVEEAIIKEIQTVIGERDIKIDDIQKLKVMENFIYESMRYQPVVDLVMRK:376
                   I                                                  II

TON-BRAIN   ALEDNDIAGTKIKKGTNIILNTGLMHKTEFFPKPEEFNHTNFEKTVPNR.YFQPFGCGPRSCVGKHIAMVMMKALLVTLL:438
TOM-BRAIN   ALEDNDIAGTKIKKGTNIILNIGLMHKTEFFPKPKEFNLTNFEKTVPSR.YFQPFGCGPRSCVGKHIAMVMMKALLVTLL:451
GF-BRAIN    ALEDDEIDGYRVAKGTNIILNIGRMHKSEFFQKPNEFNLENFEKTVPSR.YFQPFGCGPRACVGKHIAMVMTKALLVTLL:453
TON-OVARY   ALSDDIIEGYRISKGTNIILNTGRMHRTEFFLKGNQFNLEHFENNVPRPPTFQPFGSGPRACIGKHMAMVMMKSILVTLL:465
TOM-OVARY   ALSDDIIEGYRISKGTNIILNTGRMHRTEFFLKANQFNLEHFENNVPRR.YFQPFGSGPRACIGKHIAMVMMKSILVTLL:464
FL-OVARY    ALSDDIIDGYRVPKGTNIILNTGRMHRTEFFCKPNEFNLDNFEKTAPRR.YFQPFGSGPRSCVGKHIAMAMMKSILVTLL:467
MEDA-OVARY  ALFDDIIDGHRVQKGTNIILNTGRMHRTEFFHKANEFSLENFQKNTPRR.YFQPFGSGPRACVGRHIAMVMMKSILVTLL:464
RT-OVARY    ALSDDVISGYRVPKGTNIILNMGRMHRSEFFLKPNEFSLDNFEKNIPNR.FFQPFGSGPRSCVGKHIAMVMMKSILVTLL:474
GF-OVARY    ALDDDVIEGYKVKRGTNIILNVGRMHRSEFFPKPNEFSLDNFQKNVPSR.FFQPFGSGPRSCVGKHMAMVMMKSILVTLL:475
CF-OVARY    ALDDDVIEGFRVPRGTNIILNVGRMHRSEFYPKPADFSLDNFNKPVPSR.FFQPFGSGPRSCVGKHIAMVMMKAVLLMVL:475
CHICKEN     ALQDDVIDGYPVKKGTNIILNIGRMHKLEFFPKPNEFSLENFEKNVPSR.YFQPFGFGPRGCVGKFIAMVMMKAILVTLL:453
HUMAN       ALEDDVIDGYPVKKGTNIILNIGRMHRLEFFPKPNEFTLENFAKNVPYR.YFQPFGFGPRGCAGKYIAMVMMKAILVTLL:455
            III                                                IV

TON-BRAIN   SRYTVCPHQGCTLSSIKQTNNLSQQPV.EDE...HSLAVRFIPRTK..............:480
TOM-BRAIN   SRYTVCPRQGCTLNSIKQTNNLSHQPV.EDE...HSLAVRFIPRTT..............:493
GF-BRAIN    SRFTVCPRHGCTVSTIKQTNNLSMQPVEEDP...DSLAVRFIPRAQNICGDPHLGEKTEE:510
TON-OVARY   SQYSVCTHEGPILDCLPQTNNLSQQPVEHQQAETEHLHVRFLPRQGSSCQTLKDPNL...:522
TOM-OVARY   SQYSVCTHEGPILDCLPQTNNLSQQPVEHQQAETEHLHVRFLPRQRSSCQTLRDPNL...:521
FL-OVARY    SQYSVCPHEGLTLDCLPQTNNLSQQPVEHQQ.EAPHLNVRFLPRQRGSWQTL........:518
MEDA-OVARY  SQYSVCPHEGLTLDCLPQTNNLSQQPVEHHQ.EADHLSVTFLPRQRGIWESPSPF.....:518
RT-OVARY    SRYSVCPHEGLTLDCLPQTNNLSQQPVEEG..EP.HTVKFLPRHQARKQS.........:522
GF-OVARY    SRFSVCPVKGCTVDSIPQTNDLSQQPVEEPS....SLSVQLILRNAL.............:518
CF-OVARY    SRFSVCPEESCTVENIAHTNDLSQQPVEDKH....TLSVRFIPRNTHTRNRKA.........:524
CHICKEN     RRCRVQTMKGRGLNNIQKNNDLSMHPIERQP....LLEMVFTQEAQTRIRVTKVDQH...:506
HUMAN       RRFHVKTLQGQCVESIQKIHDLSLHPDETKN....MLEMIFTPRNSDRCLEH........:503
```

Table 4.2. Homology of aromatase amino acid sequences (part of exon 1, and exon 2 - 9) between different animals by pair-wise comparison (Values are percentage)

	TOM-Brain	GF-Brain	TON-Ovary	TOM-Ovary	GF-Ovary	MED-Ovary	RT-Ovary	CF-Ovary	FL-Ovary	CHI	HUM
TON-Brain	96.7	69.9	63.7	64.4	68.5	66.6	68.3	65.2	66.6	55.7	55.1
TOM-Brain		71.5	63.5	64.5	67.9	66.2	67.7	66.0	66.1	56.5	55.3
GF-Brain			59.5	59.9	62.1	61.2	63.4	63.1	62.1	55.6	55.6
TON-Ovary				95.0	66.4	78.4	71.3	61.1	77.3	53	50.5
TOM-Ovary					66.7	79.2	72.6	61.8	79.2	54.0	51.0
GF-Ovary						69.6	70.5	71.8	66.5	56.3	53.1
MED-Ovary							74.0	69.0	79.3	53.5	52.4
RT-Ovary								66.2	76.1	55.6	51.5
CF-Ovary									63.7	54.4	52.7
FL-Ovary										54.7	52.6
CHI											71.7

CF: catfish *I. punctatus*, CHI: chicken; FL: flounder *P. olivaceus*; GF: goldfish *C. auratus*; HUM: human, MEDA: medaka *O. latipes*; RT: rainbow trout *O. mykiss*; TOM: tilapia *O. mossabicus*, TON: tilapia *O. niloticus* (References for each animal are found in the text).

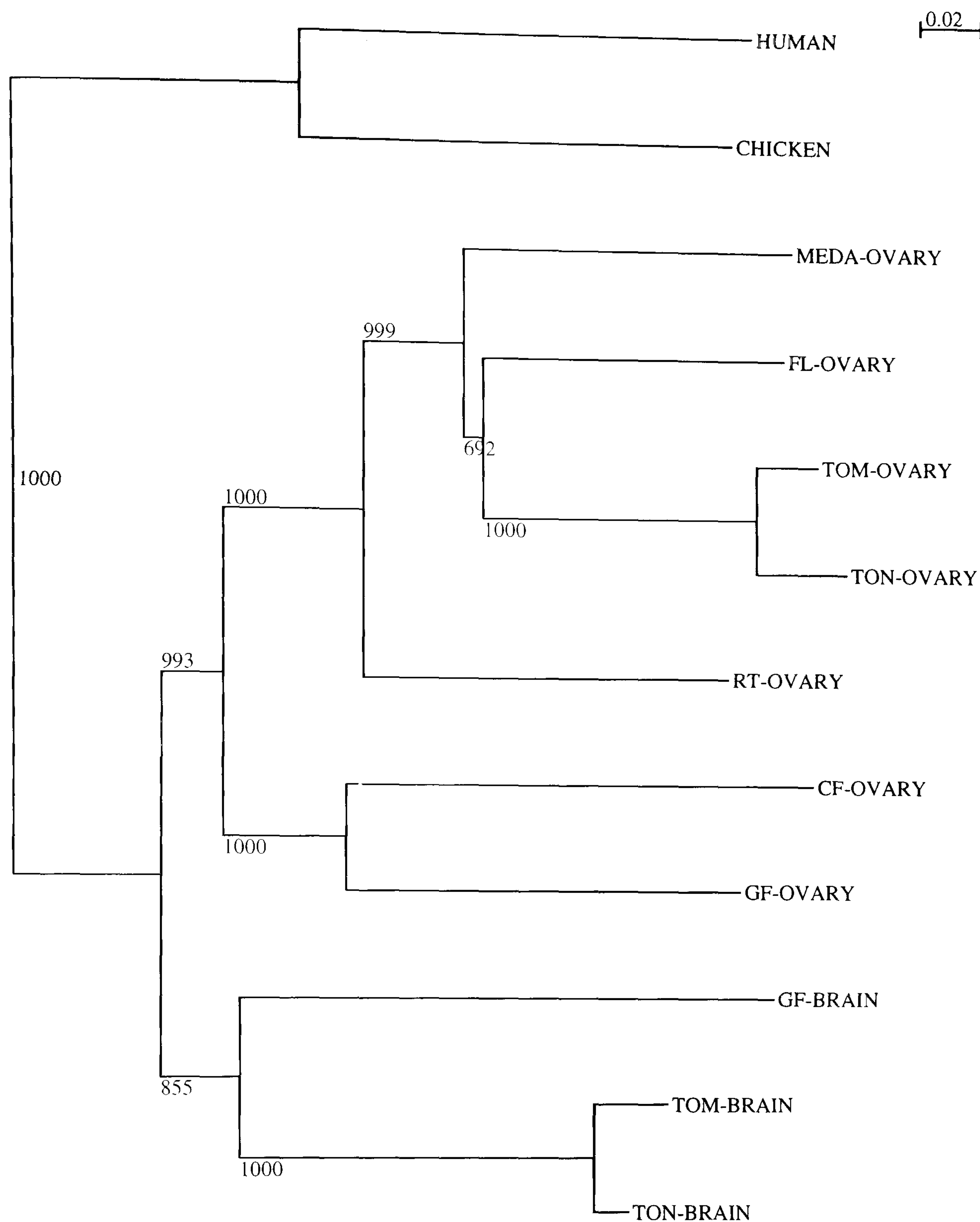


Fig. 4.5. Phylogenetic tree of P450 aromatase proteins. Tree was constructed using Clustal W multiple sequence alignment program and deduced amino acid sequences of P450 aromatase forms in chicken ovary, CF (catfish *I. punctatus*) ovary, FL (flounder *P. olivaceus*) ovary, GF (goldfish *C. auratus*) brain and ovary, human placenta, MEDA (medaka *O. latipes*) ovary, RT (rainbow trout *O. mykiss*) ovary, TOM (tilapia *O. mossabicus*) brain and ovary, and TON (tilapia *O. niloticus*) brain and ovary (for references see the text). Numbers indicate the numbers that they clustered together out of 1000 times bootstrap clustring.

By Northern transfer and hybridisation with the brain aromatase probe, one single clear transcript was detected in brain but only very faintly in ovary, indicating that brain aromatase differs from ovarian aromatase in this species (Fig. 4.6). The full length size of the *O. niloticus* brain aromatase transcript was estimated between 2.6 and 2.7kb (estimated value: 2662bp).

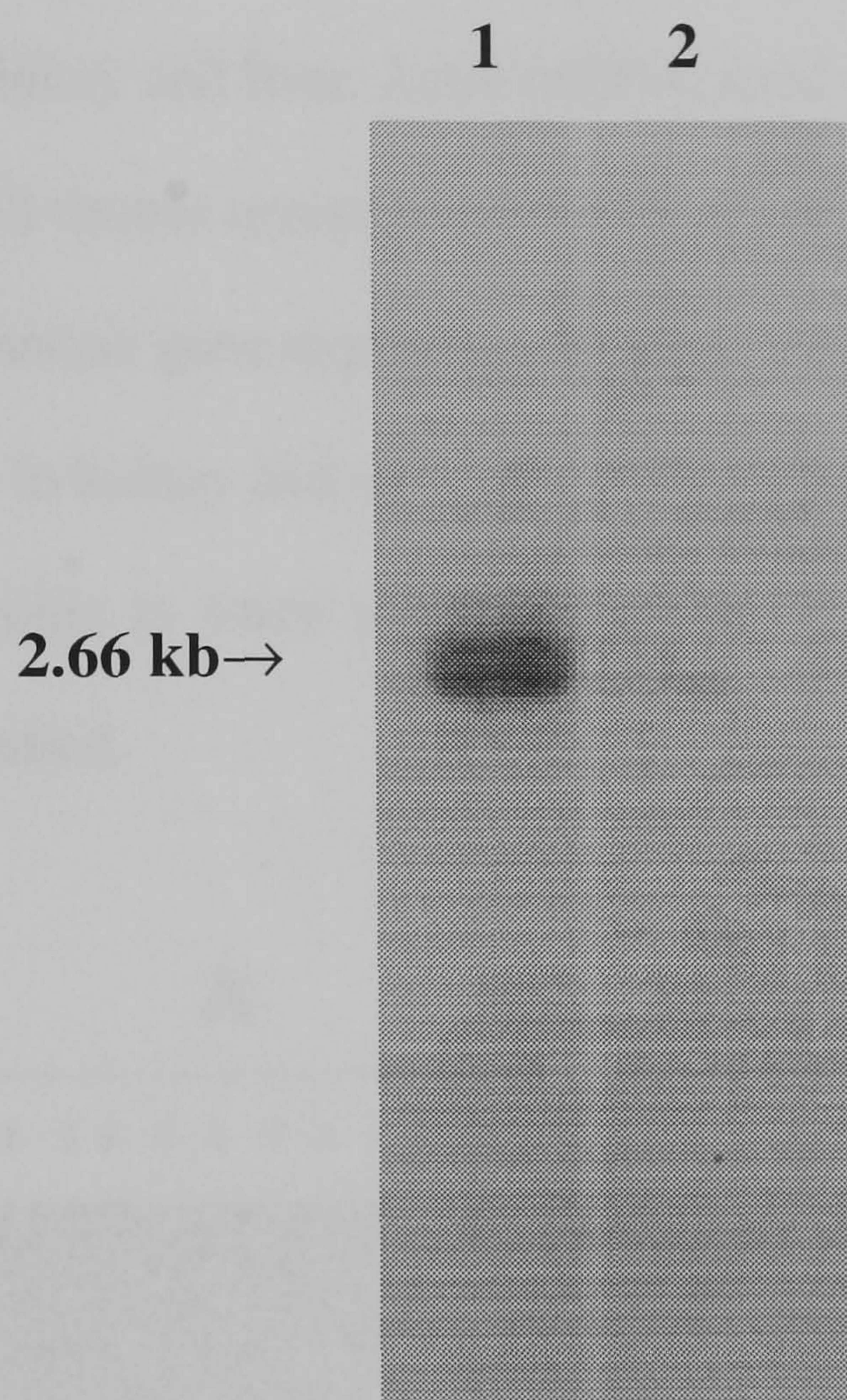


Fig. 4.6. Northern hybridisation of *O. niloticus* brain and ovarian RNA. Total RNA (10 μg) from the brain and ovary was probed with the brain aromatase probe (generated by PCR and labelled with α-^{32}P dCTP by nick translation) following Northern transfer. The size of the brain aromatase transcripts in brain tissue (lane 1) and in ovarian tissue (lane 2) was estimated as 2.6-2.7kb.

4.3.1.2. Tissue specific expression of aromatase genes

Tissue specific expression of both brain and ovarian aromatase mRNA was investigated in various adult tissues using RT-PCR (Fig. 4.7). Brain aromatase mRNA was expressed at high levels in both sexes of brain and less so in the kidney, eye, ovary and testis, but could not be detected in the liver and spleen. In contrast, ovarian aromatase mRNA was expressed strongly in the ovary and less so in the brain, spleen and testis, but could not be detected in the eye, kidney and liver. Actin mRNA, used as a control for gene expression, was expressed from all tissues investigated. No clear sexual differences were found in the pattern of either aromatase gene expression in adult tissues except for the gonads. Brain aromatase expression in kidney and ovarian aromatase gene expression in spleen appeared to be sexually dimorphic in some samples, but the pattern was not consistent in other males and females studied.

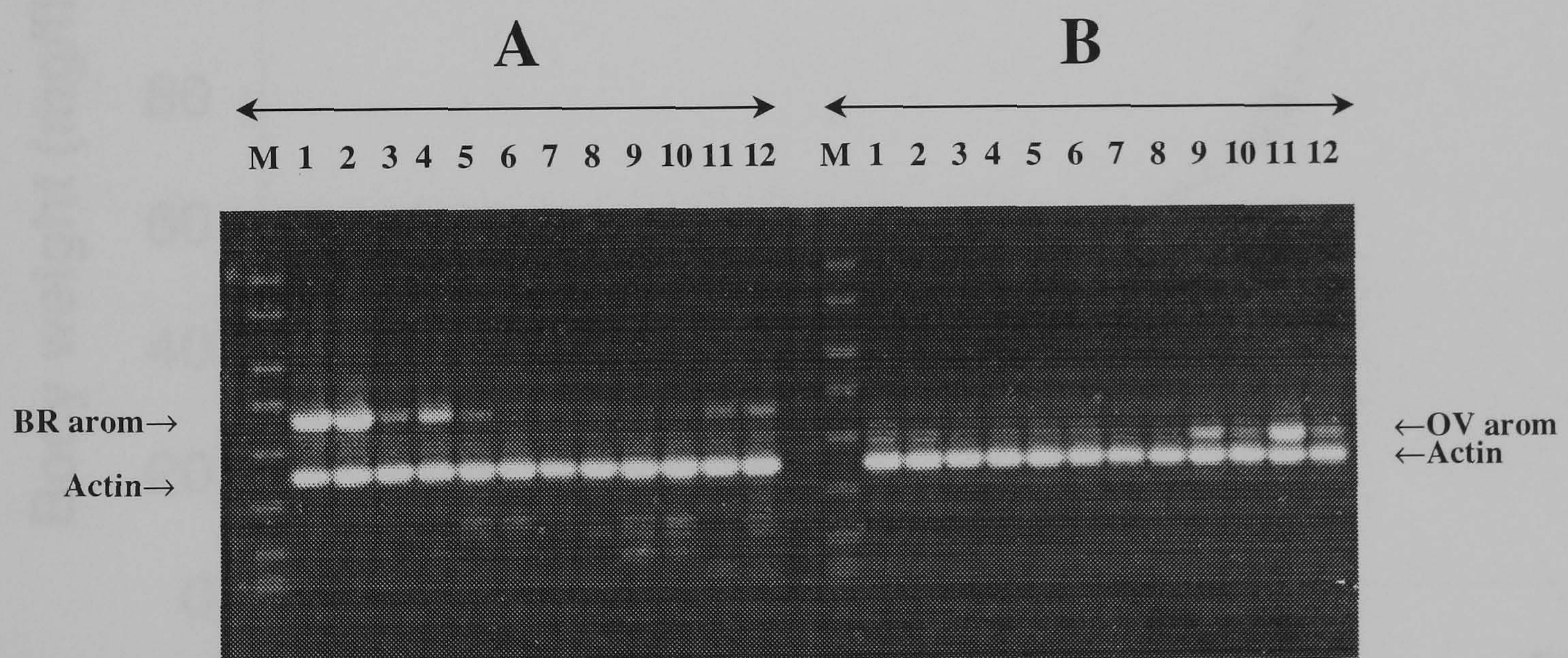


Fig. 4.7. Tissue specific aromatase gene expression in various tissues of adult *O. niloticus*. A. brain aromatase product (BR arom) and actin product (Actin) (3μl each) were loaded together (odd numbers and even numbers indicate female and male, respectively). Tissues are brain, kidney, eye, liver, spleen and gonads from left to right); B. ovarian aromatase (OV arom) and actin gene product, in the same order as for the brain aromatase and actin samples. M: size marker (50-2000bp).

4.3.2. Differential expression of brain and ovarian aromatase genes during ontogeny

Brain and ovarian aromatase gene expression was studied by semi-quantitative RT-PCR analysis in genetic male and female groups of *O. niloticus* from fertilisation to 43 dpf by which time sex is histologically discernible.

4.3.2.1. Growth and sex ratio

During the sampling period, fish grew almost in log phase after 19 dpf with a brief plateau between 23 and 31 dpf (Fig. 4.8). The final sex ratio of the sampled groups showed 84.4% females in the genetic female group (XX) and 100% males in the genetic male group (XY).

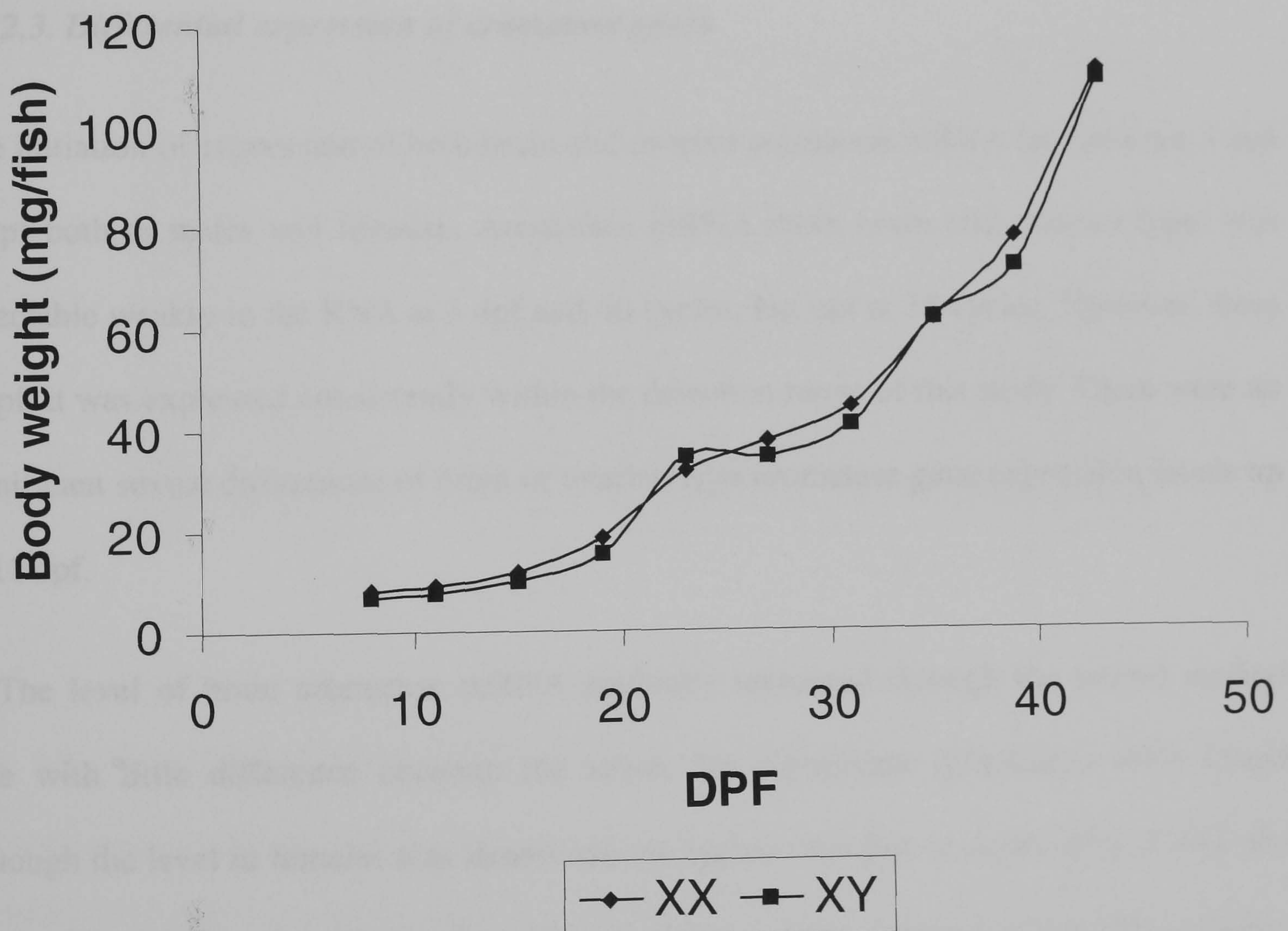


Fig. 4.8. Growth patterns of sampled groups of *O. niloticus* fry from hatch to 43 dpf. DPF: days post fertilisation.

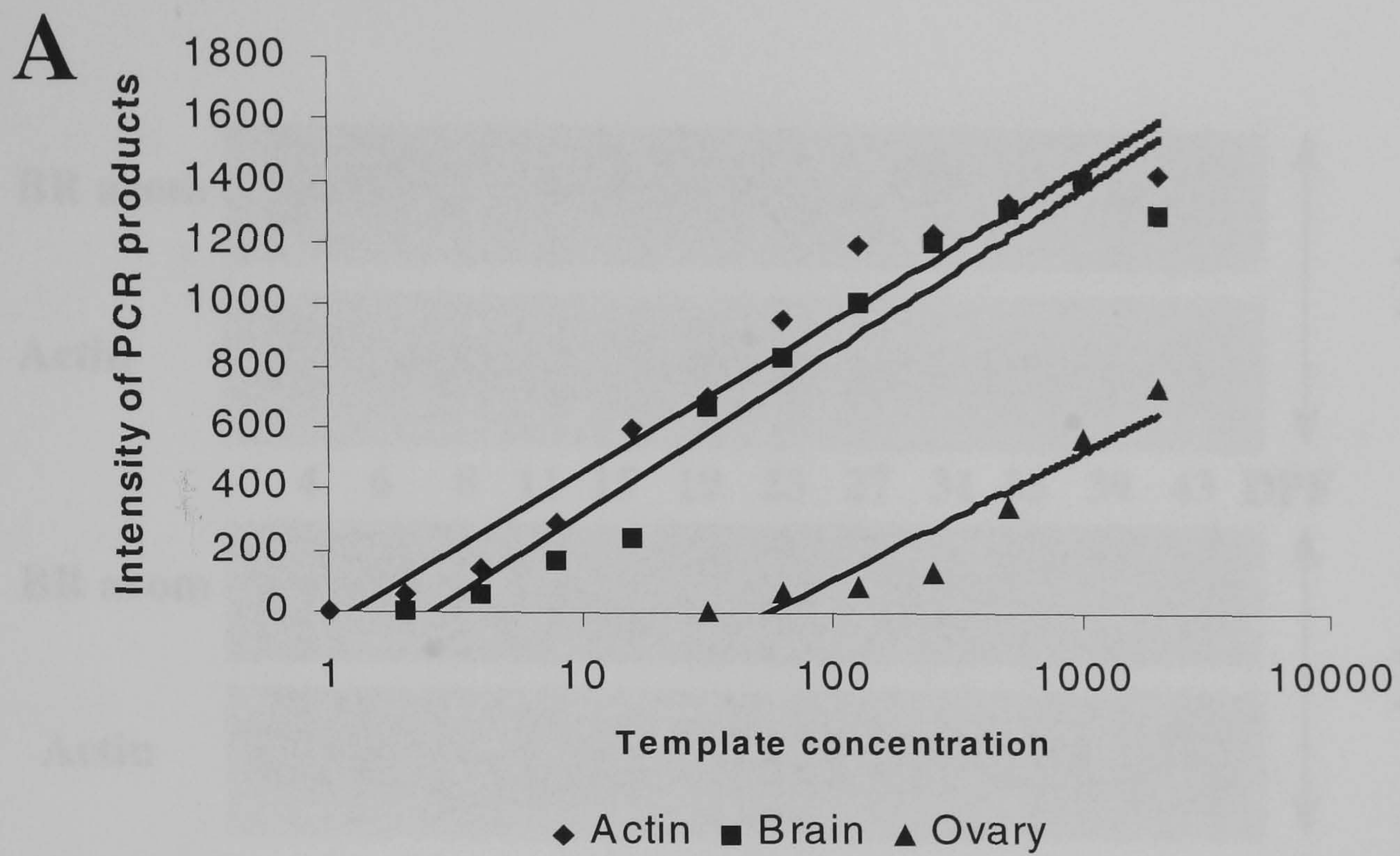
4.3.2.2. Optimisation of semi quantitative RT-PCR

PCR conditions were optimised to achieve quantitative response from three different genes (brain and ovarian aromatase, and actin as a control) (Details in *Materials and Methods*). Both brain and ovarian aromatase genes responded in proportion to cDNA concentration (template used for PCR) at 35 cycles of PCR, while the actin gene responded proportionally at 25 cycles (Fig. 4.9A and B). However, when the cDNA concentration was too high or too low, RT-PCR was not quantitative. The concentration of each cDNA sample was adjusted to stay within the semi-quantitative detection range (corresponding to between 1:2 and 1:32 dilution) (Fig. 4.2).

4.3.2.3. Differential expression of aromatase genes

The initiation of expression of both brain and ovarian aromatase mRNA lay between 3 and 4 dpf both in males and females. Aromatase mRNA (both brain and ovarian type) was detectable weakly in the RNA at 3 dpf and 40 cycles, but not at 35 cycles. However, from 4 dpf, it was expressed consistently within the detection range of this study. There were no significant sexual differences of brain or ovarian type aromatase gene expression levels up to 11 dpf.

The level of brain aromatase mRNA gradually increased through the period studied here with little difference between the sexes. No significant differences were found although the level in females was almost always higher than that in males (Fig. 4.10A and B). On the contrary, the change of ovarian aromatase mRNA expression through ontogeny was rather marked (Fig. 4.11A and B). While the expression level in females started to increase at 15 dpf, the expression in males showed down-regulation from 15 dpf resulting



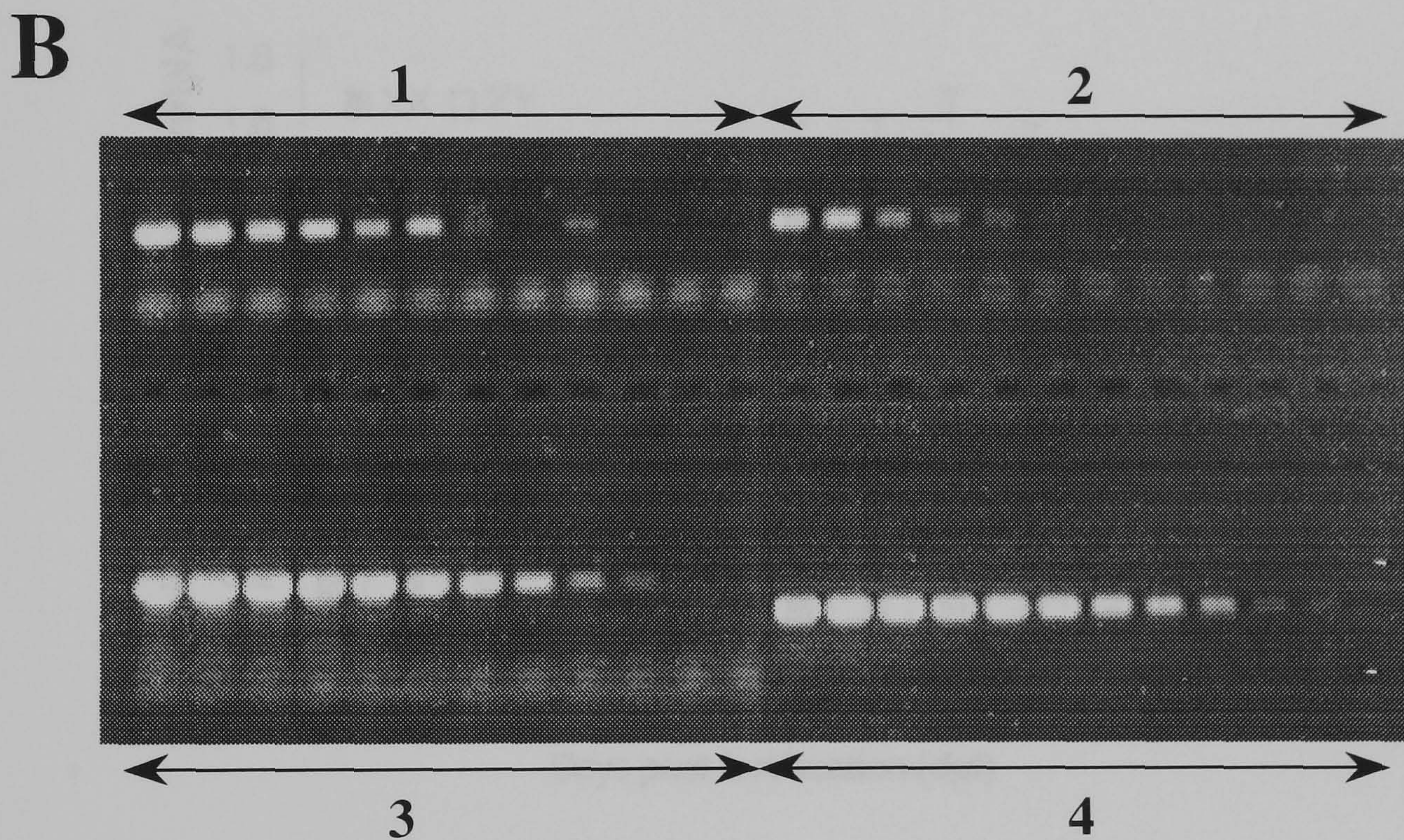


Fig. 4.9. Optimisation of PCR cycles for semi-quantitative PCR. A: The intensity of PCR products measured by image analysis system; B: PCR products run on an agarose gel. 1) 40 cycles for ovarian aromatase, 2) 35 cycles for ovarian aromatase, 3) 35 cycles for brain aromatase, 4) 25 cycles for actin gene. Each PCR was carried out with 12 serial dilution of standard cDNA sample (1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024 and 1:2048 – from left to right in each PCR).

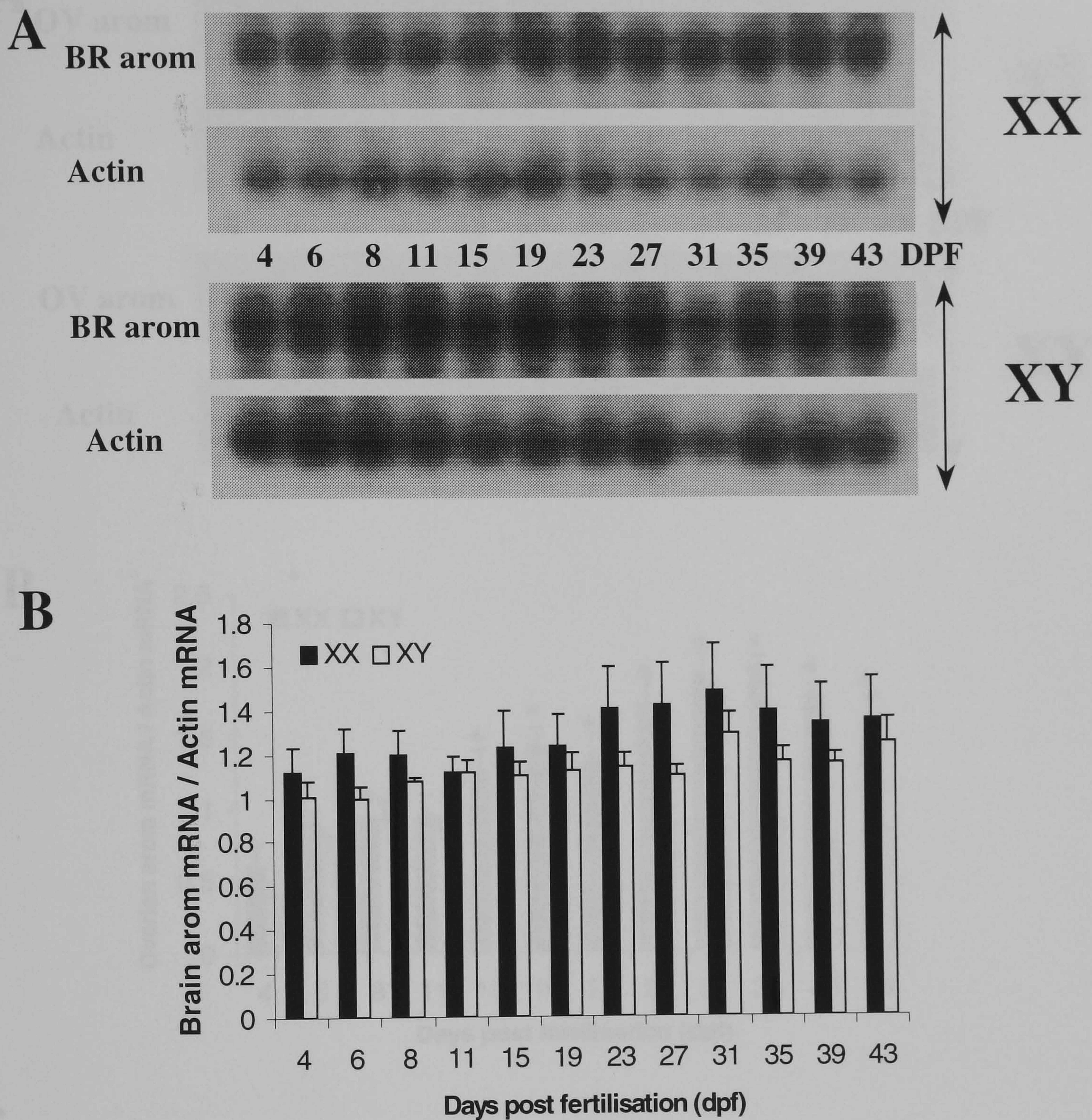
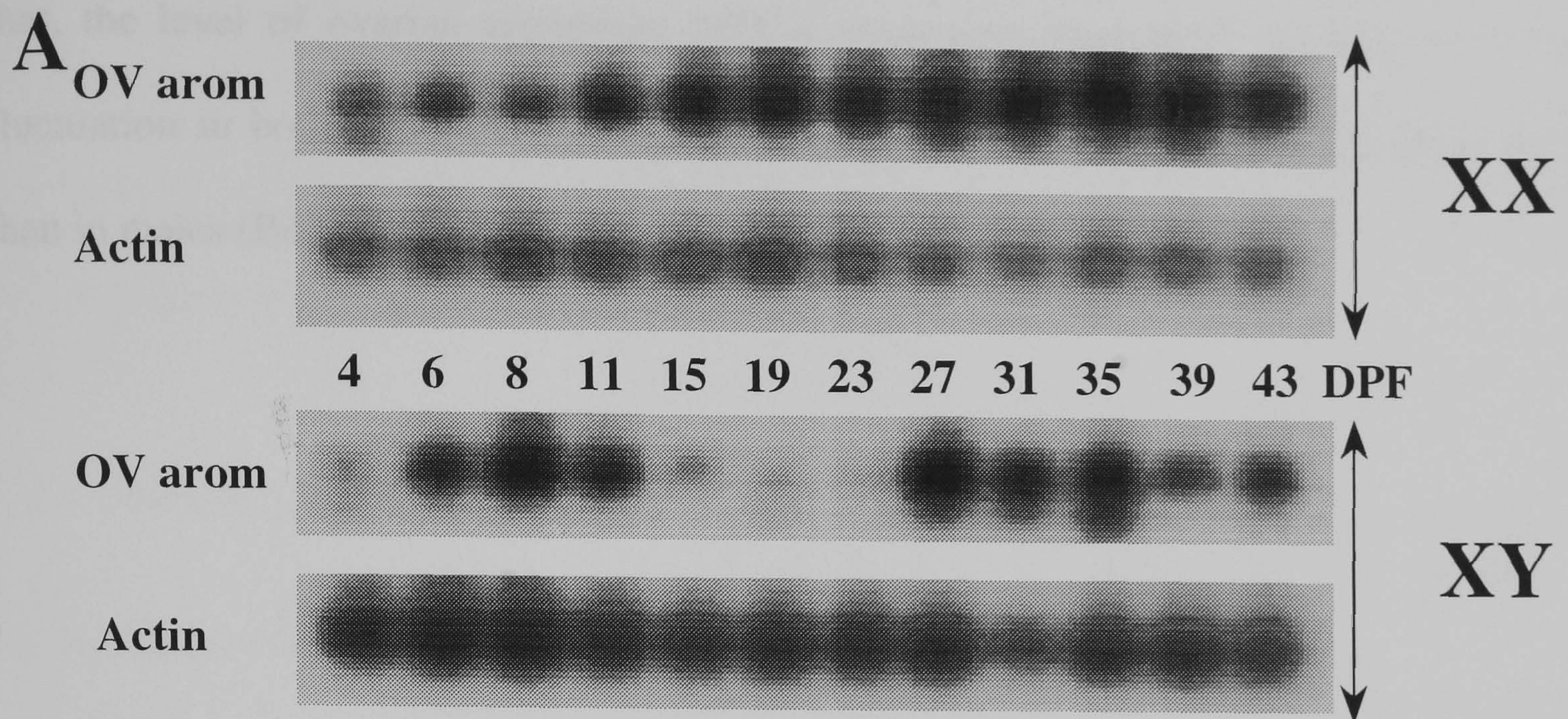


Fig. 4.10. Differential expression of brain aromatase mRNA (BR arom) during ontogeny in male and female groups. A: RT-PCR products were hybridised with gene specific probes and detected by autoradiography (representative of three RT-PCR); B: Relative expression of brain aromatase mRNA to actin mRNA (Actin). Each bar represents the mean ± S.E.



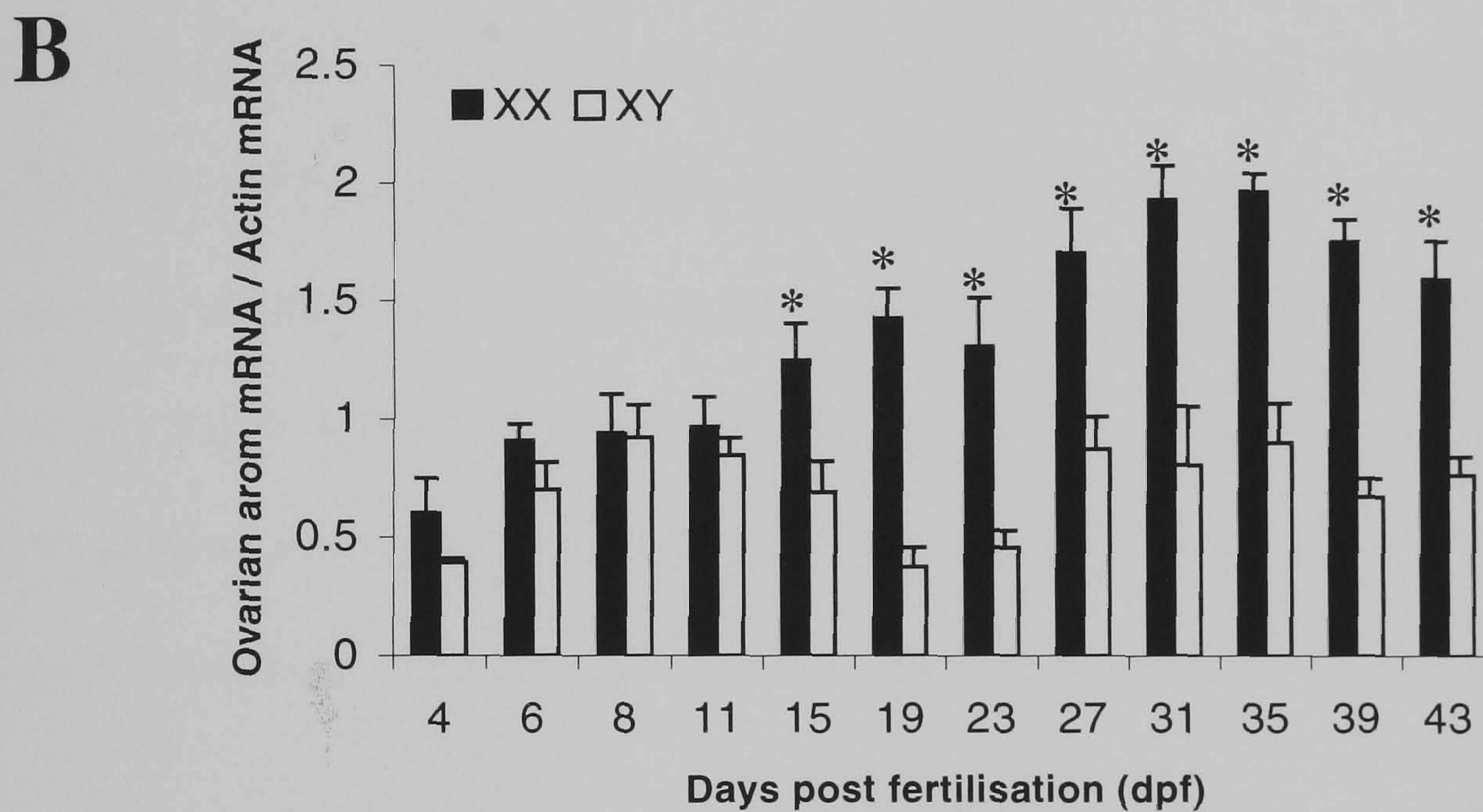


Fig. 4.11. Differential expression of ovarian aromatase mRNA (OV arom) during ontogeny in male and female groups. A: RT-PCR products were hybridised with gene specific probes and detected by autoradiography (representative of three RT-PCR); B. Relative expression of ovarian aromatase mRNA to actin mRNA (Actin). Each bar represents the mean ± S.E. * indicates significant difference between the sexes (P<0.05).

in significant differences between the sexes. The expression level in the males continued to drop through 19 and 23 dpf, and then showed some recovery at around 27 dpf. After that, the level of ovarian aromatase mRNA expression was fairly constant with some fluctuation in both sexes but the expression in females was always significantly higher than in males ($P<0.05$).

4.4. Discussion

A partial cDNA sequence of the brain type aromatase gene was identified from the brain of the Nile tilapia *O. niloticus*, and tissue specific expression in various adult tissues and the ontogeny of both brain type and ovarian type aromatase gene expression were investigated in this species using RT-PCR analysis.

4.4.1. Identification of the brain type aromatase gene and tissue specific gene expression

4.4.1.1. Brain type aromatase gene

The partial cDNA sequence of brain type aromatase identified in this study is different from that of the ovarian type aromatase identified previously (Chang *et al.* 1997) in the Nile tilapia. Within the identified region that corresponds to the putative exons 2 - 9, the homology of the amino acid sequence between brain and ovarian types was 64.3% in this species, whereas the homology of the *O. niloticus* brain aromatase gene to the *O. mossambicus* brain aromatase (Cruz and Canario 2000) and the goldfish brain aromatase gene (Tchoudakova and Callard 1998) was 96.7% and 69.9%, respectively.

In fish, aromatase genes have been cloned in rainbow trout ovary (Tanaka *et al.* 1992), catfish ovary (Trant 1994), medaka ovary (Tanaka *et al.* 1995), tilapia *O. niloticus* ovary (Chang *et al.* 1997), goldfish ovary (Tchoudakova and Callard 1998), goldfish brain (Gelinas *et al.* 1998), flounder ovary (Kitano *et al.* 1999) and tilapia *O. mossambicus* ovary (Cruz and Canario 2000) and brain (Cruz and Canario 2000). Alignment of the newly identified *O. niloticus* brain aromatase amino acid sequence to these previously reported aromatases in fish shows brain type aromatase genes are more closely related to each other in different fish species than to ovarian aromatase genes from any fish species.

This strongly suggests that brain type aromatase genes differ from ovarian aromatase genes at least in fish. In addition, both types of aromatase genes in fish clustered separately from human and chicken aromatase genes in a phylogenetic tree. This suggests that, in the evolutionary context, fish brain aromatase branched out from ovarian aromatase (or vice versa) after the divergence of bony fishes from the line of mammals and birds.

The size of the *O. niloticus* brain aromatase transcript was estimated as 2.6-2.7kb by Northern analysis. This is similar to those from *O. niloticus* ovary (2.6kb, Chang *et al.* 1997), rainbow trout ovary (2.6kb, Tanaka *et al.* 1992) and medaka ovary (2.6kb, Tanaka *et al.* 1995), but much shorter than those from catfish ovary (3.4kb, Trant 1994) and goldfish brain (3.0kb, Gelinas *et al.* 1998). There appears to be no great size difference between brain and ovarian aromatase mRNA in tilapia *O. niloticus*. However, Tchoudakova and Callard (1998) reported that brain aromatase mRNA (3.0kb) is much larger than ovarian aromatase mRNA (1.9kb) in the goldfish although they found another putative ovarian aromatase gene from the ovary (3kb) that differed from the brain aromatase gene.

Conventional approaches to the characterisation and sequencing of specific mRNA molecules frequently fail to yield full length message due to prematurely terminated cDNA molecules during cDNA library construction (Dorit *et al.* 1993). Possibly because of this reason, the 5'- cDNA sequence of brain aromatase gene could not be determined in this study. Chang *et al.* (1997) identified a 1.8kb cDNA sequence of tilapia *O. niloticus* ovarian aromatase gene. However, in their own report, the full length size of ovarian aromatase transcript was estimated as 2.6kb by Northern analysis. Neither brain nor ovarian 5'-cDNA sequence including flanking region have been clearly determined yet in

this species. For functional study of these genes, further study should determine the 5'-sequences of both genes.

4.4.1.2. Tissue specific aromatase gene expression

The presence of aromatase activity is considered as an indicator of oestrogen synthesis (Norris 1997). The aromatase activity is correlated with the expression level of aromatase mRNA (Lephart and Simpson 1990; Chang *et al.* 1997; Gelinas *et al.* 1998). Thus, detecting aromatase mRNA is commonly used to study oestrogenic activity in animal tissues (Harada and Yamada 1992; Abdelgadir *et al.* 1994; Chang *et al.* 1997; Trant *et al.* 1997; Gelinas *et al.* 1998; Kitano *et al.* 1999). Expression of aromatase genes in vertebrates has been observed in a variety of cells and tissues including the ovary, testis (Silberzahn *et al.* 1988), placenta (Kellis and Vickery 1989), brain and pituitary (Pasmanik and Callard 1989), adipose tissue (Lueprasitsakul and Longcope 1991), central nervous system (Gelinas and Callard 1993) and skin fibroblasts (Stillman *et al.* 1991). Results from the present study suggest some other putative steroidogenic tissues based on aromatase gene expression.

Tetrapod vertebrates possess a discrete adrenal gland that is known to have steroidogenic activity, mainly producing adrenocorticosteroids, and the kidney of these animals is known to be non-steroidogenic. Unlike these animals, in teleosts, the adrenal gland resides within the kidney (Milano *et al.* 1997). Hence, steroidogenic cells are found in the kidney of teleosts (Milano *et al.* 1997). Androgen and oestrogen synthesis was detected in tilapia kidney (Watts *et al.* 1995). A steroidogenic enzyme, 3β-hydroxysteroid dehydrogenase (3β-HSD) was also detected in the head kidney of rainbow trout by immunohistochemical method (Kobayashi *et al.* 1996). In spite of this evidence for steroidogenic activity, aromatase mRNA was not detected in fish kidneys examined so far

(catfish: Trant *et al.* 1997; flounder: Kitano *et al.* 1999). In these studies, the kidney RNA was examined by RT-PCR using ovarian aromatase gene-specific primers. Trant *et al.* (1997) speculated that multiple or different transcripts of aromatase are present in non-gonadal tissues in fish. In the present study, ovarian aromatase mRNA was not detected in the kidney, in agreement with the earlier studies. However, brain type aromatase mRNA was expressed in the kidney consistently in all males and females examined in this species. Interestingly, the kidney of males tended to have higher brain aromatase expression than that of females although the expression level was not quantitatively measured. This is consistent with the findings of Watts *et al.* (1995) where the kidney of male tilapia showed greater oestrogenic activity than that of female. Tchoudakova and Callard (1998) showed that both brain and ovarian forms of aromatase converted androgen to oestrogen. Taken together, it can be suggested that the brain type aromatase gene is responsible for the oestrogenic activity of the kidney in teleosts. However, the physiological role of the oestrogen that is formed in fish kidney remains unknown.

The spleen has not generally been considered as a major steroidogenic tissue. Human foetal spleen was the only spleen that expressed the aromatase gene in vertebrates until recently (Price *et al.* 1992). However, the spleen of *O. niloticus* in this study showed strong expression of the ovarian aromatase gene. The expression of the ovarian aromatase gene in fish spleen has also been reported in a flounder *P. olivaceus* (Kitano *et al.* 1999). Possible steroidogenic activity was also suggested in mouse spleen (Morohashi *et al.* 1999). SF1 (also called Ad4BP) was originally identified as a steroidogenic, tissue-specific transcription factor. Morohashi *et al.* (1999) detected SF1 immunoreactive cells in the spleen of mouse. In their study, the presence of an mRNA encoding cholesterol side-chain cleavage P450 was also detected in mouse spleen. All these results above indicate that the spleen in vertebrates is capable of producing steroid hormones from precursors,

although at the present time there is no explanation available for the function of locally produced steroids in the spleen.

The liver was classified as non-steroidogenic tissue in vertebrates (Waterman 1988). However, this classification is now in doubt, since aromatase mRNA was detected in human foetal and adult liver (Harada *et al.* 1993; Toda *et al.* 1994; see also review by Simpson *et al.* 1994). An mRNA encoding a steroidogenic enzyme, 3β-HSD was present in the liver of mouse (Bain *et al.* 1991). On the contrary, aromatase mRNA was virtually undetectable in mouse liver (Harada and Yamada 1992). In fish, no ovarian aromatase mRNA has been detected so far (catfish: Trant *et al.* 1997; goldfish: Gelinas *et al.* 1998; flounder: Kitano *et al.* 1999). In the present study, neither brain type nor ovarian type aromatase mRNA was detected in the liver of tilapia *O. niloticus*. Thus, it is unlikely that teleosts liver has oestrogenic activity, unless there is another tissue specific isoform of aromatase gene in fish liver.

In this study, low but consistent expression of brain aromatase mRNA and very weak and inconsistent expression of ovarian aromatase mRNA were detected in the eyes of tilapia. Similarly, Tchoudakova and Callard (1998) reported expression of both brain and ovarian type aromatase mRNA in goldfish retina. However, the pattern of expression of the two different genes was reversed. In goldfish, expression of ovarian aroamtase in the retina was 25 fold higher than that of brain aromatase. Direct comparison would not be appropriate since the present study was not quantitative and also the sampling methods may have been different.

As expected, brain and ovarian aromatase mRNA were most abundant in the brain and in the ovary, respectively. Brain aromatase mRNA was expressed in both ovary and testis and ovarian aromatase mRNA was expressed in both male and female brains. This

contrasted with the finding of Tchoudakova and Callard (1998). They reported that ovarian aromatase was expressed in goldfish brain, but brain aromatase was not expressed in goldfish ovary. However, in their previous report (Tchoudakova and Callard 1995), brain aromatase was clearly detected in goldfish ovary by RT-PCR using brain-derived aromatase primers. The sequence of the brain aromatase product obtained from goldfish ovary was identical to that from goldfish brain. Before obtaining this result, they had failed to hybridize ovarian RNA with brain specific aromatase probes in their preliminary study and made a new attempt using RT-PCR. Based on the RT-PCR result, they explained that the negative result they had obtained from Northern analysis was due to insufficient sensitivity (Tchoudakova and Callard 1995). Contradictorily, in their recent report (Tchoudakova and Callard 1998), they could not detect brain aromatase gene in goldfish ovary using RT-PCR. It is hard to judge which of their reports contains the correct information. It may be possible that the gene expression in the ovary might be altered at different reproductive stages or different external environments. Nevertheless, in the present study, brain aromatase mRNA was repeatedly detected from individual to individual in all ovaries and testes examined in tilapia *O. niloticus*. The expression of ovarian aromatase in the brain is in general agreement with all previous studies (Trant *et al.* 1997; Tchoudakova and Callard 1998; Kitano *et al.* 1999).

In vertebrates, locally produced oestrogen and/or gonadal-origin oestrogen in the brain play some important physiological role in sexual differentiation of brain tissue (Arnold and Gorski 1984; Naftolin 1994; Hutchison 1997), sexual behaviour (Hutchison 1991; Wingfield 1994; Gahr 1994) and reproduction (Callard *et al.* 1986; Pasmanik and Callard 1988a; Kah *et al.* 1993). The present study provides evidence of local oestrogen synthesis by both brain type and ovarian type aromatase genes in the brain of tilapia. This finding motivates further studies on the physiological role of aromatase and locally produced

oestrogen in sexual differentiation of brain tissue, sexual behaviour and reproduction in fish.

Many non-gonadal tissues appear to be capable of synthesising oestrogen locally. It is not clear whether these locally produced oestrogens in different tissues are released into circulation and contribute to the plasma oestrogen levels. If both gonadal and non-gonadal steroids are released into circulation, new mechanistic explanations would be required for many steroid-mediated biological events such as sex differentiation, sexual behaviour and reproduction in vertebrates. Additionally, tissue specific expression patterns of both brain and ovarian aromatase genes in this study suggest possible functional differences between the two genes.

4.4.2. Differential expression of brain and ovarian aromatase genes during ontogeny

Despite much evidence that fish embryos at very early developmental stages respond to AI and steroid hormones resulting in sex-reversal (Piferrer *et al.* 1994; Gale *et al.* 1999; Kwon *et al.* 2000), no attempts to investigate aromatase gene expression during these early stages had been made prior to this study. For the first time, expression of aromatase genes in fish was investigated from fertilisation through the gonadal differentiation period and beyond in this study.

Determination of phenotypic sex in *O. niloticus* appears to take place much earlier than previously proposed. The most distinctive sexual dimorphism of aromatase gene expression occurred before the reported period of the first appearance of steroid producing cells in gonadal tissues.

4.4.2.1. Initiation of aromatase gene expression and the primary aromatisation site

All studies of aromatase action on fish sex differentiation so far have focused on the gonads or presumptive gonadal area at around the time when histologically discernible gonadal tissue appears (Baroiller *et al.* 1999; Guiguen *et al.* 1999; Kitano *et al.* 1999). These studies suggested that aromatase that is differentially expressed in male and female gonadal tissue may direct sexual differentiation of fish gonads. However, this idea conflicts with many previous findings from sex-reversal experiments where steroid or AI treatment given much earlier than the stages when gonadal tissue becomes identifiable significantly affected fish sex differentiation (Piferrer *et al.* 1994; Gale *et al.* 1999; Kwon *et al.* 2000).

In this study, the timing of initiation of both brain and ovarian aromatase gene expression was estimated at around hatching (3-4 dpf in this species). Since the first sign of gonadal differentiation appears more than 20 days later (27-30 dpf) (Nakamura and Nagahama 1985 and 1989), it would not be logical to persist in the view that the observed expression at this early stage occurred in the gonads or presumptive gonadal area. The results from the tissue specific expression study suggest that the aromatisation site at this stage could be the brain, eye, kidney or spleen. Among these tissues, only the brain consistently expressed both brain type and ovarian type aromatase genes in this study. The brain has been suggested as the primordial site of aromatase expression in mammals together with the gonads (Simpson *et al.* 1994). Furthermore, the CNS in vertebrates is developed fully by the time 10-15% of embryonic life has passed (Laming 1981). Thus, the brain emerges as the most promising tissue responsible for the aromatisation that takes place at the early stage. However, other tissues including the kidney cannot be excluded yet, since the aromatase transcript in embryonic tissues may be different from that of adult

tissues. The presence of steroidogenic cells was observed in the kidney (pronephros) of larvae with and without yolk sac and also juveniles in Perciformes where tilapia belongs (Milano *et al.* 1997).

The findings from this study suggest that, in lower vertebrates, non-gonadal tissues (quite likely the brain) may be responsible for early aromatase gene expression that might direct further sexual differentiation of other tissues including the gonads. Results from reptiles support this suggestion. In reptiles, steroidogenesis occurs in the adrenals, the mesonephros and the liver, but little in the genital ridge and the gonads of very early embryos (White and Thomas 1992). Jeyasuria and Place (1998) suspected the brain as a primary aromatisation site for sex determination in reptiles. They found the presence of aromatase mRNA in the brain prior to its presence in the presumptive ovary. Further studies for localisation of aromatase gene expression using *in situ* hybridisation techniques during ontogeny would reveal the exact aromatisation site during the early stages.

4.4.2.2. Differential expression of aromatase genes during ontogeny

The significance of aromatase expression during sex differentiation has been proposed in three fish species (Guiguen *et al.* 1999; Kitano *et al.* 1999).

In tilapia, Guiguen *et al.* (1999) showed that aromatase activity in the gonads of a control group (i.e., all female group produced by crossing XX male to normal female) was much higher at 64 dpf than that of a sex-reversed male group induced by dietary administration of a steroidal aromatase inhibitor (ATD, 1,4,6-androstatriene-3-17-dione). However, this higher activity of aromatase in the female group is likely to be the result of gonadal differentiation rather than the cause. Phenotypic sex in this species was implied to

be determined between 27-30 dpf, based on histological observation (Nakamura and Nagahama 1985 and 1989), the first appearance of steroid producing cells and immunopositive reactions for steroidogenic enzymes including aromatase (Nakamura *et al.* 1997) in the presumptive gonadal area.

Differential expression of ovarian aromatase gene between females and temperature-induced sex-reversed males during the sex differentiation period was also proposed in a flounder *P. olivaceus* (Kitano *et al.* 1999). There were no differences in the expression of the aromatase gene between indifferent gonads of female and sex-reversed male. However, "after the initiation of sex differentiation", the level of aromatase gene expression increased rapidly in female flounder gonads.

In rainbow trout, aromatase gene expression was observed at 55 dpf (= 20 dph) two weeks before the first occurrence of the early oocyte meiosis (70 dpf = 35 dph) in the developing ovary (Guiguen *et al.* 1999). Aromatase expression in female gonads was at least a few hundred times higher than in male gonads during the period studied (55-127 dpf at 10°C: 550 – 1270 degree days) in their experiment. However, the previously proposed sex differentiation period in this species (45-55 dpf at 8-10°C: 360 – 440 degree days or 450 – 550 degree days) (Takashima *et al.* 1980; van den Hurk and Slof 1981) was earlier than the examined period in the study by Guiguen *et al.* (1999).

These authors' findings appear to suggest that aromatase plays an important role in maintaining or accelerating ovarian differentiation rather than initiating gonadal sex differentiation. If the expression of aromatase genes in the presumptive gonadal area is to be an initiating factor of sexual differentiation, it should take place before the occurrence of the sign of gonadal differentiation. One should also answer the question: how can a

short immersion treatment by steroids or AI in the very early stages (around hatching or first feeding) cause permanent sex change when the gonadal tissues are not discernible and when no steroid producing cells are identified in the presumptive gonadal area?

In the present study, the expression of the ovarian aromatase gene in females after the proposed gonadal differentiation period (27-43 dpf) was also significantly higher than in males. This is consistent with the previous findings mentioned above, supporting the idea that aromatase may be a maintaining or accelerating factor of ovarian differentiation. On the other hand, the expression of the ovarian aromatase gene in males was down-regulated from 15 dpf while it was up-regulated in females at the same period. It can be suggested that the down-regulation of the ovarian aromatase gene in males is reponsible for the determination of phenotypic sex in this species. Previous findings in this thesis also support this suggestion. When fish fry were fed with AI treated food for one week (11 - 17 dpf), or immersed once in a solution containing AI for 3 hours at 15 dpf, the percentage of males was significantly increased (Chapter 3). Feeding with AI at 18 – 24 dpf also resulted in some masculinising effect, but later than 24 dpf, treatment of two weeks duration was required to achieve a masculinising effect with the same AI (Kwon *et al.* 2000).

Both down-regulation of the aromatase gene and sensitivity to aromatase inhibition indicate that the decisive steroidal event(s) for phenotypic sex determination take place around 15 – 23 dpf in this species. Hines *et al.* (1999) reported the presence of sexually indifferent gonadal structure at 15 - 29 dpf and histologically identifiable ovaries at 36 dpf in the same species. Considering the timing of first SPC occurrence (27-30 dpf) in the gonadal area of this species (Nakamura *et al.* 1997), it is not likely that the decisive steroidal event takes place in the presumptive gonadal area. As discussed before, the signal

for the initiation of gonadal differentiation might come from somewhere else (Merchant-Larios and Villalpando 1990). A similar idea has been proposed in another group of lower vertebrates, reptiles. Gonadal sex in turtles is determined by incubation temperature, mediated by aromatase and steroid action. When the gonads of turtles are removed from the body and organ-cultured, they undergo differentiation *in vitro* but are not affected by male and female incubation temperatures, implying that the signal for differentiation is under temperature control but comes from a source other than the gonad itself (Merchant-Larios and Villalpando 1990). Based on the asumption that, in teleosts, events in the brain may determine the fate of the gonads (Francis 1992; Kwon *et al.* 2000), it can be hypothesised that the decisive steroidal event may take place in the brain and the signal, the final product of the decisive steroidal event, may be delivered to the indifferent gonadal area. The signal for initiation of gonadal differentiation would not necessarily be steroidal. It could be other brain hormones such as GnRH or GTH. In fact, sex reversal effects of GnRH, HCG and neuropeptide Y have been reported in hermaphrodite fish species (reviewed by Baroiller *et al.* 1999). Further studies on brain hormones would provide some useful information on the role of the brain in fish sex determination.

The sex of the fish in the sampled groups was confirmed by sexing the remaining fish in each group at the end of the experiment. The genetic male group (XY) was 100% male and the genetic female group was 84.4% female. Therefore, the dimorphic expression of ovarian aromatase gene from the examined groups can be considered as a true reflection of the ontogenic genetic difference between female (XX) and male (XY) fish. This supports the idea that sex is determined genetically in this species, although sex specific genes have not been identified. The present findings clearly suggest the involvement of the ovarian aromatase gene in the determination of phenotypic sex. The next step should be towards the regulation factors of this gene during ontogeny. A transcription factor, SF1, was

suggested to be involved in the regulation of aromatase gene expression in medaka (Watanabe *et al.* 1999). SF1 possesses a SOX9 binding site in its upstream region. Thus, it is likely that SOX9 regulates aromatase via SF1 (Nomura *et al.* 1995). However, there is no comprehensive information on the SOX9 gene in fish. Cloning genes from the SOX family and monitoring their expression during ontogeny would reveal more details of the genetic basis of the sex determining mechanism in this species.

Unlike ovarian aromatase mRNA, brain aromatase mRNA was expressed highly both in males and females through the developmental stages without showing clear sexual dimorphism. This casts a question: what is the function of brain type aromatase? Different patterns of tissue specific expression of brain and ovarian aromatase mRNA in this study implied possible functional differences between these two aromatases. Tchoudakova and Callard (1998) did not find any functional difference between brain and ovarian aromatase genes in terms of catalysing androgens into oestrogens. A finding from Osawa *et al.* (1993) provides a comprehensible explanation. According to Osawa *et al.* (1993), aromatase does not only catalyse androgens to oestrogens, but also metabolises oestrogens rendering them biologically inactive, although androgens have a higher affinity and turnover rate than oestrogens. The human placenta expresses a different aromatase transcript using an alternative promoter (reviewed by Simpson *et al.* 1994). The functional difference of the placental aromatase transcript from the ovarian one in humans is also unclear. Aromatisation yields oestrogens, but some of the oestrogen undergoes further 2-hydroxylation and is released from the active site of aromatase as the catechol oestrogen, an inactive metabolite. This pathway increases when there are high concentrations of oestrogens, such as in human pregnancy (Osawa *et al.* 1993). Placental aromatase was suggested to be responsible for the same pathway during pregnancy. This explanation might be applicable to fish, since the aromatase activity in the teleost brain is 100-1000

fold higher than in the mammalian brain and at least 10 fold higher than in the teleost ovary (Pasmanik and Callard 1988a). The fish brain might have a highly active oestrogen metabolism, and brain type aromatase might function as oestrogen 2-hydroxylase to metabolise oestrogens in fish brain. Nonetheless, at this moment, the role of the brain type aromatase in fish remains unknown.

In conclusion, the findings from this study clearly suggest that ovarian aromatase plays a decisive role in sex determination in tilapia *O. niloticus*, and that this decisive role would be achieved more likely by down-regulation of the gene expression in males rather than by up-regulation in females. The ovarian aromatase gene also appears to have a propelling effect on ovarian differentiation. The major sex determining period in this species appears to lie between 15-24 dpf. Additionally, the decisive aromatisation might take place in extragonadal tissues such as the brain but this needs to be further clarified.

CHAPTER 5

INTERACTION BETWEEN TEMPERATURE AND AROMATASE

5.1. Introduction

To a certain extent, all sorts of living organisms attain their biological identity by interacting with the surrounding environments and survive by utilising environmental resources. This biological identity includes appearance, metabolism, reproduction, behaviour, sex, psychological status, and so forth. The interaction between environments and living organisms is witnessed everywhere, forming and changing identities.

Among those identities, the sex of an organism shows one of the most interesting and dramatic interactions with its environments. Under certain environmental conditions, genetic males in some animals become females, especially during the early development, so called "sex reversal". It is even more dramatic when it happens in a gonochoristic species in which the sex is believed to be determined at fertilisation by genetic factors (e.g., XX/XY sex chromosomes). This dramatic phenomenon has been realised in many lower vertebrates including the Nile tilapia *O. niloticus* which is the subject species in this thesis.

The Nile tilapia, which has an XX/XY chromosomal genetic sex determination, also exhibits temperature-dependent sex determination (TSD) at high temperature (approximately 34-37°C) (Mair *et al.* 1990; Baroiller *et al.* 1995a,b; 1996; Abucay *et al.* 1999). TSD has also been reported in many other fish species including Atlantic silverside *Menidia menidia* (Conover and Kynard 1981; Conover and Fleisher 1986), another atherinid *Odontesthes bonariensis* (Strüssman *et al.* 1997), sockeye salmon *Oncorhynchus nerka* (Craig *et al.* 1996), channel catfish *Ictalurus punctatus* (Patiño *et al.* 1996), a cichlid, *Apistogramma sp.* (Römer and Beisenherz 1996), blue tilapia *O. aureus* (Desprez and Mélard 1998), European sea bass *Dicentrarchus labrax* L. (Blázquez *et al.* 1998) and a flounder *Paralichthys olivaceus* (Kitano *et al.* 1999; Yamamoto 1999). Thus, the effect of temperature on fish sex determination is indisputable. However, the pattern of TSD in fish

is not uniform. In some fish species, sex ratios are skewed towards male at higher temperature (e.g., tilapia *O.niloticus* and *O. aureus*, Atlantic silverside) but towards female in other species (e.g., *Apistogramma sp*, channel catfish). As an extreme, *P. olivaceus* shows 100% masculinisation at both higher and lower temperatures in some crosses (Yamamoto 1999). This complexity brings about difficulties in understanding TSD in fish.

Attempts to establish a physiological explanation for TSD in fish are impeded by the complexity of TSD pattern between different species. The complexity prevails even within one species. *O. niloticus* exemplifies this complexity. From several observations, it became obvious that high temperature increases the percentages of males in genetic females (XX) or mixed-sex groups of this species (Mair *et al.* 1990; Baroiller *et al.* 1995a,b; 1996; Abucay *et al.* 1999). On the other hand, Abucay *et al.* (1999) reported a feminising effect of high temperature in some YY genetic males. Also, the effect of high temperature varied with parental combinations and strains (Baroiller *et al.* 1995b; Abucay *et al.* 1999). So far, no satisfactory explanation has been proposed for this complexity of TSD in any fish species.

It has been demonstrated in certain reptiles that aromatase, which converts androgens into oestrogens, is expressed and active in the developing gonads at female-determining temperatures but not male-determining temperatures (Pieau *et al.*, 1994a,b; Jeyasuria and Place 1998), suggesting temperature acts through aromatase. In fish, a similar idea was proposed by investigating aromatase mRNA expression during the sex differentiation period in *P. olivaceus* (Kitano *et al.* 1999). In addition, results from Chapter 3 and 4 demonstrated that aromatase plays an important role in tilapia sex differentiation. However, it is not clear yet whether TSD is related to aromatase action in fish.

The purpose of the study described in this chapter was to confirm the temperature effect on sex ratios in different genotypes (XX, XY and YY) of tilapia *O. niloticus* and

investigate the possible involvement of aromatase action in YY feminisation using an aromatase inhibitor, Fadrozole™ used in Chapter 3 in elucidating the role of aromatase in "normal" sex differentiation. Additionally, an attempt was made to establish a genetic and physiological based explanation for the interaction between temperature and sex determination in this species.

5.2. Materials and Methods

The broodstock (XX normal female, XX neomale, YY male and YY neofemale) used in this study were selected through the earlier study and maintained as described in section 2.2 in Chapter 2.

Three different genotypes of fry were obtained by crossing XX normal female to XX neomale, XX normal female to YY male, and YY neofemale to YY male, yielding genetically all female fry (XX), all male fry (XY), and all male fry (YY), respectively (Fig. 5.1). Egg and milt collection, fertilisation and incubation of embryos were carried out as described in section 3.2 in Chapter 3. The resultant fry were subjected to different combinations of AI and temperature treatments.

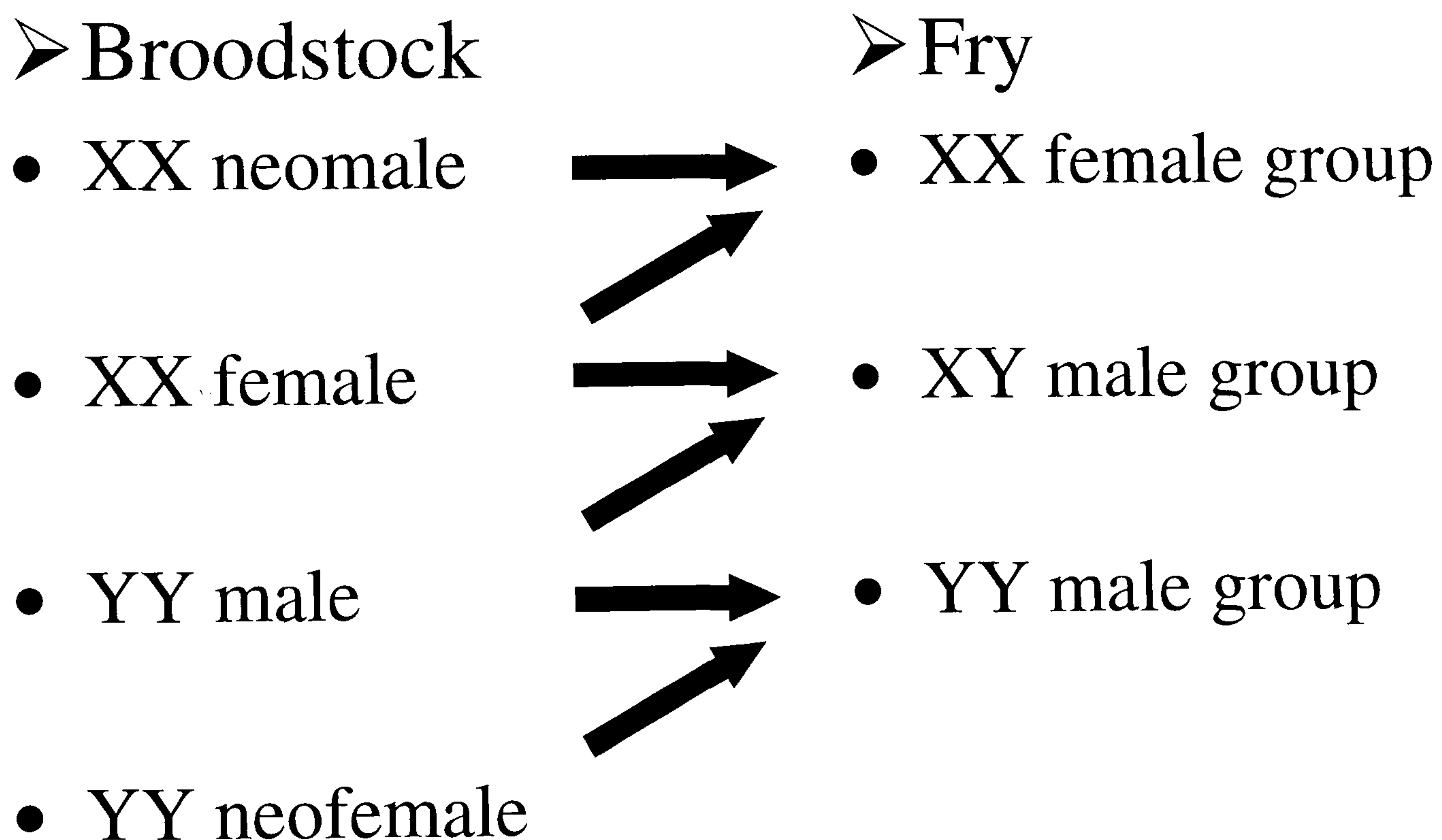


Fig. 5.1. Preparation of genetic female (XX) and genetic male (XY or YY) fry for temperature and AI treatments.

To accommodate each isolated replicate tank (approximately 5 L) for high temperature treatment in identical conditions, a glass aquarium was set up (40 × 40.5 × 120 cm). After transferring fry into each small tank and placing each small tank within the aquarium, the water temperature within the aquarium was raised from 28 to 36°C over 1 hour and maintained at 36°C by a heater/pump system until the temperature treatment was completed. For the control temperature groups (28°C), fry were transferred into the isolated replicate tanks (approximately 5 L) and the water temperature in each tank was maintained at 28±1°C by a heater. A partial water change was carried out every day to remove faeces and uneaten food and to maintain water quality. The experimental design is illustrated in Fig. 5.2. After 30 days treatment from first feeding onwards, fish were transferred to tanks in a recirculating system (26-27°C) and grown until 10-12 weeks old when they were sexed by the same method used in section 2.2 in Chapter 2.

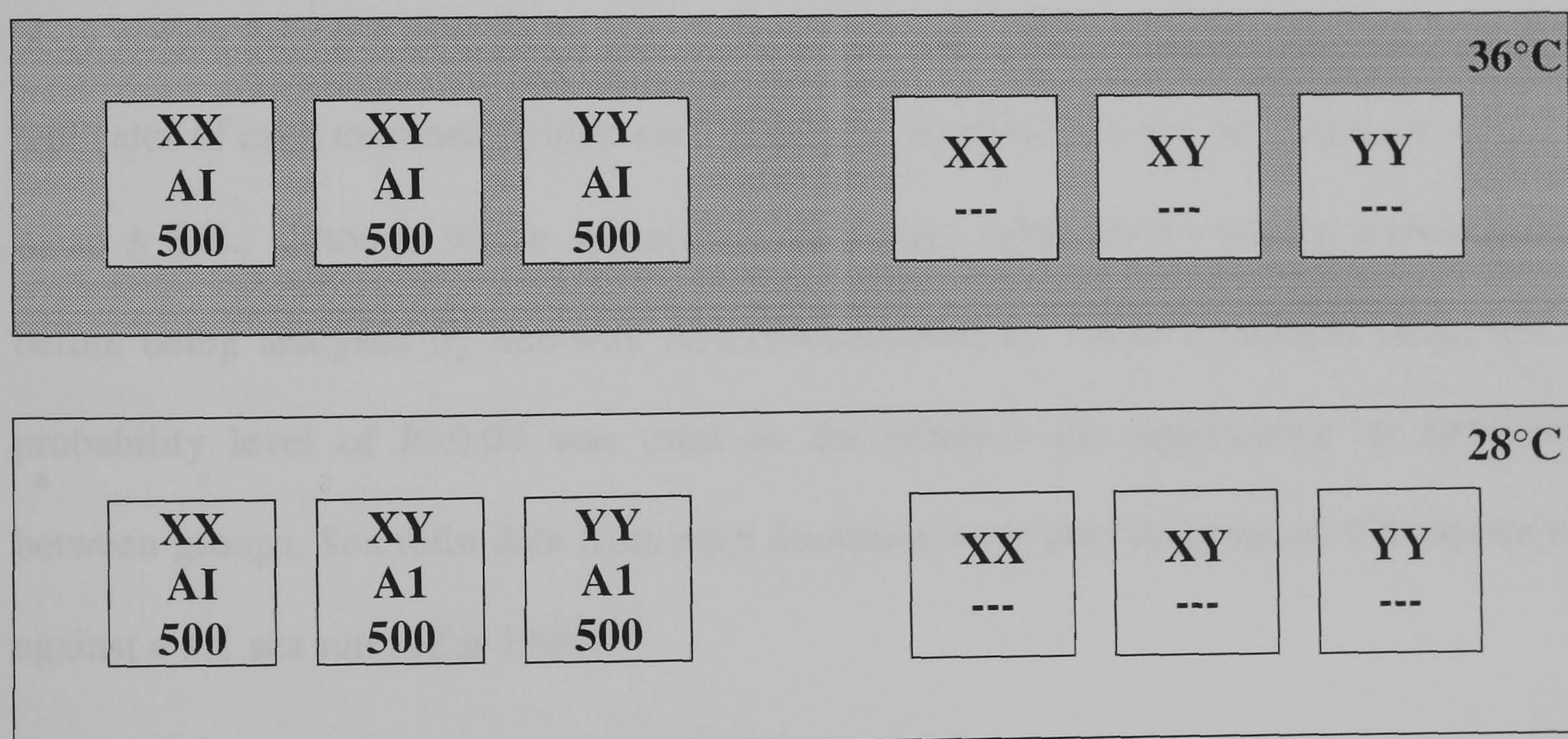


Fig. 5.2. Experimental design for the effects of temperature and AI treatments. Each genotype (XX, XY and YY) had three replicates in all treatments with the exception of the XY groups for low temperature treatment (two replicates).

The experiment for each genotype was carried out separately as female broodstock do not spawn at the same time. However, the three separate experiments for the different genotypes were conducted under as identical conditions as possible. In each experiment, tilapia fry were exposed to two different temperature (28 and 36°C) and were fed with a control diet or one that contained the chemical aromatase inhibitor (AI: Fadrozole™ CGS16949A) for four weeks from first feeding. AI was dissolved in ethanol (100%), and mixed with fry diet to a final concentration of 500 mg/kg (see Chapter 2 for details; the concentration was determined based on the previous study - Kwon *et al.* 2000). Each treatment had three replicates (except for the XY groups: both 28°C treatment with and without AI, had two replicates due to the small number of fry available of this genotype) that contained 50 to 65 fish each initially.

Differences in growth rate between treatments were not statistically compared to each other due to low survival rates in some replicates. Relative growth of total length against body weight between treatments was compared to each other using pooled data from three replicates of each treatment within each genotype. Survival and sex ratio data are shown as mean ± S.E., although where appropriate they were subjected to arcsine transformation before being analysed by one-way ANOVA followed by Tukey's multiple range test. A probability level of $P<0.05$ was used as the criterion for significance of differences between groups. Sex ratio data from each treatment were also analysed by Chi-square test against a 1:1 sex ratio (Zar 1984).

5.3. Results

5.3.1. Growth

Differences of growth rate between treatments in the different genotypes could not be properly compared to each other due to different survival rates in the replicates of the treatments. However, when analysed using results from some replicates that showed similar survival, no clear growth differences were identified. In addition, relative growth of total length against body weight from the pooled data was identical in all treatments within each genotype, indicating that there was no obvious difference in body conformation between temperature and AI treatments.

5.3.2. Survival rate

The survival rates in two replicates of the control temperature (28°C) without AI and one replicate of the control temperature with AI in the XX groups was poor and it was not possible to make a meaningful comparison with the high temperature treated groups (36°C) (Table 5.1). In the XY groups, the survival rates in control temperature groups appeared higher than that in the high temperature groups but this was not statistically different (Table 5.2). On the other hand, in the YY groups, high temperature significantly lowered survival rate compared with control temperature regardless of AI treatment ($P<0.05$, Tukey's test) (Table 5.3).

5.3.3. Sex ratios

The sex ratios of the control groups from the three different genotypes differed significantly from a 1:1 sex ratio, and verified that the fry used in this experiment conformed to the expected sex ratios for XX, XY or YY groups (Table 5.1, 5.2 and 5.3,

Table 5.1. Sex ratios of XX group *O. niloticus* after being treated (for 30 days from first feeding) at two different temperatures with or without aromatase inhibitor (AI)

Treatments	No. of replicates	No. of fish sexed	No. of males	No. of females	Survival (%)	Males (%)	$\chi 2$ values (against 1:1 sex ratio)
28 without AI	3	73	16	57	40.6 ± 24.9	28.2 ± 11.7^{a}	23.03**
28 with AI	3	96	95	1	57.8 ± 26.7	99.2 ± 0.8^{c}	92.04**
36 without AI	3	144	90	54	77.4 ± 5.8	61.8 ± 9.4^{b}	9.00*
36 with AI	3	79	79	0	41.5 ± 17.5	100.0 ± 0.0^{c}	79.00**

*P<0.05 and **P<0.001. All percentage data are shown as Mean ± SEM from triplicate values. Different alphabetical superscripts within the same column indicate significant difference (P<0.05).

Table 5.2. Sex ratios of XY group *O. niloticus* after being treated (for 30 days from first feeding) at two different temperatures with or without aromatase inhibitor (AI)

Treatments	No. of replicates	No. of fish sexed	No. of males	No. of females	Survival (%)	Males (%)	$\chi 2$ values (against 1:1 sex ratio)
28 without AI	2	84	84	0	93.3 ± 6.7	100.0 ± 0.0^{a}	84.00*
28 with AI	2	87	87	0	96.7 ± 1.1	100.0 ± 0.0^{a}	87.00*
36 without AI	3	115	112	3	76.7 ± 8.2	97.5 ± 1.3^{a}	103.31*
36 with AI	3	122	122	0	81.3 ± 4.8	100.0 ± 0.0^{a}	122.00*

*P<0.001. All percentage data are shown as Mean ± SEM from replicate values (for 28°C) or triplicate values (for 36°C). Different alphabetical superscripts within the same column indicate significant difference (P<0.05).

Table 5.3. Sex ratios of YY group *O. niloticus* after being treated (for 30 days from first feeding) at two different temperatures with or without aromatase inhibitor (AI)

Treatments	No. of replicates	No. of fish sexed	No. of males	No. of females	Survival (%)	Males (%)	χ^2 values (against 1:1 sex ratio)
28 without AI	3	126	126	0	96.4 ± 0.5[a]	100.0 ± 0.0[a]	126.00*
28 with AI	3	120	120	0	84.6 ± 5.3[ab]	100.0 ± 0.0[a]	120.00*
†36 without AI	3	62	40	14	32.3 ± 13.1[c]	60.2 ± 5.8[b]	12.52*
36 with AI	3	87	86	1	49.7 ± 9.0[bc]	98.8 ± 1.2[a]	83.05*

*P<0.001. All percentage data are shown as Mean ± SEM from triplicate values. † Intersex fish (n=8) were observed. Intersex fish were not included in the percentage of males. Different alphabetical superscripts within the same column indicate significant difference (P<0.05).

P<0.001, Chi-square test) although the control groups for XX showed high percentages of males (28.2±11.7%) implying some minor autosomal or non-genetic parental influences. As can be seen in Fig. 5.3, AI treatment of genetically female fry significantly increased the percentage of males (99.2±0.8%) in this species, as observed in Chapter 3 (P<0.05, Tukey's test). High temperature induced significant masculinisation (61.8±9.4%) in genetically female tilapia (XX) (P<0.05, Tukey's test). Interestingly, high temperature also caused significant feminisation (39.8±5.8%) in genetically male tilapia with the YY genotype (P<0.05, Tukey's test), while it did not strongly influence genetically male tilapia with XY genotype. This high temperature feminisation in YY males was significantly suppressed by the treatment with AI (P<0.05, Tukey's test). In YY groups, high temperature treatment also resulted in a small number of intersex fish (8 out of 62) which had a pair of typical testis with a few oocytes inside the testis.

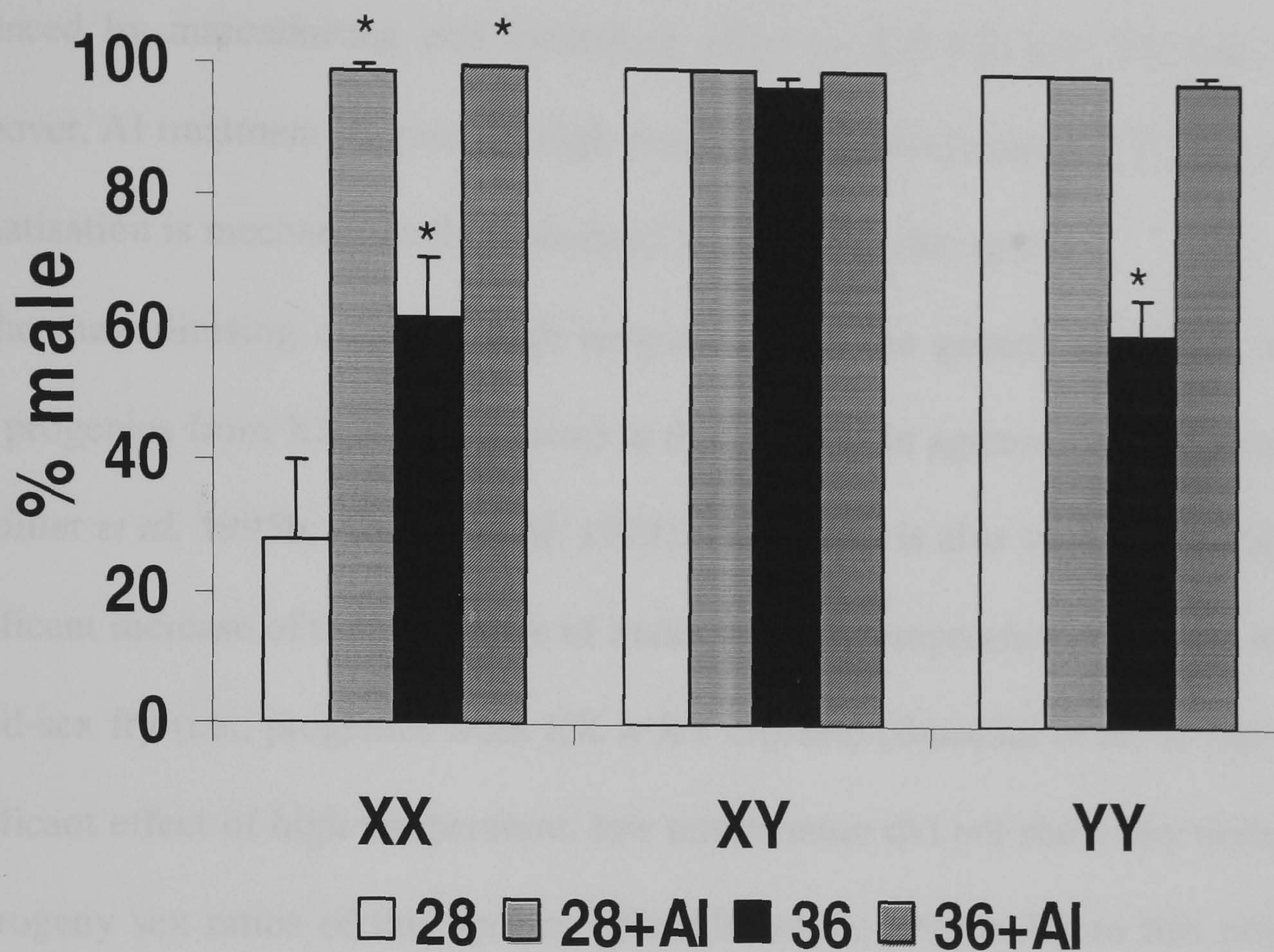


Fig. 5.3. Percentages of males after AI and high temperature (36°C) treatments. Each bar represents the mean ± S.E. * indicates significant differences between control (28°C, no AI) and each treatment ($P<0.05$) within each genotype.

5.4. Discussion

It became certain that high temperature overrides genetic sex determination in this species as evidenced by masculinising and feminising effect in XX fish and YY fish, respectively. Moreover, AI treatment suppressed high temperature feminisation in YY fish, implying that aromatisation is mechanistically associated with TSD in this species.

The masculinising effect of high temperature on the genetic female *O. niloticus* fry (i.e., progenies from XX × XX crosses) in this study is in agreement with previous studies (Baroiller *et al.* 1995b; Abucay *et al.* 1999). This effect is also supported indirectly by the significant increase of the pecentage of males by high temperature treatment in genetically mixed-sex fry (i.e., progenies from XX × XY crosses) (Baroiller *et al.* 1996b). Unlike the significant effect of high temperature, low temperature did not show any noticeable effect on progeny sex ratios of this species (Baroiller *et al.* 1995b). Up to this point, it seems likely that TSD in this species is simple unidirectional – i.e.,"only high temperature" influences "mainly genetic females" resulting in maculinisation. However, the findings from the present study and Aubcay *et al.* (1999) refute this simple unidirectional TSD in this species. When YY genetic males were exposed to high temperature, the percentages of males were significantly decreased both in the study of Abucay *et al.* (1999) and in the present study. Sex ratios of XY genetic males were also affected by high temperature although statistically not significant. These finding suggest that the pattern of TSD in this species is not unidirectional but bidirectional – i.e.,"only high temperature" influences both genetic females and males resulting in both masculinisation and feminisation.

The existence of TSD in this species is obvious and the pattern of TSD observed in this study is in agreement with Abucay *et al.* (1999). However, it is not known how the high temperature results in masculisation of genetic females and femininisation in genetic

males. Abucay *et al.* (1999) used a polygenic sex determination system to explain TSD in this species. According to their explanation: "Male and female genes may be located in many chromosomes and the determination of sex depends on the balance of these genes. The increase of temperature during the labile period of sex differentiation could result in an unexpected sex ratio among individuals carrying a differential loading of autosomal sex modifying genes." However, a polygenic model in this species has been largely refuted by sex inversion and gynogenesis experiments (Mair *et al.* 1991a). Autosomal influences on sex determination of this species has been proposed but generally minor recessive sex modifying genes that results in small deviation from the expected sex ratios. Moreover, there could be some non-genetic parental influence which might affect progeny sex ratios (Chapter 2). Taking autosomal and non-genetic parental influences into account, the XX/XY sex determining system in this species does not seem to be in doubt.

Abucay *et al.* (1999) also speculated that those fry with a higher loading of male autosomal sex modifying genes may have higher levels of endogenous androgen, and that the fish with higher endogenous androgen may be more easily sex reversed. However, in this species, there was no sexually dimorphic pattern of androgens until 57 dpf in which the sex is already determinied (Hines *et al.* 1999). Besides, Kwon *et al.* (2000) suggested that androgens aromatised during the early sex determining period may be of maternal origin.

To explain the feminisation of YY genetic males, Abucay *et al.* (1999) assumed that high level of inbreeding in one of the YY fish strains that they used may have affected the developmental stability. On the basis of this assumption, they made another assumption that the possible loss of developmental stability coupled with increases of autosomal sex modifying genes could have made sex differentiation more sensitive to environmental extremes. High temperature showed little feminising effect in crossbred YY males in the

study of Abucay *et al.* (1999). It seems to suggest that feminisation of YY fish by high temperature in the present study and in one purebred YY male in the study of Abucay *et al.* (1999) might be the result of high level of inbreeding. Significantly lower survival rates of YY males at high temperature than at control temperature in the present study may indicate that the YY fish could have lost developmental stability. However, it is not clear whether this is because of the high level of inbreeding or the absence of the X chromosome that might have some genes responsible for homeostasis against environments during early development. A graph (Fig. 5.4) was drawn using part of the data from Abucay *et al.* (1999). As shown in the graph, high temperature caused feminisation not only in the purebred YY males, but also in XY males (3 out of 5 crosses). This does not support the idea that "inbreeding" in YY males might be responsible for the high temperature-feminisation. It is more likely that the different thermosensitivity between progenies from two different YY × YY crosses observed by Abucay *et al.* (1999) resulted from strain differences.

Looking closer to the data from previous studies on TSD in this species, an interesting tendency is found. When batches of fry were split into two and treated with low (27-28°C) or high temperature (35-36°C), the sex ratios between the two temperatures showed a significant correlation (in mixed-sex group: Baroiller *et al.* 1996; in genetic female group: Abucay et. 1999). This may suggest that the expression of the minor sex modifying autosomal gene(s) is enhanced by high temperature, and that the same sex determining mechanism is involved at both high and low temperature. This suggestion is feasible when the pattern of TSD is unidirectional. However, as mentioned earlier, the pattern of TSD in this species is bidirectional (Fig. 5.3 and Fig. 5.4).

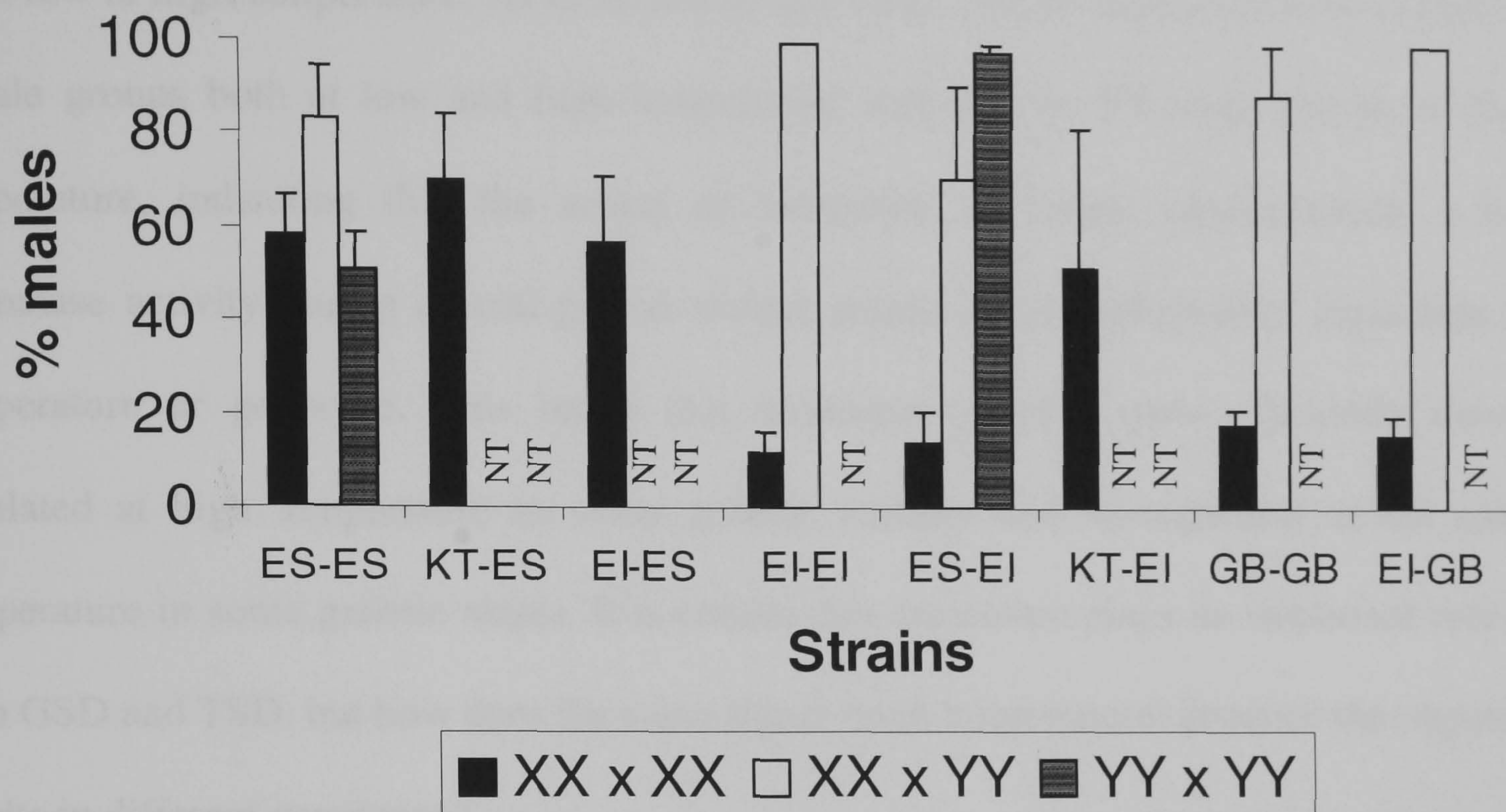


Fig. 5.4. The effect of high temperature on progeny sex ratios of different strains of *O. niloticus* with different genotypes. ES: Egypt-Swansea strain, EI: Egypt-ICLARM strain, KT: Kenya-Turkana strain, GB: Ghana-BFAR strain. ES-ES indicates the cross between ES strain females (left) and ES strain males (right) (the same in other crosses). NT: not tested. (Drawn using part of the data from Abucay *et al.* (1999), mean±SEM).

It has been clearly demonstrated that aromatase plays crucial role in genetic sex determination in the previous chapters, indicating that a certain genetic factor (a sex determining gene or its downstream genes) may suppress the expression of aromatase gene in XY genetic male but not in XX genetic female during the sex differentiation period. In reptiles, it has been frequently suggested that temperature acts through aromatase action (reviewed by Jeyasuria and Place 1998). A similar idea was also proposed in a fish species *P. olivaceous* (Kitano *et al.* 1999). Thus, it can be expected that the expression of aromatase gene in high temperature-treated XX female group might be down-regulated

during the sex differentiation period. However, the level of aromatase gene expression in temperature-treated groups was not investigated in this study. Instead, fish were treated by AI at low or high temperature. AI treatment in this study caused masculinisation in genetic female groups both at low and high temperature and also in YY male groups at high temperature, indicating that the action of aromatase is simple unidirectional – low aromatase activity during crucial period always results in masculinisation regardless of temperature or genotype. This infers that aromatase gene(s) were efficiently down-regulated at high temperature in some genetic females and up-regulated at the same temperature in some genetic males. It is certain that aromatase plays an important role in both GSD and TSD, but how does the same signal (high temperature) produce the opposite results in different genotypes?

To gain some insight into this complicated phenomenon, it is necessary to look at a broader area in biology. General features of environment/genotype interaction may provide some useful clues.

For the traits Mendel examined, a given genotype always resulted in a certain phenotype. However, in other situations, the phenotype of a given genotype varies depending on the penetrance and expressivity of that genotype (Weaver and Hendrick 1997 also below within this paragraph). Such variable expression often results from environmental factors. For example, a plant *Potentilla glandulosa* adapted to three characteristic habitats: high, middle and low altitudes. When a clone from one altitude is grown in different altitude, it changes its height. Another example is even more dramatic. Primroses have red flowers when grown at 24°C but white flowers when grown above 32°C.

The relationship between GSD and temperature in fish may be a different situation from these examples. However, if fish sex determining system evolved through the adaptation to its surrounding temperature, some extent of alteration in the expression of the sex should be expected when fish are exposed to unusual temperatures during the sex differentiation period in the same context as those examples above. Indeed, there is a good example of genotype and temperature interaction in fish sex determination. The Atlantic silverside *M. menidia* showed strong TSD against genotype in Southern populations but were unresponsive to temperature in Northern populations (Conover and Heins 1987). The authors suggested that the different thermosensitivity of sex in the species resulted from the adaptation to the local environment. A similar trend can also be found in the study of Abucay *et al.* (1999). The progenies from three strains showed different thermosensitivity in all genotypes in terms of TSD (i.e., ES-ES>GB-GB>EI-EI, see Fig. 5.4). These support the idea that fish sex determining systems might have evolved through adaptation to its surrounding temperature.

Assuming that the genetic sex determining system was adapted and fixed to a certain temperature range, it can be expected that this system may be altered or reversed partially or completely at an unusual temperature. The Nile tilapia *O. niloticus* studied in this chapter was originated from Lake Manzala, Egypt where the temperature of the peak spawning season (April-May) is expected to be well below 30°C (the recorded monthly minimum and maximum temperature in April: 20.5-25.7°C) (Payne and Collinson 1983). Rana (1990) reported the optimal incubation temperature for this strain as 25-30°C (the best incubation temperature: 28°C). It is not likely that the spawning and hatching temperature greatly differ from each other in wild habitats during the labile period because generally the labile period are considered to be 11 dpf to 35 dpf. It may be suggested that the sex determining system in this strain of *O. niloticus* was adapted and fixed to the temperature below 30°C.

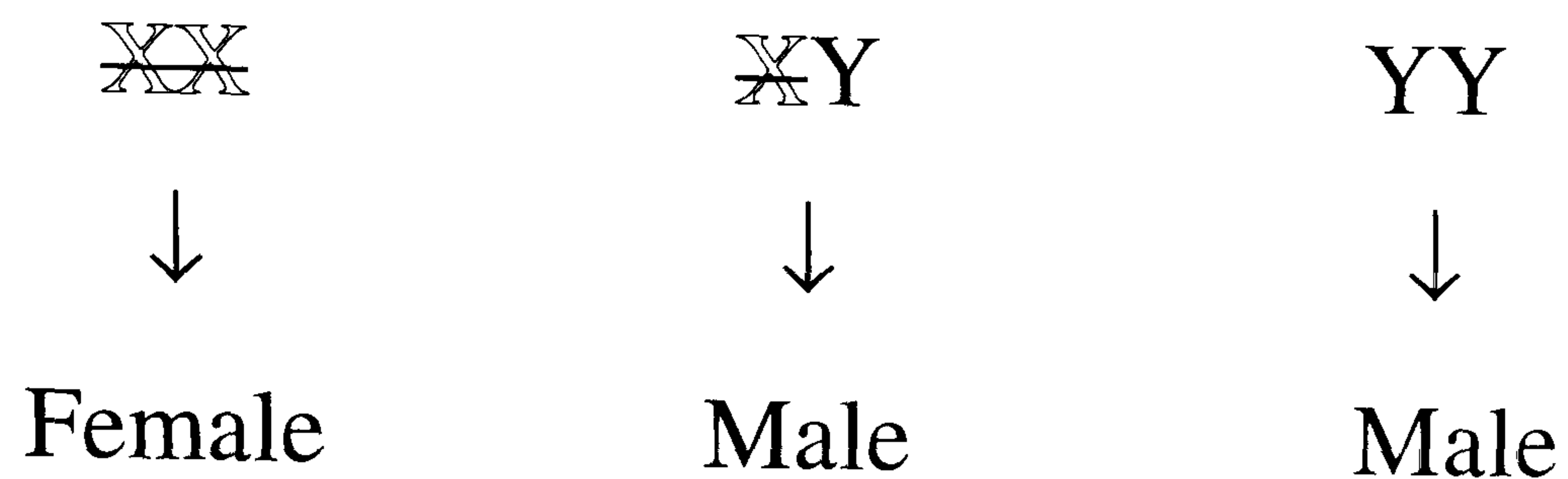
Thus, it would be reasonable to consider 28°C as a normal temperature for the sex determination of this strain and 36°C as an unusual temperature.

Inactivation of DNA sequences results in transcriptional silencing of genetic loci. The inactive DNA sequences tend to be more methylated than active sequences. Thus the methylation of DNA is thought to be responsible for many incidences of imprinting in mammals. Recently, the effect of developmental temprature on the methylation pattern of a transgene was investigated in the zebrafish *Danio rerio* (Martin and McGowan 1995). They raised transgenic siblings of zebrafish at two different temperatures (20 and 30°C). The lower temperature fish displayed a higher transgene methylation pattern than the higher temperature fish. This result implies that environmental factors may alter the status of DNA methylation for some genetic loci. This brings a new hypothesis for TSD in this species.

It is postulated that the major sex determining gene (testis determining factor-TDF) exists both on X and Y chromosomes in *O. niloticus*, and that the gene on X chromosome is inactivated at the adapted temperature (28°C or at least below about 34°C in this strain of *O. niloticus*). Thus, under the adapted temperature, the sex in this species is determined by XX/XY sex determining system. However, the polarity of this inactivation is reversed under unusual temperature (around 36°C), switching on TDF on the X chromosome and off on the Y chromosome (Fig. 5.5). Susceptibility to temperature may vary between individuals, families and strains. This theory would explain all of the results from this study including XX masculinisation, YY feminisation and less effect on XY fish at high temperature. This will also be able to explain the absence of sex specific Sry in fish (Tiersch *et al.* 1992; Ito *et al.* 1995).

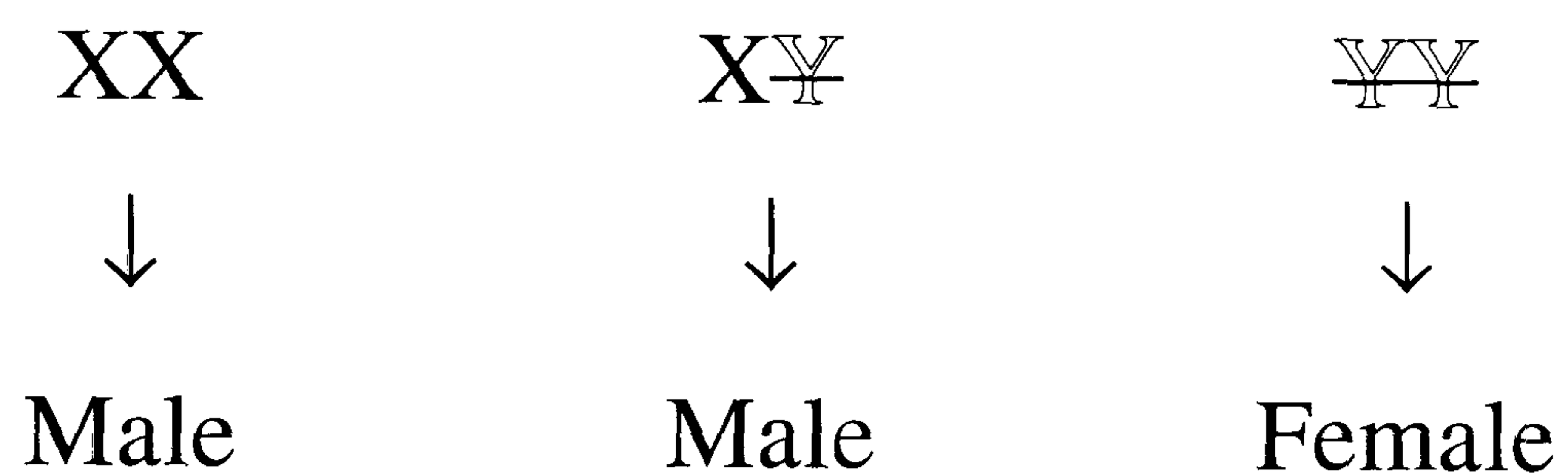
At 28°C

Monofactorial genetic sex determination



At 36°C

1.Complete reversal



2. Incomplete reversal due to the different thermosensitivity in different individuals

: This results in various genotypes in high-temperature treated groups.

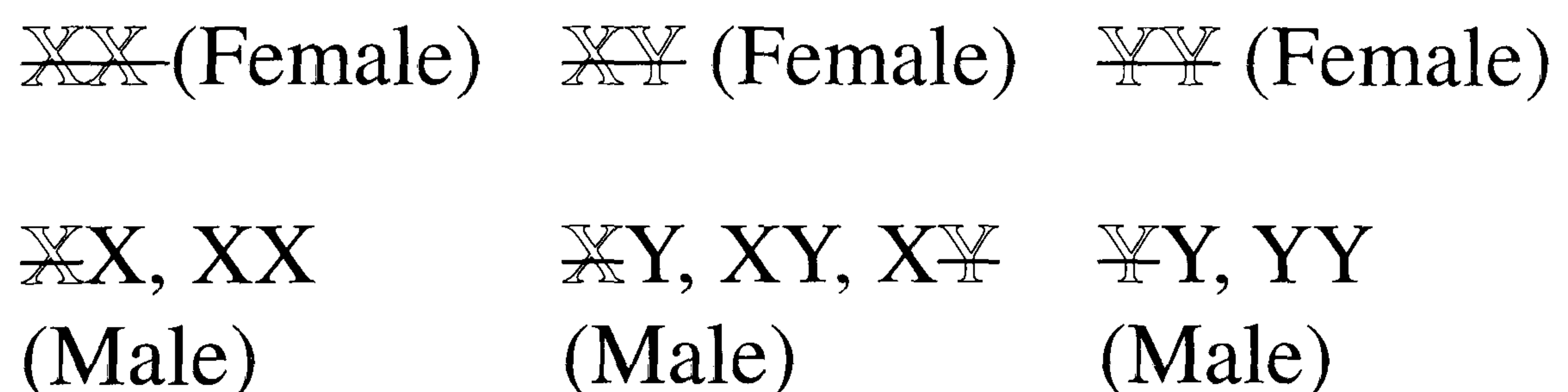


Fig. 5.5. A proposed mechanism for GSD and TSD in tilapia *O. niloticus*. TDF presents on both sex chromosomes but through adaptation to surrounding environments, TDF on X chromosome is inactivated. Unusual environments such as high temperature (36°C) may change the polarity of inactivation, resulting in sex changes. X or Y is the chromosome with inactivated TDF; **X** or **Y** is the chromosome with active TDF.

This environmental inactivation (or adaptive inactivation) in fish sex determination can be associated with other environmental factors such as pH (Römer and Beisenherz 1996) or social environment (Francis and Barlow 1993), depending on the importance of such environmental factors to the species during evolution.

It is still possible to think that high temperature simply altered the endocrine system in XX fish causing masculinisation through the hypothalamus-pituitary-gonadal axis (see also Chapter 6), and YY feminisation was caused by loss of some genetic materials (Abucay *et al.* 1999) as a separate event. However, it is less likely because this YY feminisation involves aromatisation as the process of natural female differentiation in XX fish does. Moreover, there is no direct experimental evidence for the loss of genetic material in YY fish.

In conclusion, the present finding confirms the role of aromatase in fish sex determination and the bidirectional pattern of TSD in this species. Additionally, the new explanation on TSD in this species requires further studies on environmental inactivation or the differential penetrance of sex related genes in different environments.

CHAPTER 6

GENERAL DISCUSSION

This thesis started with two premises: 1. The sex of the Nile tilapia *Oreochromis niloticus* is determined primarily by genetic factors (an XX/XY system with mild autosomal influence); 2. Sex steroid hormones are natural sex inducers in fish. The findings in this thesis confirm these premises and provide some answers for the questions raised in the beginning. This chapter discusses the answers obtained from the series of experimental approaches in this thesis and makes some suggestions on the sex determining system, the primary site of sex determination, sex determining period and further studies.

6.1. Sex determining system

The mechanism of sex determination in tilapia has been studied intensively for the last four decades (e.g., Hickling 1960; Chen 1969a; Jalabert *et al.* 1974; Avtalion and Hammerman 1978; Penman *et al.* 1987a,b; Mair *et al.* 1991a,b; Hussain *et al.* 1994; Mair *et al.* 1997). The main approach to studying sex determination has relied inevitably (at least in the past) on examining sex ratios of progenies after genetic crossbreeding experiments. The observations made from this approach contributed to the establishment of a genetic sex determining theory in this species (Mair *et al.* 1991a) although sex ratios of progeny often fail to conform to this theory. In fact, having relied on sex ratios might have been the cause of many confusing results if other factors intervene sex determining process before and after the determination of genetic sex at fertilisation.

Several factors such as sex determining genes on sex chromosomes, sex modifying genes on autosomes, parental influence, environmental influence and *a posteriori* endocrine changes have been proposed to determine or influence progeny sex ratio in fish.

If all these proposed factors are valid, the observed sex ratios have to be the result of more than three independent or associated events (Fig. 6.1).

First, as discussed in Chapter 2, physiological body condition of female and male parents including nutritional status and subsequent changes of endocrine system, might alter egg and sperm quality that could result in sex-biased mortality. It might also change the preference of eggs for sperm (e.g., X-bearing sperm or Y-bearing sperm) and the composition of X and Y carrying sperm in a semen, both resulting in unexpected sex ratios which are variance to Mendelian principles. These alterations (all termed parental influences) could occur before and/or at fertilisation.

There is another possible parental influence. The levels of maternal steroid hormones may vary with its physiological condition. The initial hormone content in fish embryo may reflect the level of maternal hormone during oogenesis since hormonal substances appear to be deposited from maternal circulation into eggs (Rothbard *et al.* 1987; Greenblatt *et al.* 1989; Lam 1994; Brooks *et al.* 1997; Kwon *et al.* 1999). Suppose some female parent has unusually high testosterone or oestrogen, resulting in eggs with unusually high levels of these steroid hormones. When these hormones are released into the embryonic circulation during early development, the concentration of the hormones may be high enough to cause sex-reversal.

Second, genetic sex would be determined by a major sex determining gene on sex chromosomes and autosomal sex modifying genes (if applicable) when two gametes form a zygote at fertilisation. The sex determining genes would, in turn, switch on many downstream genes in the cascade of sexual differentiation in different tissues during ontogeny. SOX9, SF1 and P450 aromatase genes might be included in these downstream genes.

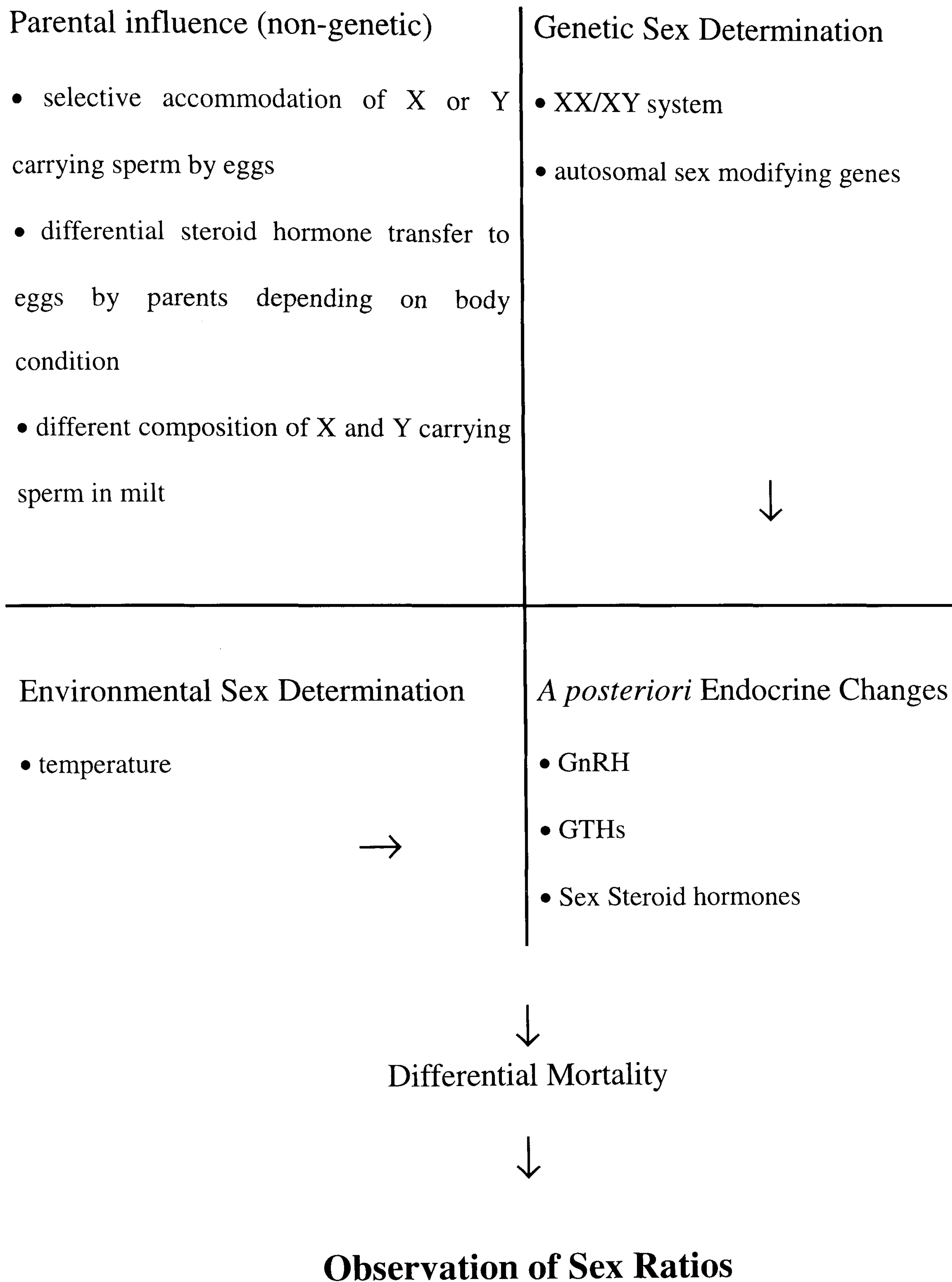


Fig. 6.1. A proposed model of sex determination in tilapia *O. niloticus*. The observed sex ratios are the results of more than three independent or associated events.

Differential expression of these genes would result in *a posteriori* biochemical and endocrinological differences between the sexes during the early developments. Eventually, these differences would direct sexual differentiation in the brain, gonads and other tissues. As evidenced by sex steroids and aromatase inhibitor treatment (Chapter 3), sex steroids are highly likely to be one of the intermediate endocrinological differences that resulted from differential steroidogenic enzyme expression (Chapter 4).

Before or after this *a posteriori* change in the endocrine system, another group of factors may strongly influence fish sex. This group is termed as environmental sex determinants (Chapter 5). Temperature is the most frequently observed environmental sex determinant in fish (Conover and Kynard 1981; Baroiller *et al.* 1996; Abucay *et al.* 1999; Baroiller *et al.* 1999; Kitano *et al.* 1999; Chapter 5). It may switch on or off some genes in the cascade of sex determining system.

The progeny sex ratio is observed after all these events that may influence or change fish sex at different stages. Accordingly, some unexpected sex ratios reported so far should perhaps actually have been expected when taking into account all of the events. It can be recommended that one should take all sex determining or modulating events into account when progeny sex ratio data are interpreted.

In conclusion, genetic sex determination is often masked by parental and temperature effects in this species as postulated in Chapter 1.

6.3. Primary aromatisation site(s) in fish

It became obvious that aromatase directs gonadal differentiation (Chapters 3, 4 and 5). What is still uncertain is by which route aromatase initiates gonadal differentiation. It is possible that the aromatase gene(s) are expressed in many different tissues during early development. There is plenty of evidence that aromatisation in gonads directs gonadal differentiation in almost all vertebrates investigated (Elbrecht and Smith 1992; Wartenburg *et al.* 1992; Yu *et al.* 1993; Piferrer *et al.* 1994; Guiguen *et al.* 1999) and local aromatisation in the brain directs sexual differentiation of the brain in mammals (Lephart 1996). Thus, it is also possible that local aromatisation in the fish brain would direct sexual differentiation of the brain.

There is no doubt that the aromatase gene(s) are expressed in gonads, but it is not likely that this expressed aromatase is the initial signal for differentiation (Chapter 4). Gonadal differentiation may be a later event in phenotypic sex determination. There might be at least two aromatisation sites (e.g., brain and gonad) for sexual differentiation. The crucial question is which organ is the initial aromatisation site and is the first to become sexually differentiated. The brain is consifered to be the primary aromatisation site in fish (Chapter 4). There is yet no direct evidence for this, but there is enough reason to speculate. The reasons are summarised as follows:

- Sexual dimorphism occurs in the fish brain (Gahr 1994).
- Both brain and ovarian type aromatase are expressed in the fish brain (Chapter 4; Tchoudakova and Callard 1995).
- Brief immersion treatment with sex steroids long before the appearance of histologically discernible gonadal differentiation sex-reverses genetic females into males (Piferrer *et al.* 1994; Gale *et al.* 1999).

6.2. The role of cytochrome P450 aromatase in fish sex differentiation

It has been proposed that androgens and oestrogens differentiate indifferent fish gonads into testis and ovary, respectively (Yamamoto 1969). A steroidogenic enzyme, cytochrome P450 aromatase, converts androgens into oestrogens. Thus, aromatase may play a crucial role in sex differentiation by functioning as a sex controlling factor further upstream than sex steroids. This was proved in three different experimental approaches in this thesis.

Inhibition of aromatase action using a chemical aromatase inhibitor sex-reversed genetic females (XX) to phenotypic males (Chapter 3).

Two different types of aromatase genes, namely brain and ovarian aromatase genes, were found in this species. Among these aromatase genes, ovarian aromatase gene showed a sexually dimorphic expression pattern during ontogeny (Chapter 4), indicating a role for aromatase in natural sex differentiation.

High temperature causes feminisation of YY males. It can be assumed that the high temperature might have turned on the switch of the oestrogenic pathway during the crucial period in this genotype. Treatment of aromatase inhibitor suppressed this high temperature feminisation of genetic males (Chapter 5).

As proved by all these consistent findings, it can be concluded that aromatase plays a crucial role in sex differentiation via steroid action. Attention should be paid now towards further upstream factors such as SOX9 and SF1.

- Brief immersion or dietary treatment with aromatase inhibitor long before the appearance of histologically discernible gonadal differentiation sex-reverses genetic females into males (Chapter 3).
- Fish brain has 100-1000 fold higher aromatase activity than mammalian brain and 10 fold higher than fish ovary (Pasmanik and Callard 1988a).
- Ovarian and brain aromatase mRNA is detected from hatching onwards at which time neither gonadal tissue nor steroid producing cells exist in the presumptive gonadal area (Chapter 4).
- Fish sex in some species is influenced by environment (Baroiller *et al.* 1999; Chapter 5). Environmental signals are likely to go through the central nervous system first rather than directly acting on the presumptive gonadal area (Francis 1992).

Suppose the brain is the initial aromatisation site and as the consequence is the first organ to become sexually differentiated. If so, how would the result of brain differentiation initiate gonadal differentiation? The classical role of GnRH and GTHs in gametogenesis and final sexual maturation may provide an explanation for this question. In gametogenesis (Xiong *et al.* 1994; Norris 1997), GnRH stimulates the secretion of GTH I. The released GTH I is delivered to the gonads and induces follicle growth in females, spermatogenesis in males and conversion of androgen to oestrogen in both sexes. This model may be applicable to gonadal differentiation since GTH I immunoreactive cells were detected in the pituitary at around hatching or before gonadal differentiation in some fish species (common carp: Van Winkoop *et al.* 1987; rainbow trout: Saga *et al.* 1993 and Feist and Schreck 1996). Moreover, GnRH immunoreactivity was also detected in several brain regions of rainbow trout around sex differentiation (Feist and Schreck 1996). In final matuation and ovulation (Nagahama *et al.* 1994; Peter and Yu 1997), GTH II becomes the

predominant gonadotropin. This GTH II induces the production of maturation-inducing hormone (e.g., 17α,20β-dihydroxy-4-pregnen-3-one: 17α,20β-DP) and a dramatic decrease of oestrogen that coincides with the suppression of aromatase mRNA expression. Unlike GTH I, GTH II immunoreactive cells have not been detected in sexually undifferentiated fish larvae. Treatments with human chorionic gonadotropin (HCG), that shows similarlity to LH (GTH II in fish) in both structure and function (Norris 1997), induced female to male sex inversions in a hermaphroditic species, the bluehead wrasse *Thalassoma bifasciatum* (Koulish and Kramer 1989). In addition, treatment of carp larvae with homologous pituitary extracts accelerated sex differentiation (Van Winkoop *et al.* 1994). Application of more sensitive methods might render the detection of GTH II in undifferentiated fish larvae in near future.

Borrowing from the gametogenesis and final maturation model, a possible scenario of phenotypic sex determination in fish (Fig. 6.2) can be established based on established findings and some assumptions. This is as yet a premature scenario but it may provide some insight to fish sex determination. An upstream sex determining gene is turned on in a region of the brain, initiating aromatisation of androgens into oestrogens. The primary aromatisation in the brain results in structural and functional differences of the brain between genetic males and females through the steroidal action. The differentiated brain starts to produce signals for further sex differentiation in other tissues including the gonads through the hypothalamus-pituitary-gonadal axis. GnRH, released from the hypothalamus, induces the production of GTH I in both sexes and GTH II in males. GTH I initiates the development of gonadal structure, inducing the expression of ovarian aromatase mRNA in the presumptive gonadal area. At the same time, GTH II in males suppresses the expression of aromatase mRNA directly or via the SOX9 gene and triggers androgen synthesis resulting in testicular differentiation. In females, GTH I keeps stimulating the

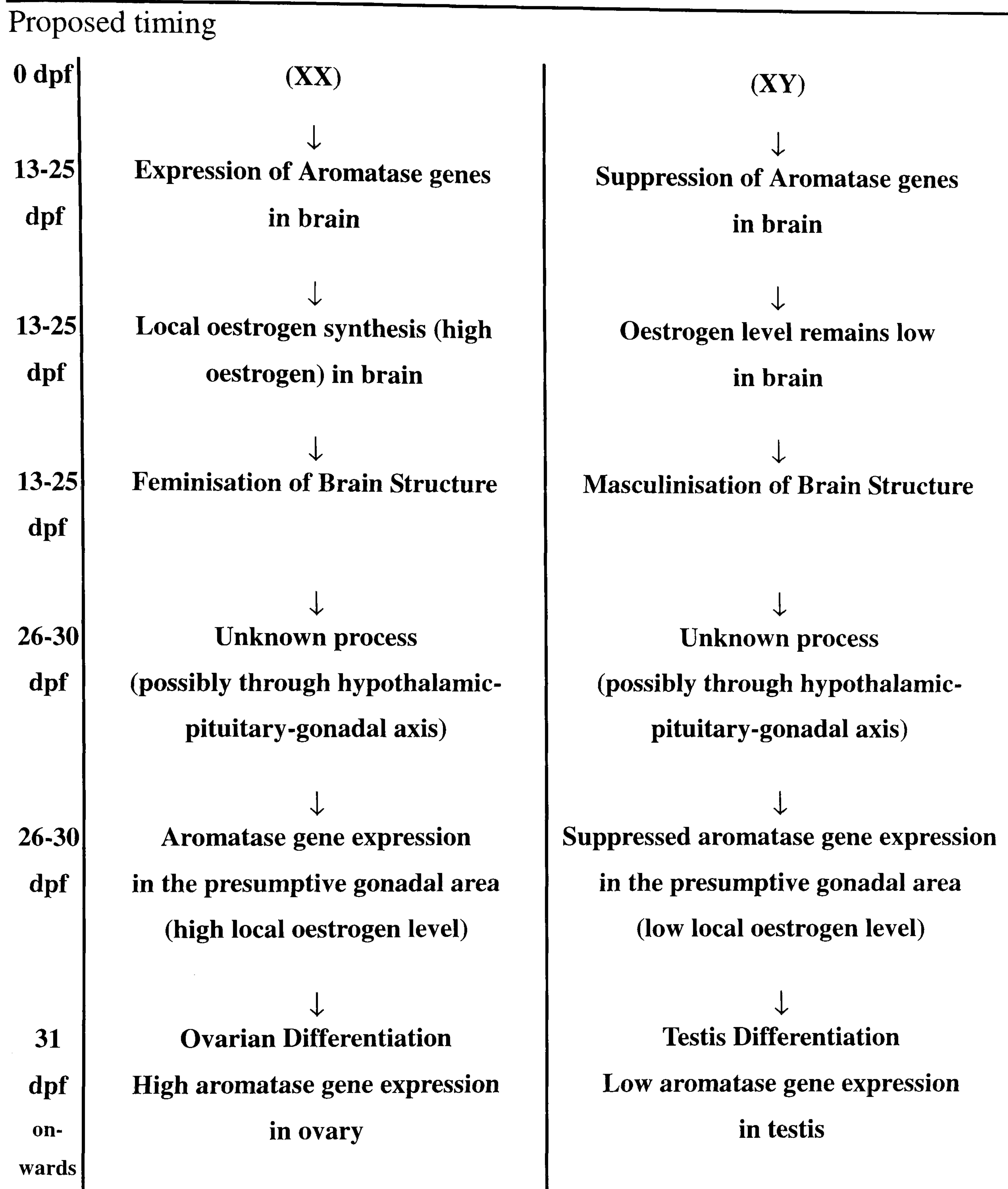


Fig. 6.2. The possible involvement of the central nervous system (CNS) in sex determination of *O. niloticus*. This model is based on the assumption that the brain is the primary site of sex determination in this species.

production of ovarian aromatase yielding high levels of local oestrogen concentration that differentiate the gonadal area into the ovaries. Additionally, GnRH is influenced by sex steroids (Norris 1997) and environmental factors such as olfactory and visual inputs (Francis 1992). It is generally known that fish have thermoreceptors in the skin and the preoptic hypothalamus. The preoptic anterior hypothalamus is a temperature sensing and regulating centre (Prosser 1991). Direct influence of temperature on GnRH and GTH II in adult fish has been reported (Gissis 1991; Okuzawa *et al.* 1994; Lin *et al.* 1996). Thus, it can be suggested that the influence of temperature on fish sex is associated with hypothalamus-pituitary-gonadal axis.

Some parts of this scenario may have an alternative. In mammals, local aromatisation in the brain results in sexually dimorphic neural organisation (Lephart 1996). This dimorphism is thought to establish gonadotropin secretion profiles along male versus female patterns (females have a phasic GTH secretion pattern whereas males have a tonic secretion pattern) (MacLusky and Naftolin 1981). If this is true in the fish brain, differential GTH secretion pattern between the sexes would provide another explanation for fish sex determination.

There appear to be two decisive aromatisation events regarding sex differentiation in *O. noloticus*. One, probably in the brain, is responsible for brain differentiation and probably produces a signal for gonadal differentiation. The other in the gonadal area is responsible for the subsequent gonadal differentiation. In Chapter 3, one week treatment with AI at 11-17 dpf effciently masculinised genetic females. Treatment at 18-24 dpf was less effective and later than this did not show masculinising effect. Treatment with AI later than 25 dpf required longer duration (two weeks) to masculinise genetic females (Kwon *et al.* 2000). AI treatment at 11-24 dpf probably overrode the first aromatisation event in sexually indifferent brain (the absence of gonadal tissues and steroid producing cells excludes the

presumptive gonadal area as the first aromatisation site), and the treatment later than 25 dpf probably overrode the second aromatisation event in the gonads where the differentiation has already begun. Thus, it is understandable that later treatment required longer duration in the experiment because it had to override an established pathway. At the present moment, it is highly likely that the result of brain differentiation directs gonadal differentiation. However, considering the plasticity of sex in this species and the susceptibility to exogenous steroids during ontogeny, it can be also suggested that the result of gonadal differentiation may interact with the central nervous system to maintain the gonadal differentiation through a positive or negative feedback system. Further studies on the involvement of central nervous system in fish sex differentiation are required.

6.4. Hormonal sex control and the sex determining period in *O. niloticus*

Defining the sex determining period or more empirically the "labile period" has been of great importance regarding hormonal sex control in fish culture since incorrect timing of hormonal treatment was suspected to result in unsuccessful sex reversal (Yamamoto 1969; Hunter and Donaldson 1983; Rosenstein and Hulata 1992). Incorrect timing may also require a higher dose and/or longer duration of hormone treatment to achieve high sex reversal. However, increasing the amount of sex steroids in fish culture is not desirable due to the potential carcinogenic effects of these chemicals (IARC Monographs 1974). Even though sex steroids administered to fish are quickly metabolised (90% in 24 hours and 99% in 3 weeks) (Goudie *et al.* 1986), fish farmers may also be exposed to these chemicals, and the farm wastewater that contains the residue and the metabolites of these sex steroids, might cause some undesirable effects on the environment. High steroid dosages also cause unwanted effects including paradoxical steroid action and high mortality of fish (Hunter and Donaldson 1983). Therefore, if using these substances is required in a necessary sex control step, the dose and duration of hormone treatment should be minimised by administering the steroids at an appropriate time based on the exact sex determining period.

Defining the sex determining period is also important in studying sex determination. Recently, two research papers were published with regards to the role of sex steroids and aromatase in fish sex determination (Guiguen *et al.* 1999; Kitano *et al.* 1999). These papers provided valuable information on fish sex differentiation. However, the results were not conclusive because the studies did not include the early developmental stages at which fish sex can be influenced by exogenous sex steroid treatment. These studies were

conducted from shortly before or around the time of the appearance of histologically discernible gonadal sex.

The sex determining period varies greatly from species to species. In this section, recent findings on sex determining periods are discussed, focusing on the Nile tilapia *O. niloticus*.

Steroid hormone treatment studies in this species began with a duration of two months from first feeding (11 dpf) (Jalabert *et al.* 1974). In subsequent studies, with much trial and error, the duration was cut down to 30 days (11 to 40 dpf) from first feeding (Nakamura and Iwahashi 1982) and this period has been generally accepted as the most appropriate for hormonal sex reversal of this species (McAndrew 1993). Immersion treatment with oestrogen before first feeding was found to be ineffective in this species (Rosenstein and Hulata 1992). On the other hand, immersion treatment after first feeding (11 and/or 13 dpf) with either MDHT (Gale *et al.* 1999) or AI (Chapter 3) was effective in masculinising genetic females. These results suggest that the sexually labile period lies between 11 and 40 dpf in this species.

Considering the fact that exogenous sex steroids induce sex changes in fish, it is expected that male and female embryo or fry might have different levels of sex steroids during the sexually labile period. On the contrary to this expectation, no clear bimodality of sex steroid hormone profiles was observed in mixed sex groups of tilapia fry until 57 dpf (Rothbard *et al.* 1987; Hines *et al.* 1999). Oestrogen levels were either very low or undetectable until 43 dpf, while testosterone levels decreased dramatically within 7 days after fertilisation (Hines *et al.* 1999). From this result, Hines *et al.*(1999) suggested that steroids may be predictive of and/or a consequence of gonadal differentiation. However, in the same study, high variablity (but no bimodality) in the distribution of testosterone level

was observed at 22 dpf, coinciding with a transient increase in 11-ketotestosterone. The authors assumed that those individuals exhibiting relatively higher levels of testosterone may represent eventual males. This assumption was perhaps undermined by the large transient increase in 11-ketotestosterone which occurred in all individuals. There is thus still an argument about the actual role of sex steroid hormones in fish sex differentiation.

The results from Hines' group appear to contradict Yamamoto's theory of sex steroids as natural sex inducers, but it is questionable whether the whole body sex steroid levels necessarily reflect small changes in local steroid synthesis in specific tissues such as the brain and the presumptive gonadal area during ontogeny. Furthermore, the results from individual sample RIA (Hines *et al.* 1999) were inconsistent with those from pooled sample RIA (Rothbard *et al.* 1987) in terms of hormone concentration. The concentration of oestrogen was comparable to that of testosterone in pooled sample RIA, while oestrogen level was undetectable in individual sample RIA when testosterone levels were high. Thus, it can be suggested that sex steroids are natural sex inducers but the steroidal events take place in a local tissue producing local changes of steroid profiles which differ from circulating levels or totals of whole body steroids. Considering the nature of steroid hormones which serve as signals for both organ to organ (as endocrines) and cell to cell (as cytocrines: local hormones) communications, it is reasonable to postulate that steroid hormones may function as cytocrines in the tissues to be differentiated. At this point, no measurable change in sex steroids in the general circulation during the determination of phenotypic sex might be expected. In fact, distinctive bimodality and high level of whole body steroids are more likely to be the consequence of gonadal sex differentiation rather than the cause. Accordingly, the absence of clear bimodality during the proposed sex determining period does not necessarily undermine Yamamoto's theory.

By histological observation, ovarian differentiation in this species was proposed to be initiated at 27-30 dpf (23-26 dph) based on the aggregation of stromal cells in the distal and proximal region of the gonad (Nakamura and Nagahama 1985). The stromal aggregation was identified as initial formation of the ovarian cavity. In the study, steroid producing cells (SPCs) were also found at the same period. Later, Nakamura and Nagahama (1989) reported that testicular differentiation and the initial appearance of SPCs in the presumed testis coincide with the ovarian differentiating period (27-30 dpf). Hines *et al.* (1999) observed ovarian differentiation at 36 dpf which is almost a week later than those previous reports. Histological sexual difference was not observed at 29 dpf in their study.

The appearance or expression of steroidogenic enzymes can be considered to be an indicator of steroidal activity, thus an indicator of sexual differentiation. A positive immunoreaction to cholesterol side chain cleavage cytochrome P450 (P450scc), 3β-hydroxysteroid dehydrogenase (3β-HSD), cytochrome 17α-hydroxylase/17,20 lyase (P450c17) and cytochrome P450 aromatase, was observed in the gonads of genetic females just prior to morphological ovarian differentiation (27-30 dpf) (Nakamura *et al.* 1997). In the same study, weak staining for P450scc, 3β-HSD and P450c17, but not cytochrome P450 aromatase, was present in the testis at 34 dpf (=30 dph). Together with the appearance of SPCs and histological differences, these results suggest sex differentiation (or at least ovarian differentiation) may be initiated before 26 dpf.

O. niloticus fry were most sensitive to AI treatment during the first week (11-17 dpf) and also effective during the second week (18-24 dpf) from first feeding in terms of sex reversal (Chapter 3; Kwon *et al.* 2000). This narrows down the sex determining period to 11-24 dpf. In addition, the observed sexually dimorphic expression of ovarian aromatase mRNA conclusively suggests that this period is indeed the major sex determining period in

this species (Chapter 4). Ovarian aromatase mRNA was significantly downregulated from 15 to 23 dpf in genetic males. A masculinising effect of a single 3 hr immersion in AI solution at 13 dpf also support this suggestion.

Therefore, it can be recommended that studies on sex determination and steroid treatments for sex control in this species should focus on this period. The duration of treatment could possibly be shortened from 30 days as currently practised to 20 days (as suggested by MacIntosh and Little 1995). Additionally, AI treatment could possibly replace the hormonal sex control technique. This chemical (Fadrozole) is currently administered to humans to treat breast cancer. However, before it could be used for fish culture, more detailed studies on treatment methods, toxicity, availability and cost are required.

6.5. Conclusions and further studies

Conclusions

- A steroidogenic enzyme, cytochrome P450 aromatase plays a crucial role in the process of sex differentiation in this species. The dimorphic expression of ovarian aromatase mRNA between genetic males and females in this thesis strongly support Yamamoto's theory that sex steroids are natural sex inducers in fish.

- The major sex differentiation period lies between 13-24 dpf in this species.

- The brain aromatase gene is clearly different from the ovarian aromatase gene, but the functional difference between these two genes remains unknown.

- The decisive aromatisation is not likely to take place in the gonadal area. The brain could be the possible primary aromatisation site in relation to sex differentiation in fish.

- Temperature also influences sex determination in this species. It is likely that TSD also acts through the pathway of sex steroids, as GSD does. Temperature and genotype interaction is obvious in this species.

- All sex ratio data from this study support the theory that the sex of the Nile tilapia *O. niloticus* is principally determined by genetic factors (an XX/XY system + autosomal genes). However, this genetic sex determination is often masked by parental and temperature influences to varying degrees.

Further studies

Further studies are recommended

1) on the role of CNS as a primary aromatisation site in sex determination using *in situ* hybridisation technique and/or RT-PCR analysis.
2) on the possible involvement of the putative upstream genes in the sex determination cascade such as SOX9, SF1 and DMRT1: the analysis of the promoter region in aromatase genes will provide some clues for the regulation of aromatase gene expression.
3) on the interaction between temperature and genotype (XX, XY, YY): the possibility of environmental alteration of gene inactivation was questioned in this thesis.
4) on temperature effect on the pattern of the aromatase gene expression during sex differentiation using individual sample.
5) on parental influences with different level of nutritional conditions.

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APPENDICES

Appendix I. Transformation Protocol

(For Epicurian Coli XL10-GoldTM Ultracompetent Cells – Stratagene; the following protocol is the manufacturer's instruction)

Appendix I-1. Protocol

1. Thaw the XL 10-Gold ultracompetent cells on ice.
2. Gently mix the cells by hand. Aliquot 100 µl of the cells into each of two prechilled 15-ml Falcon 2059 polypropylene tubes. (One tube is for the experimental transformation and one tube is for the control transformation.)
3. Add 4 µl of the β-mercaptoethanol (β-ME) mix provided with the kit to the 100 µl of cells.
4. Swirl the contents of the tube gently. Incubate the cells on ice for 10 minutes, swirling gently every 2 minutes.
5. Add 0.1-50 ng of DNA to one of the 100 µl aliquots of cells and swirl gently. As a control, add 1 µl of the pUC18 control plasmid (diluted 1:10 in high-quality water) to the other 100 µl aliquot of the cells and swirl gently.
6. Incubate the tubes on ice for 30 minutes.
7. Preheat NZY^+ broth in a 42°C water bath for use in step 10.

   Note Transformation of XL10-Gold ultracompetent cells has been optimized for use with ***NZY^+ broth***.

8. Heat-pulse the tubes in a 42°C water bath for 30 seconds. The duration of the heat pulse is critical for obtaining the highest efficiencies. Do not exceed 42°C.
9. Incubate the tubes on ice for 2 minutes.
10. Add 0.9 ml of preheated (42°C) NZY^+ broth to each tube and incubate the tubes at 37°C for 1 hour with shaking at 225-250 rpm.
11. If color screening is desired and if the agar plates were not prepared with X-gal and IPTG, spread 100 µl of 2% X-gal and 100 µl of 10 mM IPTG on the LB agar plates 30 minutes prior to plating the transformations.

Note *For consistent color development across the plate, pipet the X-gal and the IPTG into a 100 μl pool of NZY⁺ broth and then spread the mixture across the plate. Do not mix the IPTG and the X-gal before pipetting them into the pool of NZY+ broth because these chemicals may precipitate.*

12. Plate 200 μl (or less) of the transformation reaction on appropriate antibiotic plates using a sterile spreader.

Note *The cells may be concentrated by centrifuging at 1000rpm for 10 minutes if desired. Resuspended the pellet in 200 μl of NZY⁺ broth and plate.*

To plate the cells transformed with the pUC18 control plasmid, first place a 195 μl pool of NZY⁺ broth on an LB-ampicillin agar plate. Add 5 μl of the control transformation reaction to the pool of NZY⁺ broth. Use a sterile spreader to spread the mixture.

13. Incubate the plates overnight at 37°C. For blue-white color screening, incubate the plates for ≥17 hours. Colonies containing plasmids without inserts will be blue after the 17-hour (or longer) incubation. Colonies containing plasmids with inserts will remain white. The blue color can be enhanced by incubating the plates for two hours at 4°C following the overnight incubation at 37°C.

Appendix I-2. Preparation of Media and Reagents

LB-Ampicillin Agar (per Litre)

Prepare 1 litre of LB agar

Autoclave

Cool to 55°C

Add 5 ml of 10 mg/ml filter-sterilised ampicillin

Pour into petri dishes (~25 ml/100-mm plate)

LB Agar (per Litre)

10 g of NaCl

10 g of tryptone

5 g of yeast extract

20 g of agar

Adjust pH to 7.0 with 5 N NaOH

Add deionized H_2O to a final volume of 1 litre

Autoclave

Pour into petri dishes dishes (~25 ml/100-mm plate)

NZY^+ Broth (per Litre)

10 g of NZ amine (casein hydrolysate)

5 g of yeast extract

5 g of NaCl

Adjust the pH to 7.5 with NaOH

Autoclave

Add the following supplements before use

12.5 ml of 1 M $MgCl_2$ and 12.5 ml of 1 M $MgSO_4$

10 ml of a 2 M filter-sterilised glucose solution or 20 ml of 20 % (w/v) glucose

Filter sterilise

TE Buffer

10 mM tris-HCl (pH 7.5)

1 mM EDTA

Appendix II. Reagents

Appendix II-1. Electrophoresis

TBE buffer (5× Stock Solution)

54 g of tris base

27.5 g of boric acid

20 ml of 0.5 M EDTA (pH 8.0)

Dissolved in distilled water (final volume 1 litre).

Ethidium bromide stock solution (10 mg/ml)

1 g of ethidium bromide

100 ml of distilled water

Stir several hours to dissolve and keep in a dark bottle.

Gel-loading buffer (6×)

0.25% bromophenol blue

0.25% xylene cyanol FF

30% glycerol in water

(Note: Dilution with 30% glycerol once again gives better results).

Appendix II-2. RNA extraction

RNAZOL stock solution

2 M guanidinium thiocyanate

12.5 M sodium citrate (pH 7.0)

0.5% N-lauryl-sarcosinate

0.72% β-mercaptoethanol

50% water-saturated phenol

0.2 M sodium acetate

Keep in a dark bottle at room temperature (for upto 1 month).

Appendix II-3. Northern Analysis

10× MOPS solution

0.2 M MOPS

50 mM sodium acetate

1 mM EDTA

Adjust the pH to 7.0 with NaOH, autoclave and keep at room temperature.

Formamide

Deionise formamide with resin bed by stirring for 1 hour. Keep at -20°C until use.

Glyoxal

Deionise by stirring with resin bed for about 1 hour.

GFM buffer

16% deionised 6.9 M glyoxal

78% deionised formamide

6% 10× MOPS solution

Store at -70°C in aliquots.

10× Loading buffer

40% deionised formamide

50% glycerol

10% 10× MOPS solution

with smidge of bromophenol blue and xylene cyanol FF. Keep at 4°C.

Church-Gilbert solution

Solution I – 0.5 M NaH_2PO_4; Soultion II – 0.5 M Na_2HPO_4;

Solution III – titrate solution II with solution I until pH reach 7.2.

Church-Gilbert solution – 3.5 g SDS (sodium dodecyl sulfate = sodium lauryl sulfate)

1 ml of 0.5 M EDTA

Add solution III to final volume 50 ml

Dissolve at 65°C.

20× SSC

175.3 g of sodium chloride

88.2 g of sodium citrate

Dissolve in 800 ml of distilled water and adjust the pH to 7.0 with 10 N NaOH. Add water to final volume 1 litre and sterilise by autoclaving.

10% SDS

100 g of SDS

Dissolve in 900 ml of distilled water at 68°C and adjust the pH to 7.2 with a few drops of concentrated HCl. Add water to final volume 1 litre (No need to sterilise).

Appendix III. Restriction Sites in *O. niloticus* Brain Aromatase cDNA

```
                                                                                GsuI
tagagcgtcagaagtcactgctgtcctgcttcttctgctgctgttgttgctgcttttcaccacctggagacaaag base pairs
atctcgcagtcttcagtgacgacaggacgaagaagacgacgacaacaacgacgaaaagtggtggacctctgtttc 1 to 75
                                                                                BpmI

                         Psp5II
                         EcoO109I                 AtsI
                    SexAI                         Tth111I          SfcI
aaaacagtcacacataccaggtcctttcttcttagcaggactcggtccaattctctcctacagcagattcatctg base pairs
ttttgtcagtgtgtatggtccaggaaagaagaatcgtcctgagccaggttaagagaggatgtcgtctaagtagac 76 to 150
                         PpuMI                    AspI             BstSFI
                         DraII

gtctgggattggaacagcgtgtaactactacaacaacaaatatgggagcattgtgcgggtgtggataaacggaga base pairs
cagaccctaaccttgtcgcacattgatgatgttgttgtttataccctcgtaacacgcccacacctatttgcctct 151 to 225

                             BspMI
         BsaI               DraII
      BseRI                 PpuMI       Eco57I                BseRI              GsuI
ggagaccctgatttgagcaggtcctctgaagtgtaccacgttttgaggagtgcccactacacctccagatttgg base pairs
cctctgggactaaaactcgtccaggagacttcacatggtgcaaaactcctcacgggtgatgtggaggtctaaacc 226 to 300
         Eco31I             EcoO109I                                         BpmI
                            Psp5II

                Eco88I
                XhoI  PaeR7I
                Sfr274I      MslI  NspI
cagcaaaaaaggactcgagtgcatcggcatgtacggaaatggtatcattttcaacagtgatgtcctgctttggaa base pairs
gtcgtttttttcctgagctcacgtagccgtacatgcctttaccatagtaaaagttgtcactacaggacgaaacctt 301 to 375
                Ama87I
                BcoI  BsoBI
                AvaI

                                                                                     Eco24I
                                                      PstI                           AspHI
                                                 SfcI                              Ecl136II
aaaagtgagaacatactttctaaagctctgactggacccggcctgcagaggaccgtaggaatctgtgtgagctc base pairs
ttttcactcttgtatgaaaagatttcgagactgacctgggccggacgtctcctggcatccttagacacactcgag 376 to 450
                                                 BstSFI                            EcoICRI
                                                                                     Bbv12I
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     SstI
    FriOI                                                   NspI
     BanII  BstXI                                       BspLU11I
cacagccaaacacctggacaacttacaggacatgactgacccctctggacatgtagatgctctcaatctcctgag base pairs
gtgtcggtttgtggacctgttgaatgtcctgtactgactggggagacctgtacatctacgagagttagaggactc 451 to 525
    SacI                                                AflIII
     Alw21I                                                  MslI
       BsiHKAI

                         Bse118I
        BstXI            BssAI                                 Asp700I      AcsI
agccatcgtgttggacatctccaaccggctgttcctcagagtgccgttaaatgagaaagacttcttgacgaaaat base pairs
tcggtagcacaacctgtagaggttggccgacaaggagtctcacggcaatttactctttctgaagaactgcttta 526 to 600
                         BsrFI                                 XmnI         ApoI
                         Cfr10I

                                                                       BsrGI
                                                                       SspBI
tcacaactactttgatacctggcaaacagttctaataaaccagatatattcttcaaggttggatggctgtacaa base pairs
agtgttgatgaaactatggaccgtttgtcaagattatttggtctatataagaagttccaacctaccgacatgtt 601 to 675
                                                                       Bsp1407I

                          AlwNI           BsrDI                            BspHI
caagcataagagagcagcacaggagctgcaagatgcaatggagagcctgcttgaagttaagagaaagatgattca base pairs
gttcgtattctctcgtcgtgtcctcgacgttctacgttacctctcggacgaacttcaattctctttctactaagt 676 to 750
                                                                           RcaI

                             Eco24I SstI        Eco24I SstI
                             AspHI FriOI        AspHI FriOI
                           Ecl136II BanII     Ecl136II BanII     BstDSI
tgaagccgagaagctggacgacgagctcgactttgcaacagagctcatcttcgcccagaaccacggagagctatc base pairs
acttcggctcttcgacctgctgctcgagctgaaacgttgtctcgagtagaagcgggtcttggtgcctctcgatag 751 to 825
                           EcoICRI SacI       EcoICRI SacI       DsaI
                             Bbv12I Alw21I      Bbv12I Alw21I
                             Psp124BI BsiHKAI   Psp124BI BsiHKAI

                                     Bse8I
                                     BsrBRI                                EarI
                                     MamI          AlwNI                   Eam11
agcagataacgtcaggcagtgtgtgctagagatggtgatcgcagcccctgacacactttccatcagcctcttctt base pairs
tcgtctattgcagtccgtcacacacgatctctaccactagcgtcggggactgtgtgaaaggtagtcggagaagaa 826 to 900
                                     BsaBI                                 Ksp63
                                     Bsh1365I
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    MslI                                      PstI
  04I                                     SfcI             BseRI
catgccgatgctgctgaaacagaacccggacatagagctgcagctagtggaggagatgaacaccatcttgaatga base pairs
gtacggctacgacgactttgtcttgggcctgtatctcgacgtcgatcacctcctctacttgtggtagaacttact 901 to 975
  2I                                      BstSFI

                 BspXI ClaI
                 Bsp106I
                 BanIII             Eco57I
aaaagacgtggaaaatatcgattaccaaagcctgaaggtgatggagagcttcatcaacgagtctttgaggtttca base pairs
ttttctgcaccttttatagctaatggtttcggacttccactacctctcgaagtagttgctcagaaactccaaagt 976 to 1050
                 BspDI BseCI
                 Bsa29I
                 BscI Bsu15I

                                                                      BshNI
                                     GsuI                             Eco64I
tcctgtggtcggtttcacaatgaggaaagctctggaggacaacgacatcgcaggcacaaaaatcaagaagggcac base pairs
aggacaccagccaaagtgttactcctttcgagacctcctgttgctgtagcgtccgtgtttttagttcttcccgtg 1051 to 1125
                                     BpmI                             BanI
                                                                      AccB1I

                                      ApoI                   Ksp632I
                                      AcsI                 Eco57I
caacatcattctcaacactggcctcatgcacaaaaccgaattcttccccaaacctgaagagttcaaccacacgaa base pairs
gttgtagtaagagttgtgaccggagtacgtgttttggcttaagaaggggtttggacttctcaagttggtgtgctt 1126 to 1200
                                      EcoRI                  Eam1104I
                                                             EarI

            BshNI
            Asp718I
            Eco64I                    BstXI
ctttgaaaaaacggtacccaatcgttacttccagccctttggctgcgggcctcgttcctgtgtgggcaaacacat base pairs
gaactttttttgccatgggttagcaatgaaggtcgggaaaccgacgcccggagcaaggacacacccgtttgtgta 1201 to 1275
            BanI KpnI
            Acc65I
            AccB1I

  StyI DsaI
  Eco130I
 MslI NcoI                                            DraIII   EcoNI
cgccatggtgatgatgaaggccatcctggtcactctcctgtctcggtacactgtgtgtcctcatcaaggctgcac base pairs
gcggtaccactactacttccggtaggaccagtgagaggacagagccatgtgacacacaggagtagttccgacgtg 1276 to 1350
 BcgI EcoT14I
  ErhI BstDSI
  BssT1I Bsp19I
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                                                         Alw21I  BalI
                                        Bse118I     BbsI AspHI   MscI
BsgI                                    BssAI       BpuAI      EaeI
actcagcagcatcaagcagaccaacaacctgtcacagcagccggtggaagacgagcacagcctggccatgcgctt base pairs
tgagtcgtcgtagttcgtctggttgttggacagtgtcgtcggccaccttctgctcgtgtcggaccggtacgcgaa 1351 to 1425
                                        BsrFI       Bbv16II    CfrI
                                        Cfr10I      BpiI Bbv12I  MluNI
                                                         BsiHKAI

                                                     HaeII
                                                   AfeI
                                                   Aor51HI         Eco57I
catcccacgaacgaaataaccccacgacaacaagctgcgaaacataacagcgctttttacacttcagatgaaagc base pairs
gtagggtgcttgctttattggggtgctgttgttcgacgctttgtattgtcgcgaaaaatgtgaagtctactttcg 1426 to 1500
                                                   Eco47III
                                                     Bsp143II
                                                     BstH2I

                    SseBI
                    AatI
                    Pme55I            EcoNI                AcsI
atcttgaaatgggtgtaaaggccttaatgaagtcctgtactaggaaatactaacagtaaatttgtattaacagca base pairs
tagaactttacccacatttccggaattacttcaggacatgatcctttatgattgtcatttaaacataattgtcgt 1501 to 1575
                    StuI                                   ApoI
                    Eco147I

   NspI
BspLU11I  AcsI                  DraI
catgtacgccaaattcaactttactaatctttaaaaaaatacacttgttaagtagagttttctgattcatgtttt base pairs
gtacatgcggtttaagttgaaatgattagaaatttttttatgtgaacaattcatctcaaaagactaagtacaaaa 1576 to 1650
AflIII    ApoI

                               MflI
                               BstYI
gacaatgtaaacagctcatacttttaataaagatccatttgcaaaaaaaaaaaaaa  base pairs
ctgttacatttgtcgagtatgaaaattatttctaggtaaacgttttttttttttttt  1651 to 1707
                               BstX2I
                               XhoII
```

Appendix IV. List of Communications

Oral Presentations

Kwon, J.Y., McAndrew, B.J. and Penman, D.J. 1999. Inhibition of aromatase activity suppresses high-temperature feminisation of genetic male Nile Tilapia *Oreochromis niloticus*. FSBI Annual International Symposium (St Andrews, Scotland, July 4-8, 1999).

Kwon, J.Y., Penman, D.J. and McAndrew, B.J. 2000. Differential expression of brain and ovarian cytochrome P450 aromatase genes during ontogeny in the Nile Tilapia, *Oreochromis niloticus*. Second International Symposium on Vertebrate Sex Determination Mechanism (Honolulu, Hawaii, April 10-14, 2000).

Poster Presentation

Kwon, J.Y., McAndrew, B.J. and Penman, D.J. 1999. Inhibition of aromatase activity suppresses high-temperature feminisation of genetic male Nile Tilapia *Oreochromis niloticus*. Sixth International Symposium on Fish Reproductive Physiology (Bergen, Norway, July 4-8, 1999).

Publication

Kwon, J.Y., Haghpanah, V., Kogson-Hurtado, L.M., McAndrew, B.J. and Penman, D.J. 2000. Masculinisation of genetic female Nile tilapia (*Oreochromis niloticus*) by dietary administration of an aromatase inhibitor during sexual differentiation. *Journal of Experimental Zoology* (in press).