THE SYNAPTONEMAL COMPLEX AND ANALYSIS OF SEX CHROMOSOMES IN THE GENUS *Oreochromis*

A Thesis Submitted for the Degree of Doctor of Philosophy to the University of Stirling

By

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DEDICATION

To My Dear Wife;

Danitzia

To My Dear Son;

Santiago

My Mother; ***María del Carmen Ramos Parajón***

My Uncle in heaven; ***Gabriel Ramos Parajón***

My Parents in law; ***Lilia*** **and** ***José***

DECLARATION

I hereby declare that this Thesis has been composed entirely by my own investigation. It has neither been accepted nor submitted for any other degrees. All information from other sources has been properly acknowledged.

Candidate

Supervisor (1)

Supervisor (2)

Date 28-05-2002

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ABSTRACT

Four species of fish from the genus *Oreochromis* (Cichlidae) were studied cytogenetically through meiotic prophase I using TEM. The female synaptonemal complex complement length (SCCL) in these species was around 1.4 times longer than that of the males. The SCCL of neomales (hormonally masculinized genetic females) was reduced significantly compared to control females, while the SCCL of genetic males given the standard hormonal masculinization treatment was increased slightly compared to control males.

Unpaired lateral elements were observed in the heterogametic sex of *O. niloticus* (XY: unsynapsed sub-terminal and terminal region of bivalent 1). In *O. aureus*, two distinct regions of restricted pairing were present only in the heterogametic sex. The first, a subterminal region of the largest bivalent, was located near to the region of unpairing found in the closely related *O. niloticus,* while the second was in a small bivalent, most of which was unpaired. These results suggest that *O. aureus* has two pairs of sex chromosomes. In *O. mossambicus*, unpaired lateral elements were not observed, nevertheless, a region of bivalent 1 with diffuse chromatin and differential staining was observed in one XY male, perhaps near to the kinetochore. A similar pattern was observed in female *O. niloticus* x male *O. mossambicus* hybrids. The recombination rates between the centromere and sex loci in *O. mossambicus* also suggested that sex genes in this species are located close to the centromere. An autosomal influence on sex determination was also observed.

During meiosis in the female *O. niloticus* (2n = 44) x male *O. karongae* (2n = 38) hybrid (2n = 41), three trivalents were observed in some spreads. In other spreads failures in chromosome synapsis were observed as aberrant pairing and multivalents. The *O. karongae* karyotype appears to have arisen from the typical *Oreochromis* one by three fusions.

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CHAPTER 1

GENERAL INTRODUCTION

1.1. Introduction

Primary sex determination mechanisms control whether an individual develops testes or ovaries and will become male or female (Bull, 1983; Hayes, 1998). Usually, primary sex determination refers to those genes that are harboured in sex chromosomes of higher vertebrates as most birds and mammals (Solari, 1994).

Fish, however, present a high degree of plasticity in sex determination. In teleostean fish the capability to sex reverse many species shows the sexual bi-potentiality of undifferentiated germ cells, capable to produce fertile oocytes or fertile sperm, according to external stimuli.

Fish appeared about 500 million years ago. The evolution of sex in fish has generated patterns of sexuality such as simultaneous hermaphrodites (functional ovaries and testes at the same time), sequential hermaphrodite fish and gonochorist individuals (i.e. separate sexes) as most teleostean fish now are. In fish, amphibians and reptilians, genetic sex genes are responsible for determining the genotypic sex but not necessarily the phenotypic sex. The above information gives us an understanding that sex genes are present in fish in undifferentiated sex chromosomes, morphologically differentiated sex chromosomes and even in autosomes, where they act only as a genetic sex determination mechanism.

The interest in sex determination in *Oreochromis* species has been strongly stimulated by scientific purposes and the necessity to farm all-male populations in culture conditions. Males naturally grow faster than females and the coexistence of both sexes in the same ponds rapidly leads to undesirable overpopulation by fingerlings (Hickling

1960, 1963). All-male individuals have been produced by oral administration of natural or synthetic androgens (Tayamen and Shelton, 1978). Direct sex reversal is commonly used in Asia and with the right hatchery set up can regularly achieve almost 100 % males on a large scale. However, direct sex reversal is not accepted in some countries because of the use of hormones in food production. Another method to control undesirable reproduction of cultured tilapia is the production of YY male broodstock through sex reversal and gynogenetic techniques (Scott *et al.*, 1989; Mair *et al.*, 1993, 1995, 1997; Varadaraj and Pandian, 1989; Myers *et al.*, 1995). When YY males are crossed with normal XX females the progeny are 100 % males, or nearly 100 % if environmental or autosomal influences took place. The initial phase in the generation of YY super males is complex and time consuming, and some super males do not yield the predicted Mendelian inheritance (Mair *et al.*, 1991a). Therefore, further research is needed to understand the sex determination mechanisms operating in tilapias, to be able to develop new reliable sex control techniques. One topic in sex determination research involves the cytogenetic analysis of putative sex chromosomes.

The existing knowledge of sex chromosomes in fish is limited. About 1000 species of fish have been analysed by karyotyping of mitotic chromosomes and the presence of morphologically differentiated sex chromosomes has been found in only about 31 species (3 %) (Solari, 1994; Foresti de Almeida Toledo and Foresti, 2001). In contrast only 16 species have been analysed through meiosis using transmission electron microscope. Meiotic analysis has three main aims. First to observe synapsis of homologous chromosomes through the formation of the synaptonemal complex (SC), second to observe the degree of homology in hybrids from different parental species and third, to

observe the absence of pairing in the heterogametic sex independently of morphologically undifferentiated or differentiated sex chromosomes. The last scenario has been found in 5 species: the mosquito fish, *Gambusia affinis* (Wise *et al.*, 1987), *Mastacembelus sinensis* (Liu and Yu, 1991), the Nile tilapia, *Oreochromis niloticus* (Foresti *et al.*, 1993; Carrasco *et al.*, 1999), the rainbow trout, *Oncorhynchus mykiss* (Oliveira *et al.*, 1995) and the guppy, *Poecila reticulata* (Traut and Winking, 2001), which represent 31 % of those species analysed. From those species that do not present unpaired regions related to sex determination (absence of recombination), what meiotic constraint mechanism, if any is occurring?

Ironically, the study of sex determination in tilapia began as a consequence of tilapia culture (see below). Since then tilapia culture and sex determination studies have been hand in hand for 40 years. However, sex determination in fish is still far from being entirely understood.

1.1.1. Fish aquaculture

Before the 1960's aquaculture was an insignificant small-scale activity in global terms. By 1992 aquaculture had increased to about 16.6 million tonnes from about 1 million tonnes in 1967 (FAO, 1992; 1993 cited in Pillay, 1994). By 1998, aquaculture production of fish, crustacean and molluscs presented a breakdown by environment as follows: 58.7 % (18.1 million tonnes) of freshwater culture, 35 % (10.8 million tonnes) of marine culture and 6.3 % (1.9 million tonnes) of brackish water culture (FAO, 2000). China

(Asia) continues being the leader in production with 27.07 million tonnes, followed by India (2.0 million tonnes) and Japan (1.29 million tonnes).

In fresh water aquaculture production, finfish represent 98 % and crustaceans 2 % (FAO, 2000). Tilapias (principally *Oreochromis* species) are of major economic importance to aquaculture in tropical and subtropical regions, contributing nearly a million tonnes to world aquaculture production (nearly 31 million tonnes) in 1997, which means that tilapia represents about 3.0 % of world aquaculture production (FAO, 2000).

According to FAO (1999), the main tilapia species cultivated and their production in 1997 in ascending order are *Oreochromis niloticus* (742,000 mt), tilapia hybrids (127,000 mt), *O. mossambicus* (61,000 mt), *O. aureus* (11,000 mt), *O. andersonii* (3,000 mt), *Tilapia rendalli* (1,000 mt) and *O. macrochir* (400 mt).

The Nile tilapia, *O. niloticus*, is the most important species in aquaculture representing more than 75.0 % in 1996 of the total production from fisheries and aquaculture (Young and Muir, 2000). This production has grown about 18 % annually over that period (FAO 1996 cited in Young and Muir, 2000).

The Mozambique tilapia, *O. mossambicus*, is the second most important species in aquaculture. In 1996 it represented 6.25 % of the total production from fisheries and aquaculture as pure species (Young and Muir, 2000). Additionally, it is also possibly the major contributor of the various hybrid tilapia populations around the world, particularly the red tilapias (Penman and McAndrew, 2000).

The blue tilapia, *O. aureus*, is currently an important species in aquaculture. However, it only represented 1.48 % in 1996 of the total production from fisheries and aquaculture (Young and Muir, 2000). The production of the blue tilapia has decrease about 2.3 %

annually over that period (FAO 1996 cited in Young and Muir, 2000). The blue tilapia shows the lowest growth rate whilst the Nile tilapia followed by the Mozambique tilapia show the highest growth rate (McAndrew and Majumdar, 1989). In the blue tilapia the sex ratios are sometimes skewed to females, which involves reproduction in the ponds for culture. Nevertheless this is the third most cultivated tilapia species.

In recent years interest in the potential of other species has increased (Msiska and Costa-Pierce, 1997a,b). *O. karongae* is one of these recently identified candidates and, with *O. lidole* and *O. squamipinnis*, forms part of a closely related species complex which is collectively known as the chambo in Malawi (Turner and Robinson, 1991).

1.1.2. General features of tilapia

Tilapias belong to the Family Cichidae, Tribe Tilapiini. There are three main genera that have been classified according to differences in reproduction, feeding habits, and biogeographical distribution: *Tilapia* as substrate spawners and guarders, *Sarotherodon* as paternal or biparental mouthbrooders and *Oreochromis* as maternal mouthbrooders (Trewavas, 1983). Tilapias can tolerate a wide range of environmental conditions in the wild such as high salinity, low oxygen, high density and generally poor water quality. In culture conditions they are amenable to handling and captivity, they are preadapted to stress of culture, disease resistant and the cost of food to rear tilapia is low. Tilapia has a short reproductive cycle that permits an easy acquisition of fry all year and can be cultivated from extensive to intensive farming systems (Pullin, 1996). Due to all these attributes mentioned above, tilapias are also of major interest for scientific research, for

example on their biology and taxonomy, evolution, reproductive biology and genetics (Rana, 1988; McAndrew, 2000; Turner, 2000; Penman and McAndrew, 2000).

Tilapias are prolific breeders. They reach sexual maturity and can start to reproduce at about four months of age, at sizes from 6 to 15 cm (Babiker and Ibrahim, 1979; Dadzie and Wanglia, 1980; Wohlfarth and Hulata, 1983). The introduction of non-endemic species into some lakes has depopulated endemic ones by competition, such is the case of Lake Victoria in Africa in which the endemic tilapias *O. esculentus* and *O. variabilis* (that originally occupied different biotopes without reproductive competition with each other), have become replaced in catches by introduced *O. niloticus* and *T. zillii*. *O. esculentus* has almost vanished from the lake whilst *O. variabilis* has become very rare in catches (Lowe-McConnell, 2000).

1.1.3. Tilapia culture

During the last 30 years, the major benefit of tilapia culture has been the production of a high quality meal (protein) at a low cost, to improve nutrition regimes in humans. However, since some farmers export the production (from example from Latin America to USA), tilapia culture can be a profitable business too. Well-established tilapia culture technology is available nowadays. Furthermore, in recent years, there has been a demand for the red tilapia (McAndrew *et al.*, 1988). This phenotype has started to compete in the fish market with marine fish such as the sea bream and snappers, because "for the eyes" of the consumer the important feature is the skin colour, which is related to "good taste and quality".

Nevertheless, the knowledge of how to culture tilapia was acquired by the Egyptians more than 2000 years ago (Penman, 1989). Technically, Chimits (1955, 1957) made the first reviews of documents of tilapia and their culture for the FAO. However, it was known before this, that the culture of tilapia had the disadvantage of precocious maturity and high fry survival that led to fishponds being over populated with small fish of low commercial value. However, it was quite noticeable that in tilapia culture males grow naturally faster than females (sex dimorphism). Thus, hand sexing was practised as a technique to help production of commercial table size fish by getting rid of females during the first 3 months of culture and thus preventing reproduction. Of course, selecting males by eye is quite laborious and not very accurate. In 1963, Hickling published what would be the first document about the cultivation of tilapia that contemplated all the difficulties of reproduction in ponds.

The first report of a genetic solution to reproduction in ponds was discovered by Hickling (1960), by producing the "Malacca hybrids". A cross between XX female *O. mossambicus* x ZZ male *O. hornorum* gave all-male progeny, and thus reproduction could be prevented by the absence of females in the ponds. This was also the first report to suggest that knowledge of sex determination systems could be of great importance for tilapia culture. Since then, sex determination in tilapia has been one important focus of aquaculture and scientific research.

1.1.4. Monosex culture and hormonal sex-reversal

There are attributes to single sex production systems. (1) Size dimorphism: in many fish species, such as marine flatfish, the female is substantially larger than the male, e.g. the hirame, *Paralichthys olivaceus* (Tabata, 1991), the Atlantic halibut, *Hippoglossus hippoglossus* (Björnsson, 1995), the starry flounder, *Platichthys stellatus* (Tokranov, 1996) and the turbot, *Scophthalmus maximus* (Imsland *et al.*, 1997). Alternatively, in many cichlid species such as tilapia and in some catfish the males are the larger sex (Lowe-McConnell, 1987). (2) Sexual maturation earlier in some species: in the rainbow trout, the development of secondary sexual characteristics, such as dark colour and a hooked lower jaw in males is accompanied by a cessation of growth (Bye and Lincon, 1986). Such male fish become more aggressive and the texture of the flesh rapidly deteriorates. Monosex female culture of salmonids also reduces the cost of broodstock maintenance (Hunter and Donaldson, 1983). In tilapia, precocious maturity of females (see 1.1.3. Tilapia culture). (3) One or other sex is more valuable: in ornamental species (guppy, Siamese fighting fish, platy etc.) where males have bright coloration and long fin shapes, gives males much higher prices than females that not exhibit such characteristics (Piferrer and Lim, 1997).

A major development of tilapia culture in the developing world involves the direct production of all-male stock, by using hormones to directly change sexual phenotype. Hormones may be administered by feeding or immersion, of which the first is favoured (Maclean, 1998). A range of different hormones has been used in tilapias. Androgen treatments are now used routinely to produce masculinized batches of fry by direct

administration of the hormone in the food (30-40 mg/kg 17α methyltestosterone for 30 days starting at the swim-up stage). It has been reported that it is more difficult to produce all-female populations by direct administration of the hormone diethylstilbestrol and ethynylestradiol in doses of about 100 to 1000 mg/kg for 20 to 40 days (see McAndrew, 1993 for review). It is not so much the concentration in the feed that is the determining factor, but rather the actual quantity of hormone consumed; the latter is dependent on the feeding rate and how well the fry consume the hormone feed mixture. The feed should be given at least two, but preferably up to six rations daily. Hormone feeding must cover the period of development during which sexual differentiation takes place (Macintosh and Little, 1995). To masculinize fry of *O. niloticus* (XX, XY system) it is possible to delay hormone administration for up to 10 days after first feeding, but with the risk to obtain less than 100% sex reversal (Macintosh and Little, 1995). In the case of *O. mossambicus* (XX, XY system), the feeding with the hormone has to start from the ninth day after hatching at the latest, otherwise incomplete sex reversal is achieved (Pandian and Varadaraj, 1988). In these species, temperature, photoperiod, feeding rate, water quality, and storage conditions of the hormone can modify sex reversal (Macintosh and Little, 1995; Varadaraj *et al.*, 1994). In *O. aureus* (ZW, ZZ system), the use of pseudofemale (ZZ) spawners is an alternative technique for producing monosex genetically male tilapia offspring (Desprez *et al.*, 1995). High percentages of females have been achieved with 17α-ethynylestradiol at 100 mg/kg food, together with methallibure at 100 mg/kg for 42 days (Mair *et al.*, 1987a). Alternatively, doses of 150 mg/kg of 17α-ethynylestradiol for 40 days have been successful (Melard, 1995; Desprez *et al.*, 1995).

A steroidogenic enzyme, cytochrome P450 aromatase, which catalyses androgens into oestrogens is implicated in sexual differentiation of many vertebrates including fish. Inhibition of aromatase action by a chemical inhibitor mimics the sex-reversal effect of androgen treatments in tilapias (Kwon, 2000; Kwon *et al.*, 2001).

An indirect method in the production of all-male stock requires initially to sex reverse males into females. Neofemales (XY females) lay eggs, half of which are Y male-determining. When neofemales are crossed with XY males, a proportion of YY supermales is produced. These YY males can be used as broodstock to produce genetically all-male (XY); when they are crossed with XX females (Scott *et al.*, 1989; Mair *et al.*, 1995).

1.1.5. A brief review of studies in sex determination in tilapia

The first study on sex determination in tilapia was made by Hickling (1960) with the hybrid cross between a female *Oreochromis mossambicus* and a male *O. hornorum*, which produced all-male F1 hybrids. Clemens and Inslee (1968) made the first study in sex determination in tilapia involving hormones in *O. mossambicus*. The aim of their study was to obtain male monosex progeny by sex reversal with methyltestosterone. Clemens and Inslee were the first authors to achieve sex reversal in tilapia and also found indirectly that *O. mossambicus* had an XX/XY sex determination system, after observing female progeny when crossing neomales (XX males) with XX females. One year later a paper was published by Chen (1969) that extended the hybrid crosses between the two species used by Hickling. Chen titled his publication as preliminary studies of sex

determination in tilapia. He found that an XX female *O. mossambicus* x ZZ male *O. hornorum* gave 100 % male (ZX) F1 progeny hybrids and the reciprocal cross (WZ female *O. hornorum* x XY male *O. mossambicus*) gave a 1:3 female:male ratio, the females having a WX genotype and three different genotypes existing in such males: YW, XZ and YZ. Then Chen made backcrosses between the F1 male hybrids and the females of either parent species (XX and WZ), obtaining the expected 1:1 sex ratio in XX female *O. mossambicus* x F1 male YW and XZ and WZ female *O. hornorum* x F1 male YW and XZ. The backcross XX female *O. mossambicus* x F1 male YZ gave all-male progeny and this last cross could explain the cross between WZ female *O. hornorum* x F1 male YZ giving 100 % males (should give 1:3 female:male). Thus, Chen postulated that the male-determining genes, the Z and Y chromosomes were dominant to the female-determining genes of the X and W chromosomes, respectively. However, when Chen observed the F2 hybrids, they resulted in a sex ratio different to the expected (e.g. the cross between WX female hybrid and XZ male hybrid is expected to be 1:1 under the Z dominance, however, under the W dominance is 3:1).

Chen's model was corroborated by Jabalert *et al.* (1971), from crossing XX females of *O. niloticus* with ZZ males of *O. macrochir* and the backcrosses to either parent species. Chen's model also was corroborated by Pruginin *et al.* (1975) crossing XX females of *O. niloticus* with ZZ males of *O. aureus* and obtaining only male progeny in some strains. These results agreed with the dominance of the Y and Z chromosomes.

Using the same experimental procedure as Clemens and Inslee (1968) in *O. mossambicus*, Jabalert *et al.* (1974) found female homogamety and male heterogamety in *O. niloticus* and Guerrero (1975) found that in *O. aureus* females are the heterogametic

sex. The work of Guerrero was the first publication that contradicted Chen's model in terms that the W chromosome was dominant to the Z chromosome. Therefore in the three species mentioned above, the sex-determining mechanisms were based on monofactorial systems. Avtalion and Hammerman (1978) and Hammerman and Avtalion (1979) proposed a theory of autosomal influence in which the Y and Z chromosomes were identical, with the additional presence of an autosomal locus with alleles "A" and "a". In this sex determination system, tilapias could have one out of six of the following gonosomes XX, XY, WX, WW, WY and YY. Each genotype could have the autosomal influence of two alleles as AA, Aa and aa. Therefore, 18 genotypes were predicted. This complicated theory was based on pure species having homozygous autosomal alleles. When a hybrid was produced these alleles influenced sex determination. Thus a female of *O. niloticus* or *O. mossambicus* would be AAXX whilst the males of both species would be AAXY. The females of *O. aureus* would be aaWY and the males aaYY. Therefore, the cross between female *O. niloticus* x male *O. aureus* (AAXX x aaYY) would give 100 % males from pure parental species. The unexpected presence of females in this cross was explained by hybrid contamination (Pruginin *et al.*, 1975). From the early 1980's several authors began to study hybrid crosses from pure parental species. The large variation observed in the sex ratios and the inconsistency to obtain 100 % males between XX x ZZ individuals (e.g. Hulata *et al.*, 1983; Majumdar and McAndrew, 1983; Mair *et al.*, 1991b) proved that the Avtalion and Hammerman theory did not provide a complete explanation of sex determination in tilapias. Therefore, sex determination from tilapia hybrids was difficult to explain due to the high variability in the sex ratios from intra- and

inter-specific crosses, so a possible hypothesis was that sex in tilapias was determined by polygenic systems (Majumdar, 1984; Wohlfarth and Wedekind, 1991).

Most studies of progeny sex ratios in *O. niloticus* showed that there was not a significant deviation from 1:1 (Pruginin *et al.*, 1975; Tayamen and Shelton, 1978; Majumdar and McAndrew, 1983; Majumdar, 1984). Nevertheless, Shelton *et al.* (1983) analysed sex ratios for 71 progeny groups; 31 females spawned with 12 males. Of the total number of progeny sexed, 54.7 % (a range of 31 to 77 %) were males. One female for example, spawned four times with two different males and the sex ratios were skewed each time (percent male = 45, 31, 32 and 38). Thus, considerable variation in sex ratio was demonstrated between pair spawns, however, yet an overall population sex ratio of 1:1 resulted through random matings or the summation of pair spawnings. Their conclusion was that females exerted a greater influence on progeny sex ratios, suggesting autosomal influences or a multi-gene system in tilapia sex determination. As a confirmation of female influence on the sex ratios, Calhoun and Shelton (1983) observed that in *O. niloticus* the progeny sex ratios of neomales crossed with some females were 100 % females. However, when they crossed the neomales with other females, a small proportion of male progeny (0-13%) was observed.

After the latest evidence of autosomal genes influencing sex presented by Calhoun and Shelton (1983), sex-determination studies in tilapia were directed towards chromosome set manipulation. Gynogenesis from XX females *O. niloticus* showed only female gynogens supporting the female homogamety in this species (Penman *et al.*, 1987). However, Mair *et al.* (1991a) and Hussain *et al.* (1994) observed a low percentage (4-17%) of gynogenetic males from XX females. Hussain *et al.* (1994) named a putative

sex-determining locus as sex determining locus 2 (SDL2). Thus, after all these studies, genetic sex-determining mechanism in tilapia based in a monofactorial system with some autosomal genes influencing the sex is generally accepted.

Gynogenetic studies of neomales of *O. niloticus* (Mair *et al.*, 1991a) and WZ *O. aureus* females (Avtalion and Don, 1990; Mair *et al.*, 1991b) showed recombination between the centromere and the sex-determining locus. It was calculated that sex genes were located distantly from the centromere. The observations of Foresti *et al.* (1993) and Carrasco *et al.* (1999) provided cytogenetic evidence that a distal region, possibly the opposite end from the centromere in bivalent 1 is unpaired, which implies a constraint of recombination during meiosis and this restriction is only observed in XY males (i.e. not in XX or YY males). This confirmed the monofactorial system previously proposed and showed the situation where morphological undifferentiated sex chromosomes were operating in *O. niloticus*.

1.1.6. Autosomal and environmental sex determination in tilapias

Sex determination has a high complexity beyond sex chromosomes. Autosomal effects present significant heterogeneity in sex ratios in normal pair crosses. Autosomal influences on sex are obvious when males are observed in meiogynes and mitogynes from XX females or in XX x XX crosses. In the same way, when females are observed in YY x XX and YY x YY crosses (Mair *et al.*, 1991a; Mair *et al.*, 1997; Hussain *et al.*, 1994; Sarder *et al.*, 1999).

Another factor affecting sex differentiation is temperature. In the case of tilapias several studies have been carried out on this matter (Mair *et al.*, 1990; Baroiller *et al.*, 1995a,b, 1996, 1999; Desprez and Mélard, 1998; Abucay *et al.*, 1999; Wang and Tsai, 2000; Kwon, 2000). In temperature sex determination (TSD), the threshold of reversion is at approximately 35 °C. TSD affects both genetic sexes: XX females are sex reversed to males and XY males are sex reversed to females. However, mixed batches tend to show skew to an excess of males at high temperatures, which means there is a stronger effect of temperature on XX females. The YY genotype is also affected more strongly than XY males. TSD represents a possible practical effect on sex ratios in genetic male tilapia production (GMT).

Parental effect have been observed in the last two decades (Shelton *et al.*, 1983; Tuan *et al.*, 1999; Sarder *et al.*, 1999; Kwon, 2000). However, it is still not known if parental effects are genetic.

1.2. Background

1.2.1. Genome of eukaryotic organisms

The genome of eukaryotic organisms is organised into chromosomes that are the structures that carry genes. A gene is a segment of the genome that is transcribed into RNA. RNAs are translated into proteins in the cytoplasm. Many genes in eukaryotes are discontinuous along the genome: they are split into exons (coding sequences) and introns (non-coding regions). The coding regions of genes are interrupted by intron sequences, so

a gene may hence take up many kilobases of sequence, and different genes are separated by long stretches of sequence for which the function is unknown. The introns are removed from the primary transcript by splicing to produce the functional RNA molecule, which means that they are cut out from central regions of the pre-mRNA and the outer portions joined in the nucleus before the mature mRNA is exported to the cytoplasm. Most of these noncoding DNAs consist of multiple repeats of similar or identical copies of a few different types of sequence. About one third of the total genomic DNA is composed of repetitive noncoding DNA. These copies may follow one another directly as tandem repeats e.g. satellite DNA, or they can be interspersed as multiple copies throughout the genome. Satellite DNAs are categorised according to the length of the repeating sequence into minisatellites and microsatellites. A single genome can contain several different types of satellite DNA, each with a different repeat unit (from < 5 to >200bp). Minisatellites form clusters up to 20 kb in length, with repeat units generally up to 25 bp e.g. telomeric DNA consists of up to hundreds of copies of a short repeated sequence 5'-TTAGGG-3', some repeats are in the 100's bp. These repeats are the basis of the DNA fingerprinting technique using southern blotting (Jeffreys *et al.*, 1985), used to identify individuals and their familial relationships. Microsatellite (Litt and Luty, 1989; Weber and May, 1989) clusters are shorter; usually < 150 bp, and the repeat unit is usually 4 bp or less. Many of them are highly variable; the number of repeat units in the array is different in different members of a species and the flanking DNA regions at both sides of the microsatellite become essentially the same between individuals. Microsatellites are expressed as alleles, they are observed as individual polymorphic bands or a given name "variable number of tandem repeat polymorphism"

(VNTR). If enough microsatellites are analysed then a unique genetic profile (like fingerprinting) can be established for every individual, this variability is due to "slippage", which sometimes occurs when a microsatellite is copied during DNA replication, leading to insertion or less frequently a deletion of one or more repeat units. Microsatellites have become an important tool in the analysis of population structure in many animal and plant species. The advantage of this type of marker is that they can be visualised using PCR techniques once suitable primer sites have been identified in the flanking unique DNA cluster adjacent of the repeat sequence (Turner *et al.*, 1998; Winter *et al.*, 1998; Brown, 1999).

1.2.2. Cell cycle

Cells proliferate through a regular cycle of events in which the genetic material is duplicated and divided equally between two daughter cells. Except in the production of gametes (meiosis), the nuclei of eukaryote cells divide by mitosis after interphase and immediately the cytoplasm divides by cytokinesis. The interphase of the cell cycle is generally divided into three phases: (1) G1 (Gap 1), (2) DNA replication, S phase or DNA synthetic (see section 1.2.2.1) and (3) G2 (Gap 2). Interphase is the period between successive cell divisions, when the cell grows and prepares for mitosis, the DNA replicates itself, and there is a great activity of synthesis of proteins, some of which will form the mitotic spindle. Interphase takes about 90 % and mitosis 10 % of the time of the cell cycle. In meiosis the times are different, in the ovary, oocytes are arrested at the end of prophase I (some invertebrates) or after the first meiotic division (vertebrates) and is

not until ovulation and fertilisation occurs in vertebrates that cell division is restarted. In contrast, spermatocytes divide continuously in testes (Winter *et al.*, 1998; Miller and Therman, 2001).

1.2.2.1. DNA replication

During DNA synthesis a cell copies its DNA prior to dividing. Replication is necessary so that the genetic information present in cells can be passed on to daughter cells following cell division. Enzymes called DNA polymerases copy the DNA. These enzymes act on single-stranded DNA synthesising a new strand complementary to the original strand. DNA synthesis always occurs in the 5' → 3' direction. Replication is semi-conservative, which means that each copied DNA molecule contains one strand derived from the parent molecule and one newly synthesised strand (Winter *et al.*, 1998; Miller and Therman, 2001).

1.2.2.2. Composition of chromosomes

There are at least three basic components of chromosomes: (1) The primary constriction of a chromosome, the centromere, is associated with the special structure kinetochore in which microtubules of the mitotic spindle become engaged. Centromeres in vertebrate chromosomes are observed as sites rich in constitutive heterochromatin which is characterised by its content of satellite DNA (stretches of DNA formed by highly repeated, short nucleotide sequences) and several specific proteins; (2) Replication

origins (autonomously replicating sequences, ARS), which are homologous to the sites of DNA replication initiation; and (3) Telomeres, the specialised end-regions of chromosomes, which have characteristic properties, such as stability and affinity for the nuclear envelope during meiotic prophase. In vertebrates telomeres share a terminal, repeated DNA sequence, TTAGGG (Solari, 1994). Chromosomes are composed of DNA, proteins (histones and nonhistones) and a small amount of RNA is also present but this is effectively only in transit to the cytoplasm (Winter *et al.*, 1998). Histones surround the DNA double helices, and when they are associated with DNA are called chromatin. There are five types of histones in eukaryotic cells, differentiated into two groups. The first group comprises the nucleosomal histones, which are small proteins responsible for folding and packing the DNA into units called nucleosomes that together form the chromatin solenoid. They are designated histones H2A, H2B, H3 and H4. The second group consists of the H1 histones of which there are several different but closely related varieties in each cell. H1 histones appear to be responsible for packing nucleosomes into a chromatin fiber. Nonhistone proteins make up the binding of chromatin fibers onto a chromosomal scaffold, mainly topoisomerase II. In this way the long DNA molecule is highly condensed or compacted into chromosomes (Alberts *et al.*, 1994).

1.2.2.3. Structure of the spindle

The spindle is characterised by fibres. These fibres consist of microtubules, which are in turn formed by the assembly of multiple protein dimers consisting of two closely related polypeptides, α- and β-tubulin. The assembled tubule is in equilibrium with a cytosolic

pool of tubulin subunits. There is a continuous flux of tubulin in and out of the assembled form, which allow the tubules to disassemble and reassemble in response to cellular conditions (Soifer, 1986; Mitchison, 1988; Hyams and Loydl, 1994). There are two kinds of microtubules in the spindle fibres. Those called kinetochore microtubules have one end in one of the cytoplasmatic poles, near the centrosomal or centriolar region. The other end is anchored in a region of the centromere of the chromosome formed by the interaction of special proteins with particular repetitive sequences of DNA (Murray and Szostak, 1985; Mitchison, 1988). The second type of microtubules is called non-kinetochore microtubules, which grow from the centrosome but have the other end free. These structures interact with one another providing a framework to the spindle and maintaining the separation of the two cellular poles during chromosome disjunction (Murray and Szostak, 1985; Gerace and Burke, 1988; Mitchison, 1988; Lewin, 1997).

1.2.2.4. Mitosis

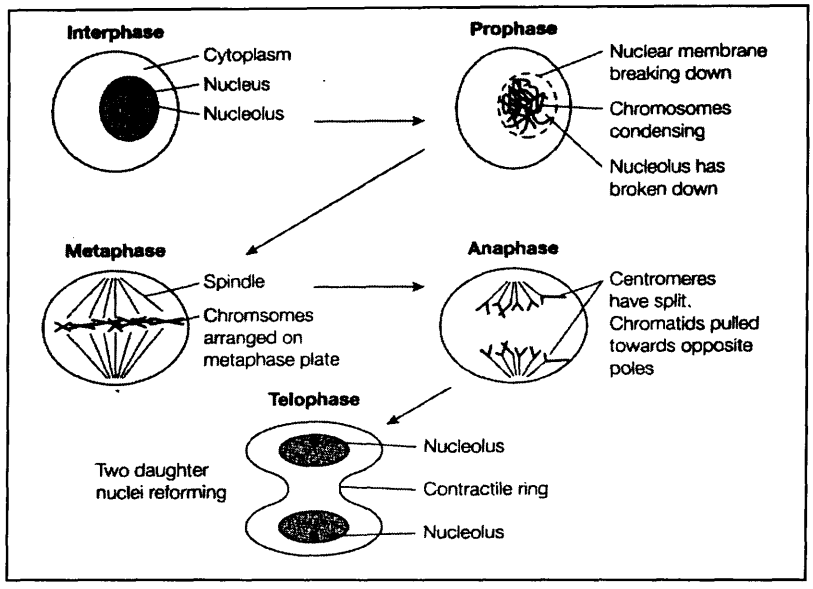
Mitosis is a biological process that involves the period of division of a cell. It has been described by four stages subsequent to the interphase stage (Fig. 1.1. a and b). After interphase, it is possible to observe the coiling or the "condensation" of the chromosomes as thread like structures. This period is called prophase. Each chromosome appears split into two duplicates (chromatids) that are connected at the centromere. The centrosome splits in two and each one move to opposite side of the nucleus forming the centrioles. The nucleolus disappears and the breakdown of the nuclear membrane begins. The next stage is metaphase, and it is characterised by the complete breakdown of the nuclear

membrane. Each chromosome is attached to the centromere and to the mitotic spindle. The centromere has split in two in each chromosome. Centrioles form a network of spindle microtubular fibres between each other. All chromosomes are moved and arranged midway between the two poles of the spindle (metaphase plate). Then, the centromere of each chromatid on the metaphase plate separates from its sister centromere and the two chromatids move toward opposite poles of the spindle through the microtubules. This separation process is called anaphase. The last stage of mitosis is telophase: in this period each of the two polar groups of chromatids undergoes a reversion to the more extended and swollen interphase state. The nuclear membrane is re-established and the nucleoli reformed. Division of the cytoplasmic portion of the cell (cytokinesis) is then completed (Strickberger, 1976).

1.2.3. Gametogenesis in rainbow trout and tilapia

Gametogenesis is a process of cellular differentiation that results in the transformation of undifferentiated gonial cells into gametes. The generation of sex cells involves a series of structural changes from the initial cells (oogonia in female and spermatogonia in testes). In the ovary, the primordial germ cells divide through mitosis and become primary oogonia, then primary cells enter another mitosis and become secondary germ cells. Secondary cells enter into meiosis (Bromage and Cumaranatunga, 1988). In testes, spermatogonia divide through mitosis and become primary cells that enter directly into meiosis (Billard, 1986, 1992). The relationship between meiosis and gametogenesis is represented in Table 1.1.

a



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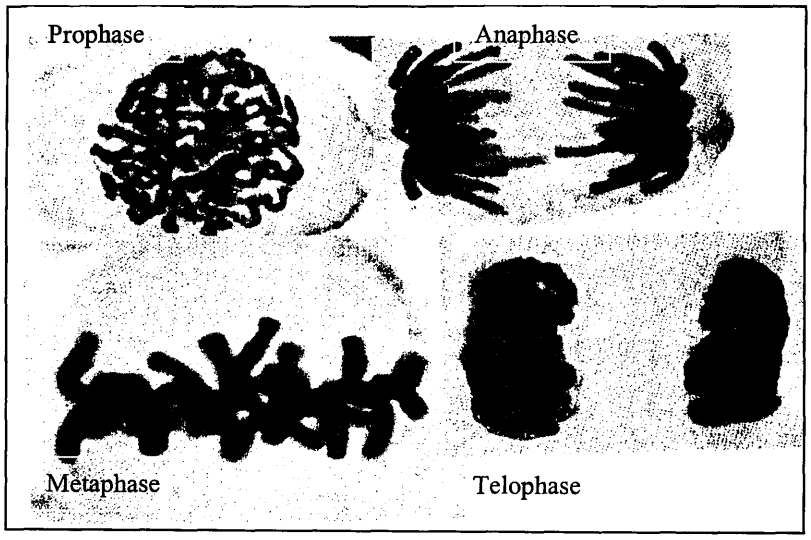


Figure 1.1. Representation of mitosis. (a) Schematic representation (Taken from Turner *et al.*, 1998). (b) Photographic representation in plants (Taken from Darnell *et al.*, 1986).

Table 1.1. The relationship between meiosis and gametogenesis in the rainbow trout and tilapia. After: Billard (1986, 1992); Bromage and Cumaranatunga (1988); Coward (1997).

Spermatogenesis	**Oogenesis**
Spermatogonia	Oogonia
Mitosis	
Primary spermatocyte	Primary oogonia
	Mitosis
	Secondary oogonia
	Stage 1 to 6 oocytes
First meiotic division	
	1st polar body
	Ovulation
Secondary spermatocyte	Fertilisation (Stage 7)
Second meiotic division	
Spermatid	2nd polar body
Spermatozoa	

1.2.4. Meiosis

Meiosis involves three important processes (Strickberger, 1976; Loidl 1994): pairing of the homologous chromosomes (synapsis), genetic recombination and segregation of homologous chromosomes. The particular features of meiosis are centred on its long and complex first prophase, where chromosome synapsis and genetic recombination occur during zygotene and pachytene, respectively. The differential region of the gonosomal pair in the heterogametic sex is inhibited from undergoing meiotic recombination. At the same time, in the homogametic sex of the same species, there is no recombination restriction. Thus, a dichotomous difference is established between the gonosome that finds its partner and is free to recombine in the homogametic sex (chromosome X or Z) and the gonosome that has recombination restriction in the heterogametic sex and is transmitted exclusively through this sex (chromosome Y or W). The presence of recombination restriction in homomorphic gonosomes demonstrates that this restriction is a primitive phenomenon previous to gonosome differentiation (Solari, 1994).

A reduction of the number of chromosomes in each gamete to half the usual number occurs during meiosis. Chromosomes are separated during the formation of sex cells and their numbers reduced from diploid to the haploid condition. Fertilisation marks the event in which two haploid nuclei join to reform a diploid cell. Each individual chromosome usually has a pairing mate or homologue.

There is a different process for the independent segregation of the sex chromosomes. In several rodent species of the genus *Apodemus* segregation of sex chromosomes occurs during the second meiotic division, a process called post-reduction. In this case the

existence of an interstitial pseudoautosomal region with a single obligatory crossing over makes sure that by the end of meiosis I, each daughter cell contains an XY composition even when segregation has taken place and chromosome number has been reduced to half. Therefore, the separation of the X and the Y takes place in the second meiotic division (Stitou *et al.*, 2001).

After interphase (Fig.1.2-1) the first meiotic prophase has been described by five stages (Strickberger, 1976):

1.2.4.1. Leptotene

Leptotene (thin strings): In leptotene the chromosomes appear as long slender threads with many bead-like structures (chromomeres) along their length. Although not observed through the microscope, there is biochemical evidence that replication of chromosomal material has already occurred (Fig. 1.2-2). In this stage, chromosomes are attached by their ends to the nuclear envelope (Fig. 1.4a).

1.2.4.2. Zygotene

Zygotene (to bring a pair together): Presynaptic alignment occurs at late leptotene/early zygotene in which homologues are aligned in parallel. Presynaptic alignment overlaps in time with the development of the synaptonemal complex (Moses, 1956; Fawcett, 1956), which usually begins near the chromosome ends by the appearance of central elements and transversal filaments between the axial elements (Fig. 1.4-b). In this period the

homologous chromosomes attract each other and enter into a very close zipper-like pairing called synapsis (Fig.1.2-3). At the sites at which they converge "early nodules" may or may not be observed.

1.2.4.3. Pachytene

Pachytene (Thick): During pachytene the chromosomes show a progressive shortening and coiling (Fig. 1.2-4). The two sister chromatids of a homologous chromosome are associated with the two sister chromatids of their homologous partner. This association is called a bivalent. A series of exchanges of genetic material occurs between non-sister chromatids, which is called crossing over or recombination. In this period the synaptonemal complex is observed through the electron microscope. It appears as a ribbon-like group of three longitudinal components organised into two dense lateral elements and a thin central element composed primarily of proteins (Fig. 1.4 and 1.5). This structure may function to pull chromosomes together, helping them to pair more precisely and efficiently.

1.2.4.4. Diplotene

Diplotene (double): At this stage, distinctly visible separations occur between homologous chromosomes except for specific regions where an actual physical crossing over appears to have taken place between homologous chromatids (Fig.1.2-5). These crossed areas or chiasmata (singular: chiasma), are x shaped attachments between the

chromosomes and seem to be the only remaining force holding each bivalent together until metaphase.

1.2.4.5. Diakinesis

Diakinesis (separation): The last stage of first prophase is characterised by the continuing coiling and contraction of the chromosomes until they are thick, heavy stained bodies. The bivalents usually migrate close to the nuclear membrane and become evenly distributed (Fig.1.2-6). The nucleolus* disappears or detaches from its associated chromosome. The nuclear membrane dissolves and the bivalents attach themselves by their centromeres to the rapidly formed spindle.

* The nucleolus is a sub-organelle of the nucleus. The nucleolus interacts with a region of one or more chromosomes termed the nucleolar organiser regions (NORs). This region contains many copies of the DNA that directs the synthesis of ribosomal RNA, most of which is synthesised in the nucleolus. The finished or partly finished ribosomal subunit passes through a nuclear pore into the cytoplasm (Darnell *et al.*, 1986).

1.2.4.6. First metaphase

At this stage, chromosomes have reached their most condensed state. The chiasmata have moved toward the ends of each chromosome (termination), leaving a single attachment between the paired arms of homologous chromosomes, which now lie on each

side of the equatorial plate of the spindle, stretched by their respective centromeres toward opposite poles (Strickberger, 1976) (Fig.1.2-7).

1.2.4.7. First anaphase

Although previously duplicated along its entire length, each homologous chromosome still maintains only a single functional centromere for both of its sister chromatids. The separation or disjunction of one homologous chromosome from the other toward the opposite poles therefore results in this single centromere dragging both chromatids along with it (Fig. 1.2-8). The chiasmata slip off the ends of the chromosome as they are pulled apart, and the moving chromatids are now bound together at only one point, the centromere. If genetic exchange has occurred during meiosis, each separating chromosome carries part of its homologue, resulting in an equal division of the exchanged chromosome material to both daughter cells. It is a matter of chance which of the parental homologous chromosomes are separated to each daughter cell: the more pairs of chromosomes a organism has, the greater the chances that a gamete will contain material from both parents. Thus the random distribution of parental chromosomes, as well as the crossing over between them, leads to a most important source of differences or variability among the gametes of sexual reproducing organisms (Strickberger, 1976).

1.2.4.8. First telophase and interphase

Once each of the two groups of chromosomes reaches its respective spindle pole, a nuclear membrane is formed around them, and the chromosomes pass into a short interphase before the second meiotic division begins (Fig. 1.2-9). During the short interphase, chromosomes are not physically extended, and there is no formation of a single nucleolus. Cytokinesis may occur during this stage or may be postponed until simultaneous formation of four daughter cells at the end of the second meiotic division (Strickberger, 1976).

1.2.4.9. Second meiotic division

The second meiotic division is similar to a mitotic division. It has the following features. The chromosomes enter into the prophase of the second meiotic division as two sister chromatids connected together at their centromere region at metaphase II (Fig. 1.2-10). As soon as these centromeres divide, each chromatid separates from its sister and moves to the opposite pole in the second anaphase (Fig. 1.2-11). The second telophase and cytokinesis follow rapidly, giving rise to four haploid cells from each initial diploid cell that entered meiosis (Fig. 1.2-12). In the case of spermatogenesis, four final functional sperm cells are formed. In oogenesis, only one functional ovum cell is formed. The first polar body is expelled during the first meiotic division, after ovulation and fertilisation, the functional oocyte divides at the second meiotic stage where the second polar body is extruded (Strickberger, 1976). The first and second polar bodies have been extruded from the functional oocyte with a minimum quantity of yolk (Fig. 1.3).

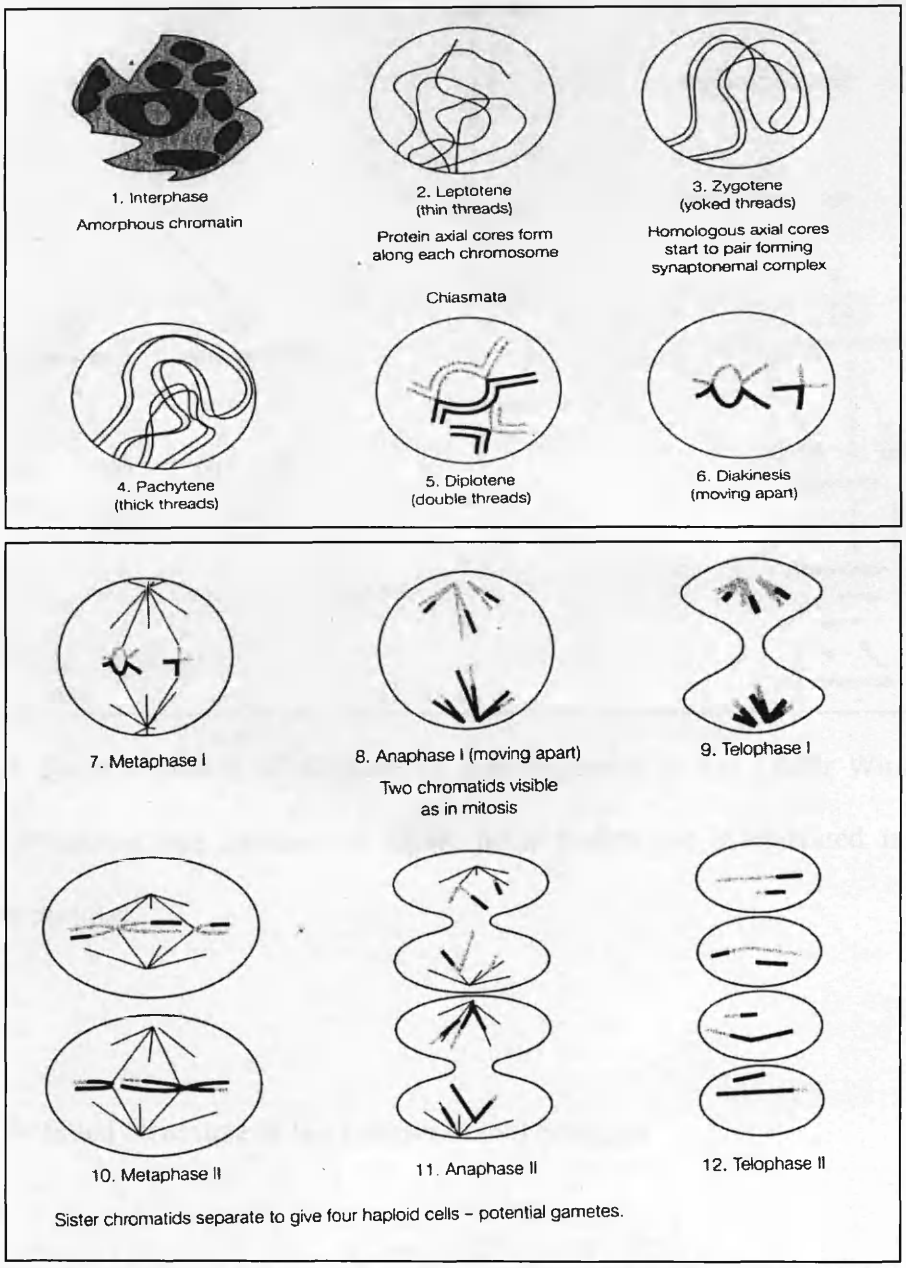


Figure 1.2. Diagrams 1-9 represent a nucleus undergoing meiosis in testes. Only two pairs of chromosomes are shown. One of each pair is darker. After meiosis I, the cells go into a transient interphase, and prophase of meiosis without DNA replication. Diagrams 10-12 represent the major stages of meiosis II. The result is the formation of four haploid nuclei, each of which carries recombined chromosomes (Taken from Winter *et al.*, 1998).

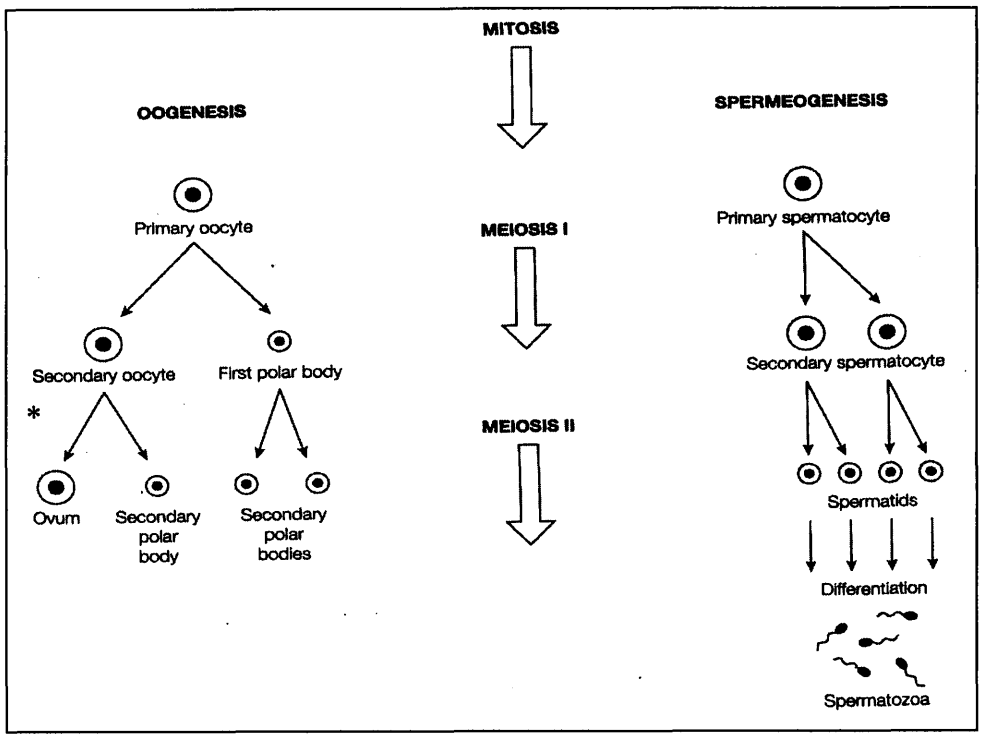


Figure 1.3. General pattern of divisions in gametogenesis in fish (After Winter *et al.*, 1998). * Ovulation and fertilisation. Note: polar bodies are exaggerated in size for illustration purpose.

1.2.4.10. Detailed structure of the synaptonemal complex

Synaptonemal complexes are meiosis-specific nuclear organelles involved at the meiotic prophase in chromosome rearrangements such as chromosome pairing and recombination. One synaptonemal complex is formed between a pair of homologous chromosomes (Fig. 1.4 and 1.5). They consist of two proteinaceous axes, called axial elements (before synapsis) or lateral elements (when synapsed), along each homologue.

They are connected along their length by numerous transverse filaments. Chromatin loops come out from the lateral elements, however, only a small fraction of the chromosomal DNA projects into the space between them (Loidl, 1994). A third longitudinal structure, called the central region exists on the transverse filaments between the two lateral elements (Schmekel *et al.*, 1993; Meuwissen *et al.*, 1997). The central region, which is composed of proteins and RNA, synthesises recombination nodules (von Wettstein *et al.*, 1984). These nodules have been described in female *Drosophila melanogaster* as dense staining, spheroid structures. The total numbers of nodules per nucleus and their locations along the euchromatic portion of the bivalent arms correspond quite closely to the numbers and locations of genetically detected exchange events, suggesting that the recombination nodule performs a role in the recombination process (Carpenter, 1979). Spermatocytes at meiotic metaphase I and anaphase I have a characteristic centromeric filament in a variety of vertebrate organisms. It is suggested that this structure is a remnant of a lateral element of the synaptonemal complex, which is located specifically at both centromeric regions of each bivalent. This filament is not found at the second meiotic division or at the centromeres of mitotic chromosomes. It is assumed that this centromeric filament joins the two sister chromatids of each homologue at the centromere and thus ensures the proper co-orientation of sister kinetochores at metaphase I (Solari and Tandler, 1991). In yeast, several meiosis-specific genes that encode structural components of the synaptonemal complex have been identified. The Zip 1 protein is a component of the central region. In the absence of Zip 1, crossover interference is abolished, resulting in a random distribution of crossovers along and among chromosomes. The Zip 2 protein localises the sites of synapsis initiation and

promotes the polymerisation of Zip 1 onto chromosomes. The Red 1 and Mek 1 are associated with the lateral elements. In the absence of Red 1, sister chromatids separate precociously, leading to chromosome mis-segregation and non-viable meiotic products. The Ndj 1 and Hop 2 proteins are both involved in the mechanisms by which the homologous chromosomes pair with each other prior to synaptonemal complex formation. Loss of the Ndj 1 protein leads to delayed and incomplete pairing. In the absence of Hop 2, synapsis takes place between non-homologous chromosomes (Roeder *et al.*, 1988).

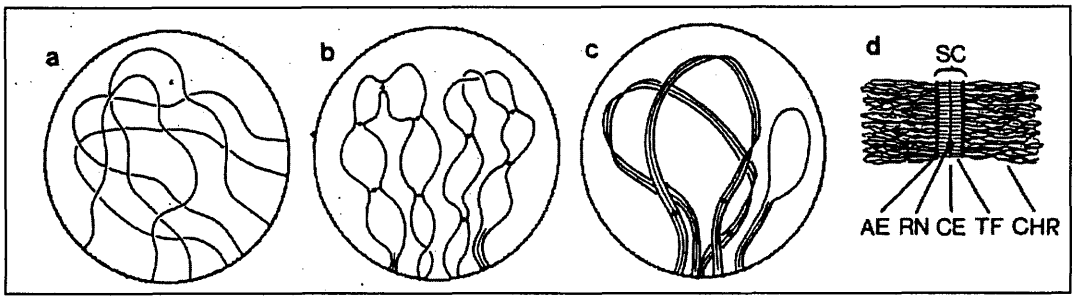
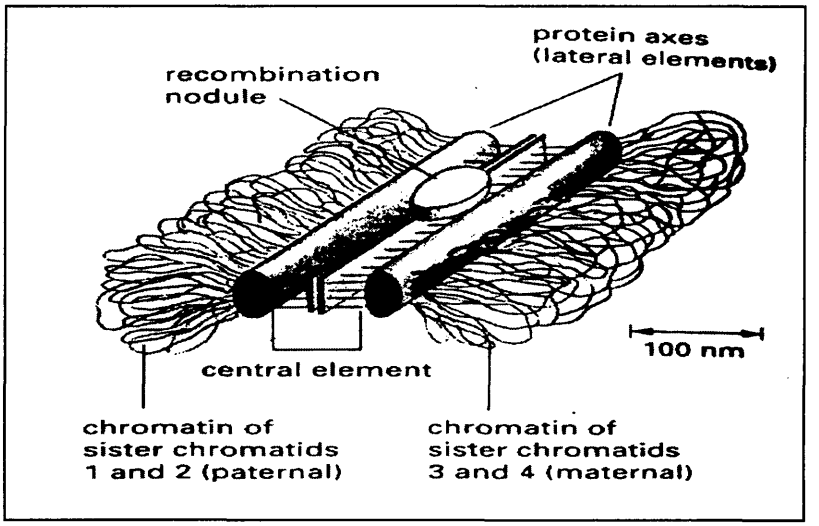


Figure 1.4. Hypothetical sequence of homologous alignment and synapsis. (a) Chromosomes attached with their ends to the nuclear envelope (leptotene). (b) Presynaptic alignment in which homologues are roughly in parallel. At sites where they converge "early nodules" may be present (zygotene). (c) At late zygotene-pachytene most homologous chromosomes are synapsed along their entire length. Recombination nodules occur at the sites of crossovers where chiasmata will be formed subsequently. (d) Segment of a pachytene synaptonemal complex (SC). Axial elements (AE). Chromatin loops (CHR). Recombination nodules (RN). Central elements (CE). Transversal filaments (TF). (Taken from Loidl, 1994).

a



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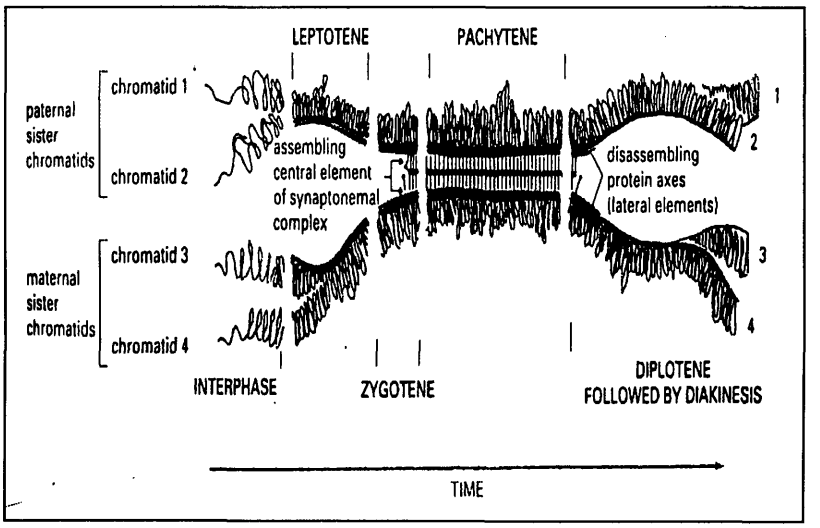


Figure 1.5. Synaptonemal complex structure. (a) Cross section of synaptonemal complex during pachytene. (b) Time of chromosome synapsis and desynapsis during meiotic prophase I (Taken from Alberts *et al.*, 1994).

1.2.5. Types of chromosomal sex determining mechanisms in vertebrates

In 1891, H. Henking (cited in Solari, 1994) published a morphological study on spermatogenesis of the hemipteran insect *Pyrrhocoris apterus* in which a chromatin body was described as an "X chromosome" because of its unusual staining during meiotic prophase and its peculiar behaviour at meiotic divisions. "X" (for its unknown nature) has been used to name a biological element, the X chromosome. The observations of Henking and other cytologists were finally interpreted by C.C. McClung in 1901 and by E.B. Wilson in 1909 (both cited in Solari, 1994). They assumed that the presence of either one or two of the "X chromosomes" determined the binary decision towards male or female in X0-XX systems (as are present in a number of insects, in which males have an odd number of chromosomes and females have an even number of chromosomes).

Differential staining during meiotic prophase is a widespread feature of sex chromosomes among individuals of the male sex in mammals and insects (White, 1973).

Among vertebrates the main types of chromosomal sex-mechanisms are the XX-XY (heterogametic males), and the ZW-ZZ (heterogametic females) systems. The recognition of sex chromosomes is usually possible only in the heterogametic sex. This scheme was based mainly on observations of insects and higher vertebrates, especially birds and mammals. The fact that recognisable sex chromosomes are not observed in many taxa in which sexes are generally separated (for instance in most fish and amphibians) is evidence that sex chromosomes *per se* are not essential for sex determination, but their specific gene contents are related to sex. Therefore, in some organisms, sex determining

genes are located in conspicuous "sex chromosomes" while in other types of organisms, sex determining genes are harboured in non-distinguishable chromosomes (Solari, 1994).

Taxa having non-differentiated sex chromosomes usually have labile sex-determination mechanisms, where the phenotypic sex of new born individuals or adults can be reverted by hormone administration, by temperature and other environmental sex determination (ESD). That is, the sex may be determined in nature by environmental stimuli. On the other hand, vertebrates having differentiated sex chromosomes have more stable sex-determination mechanisms, and this is visible for example in mammals. Thus, sex chromosomes do not determine the sex of organisms by themselves, but their peculiarities are associated with the harbouring of genes acting on sex determination (Solari, 1994).

A number of differences may be observed between autosomes and sex chromosomes. Many reptilians and most avian and mammalian species possess an unequally sized or otherwise morphologically differentiated chromosomal pair in the heterogametic sex. In some cases it is possible to differentiate them through the observation of specific heterochromatin bands (Solari, 1994).

1.2.5.1. The evolution of sex chromosomes

The most primitive mechanism for the determination of separated sexes was possibly a single allelic difference at a single locus (Aa versus AA). The presence of the allele "a" was restricted to one sex, and mutations that accumulate in the vicinity of this allele tended to have sex-linked expression (Lucchesi, 1994). Probably the simplest sex

determining mechanism is found in yeast (*Sacaromices cerevisiae*). A gene on chromosome 3 has two alleles a and α. Most of the life cycle of yeast is haploid and each yeast cell will carry one of the alleles. This determines mating type. Only yeast cells of opposite mating types can fuse to form diploids which undergo meiosis and release new haploid spores (Winter *et al.*, 1998).

It is generally considered that sex chromosomes evolved from a pair of homologous autosomes (Ohno, 1967, cited in Bull, 1983) and during the differentiation process both retained a small region of homology known as the pseudo-autosomal region (Winter *et al.*, 1998). This region is required to permit the two chromosomes to pair and segregate accurately at meiosis.

Muller (1918) (cited in Bull, 1983; Solari, 1994; Charlesworth and Charlesworth, 2000), proposed that the ancestral X and Y chromosomes were identical in genetic content. From Muller's theory it is believed that in vertebrates the sex chromosome that is limited to the heterogametic sex (Y or W) generally is found to carry few genes and to accumulate large amounts of satellite DNA sequences and constitutive heterochromatin (Bull, 1983; Solari, 1994; Turner *et al.*, 1998; Charlesworth and Charlesworth, 2000).

Sex chromosomes heteromorphism is a late consequence of sex chromosomes specialisation, involving the progressive loss of functional genes or gene-inactivation of the Y or W chromosomes by the acquisition of heterochromatin and the accumulation of lethal or deleterious mutations. The evolution of sex chromosomes is thought to begin initially with the presence of chromosome inversions and mutations at specific loci. A change in recombination patterns is considered as an early step towards differentiation, where sex is subjected to special constraints regarding the amount and location of

recombination events during meiosis. Then come the accumulation of heterochromatin (intermediary step), the accumulation of transposable elements (Steinemann and Steinemann 1997) and a progressive loss of copious amounts of euchromatin in the Y or W chromosome. The process finally results in the change from apparently equal elements to grossly heteromorphic, the complete isolation of the Y or W chromosome (Solari, 1994). Intermediate states of slightly heteromorphic sex pairs have been described in fish, amphibians and reptiles (Bull, 1983; Solari, 1994). They could be interpreted as the transitions from a primitive homomorphic sex pair leading to a heteromorphic state (Pigozzi, 1999). Therefore, the evolution of sex chromosomes originates from the absence of recombination but it is not the result of morphological changes (Pigozzi, 1999).

1.2.5.1.1. Muller's ratchet (Reviewed by Bull, 1983; Solari, 1994; Charlesworth and Charlesworth, 2000).

This phenomenon has been described as a basic evolutionary mechanism in sexual populations initially having asexual reproduction.

In the absence of recombination between the X and Y chromosomes or any other pairs, individuals from a population are obliged to inherit the full load of mild deleterious mutations present in his/her parent. Then, only a slight selective difference exists between chromosomes with no mutations and those with one, so, there is an equally slight fitness difference between Y chromosomes with two mutations and those with only one, and so forth. In a finite population with a sufficient rate of mutation to deleterious alleles, there

is an appreciable chance that the class of Y-chromosomes with the lowest number of mutations (zero class) will be lost. Loss of the lowest class can occur with a moderately large probability if the mutation rate is sufficiently high relative to population size. Once the lowest class is lost, it is not likely to reappear since it can not regenerate by recombination (The Y does not recombine), then the "ratchet" accumulates one loss (those individuals of the lowest class). The ratchet works because each loss of the class with fewest mutations is irreversible. A perpetually increasing accumulation of deleterious genes therefore occurs in the population. This process seems to be the most plausible explanation for the degeneration of the Y-chromosome. If the population is maintained in equilibrium, then the ratchet does not operate. In resume, the Muller's ratchet mechanism applied to the Y chromosome assumes that the Y-chromosome was active and had genes homologous to those of the X chromosome, but recombination was avoided in at least part of this chromosome. The loss of fitness given by a mutation in the non-recombining part of the Y chromosome in an XY individual is feasible, although this theory can be only evident under computer simulations.

1.2.5.1.2. Genetic hitchhiking (Reviewed by Solari, 1994; Charlesworth and Charlesworth, 2000).

Another mechanism operating in a population is known as "genetic hitchhiking" (Rice, 1987: cited in Solari, 1994). The conditions of this model are similar to those found in Muller's ratchet. If a new mutation occurs in the differential region of the Y-chromosome and is beneficial for the individual, the frequency of this new allele may increase. This

beneficial allele presents an advantage over Y-chromosomes having the fewest mutations. Therefore, this particular Y-genome may rapidly accumulate in the population. This allele enhances the effect of Muller's ratchet (if it operates) by increasing the proportion of more heavily mutated Y-chromosomes, and presents a high frequency of mutations in a small number of loci of the particular Y-chromosome carrying the beneficial allele. These mildly deleterious mutations have been given a "hitch" by the increase in frequency and fixation of the beneficial allele.

1.2.6. Techniques for sex determination analysis

1.2.6.1. Sex reversal and progeny testing

Most sex determining systems tend to produce balanced sex ratios (Fisher, 1930). Crosses between females and males of any species usually gives a sex ratio not significantly different from 1:1 female:male. Because fish present a labile mechanism of sex determination, one sex can be reverted to the opposite sex with the use of hormones or environmental stimuli such as temperature. For example, if a hormone such as methyltestosterone is used to reverse females into males, then, that batch will have a mixture of males types; those with a genotype XY or ZZ and those with a genotype XX or WZ. All males are progeny tested by crossing them with genetic females (XX or WZ). The expected sex ratio from genetic males (XY or ZZ) must be not significantly different from a 1:1, whilst the expected sex ratio from neomales (genetic females) must be 1:0 female:male in a XX/XY system, or a sex ratio not significantly different from a 3:1 in a

WZ /ZZ system. Therefore, sex reversal and progeny testing is the easiest technique to identify a sex determination system in fish (Penman, 1992).

1.2.6.2. Genetic chromosomal set manipulations in fish

1.2.6.2.1. Induced triploidy

Although triploidy was not used in the present thesis, it is convenient to briefly explain it here. Triploidy can be a tool for sex determination studies, but the difference between triploidy and gynogenesis (or androgenesis) has to be made clear. It should also be noted that a failure during sperm UV radiation for gynogenesis could instead induce triploid individuals.

Triploidy is a form of polyploidy characterised by the presence of three sets of homologous chromosomes in the genome. It occurs naturally in certain animals and plants (Fankhauser, 1945). Induced triploidy affects the sexes differently in teleosts. Females show a large reduction in ovarian size with the presence of only a few developing oocytes. In mature males, the testes are smaller but not generally reduced to the same condition as in females and contain more connective tissue and cell-free spaces due to the degeneration of spermatocytes. Male triploids generally produce some aneuploid sperm and present relatively normal secondary sexual characteristics, however, secondary sexual characteristics are absent in female triploids. These physiological conditions have been observed in salmonids, bass, bream, carps, catfish, cichlids, flatfish and others (see Carrasco, 1988 for review). The main goal for induced triploidy is to

achieve sterility in the species being used, which is caused by the abnormal segregation of chromosomes during meiosis. This is generally only successful in females in teleosts. Sterile animals cannot reproduce at all; male triploids can produce some sperm and fertilise eggs to some extent. In this way induced triploidy could be important in conserving or protecting endemic species in specific areas; rearing sterile individuals may be advantageous when a species is grown out of its geographical distribution (Dumas and Campos-Ramos, 1999).

On the other hand, it has also been used to study sex determination systems in tilapias (Penman *et al.*, 1987). Triploidy can be induced by retention of the second polar body, resulting in a zygote with one parental and two maternal chromosome sets. In tilapias, applying a heat shock of 41.0 °C for 3.5 minutes commencing 5 minutes after fertilisation to eggs fertilised with functional spermatozoa induces high rates of triploidy (Chourrout and Itskovich, 1983; Penman *et al.*, 1987; Hussain *et. al.*, 1991).

1.2.6.2.2. Gynogenesis and androgenesis

These two genetic phenomena define the development of individuals which have all their chromosomal genetic endowment derived either from the father (androgenesis, apart from the mitochondrial DNA (mt DNA)) or the mother (gynogenesis) only. The first one involves elimination by irradiation of egg-derived DNA prior to fertilisation, then the suppression of the first cleavage division. The progeny have a chromosome set derived by the duplication of the original male haploid set and is then complete of paternal origin. The second one can be induced by irradiation or chemical treatment of sperm prior to fertilisation: the sperm genome is inactivated without killing the sperm motility, so that

fertilisation can still occur. This process is followed by treatment of the activated eggs by pressure or heat exposure to suppress the second meiotic division, producing meiotic gynogenetics by retention of the 2nd polar body, or the first cleavage division, producing mitotic gynogenetics retaining two haploid sets in the same cell (Maclean, 1998). Therefore, gynogenesis is a process where only the maternal genome is inherited by the progeny. This is because the sperm fertilises the egg but its genetic material has been previously destroyed or inactivated with agents such as ultraviolet light (UV), gamma rays or chemical mutagens (Thorgaard, 1983a). Because the paternal genome has been eliminated, it is necessary to restore diploidy, otherwise the resulting individuals will be haploid possessing only a half of the diploid genome. Haploid individuals rarely hatch and usually die fairly early during development. To restore diploidization it is necessary to apply a physical (temperature or pressure) shock or chemical agent such as dimethylsulphate (DMS) to inhibit the formation of the spindle during cell division.

Hertwig described a "Hertwig effect" in 1911 (cited in Thorgaard, 1983a), after irradiating frog sperm with radium gamma rays, prior to fertilisation of frog ova. He observed that if eggs were fertilised with sperm given increasingly higher irradiation dosage of gamma rays, a higher haploid embryo production was observed than those irradiated with a lower dosage. This was attributed to the complete inactivation of the sperm genome at a high dose that resulted in more haploid embryo survival. The presence of sperm chromosome fragments at the lower dose was lethal to the embryos. Since then, ionising rays, ultraviolet rays and chemical mutagens have been used to produce haploid gynogenetic fish (Thorgaard, 1983a; Chourrout, 1987; Ihssen *et al.*, 1990). Later on it was observed that ionising radiation such as X rays and ^{60}Co gamma rays had a good

penetration into the sperm genome without affecting the sperm's mobility to fertilise the egg. However, chromosome paternal fragments could pass into the embryo (Ijiri, 1980; Chourrout and Quillet, 1982; Onozato, 1984). The same phenomenon was observed using chemical mutagens such as DMS (Tsoy, 1969; Chourrout, 1986). In contrast to gamma irradiation and chemical mutagens, ultraviolet irradiation was the easiest, least expensive and safest to work with, and resulted in no residual chromosome fragments (Thorgaard, 1983a; Chourrout, 1984, 1986; Ihssen *et al.*, 1990; Myers *et al.*, 1995). In some fish, spontaneous diploidization of eggs fertilised with UV-sperm has been reported e.g. common carp, *Cyprinus carpio* (Nagy *et al.*, 1978).

In tilapias, two kinds of gynogynes have been produced by inducing diploidization of eggs fertilised with UV irradiated sperm at two different times after fertilisation. Applying a heat shock of 41.2 °C for duration of 3.5 minutes, commencing 5 minutes after fertilisation produced meiotic gynogenetics (retention of the second polar body in meiosis) (Mair *et al.*, 1987b, 1991a,b). Meiotic gynogenetics have been induced successfully in tilapias (Chourrout and Itskovich, 1983; Penman *et al.*, 1987; Don and Avtalion, 1988; Varadaraj and Pandian, 1989; Varadaraj, 1990. Mair *et al.*, 1991a,b; Hussain *et al.*, 1993; Peruzzi *et al.*, 1993).

Mitotic gynogenetics (suppression of the first mitosis) are produced by application of the same heat shock, commencing 20-35 minutes after fertilisation (Myers *et al.*, 1995). Androgenesis has been achieved by the de-nucleation of tilapia eggs using ultra violet irradiation for 5 to 8 minutes with a total dose of 450-720 J/m^2. Then, the mitotic inhibition is achieved using a heat shock of 42.5 °C for 3-4 minutes applied at 2.5 minutes intervals from 22.5 to 30 minutes after fertilisation (Myers *et al.*, 1995).

Gynogenesis or androgenesis are useful to produce inbred lines of fish. Combined with the sex reversal technique is possible to "fix" a characteristic, for example an exotic coloration of a male (previously sex reversed to female), for the exotic aquarium fish industry or a red colour that attracts the attention of consumers in the market.

In fish biology meiotic gynogenetics are useful in finding the sex determination system of a particular species. It is expected that induced gynogenesis in a XX female (XX/XY system) give female progeny only. Induced gynogenesis in a WZ female (WZ/ZZ sytem) gives both females and males in a 1:1 sex ratio. Both cases consider no recombination between sex chromosomes.

Gynogenetic individuals are highly inbred in a single generation. Meiotic gynogenetic are about 30 % inbreed in a single generation and can be used to speed up the production of inbred strains (Nagy and Csányi, 1982) or in the analysis of traits such as sex determination mechanisms in carps (Stanley, 1976; Mirza and Shelton, 1988; Castelli, 1994) and tilapias (Penman *et al.*, 1987; Mair *et al.*, 1991 a,b). Mitotic gynogenetic fish are 100 % homozygous in the first generation and individuals can be used to establish inbred clonal lines. Eggs from a mitotic gynogenetic female or sperm from a mitotic gynogenetic male can be put through meiotic gynogenesis or androgenesis respectively, to generate viable isogenic lines. Isogenic lines can be produced from crosses between clones (outbred clonal lines) (Streisinger *et al.*, 1981; Quillet *et al.*, 1991).

Meiotic gynogenetics are formed from the two sets of chromosomes originated from sister chromatids in the eggs of the female (2^{nd} meiosis division, retention of the second polar body). If there were no recombination then the offspring will get the paternal or maternal chromosomes and individually would be homozygous, but different to each

other, however recombination is usual and ensures exchange between paternal and maternal chromosomes. Meiotic gynogenetic analysis of such individuals can identify heterozygous loci, the product of recombination. The frequency of heterozygotes in the offspring will reflect the effective crossover rate, between non-sister chromatids and the level of heterozygosity in the mother. Multiple crossovers per chromosome arm (i.e. mammals) do not appear to be the rule in fish, in many fish species there is a high level of chiasma interference (Allendorf *et al.*, 1986). Thus for most chromosomes there appears to be only one chiasma per meiosis and therefore the probability of any recombinant event is proportional to the proximity of a locus to its centromere (Hussain *et al.*, 1994). The recombination rate (r) in a meiotic gynogenetic batch for a particular locus, with dominance is calculated as:

r = (A-B)/A+B. Where:

r = proportion of recombinants in the progeny.

A = number of individuals with a dominant phenotype (e.g. RR; Rr) in the progeny, and

B = number of individuals with recessive phenotype in the progeny (e.g. rr).

Examples:

Induced gynogenesis in a XY neofemale is useful to estimate the gene-centromere recombination between a sex locus and the centromere. The location of a sex locus in its respective chromosome from the observed proportion of XX females (rr); XY male recombinants (Rr) and YY males (RR) can be calculated.

Before the first meiotic division, there will have been chromatid exchanges between homologous chromosomes. If one the Y chromatids has exchanged "sex genes" DNA material with one of the X chromatids, the result after retaining the second polar body

will be reflected in an excess of males in the meiogynes progeny (XY recombinants as well as XX and YY non-recombinants). This excess of males can be interpreted as the "distance" between the centromere and the sex genes on the chromosome arm. This means that the further away the sex genes are located from the centromere, the more males will be observed in the progeny. This is because the probability that a crossing over occurs involving a locus far away from the centromere is higher than if it occurs near to it. If an imaginary chromosome length is 100 arbitrary units, and a locus is located at unit 10, it will have 10/100 = .1 or 10 % of probability to exchange DNA material. If it is located at unit 80, it will have 80 % of probability to cross over. In the case of no recombination between the Y and X chromosomes, the expected proportions of males and females will be similar, indicating that the sex genes did not cross over, or in other words, there was a small probability to exchange DNA with its non-sister chromatid.

Different types and proportions of male and female may be formed through meiotic gynogenesis. If there is no recombination, by the end of meiosis I the X and Y chromosomes will segregate independently to different daughter cells. Because DNA was semi-conservatively duplicated during the Synthesis phase "S", there are two sister chromatids that will be retained during the second meiotic division (2^{nd} polar body retention). If the chromosome was X, then it will retain its partner X chromatid, being a XX female. In the same form, if it was a Y, then it will be a YY male. If recombination occurred in all the meiotic events, all the progeny will be XY male individuals. If recombination occurred in some proportion of all the meiotic events, then some will be XX females, some will be YY males and those that recombined will be XY male individuals.

The recombination rate (r) between the sex determining locus region and the centromere of the chromosome can be calculated as the subtraction of the number of females from the number of males, divided by the total number of individuals. The proportion of YY and XY males can also be checked if these fish are crossed individually with normal XX females. For example, if ten males and ten females are produced from an XY neofemale, then r = 0, and it would be expected all of the males to be YY and the 10 females to be XX. If 20 males and ten females meiogynes were produced then, r = 0.33 which means that 33 % of the fish (10 males) would be XY recombinants, the rest (10 males) would be YY, and all the females would be XX. If only 20 males are obtained in the meiogyne progeny, then r = 1, or 100 % which means that a crossing over occurred in every meiotic event between the sex chromosomes, and all the males would be expected to be recombinants (XY).

Another example of induced gynogenesis is to estimate the gene-centromere recombination between colour (i.e. red (RR) and white (rr)) and the centromere. By crossing a pure RR red-male x a wild rr white-female, the F1 progeny become heterozygous Rr (XY Rr males and XX Rr females, all showing an intermediate coloration). Induced gynogenesis in a XX Rr female would give the distribution of body colour pattern and frequency of heterozygotes from the difference of the observed number of XX RR (red) and the observed number of XX rr (white) divided by the total (example taken from Hussain *et al.*, 1994).

One of the red hybrid tilapia strains has been used to detect contamination in meiotic gynogenetics from XX females (Varadaraj, 1990). In *O. niloticus* the blond colour type identified at pigmentation stage has been used for a similar purpose (Myers *et al.*, 1995).

In other species colour has been used in gynogenesis for maternal confirmation in zebra fish (Streisinger *et al.*, 1981) and rainbow trout (Thorgaard *et al.*, 1985). Colour has been used in androgenesis for paternal confirmation in rainbow trout (Parsons and Thorgaard, 1985), in carp (Bongers *et al.*, 1994), and a carp hybrid (Grunina *et al.*, 1991).

Induced gynogenetic individuals can be detected with allozymes or microsatellites where alleles show co-dominance. The enzyme adenosine deaminase (ADA) is an allozyme marker. The ADA locus is distally placed on its respective chromosome, so, never becomes homozygous in eggs from heterozygous females. Therefore, ADA is useful to identify meiotic gynogenetics and mitotic gynogenetics (Hussain *et al.*, 1994). In the same Mendelian fashion of allozymes, those microsatellites having a high recombination frequency never become homozygous in eggs from heterozygous females. Microsatellites present polymorphic bands (alleles) that can be used for the identification of meiotic gynogenetics and mitotic gynogenetics, for fingerprinting analysis (Wright, 1993) and gene-centromere map distances (Kocher *et al.*, 1998; Sakamoto *et al.*, 2000).

1.2.6.3. Sex chromosomes studied using mitotic spreads

Most fish species have undifferentiated sex chromosomes. It is not uncommon to find that when a species has differentiated sex chromosomes, there are other closely related species displaying homomorphic chromosomes (see examples below). Fish show natural hermaphroditism (both male and female gonads in the same individual) in a significant number of species, while separate sexes (gonochorism) are present in the majority of teleostean species. Among 1000 karyologically defined teleostean fish, of which 900 are

neotropical freshwater fish, morphologically differentiated sex chromosomes have been reported in about 31 species. Generally, among fish the sex chromosomes types are XY-XX, ZZ-ZW, X0-XX, and multiple gonosomes systems (Solari, 1994; Foresti de Almeida Toledo and Foresti, 2001).

1.2.6.3.1. Examples of XY-XX system in fish

Cytogenetic differences between X and Y chromosomes found in fish:

In females of the lake trout, *Salvelinus namaycush*, the second largest chromosome shows a terminal, quinacrine-bright heterochromatic band in the short arm of both homologues, while in males only one homologue has this band (Phillips and Ihsen, 1985).

In several domestic populations of the rainbow trout, *Oncorhynchus mykiss*, males present a sex chromosome pair XY formed by subtelocentric chromosomes with short arms of different sizes (Thorgaard, 1983b; Ueda and Ojima, 1984). However, sometimes sex chromosomes are unrecognisable (Thorgaard, 1977). The X chromosome is usually larger than the Y chromosome but polymorphism has been observed, thus, it is not know if it is a true sex chromosome or it presents polymorphism caused by constitutive heterochromatin (Hartley, 1987).

In the guppy *Poecilia reticulata* the largest chromosome in males present a difference in the C-band between the telomeres (Nanda *et al.*, 1990, 1992). In *Eigenmannia virescens* (Almeida-Toledo *et al.*, 1988) and *Hoplias malabaricus* (Born and Bertollo, 2000) the heterochromatinization occurred in the X chromosome, contrary to the Y-heterochromatic band present in the guppy fish.

1.2.6.3.2. Examples of ZZ-ZW system in fish

The ZZ-ZW system with female heterogamety is the most frequent among Neotropical fish (Foresti de Almeida Toledo and Foresti, 2001).

In the genus *Leporinus*, the species *L. silvestrii* and *L. obtusidens* present heteromorphic sex chromosomes. The W is a large submetacentric chromosome and the Z is about half of the size of the W (Galetti *et al.*, 1981).

Conversely in *Anguilla japonica*, the Z is the largest metacentric chromosome and the W the smallest (Park and Kang, 1979).

In the rudd *Scardinius erythrophthalmus* there appears to be a ZW/ZW'/ZZ system. In this case, of the 33 gonadal females analysed, 16 had a karyotype similar to the males (ZW' females) and 17 had a heteromorphic pair of chromosomes, including a large metacentric Z chromosome and a small acrocentric W chromosome (ZW females) (Koehler *et al.*, 1995).

In females of the mailed catfish *Loricariichthys platymetopon* a metacentric chromosome has no homology with any other chromosome in the karyotype (W-chromosome) and an acrocentric chromosome is observed in both female and male (Z-chromosome (Denise *et al.*, 1995).

1.2.6.3.3. The XO-XX system in fish

In a few teleostean fish (at least six species), a hypothetical X0-XX gonosomal system has been described. In these species the diploid number of males differs from that of

females, but the evidence is too scarce to define this gonosomal type as different from some multiple gonosomal systems (Solari, 1994).

1.2.6.3.4. Examples of multiple gonosomal system in fish

Multiple sex chromosomes systems usually arise as a result of rearrengements involving sex chromosomes and autosomes. Either by centric fusion, mutual translocation between metacentric chromosomes and chromosome dissociation (Foresti de Almeida Toledo and Foresti, 2001). The species *Monodactylus sebae* is one of the few cases of the XXY type multiple sex chromosome mechanism. The karyotype of the female consists of 2n=48 acrocentric chromosomes. In contrast, the male karyotype has 2n=47 chromosomes comprising a large metacentric and 46 acrocentric chromosomes. In meiotic analysis of the male, 22 bivalents and one trivalent are observed. In this case, a fusion of two acrocentic chromosomes forming the Y metacentric chromosome has occurred (Suzuki *et al.*, 1988). In the Neotropical fish *Apareiodon affinis* (Moreira-Filho *et al.*, 1980) the female has 2n = 55 chromosomes with ZW1W2 whilst the male consist of 2n = 54 with ZZ chromosomes, a closely related species (*Parodon hilarii*), the corresponding W1W2 pair is homomorphic in both males and females (Moreira-Filho *et al.*, 1993).

According to Bertollo and Mestriner (1998), *Hoplias malabaricus* presents a X1X2Y sex chromosome system. However, this species as mentioned before, is also considered to have a XX/XY system.

1.2.6.3.5. DNA markers for sex chromosomes in fish

Sex chromosome-specific paint probes have been prepared for lake trout *Salvelinus namaycush*. The probes go to both X and Y chromosomes, suggesting that most of the chromosome is pseudoautosomal. The Yp appears to paint a slightly larger region on the Y chromosome than in the X chromosome (Reed *et al.*, 1995).

In the rainbow trout *Oncorhynchus mykiss* from the Mount Lassen and Scottish strains, two RAPD markers generate polymorphic bands amplifying preferentially in males. Fluorescent *in situ* hybridisation of one of these markers reveals a brightly defined signal on a chromosome that could morphologically be classified as the Y-chromosome (Iturra *et al.*, 1998).

In the chinook salmon *Oncorhynchus tshawytscha* a rapid PCR-based test for the Y-chromosomal DNA (OtY1 probe) is capable for determining the genetic sex of this species (Devlin *et al.*, 1994).

A PCR-based sex test for the platyfish *Xiphophorus maculatus* using Y-sex chromosome-linked molecular markers can identified male and female embryos prior to birth (Coughlan *et al.*, 1998).

In the medaka fish *Orzias latipes*, two sex linked markers named Sex Linked 1 (SL1) and Sex Linked 2 (SL2) have been isolated using genomic differences between inbred strains. Fluorescent *in situ* hybridisation of SL2 shows strong hybridisation signals in a couple of submetacentric chromosomes. The SL2 marker was repetitive in the genomes of three species of *Oryzas* that are karyologically related to *O. latipes* and was not detected in other *Oryzias* species (Matsuda *et al.*, 1997,1998).

1.2.6.4. Meiotic analysis in fish

In fish, the application of the synaptonemal complex (SC) analysis with the high power resolution of the electron microscope, has permitted the visualisation of chromosome pairing and the analysis of chromosome aberration, sex chromosomes if they are recognisable and karyotype morphology (Wise and Nail, 1987; Liu and Yu, 1991; Foresti *et al.*, 1993; Oliveira *et al.*, 1995; Mestriner *et al.*, 1995; Rodionova *et al.*, 1996; Van Eenennaam *et al.*, 1998; Dias *et al.*, 1998; Carrasco *et al.*, 1999; Cuñado *et al.*, 2001; Truat and Winking, 2001). It is during the pachytene stages of first prophase of meiosis that SC data have been analysed widely in animal and plants (see Von Wettstein *et al.*, 1984 for review).

Wise and Nail (1987) showed the advantages of analysing chromosomes and karyotypes in the mosquito fish *Gambussia affinis* through the whole mount technique described by Counce and Meyer (1973) and Dresser and Moses (1979). In this technique meiotic cells are used and homologous pairing can be seen directly, obviating the need to match homologues. Another advantage is that the same preparations can be viewed using both light and electron microscopy. Their observations on SCs of this species showed one bivalent with a region of incomplete pairing. All SCs showed thickened attachment plaques at one end of each bivalent.

Liu and Yu (1991) made the first study to look for sex chromosomes of fish through analysis of the SC. From six species they found sex chromosomes in only one: *Mastacembelus sinensis*. They observed the X and Y chromosomes partly paired, with

remnants of the sex vesicle, which disappeared gradually. Liu and Yu (1991) stated that the SC of sex chromosomes broke up and disappeared at late pachytene.

In the rainbow trout *Oncorhynchus mykiss*, Oliveira *et al.* (1995) observed that silver staining does not differentiate the central elements of the SCs. Synaptic initiation appeared to be restricted to one site in each lateral element, generally located in a subterminal or central position, presumably without any pre-synaptic alignment. Synapsis started after lateral elements formation and concluded first in the extremity with attachment plaques whilst it advances towards the other extremity. At the end of zygotene two types of SCs could be identified: 1) those with attachment plaques in one terminal region and the other terminal region slender and weakly stained (probably uniarmed chromosomes), and 2) those with attachment plaques at one end, and the other terminal region well characterised (probably biarmed chromosomes). Synapsis of the lateral elements of the sex chromosomes of the male occurred only after all autosome arms had synapsed, as has also been observed in birds and mammals (Solari, 1994). In the rainbow trout synapsis started in the subterminal region near the extremity with attachment plaques and progressed toward both extremities. Then a partially synapsis occurred between both lateral elements and finally the Y chromosome paired along its entire length with the X chromosome, showing a difference in size.

Mestriner *et al.* (1995) analysed the sex system of *Leporinus lacustris* through mitotic chromosome banding and meiotic SCs. The data obtained by the authors did not support the presence of sexual heteromorphism in the karyotype of this species.

In the genus *Poecilia*, Rodionova *et al.* (1996) made a SC analysis of interspecific hybrids of the species *P. reticulatus* (guppy), *P. sphenops* (black molly), and *P. velifera*

(marble molly). Meiotic chromosomes of the pure species and black molly x marble molly hybrid showed complete pairing at pachytene, without any indication of chromosomal heteromorphism. Most of the pachytene cells of the hybrid between the marble molly and the guppy showed various signs of pairing failure as univalents, interlocks, multiple non-homologous pairing and end to end associations, however few cells were found with completely paired homomorphic bivalents. The largest bivalent (chromosome 1) in males of the guppy showed lateral elements of equal length, uniformly stained and completely paired with each other in the majority of the pachytene cells. No bivalent had axes of unequal size. Some bivalents displayed a temporary asynapsis of the terminal segments at early and late pachytene, but usually there were several of these. In conclusion, there was no evidence of the observations of sex chromosomes in any of the species, although it was reported in guppies that there is a difference in the C band patterns between the telomeres of the largest chromosome, with thus species having an XX/XY system.

Dias *et al.* (1998) studied synapsis in supernumerary chromosomes of *Prochilodus lineatus*. The observations of testicular pachytene cells revealed the presence of continuous filaments of variable sizes that correspond to the number of bivalents of the regular set of chromosomes. The supernumerary chromosomes of small size were observed as isolated and well-stained bodies.

The SC analysis in spermatocytes of white sturgeon, *Acipenser transmontanus* Richardson by Van Eenennaam *et al.* (1998) revealed the same features observed in previous studies of fish. Zygotene nuclei were scarce, and were characterised by incomplete synapsis, some unpaired univalents, interlocks, and diffuse lightly stained

nucleoli. Most pachytene nuclei had prominent darkly staining nucleoli that were usually detached from the synaptonemal complex. Overall, they found no distinguishable bivalent that consistently exhibited asynaptic behaviour or that that had axes of unequal length that could be associated with heteromorphic sex chromosomes.

Cuñado *et al.* (2001) analysed synapsis in diploid and triploid males and females of turbot, *Scophthalmus maximus*. They did not find any bivalent exhibiting the atypical synaptic behaviour that is often associated with heteromorphic sex chromosomes.

Truat and Winking (2001) analysed three species: the zebra fish *Danio rerio*, the platy fish *Xiphophorus maculatus* and the guppy fish *Poecila reticulata*. The authors suggested that the three species represent basic steps of sex chromosome differentiation: (1) the zebra fish with an all-autosome karyotype; (2) the platy fish with genetically defined sex chromosomes without any differentiation at pachytene stage and (3) the guppy fish with genetically and cytogenetically differentiated sex chromosomes.

The work of Foresti *et al.* (1993) and Carrasco *et al.* (1999) in the Nile tilapia were mentioned at the end of section 1.1.5 and they are described in detail in Chapter 4.

1.3. General research aims

This thesis mainly focuses on the analysis of the synaptonemal complex and the observation of sex chromosomes during pachytene stage in *O niloticus*, *O. mossambicus*, *O.aureus* and *O. karongae*.

The general aims and working hypothesis were:

1) To give a full description of meiotic prophase I in tilapias of the genus *Oreochromis* with the hypothesis that Prophase I in fish is similar to eukaryotes.
2) To give an extension of the observations of undifferentiated sex chromosomes in *O. niloticus* with the hypothesis that the unsynapsed terminal region in bivalent 1 is an early pachytene stage.
3) The presence of putative sex chromosomes in the Nile tilapia gives the hypothesis to identify putative sex chromosomes in *O. aureus* as well.
4) To investigate further sex determination in *O. mossambicus* with the hypothesis that there is no recombination between the centromere and sex locus (loci) in this species.
5) To analyse the synaptonemal complex and sex chromosomes of interspecific hybrids of tilapia of the genus *Oreochromis* with the hypothesis that hybrids show complete homology judged by complete synapsis of bivalents in pachytene stage.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1. Materials and methods

2.1.1. Species studied

Tilapia individuals used in this study came from populations derived from the River Zambezi, Zimbabwe (*O. mossambicus*) and Lake Manzala, Egypt (*O. niloticus* and *O. aureus*) and Lake Malawi (*O.N. karongae*). These species are held in the tilapia reference collection at the Institute of Aquaculture, University of Stirling. The species broodstock have been maintained as isolated populations for at least 20 years. Each species broodstock has been shown to be pure through the analysis of enzymes observed in electrophoresis (McAndrew and Majumdar, 1983; Sodsuk and McAndrew, 1991). More recently, microsatellite loci have also been used to identify pure broodstock (Sobolewska, 1999).

2.1.2. Fish maintenance

Fish were maintained in two different places: the tropical aquarium where all the species were held and bred to maintain each particular stock and the genetics aquarium where fish were used for experimental purposes. Both aquaria are sealed and isolated from the external environment, especially for wintertime. Lighting in each laboratory consists of conventional 2000-lux lamps attached to the ceiling that are regulated by an automatic timer to 12 hour light : 12 hour dark. The dynamics inside the fish laboratory consists of a series of isolated recirculating water systems. Most of the systems have similar dimensions but they can vary due to space availability. Each system consists of several

components. The header tank contains an electrical heater that maintains the water temperature at 27 °C. The header tank is connected to a pipe that runs along one side of the fish holding tanks. At each tank a "T" connection is made on the pipe that feeds water into the tank. Each tank has a central pipe drainage connected to the bottom-center of the tank that maintains the water level inside. The water flows from the header tank to the rearing tanks, then overflows via the central pipe of the rearing tank and is discharged to a drain canal that is placed below the battery of tanks. This drain canal discharges into a series of interconnected settling tanks ending in a sump tank attached to a 0.25 HP pump that sent the water back to the header tank. Water flow is regulated with conventional PVC valves between the different tanks. Between the header tank and the rearing tank battery, there is a tank filled with crushed cockleshells that maintains the water at pH 8.0. The settling tanks are filled with rows of long brushes and floating plastic rings. These materials assist the settlement of solid waste and to act as a surface where nitrifying bacteria grows. The principle of a recirculating system is to maintain low levels of ammonia and nitrites and a reasonable level of nitrates. There are bacteria that degrade ammonia to nitrites and another one that degrades nitrites to nitrates. Ammonia and nitrites are very toxic for aquatic organisms whilst nitrates are not, therefore to ensure the low levels of toxic compounds, ammonia, nitrite and nitrate were measured with a colourimetric kit once a week. There is a slow turnover of water via trickle input from a cold water supply and replacement of water after the settling tanks are cleaned. This is enough to dilute the nitrate to an acceptable level. There is an alarm system that detects a drop of temperature or water level. In general terms there are three system categories that are in concordance with the life cycle of fish.

2.1.2.1. An incubator system for eggs

This system consists in a battery of small perspex cells of 12 x 12 x 20 cm each. Along one side of the battery each cell has an aperture of 7 cm long, 2cm wide located 3 cm below the top edge of the perspex. This aperture is covered with plastic net material of 0.5 mm. Water in this system passes by gravity from a 100 l tank trough a 30 W UV light sterilisation unit and then via a 2.5 cm PVC pipe that runs along the top of the perspex cells. In this PVC pipe a regulated plastic valve is positioned at the centre of each cell. To simulate oral incubation of the eggs, individual egg batches were held inside 750 ml plastic containers with rounded bottom and placed inside each cell. To keep the eggs in gentle and continuous motion, a 1.5 x 15 cm long plastic pipe (adapted from a 1 ml pipette) was connected to the valve and placed inside the container. This allowed the water to flow from the bottom of the container (lifting the eggs) and overflow the container, passing into the perspex cell and through the aperture of the wall to a drainpipe and hence into a 180 l settling tank. A water pump connected to the settling tank returns the water to the header tank. When fry swam up, the container with the fry was taken out and placed in the nursery systems.

2.1.2.2. Nursery systems and juvenile rearing

In these systems free swimming fry were pre-reared for up to a month from first feeding. Depending on the number of fry, type of experiment, space available, etc., different type of tanks can be used: small (6 l), medium (10 l) or large (20 l) rectangular

transparent tanks and cylindrical non-transparent (20 l) tanks were available. Transparent tanks allowed the observation of fish as well as the particular conditions of the tank like bottom sediments, dead fish etc. As fish grew they were moved increasingly to larger tanks. The most important feature when rearing fry was to use a 0.5 mm net mesh attached to the central drainpipe to prevent the escape of fry via the overflow.

2.1.2.3. Juvenile- adult systems

Fingerling fish were moved into larger tanks 20-200 l and reared until they reached about 20 g. Fish broodstock were maintained in tanks at low densities (about 3 fish/20 l water).

2.1.2.4. Breeding facilities

Female and male broodstock were kept individually in glass tanks (120 x 40 x 30 cm). Each glass tank had three vertical separators, made of translucent perspex (0.5 cm thick). Each separator had holes in it to permit the water to flow more efficiently. Perspex covers were placed above the aquarium to prevent fish from jumping out of the tank. Therefore in each aquarium up to four adult fish were kept for experimental breeding purposes. In this type of aquarium it was possible to observe fish through the glass without handling. The daily observations were health (body, fins eyes, mouth, coloration), feeding behaviour, reproductive behaviour and papilla extension.

2.1.2.5. Labelling of tanks

Each tank was always labelled with the basic information of the user (student) including the student's name and supervisor's names, Home Office Project Licence number, PIT tag number, fish species, date of hatching, feeding regime, food size employed and any other specification such as genotype or nature of the experiment. For female broodstock a sheet of paper for recordings spawning dates and the quality of the spawn was used.

2.1.2.6. Feeding

Fish were fed twice a day *ad libitum* with commercial trout feed. The swimming fry were fed with micronized food sieved to 0.5 mm or less. Juvenile fish were feed with 2 mm particle size. Adult fish were feed with 4 mm particle size.

2.1.2.7. Fish handling and Anaesthesia

Fish handling and anaesthesia in all experiments described is this thesis conformed to the regulated procedures on living animals established by the United Kingdom (Scientific Procedures) Act of 1986. A training Course (ScotPil) co-ordinated by the Home Office was taken and a Certificate and a Personal Identification Licence held by a Project licence was obtained to handle fish. Handling stress during blood collection for DNA extraction, egg stripping, tagging or sperm collection was minimised by anaesthetising the fish in benzocaine (ethyl-p-aminobenzoate, Sigma) at a 1;10,000 dilution in water.

After completing any handling task, the fish were transferred into clean aerated water where they generally recovered within 3-5 minutes.

2.1.2.8. Tagging of fish

Fish were tagged at 4 months old using transponder tags (Avid. Inc. California, USA). Each tag has a 10-digit code that is read by an Avid tag reader. The procedure involved anaesthetising the fish, then making a small incision (about 3 mm) in the belly, just above and anterior to the anus, using a sharp sterile blade. To make the incision, first a scale was removed and then the body wall was cut carefully without damaging the internal organs. The tag was introduced into the body cavity and a small amount of Orahesive protective power (E.R. Squibb and Sons Ltd., Middlesex, UK) was placed on the incision to assist healing. The tilapia skin healed within 3 days without signs of damage.

2.1.2.9. Stripping and fertilisation of eggs

Females ready to spawn (showing swollen urogenital papilla) were first anaesthetised. The female was taken out from the anaesthetic and wrapped in wet paper around its head and anterior body. The female was stripped by applying a gentle pressure and squeezing the belly from the pectoral fin to the anus, always in this direction, while being held in a 20-l bucket containing aquarium water. The female was returned to her aquarium space to recover from the anaesthetic. The eggs were collected from the bucket (this facilitated cleaning the eggs of any ovarian tissue or fluids) using a plastic 2-ml pipette and placed

in a 10 cm petri dish. The male was anaesthetised and placed horizontally in the bench (still inside the net) covered with wet paper. Gentle pressure on the belly (no stripping or squeezing) was used to express milt into a capillary tube placed just on the urogenital aperture of the papilla. The milt was directly dropped into the Petri dish containing the eggs and mixed. After 2 minutes the eggs were transferred to the incubation system. If sperm needed to be diluted or kept for later use (to obtain concentration, UV radiation etc.), then it was placed in cold Hank's solution at 1 part of sperm : 4 parts of physiological solution.

2.1.3. Preparation of diets for sex reversal

Diets used for sex reversal of tilapia were prepared taking strict precautions when handling the hormones, wearing a protective laboratory coat, close-fitting mask, surgical gloves and performing all manipulations in the fume cupboard.

For each hormone, a 10 mg/ml stock solution of the steroid was prepared. In the case of MT and DES, 100% ethanol was used as a solvent. In the case of ET, a mixture of ethanol: chloroform (1:1) was prepared as a solvent, dissolving the hormone in a warm bath at 33 °C. Commercial trout pellets were ground using a blender, sieved to less than 500 μm particle size, weighed and spread on aluminium foil-covered trays. The desired amount of steroid was added to 10 ml of ethanol in a small hand sprayer, then the steroid solution was sprayed onto the food whilst continuously mixing it with a plastic teaspoon. Once finished, 5 ml of ethanol were added to the hand sprayer and sprayed again onto the food to ensure no traces of the hormone remained in the plastic container attached to the

sprayer. Control food was sprayed only with equal volumes of ethanol in a similar way. Food was dried overnight and, when dry, it was stored in sealed plastic containers, which were covered with aluminium foil and maintained at 4 °C.

2.1.4. Sexing juvenile fish by dissection

Three month old fish were killed with anaesthesia (by immersion in a 0.01% benzocaine solution) followed by destruction of the brain before dissection. Fish were dissected by opening them ventrally from the anus to the pectoral fin and then cutting laterally around the pectoral fin upwards above the lateral line. This procedure allowed opening the belly from one side and removing the digestive tract, which left the gonads, attached to the upper wall of the body cavity free to dissect them. Then the gonads were taken out using small dissecting tweezers and placed on a glass slide. A drop of 2 % orcein dissolved in 40 % acetic acid was placed in the gonad and a cover glass positioned. A gentle squash was made using one finger and the glass slide was placed under the microscope and observed using a 4X or 10 X objective. Ovaries were observed as a tissue full of large and rounded cells with a nucleus (ova) while testes were observed as a flat, granular tissue. Guerrero and Shelton (1974) described this technique.

2.1.5. Preparation of synaptonemal complex (SC) spreads

The preparation of SC specimens from testes was carried out according to the protocol of Foresti *et al.* (1993) with some modifications. Fish were killed by immersion in

anaesthetic (0.01% benzocaine solution) followed by destruction of the brain before dissection. Testes or ovaries were placed in a small Petri dish containing Hanks' saline solution (Sigma-Aldrich) at 4 °C, then the gonad was minced with two razor blades and the resulting cell suspension was transferred to a 1.5 ml centrifuge tube where it was allowed to settle for 20 min.

In the case of testes, the cell suspension was made in 1.5 ml of cold Hank's solution, then 1ml of the cell suspension was taken and transferred to a 1.5 ml centrifuge tube where it was allowed to settle for 20 min. The supernatant was transferred to another tube and centrifuged at 1000 RPM for 2 min, then the supernatant was transferred to another tube and centrifuged again at the same speed for another 5 min. The tiny pellet formed was taken out carefully in 20 µl and pipetted into another tube with a solution containing 40 µl of 0.2M sucrose and 60 µl of 0.2% "Lipsol" detergent (each buffered to pH 8.5 with 0.01M sodium tetraborate) and gently shaken. After 10 min the cells were fixed with 80 µl of 4% paraformaldehyde (buffered to pH 8.5 with 0.2M sodium tetraborate), shaken gently, and kept at 4 °C. In young females (about 2-3 months old) the ovaries were very small, so the cell suspension was made in 100 µl of cold Hank's solution and allowed to settle in a 1.5 ml centrifuge tube for 20 min. The cell suspension, without any solid pieces of tissue, was pipetted into another tube containing 50 µl of sucrose and 200 µl of buffered Lipsol and after 10 min 100 µl of the buffered fixative were added.

Microscope slides were cleaned and plastic coated with a 0.75% solution of pioloform or formvar (Agar Scientific) in chloroform, then rinsed in a solution of 0.4% Photoflo (Kodak). About 100-250 µl of the fixed cell suspension was pipetted onto one of these plastic-coated slides. The slide was then air-dried horizontally for about 4 h in a fume

cupboard, rinsed for 1 min in Photoflu solution, air-dried, and stained with 50% silver nitrate by the method of Howell and Black (1980). Briefly, two separate solutions were prepared. For the first (developer), 0.5 g of gelatin were dissolved in 25 ml of warm distilled water, then 250 µl of formic acid were added. The second one was a 50% silver nitrate (Sigma-Aldrich) solution in distilled water. Then, 25 µl of the first and 50 µl of the second solutions were dropped onto the slide, which was then covered with a cover glass and incubated at 70 °C until the solution faded to yellow and then to golden-brown colour (about 3 min). The slide was then rinsed twice in distilled water, allowing the cover glass to fall off during the first rinse, and air-dried. After localisation and marking (if necessary) of pachytene cells under a light microscope, the plastic film was floated off in distilled water, and 50-mesh electron microscope copper grids (Agar Scientific) were carefully positioned over the marks. The plastic film was picked up with a piece of parafilm (making a sandwich) and left to dry. The grids were detached from the parafilm-plastic sandwich by cutting the plastic around each grid with a sharp blade without cutting the parafilm. Grids were stored in grid boxes (Agar Scientific). Each grid was placed in the specimen holder and introduced in the transmission electron microscope for examination at 80 kV using a Philips 301 transmission electron microscope. SC spreads were photographed and then SC photographs were scanned. Each bivalent of a nucleus was measured, then bivalent lengths were added to obtain the synaptonemal complex complement length (SSCL), using Image Pro Plus 3.0 software.

2.1.6. Blood sampling

After each fish was anaesthetised, blood was sampled from the caudal vein using 23 g (0.5 mm) size sterile needles and 1 ml syringes. To avoid blood clotting, syringes were prepared with 0.1 ml of modified Cortland's saline (without $MgCl_2$ and added 10 mM EDTA) prior to the blood sample. A scale was removed and the needle was introduced just below the lateral line in the caudal peduncle. While introducing the needle, the vertebrate was touched and then the needle was re-orientated below the vertebrate making a gentle suction pressure with the syringe until blood started to come out. After finishing blood sampling, the needle was taken out and a small amount of Orahesive protective power (E.R. Squibb and Sons Ltd., Middlesex, UK) was placed on the needle entry point to assist healing. The tilapia skin was healed within one day without any signs of damage.

2.1.7. DNA extraction

Blood samples were taken from fish and maintained on ice. Each blood sample was placed into a 1.5-ml autoclaved tube and centrifuged at 5000 RPM for 5min. The supernatant was discarded and the blood cell pellet was stored at –20 °C. For DNA extraction, 10 µl of thawed blood cells were mixed with 440 µl of TEN buffer, then 10 µl of proteinase K (from a 20 mg/ml stock solution) and 50 µl of SDS (from a 10 % stock solution) were added, mixed well and incubated overnight in a rotating cylinder in an oven at 55 °C. The next morning 10 µl of RNAse (from a 20 mg/ml stock solution) were

added and incubated at 37 °C for one hour. Then 500 µl of buffered phenol were added and mixed gently using a rotator for 20 min, then 500 µl of chloroform were added and mixed gently for another 20 min. The tubes were centrifuged at 10,000 RPM for 15 min. The supernatant was transferred to another 1.5 ml sterile tube and 1 volume of isopropanol at –20 °C was added and shaken vigorously until the white mass of precipitated DNA was visible. The DNA precipitate was washed twice in 70 % ethanol (30 min each). The 70 % ethanol was poured off and the DNA precipitate was left to air dry at room temperature. DNA was resuspended in TE buffer and stored at 4 °C. After 5 days the DNA concentration was assessed using a spectrophotometer. The DNA molecular weight was checked by running 2 µl of each sample along with a standard marker (λ α Hind III) in a 0.7 % agarose gel with ethidium bromide (2 µl/100 ml from a 10 mg/ml stock solution). Electrophoresis was run at 4 V/cm for one hour in TAE buffer. DNA was visualised using an UV transilluminator.

2.1.8. PCR amplification of microsatellite DNA loci

PCRs were carried out in 0.5 tubes using 25 µl total volume reaction, which consisted of 2.5 µl universal buffer (10 X), 1.5 mM $MgCL_2$, 200 µM dNTPs, 1 µM of each primer, *Taq* polymerase (0.1 units/µl) and about 100 ng of DNA, using a thermal cycler with the following programme: initial denaturation at 96 °C for 5 min, then 25 cycles of 95 °C for 1 min, annealing temperature of 52 °C for 30 sec, and extension at 72 °C for 1 min. One primer was 5' end labelled with a fluorescent dye.

The PCR products were diluted 1/10 and 1.5 µl of the diluted product was mixed with 2.5 µl of loading buffer mix containing1.3 µl of deionised formamide, 0.4 µl of size standard GeneScan 500 Tamra (Applied Biosystems Inc., Foster City, CA) and 0.3 µl of blue dextran. After denaturation at 90 °C for 2 min, the entire solution was loaded on a 5 % acrylamide (Long Ranger) gel on an ABI 377 automated DNA sequencer. Genotyper 2.1 software was used to analyse the genotypes of the microsatellite loci.

2.1.9. Multilocus DNA fingerprinting

Fingerprinting was performed using NICE probe 33.15 (Jeffreys *et al.*, 1985). About 10-25 µg of high molecular weight DNA were digested using the restriction enzyme *Hinf* I. This enzyme does not have a strict control over the position of the cut relative to the recognition sequence. Furthermore it is an enzyme with degenerate recognition sequences. *Hinf* I (from *Haemophilus influenzae*) recognises 5'-GANTC-3' and so cuts at 5'-GAATC-3', 5'GATTC-3', 5'-GAGTC-3' and 5'-GACTC-3', cutting the two DNA strands at different positions. The resulting DNA fragments have short single-stranded overhangs at each end (called sticky or cohesive ends), usually two or four nucleotides apart (Brown, 1999). Digestion was carried out in 100 µl in a 1.5 ml sterile tube containing 10 µl of 10X universal buffer, 10 µl of spermidine trihydrochoride (from a stock 400 mM solution), 1 µl of acelytated BSA (from a stock 10 mg/ml solution), 0.5 units of enzyme per µg of DNA and sterile distilled water to raise the volume to 100 µl. The mixture was shaken gently, spun for 15 sec and incubated at 37 °C for 16 hours using a warm bath with slight agitation. After this incubation, 400 µl of TE were added

and DNA was re-extracted using the phenol-chloroform procedure (explained above), then the DNA was precipitated by adding 1/10 of sodium acetate (3M) and 2.2 volumes of chilled ethanol and mixed thoroughly. The tubes were maintained at – 70 °C for one hour and then centrifuged at 10,000 RPM for 10 min. Each DNA pellet was washed twice with 70 % ethanol, the ethanol was poured off, the pellet air dried and resuspended in 20 µl of TE.

The DNA concentration was obtained using the spectrophotometer and volume equivalent to 5 µg of restricted DNA was run through electrophoresis in 0.7 % agarose gel in 0.5 X TPE buffer for 24 hours. The restricted DNA was transferred to a non-charged membrane (Southern transfer) by alkaline vacuum blotting (Vacu Gene^{TM}, Pharmacia LKB). Initially a 20 X 20-cm non-charged nylon membrane (Hybond-N, Sartorius Ltd) was wetted with deionised water and placed on a wetted porous screen, which fits the tray of the unit that is connected to a vacuum pump. Then a plastic mask with a central window 5-mm smaller than the membrane was placed to overlap the membrane and then locked with a top-frame with clamps. The gel was placed onto the centre of the window to overlap the membrane and the pump was turned on. To denature the restricted DNA (single stranded), about 100 ml of 0.2 N HCl solution were poured onto the centre of the gel for 30 min and then the reminder was pipetted out from the vacuum unit. Then 1 l of 0.4 M NaOH solution was poured on the gel, covering it during 1 hour and then poured out. The pump was turned off and the gel was discarded after marking the points of the wells with a blunted pencil. The membrane was neutralised by washing it for 10 min in a tray containing 500 ml of 2 X SSC with constant agitation, air

dried for 30 min, and placed between two sheets of filter paper. To fix the DNA the membrane was incubated at 80 °C for two hours. It was then stored at room temperature.

Hybridisation with the NICE™ probe 33.15 (Cellmark Diagnostic) involved several steps. The membrane was wetted in a tray containing 250 ml of 1 X SSC, then placed inside a cylindrical canister and pre-washed by pouring in 50 ml of 0.1 X SSC, 0.5 % SDS pre-warmed at 65 °C. The canister was placed inside a hybridiser (HB-1, TechNe) and left for one hour at 65 °C with a slow rotating motion. The pre-wash solution was then replaced by 50 ml of pre-warmed (50 °C) pre-hybridisation solution consisting of 99 ml of 0.5 M Na_2HPO_4 and 1 ml of 10 % SDS, and left for 20 min. The pre-hybridisation solution was then replaced by 20 ml of pre-warmed (50 °C) hybridisation solution consisting of 18 ml of pre-hybridisation solution plus 2 ml of 10 % casein solution and 5 µl of NICE probe, and left for 20 min. The membrane was rinsed twice for 10 min each with 50 ml of pre-warmed (50 °C) wash solution I consisting of 19.2 ml of 0.5 M Na_2HPO_4, 1.2 ml of 10 % SDS and 99.6 ml of deionised water. Finally the membrane was rinsed twice for 10 min each with 50 ml of wash solution II at room temperature, consisting of 13.8 g/l of Maleic acid, 8.7 g/l NaCl, pH 7.5. The membrane was removed from the canister and placed with the DNA side up on a glass plate, then trimmed with a sharp blade to fit the size of a light proof cassette. Then about 4 ml of Lumi Phos™ 350 (Cellmark Diagnostics) were sprayed uniformly over the membrane. The membrane was then wrapped using Saran food wrap (Dow Chemical Company ™) and placed inside the cassette.

A sheet of autoradiography film (Hyperfilm MP™, Amersham) was placed into the cassette in a dark room and the cassette incubated at 37 °C for 4 hours. Finally the film was developed in a dark room using D19 (Kodak), then fixed (Kodak fixer) and air-dried.

APTER 3

PTION OF MEIOTIC PROPHASE I IN FILAPIAS OF
Oreochromis THROUGH A SYNAPTONEMAL COMI
TES

CHAPTER 3

DESCRIPTION OF MEIOTIC PROPHASE I IN TILAPIAS OF THE GENUS *Oreochromis* THROUGH A SYNAPTONEMAL COMPLEX ANALYSIS

3.1. Introduction

Analysis of mitotic chromosomes in tilapias has not shown any evidence of differentiated sex chromosomes (e.g. Majumdar and McAndrew, 1986). However, the observation of pairing anomalies in the synaptonemal complex (SC) during meiosis has led to the identification of putative sex chromosomes in the Nile tilapia, *O. niloticus* (Foresti *et al.*, 1993; Carrasco *et al.*, 1999).

During meiosis, several proteins scaffold the formation of the SC (see Chapter 1), a ribbon-like structure composed of two lateral elements (LEs) and a central region that mediates in chromosomal pairing and recombination of meiotic prophase I (Loidl, 1994). Pairing anomalies are frequently observed in differentiated sex chromosomes of other vertebrates (Solari, 1994). As reviewed in Chapter 1, male heterogamety/female homogamety has been found in *O. mossambicus* (Clemens and Inslee, 1968; Penman *et al.*, 1987) and *O. niloticus* (Jalabert *et al.*, 1974; Penman *et al.*, 1987; Mair *et al.*, 1991a), where sex is predominantly determined by a monofactorial system. In *O. aureus* sex appears to be determined by a multifactorial mechanism with an underlying primary mechanism of female heterogamety/male homogamety (Guerrero, 1975; Penman *et al.*, 1987; Avtalion and Don, 1990; Mair *et al.*, 1991b).

There is only one description of prophase I in cichlids (Liu and Yu, 1991). In general, Liu and Yu (1991) described prophase I as following the same synapsis pattern as other eukaryote organisms. However, this paper stated that in fish, the sites of synapsis recognition of the homologous chromosomes at zygotene depend mainly on chromosome morphology. Also, it was not clear about the patterns of desynapsis and the breakage and

disappearance of the LEs during the pachytene (sex chromosomes) and diplotene stages in fish meiosis. In addition, this study did not analyse prophase I in both sexes.

3.2. Aims

The aims of this research were:

1) To describe meiotic prophase I in tilapias.
2) To analyse meiotic prophase I stages in males, females and neomales (females sex-reversed to males) of three *Oreochromis* species.
3) To observe the pairing process of the synaptonemal complexes focusing on the identification of non-homologous regions as unpaired lateral elements that could be related to sex chromosomes.
4) To compare the total length of synaptonemal complexes between genotypes and species.
5) To see how this adds to our understanding of the description of sex chromosomes and sex determination in *Oreochromis*.

3.3. Materials and methods

3.3.1. Species studied

The fish used in this study came from populations derived from the River Zambezi, Zimbabwe (*O. mossambicus*) and Lake Manzala, Egypt (*O. niloticus* and *O. aureus*). These species are held in the Tilapia Reference Collection at the Institute of Aquaculture, University of Stirling.

3.3.2. Fish used for SC analysis

Males: six *O. mossambicus* and six *O. aureus* males between 30 and 50 g of body weight were studied. They had been previously crossed with normal females and the progeny were sexed to ensure that the sex ratio fitted with the expected 1:1 ratio. For each male, 15 spermatocytes, ten of them at pachytene stage, were analysed, giving a total of 90 cells per species.

Females: six females of each of the three species were studied at 70 to 90 days after hatching. These could not be progeny tested (SC spreads in females can only be prepared from pre-vitellogenic ovaries) but it was assumed that they were they were XX (*O. niloticus* and *O. mossambicus*) or ZW (*O. aureus*). For each female, two oocytes at pachytene stage were analysed. A total of 12 cells were examined for each of the three species.

Neomales: six XX neomales (*O. mossambicus*) and six WZ neomales (*O. aureus*) were studied. The fry were previously sex-reversed with 17 ∝ Ethynyltestosterone (ET) or 17∝-Methyltestosterone (MT) (Sigma-Aldrich) at 50 mg/kg, during 60 days from first feeding (see Chapter 2; section 2.1.3.). The treated *O. mossambicus* males were crossed with normal (XX) females to check if the progeny sex ratio was 1:0 female: male that would indicate a putative neomale or 1:1 that would indicate a normal male. Treated *O. aureus* males were crossed with a ZZ neofemale previously sex reversed with 17 ∝ Ethynylestradiol (Sigma-Aldrich) at 150 mg/kg during 35 days (Melard, 1995), to compare to an expected sex ratio of 1:1 female : male that would indicate a putative neomale or 0:1 that would indicate a normal male. Some eggs from the ZZ neofemale *O. aureus* where also crossed to a test control ZZ male, which was expected to give a sex-ratio of 0:1 female: male. The number of cells analysed was as for normal males, as described above.

Males exposed to MT or ET hormone (males-T): six progeny tested genetic males of *O. mossambicus* and five progeny tested genetic males of *O. aureus* from the groups treated with the hormones MT or ET were analysed at pachytene stage (10 nuclei).

O. niloticus controls: one *O. niloticus* progeny tested XY male was used as a control for the SC silver stain technique and the observation of the unpaired region in the subterminal or terminal position of the largest chromosome pair (Foresti *et al.*, 1993; Carrasco *et al.*, 1999).

3.3.3. Preparation of SC spreads (transmission electron microscopy)

Fish were killed with anaesthesia (by immersion in a 0.01% benzocaine solution) followed by destruction of the brain before dissection. Preparation of fish SC spreads for the observation in the transmission electron microscope and posterior analysis were made accordingly with the protocol showed in Chapter 2, section 2.1.4.

3.3.4. Preparation of SC spreads (light microscopy)

Minced cell suspension was incubated in hypotonic 75 mM KCl for 15 minutes, fixed in four changes of 3:1 methanol:acetic acid and stored at –20°C until required. Cell suspensions were dropped onto ethanol-cleaned glass slides, incubated in modified Giemsa stain (2% v/v) (Sigma) for five minutes, rinsed in distilled water, air-dried and the slides then mounted. Metaphase images were captured and enhanced using a Cytovision image analysis system (Applied Imaging).

3.3.5. Identification of meiotic stages

The criteria for identification of meiotic stages were based on SCs from the Chinese hamster *Cricetulus griseus* (Moses, 1977), the boar *Sus scrofa domestica* (Villagomez, 1993), the rainbow trout *Oncorhynchus mykiss* (Oliveira *et al.*, 1995), and on the general descriptions of meiosis in Strickberger (1976), von Wettstein *et al.* (1984), Macgregor and Varley (1988), Loidl (1990; 1994) and Miller and Therman (2001).

3.3.6. Statistical analysis of SCCL

The bivalent lengths at pachytene stage of each nucleus were added to allow comparison of the differences in SCCL between sexual genotypes/phenotypes in each species through a one-way analysis of variance (ANOVA), assessing normality by Kolmogorov-Smirnov test and homogeneity of variance by Bartlett's test. Further mean differences were assessed by Tukey's pairwise comparisons. Because the 1C DNA content of these species is known, it was also possible to calculate the packing density. Packing density is equal to 1C DNA content (pg)/one chromatid/micrometer of SC. The values for 1C DNA contents used in these calculations were taken from Majumdar and McAndrew (1986): *O. niloticus* = 0.95 pg; *O. mossambicus* = 1.00 pg; and *O. aureus* = 1.21 pg. However, it should be mentioned that the DNA contents per nucleus of *O. niloticus* and *O. aureus* have also been calculated to be 2.27 pg and 2.22 pg respectively, corresponding to 1.14 and 1.11 pg respectively for 1C DNA (Fan *et al.*, 1995). Using these latter values would have tended to make the calculated packing densities of these two species more similar.

3.4. Results

The three *Oreochromis* species presented all the main characteristic features of prophase I that have been observed in eukaryotes. Apart from unpairing related to the putative sex chromosomes and analysis of SCCL (which are dealt with separately below), the three species showed no observable differences in the stages of meiosis. SCs from females showed features of prophase I observed in females but not in males, such as polarization of the LEs, a large nucleolus and higher SCCL (see below).

The general observation of meiotic cells in testes is shown in Fig.3.1.A, in which the different cell sizes of spermatogenesis and spermiogenesis can be recognized. Primary spermatocytes (Fig. 3.1.A: a) form from spermatogonia through mitosis. Subsequently these cells undergo meiosis and the first division occurs, forming from each cell two daughter cells called secondary spermatocytes (Fig. 3.1.A: b). They are about half of the size of the primary spermatocytes and undergo the second meiotic division forming two spermatids (Fig.3.1.A: c). These cells evolve into spermatozoa through spermiogenesis, developing the flagellum (Fig. 3.1.A: d) (Pankhurst, 1998).

3.4.1. Leptotene stage

The early stage of leptotene in which axial elements begin to appear as threads or fragments randomly distributed in the nucleus is shown in Fig. 3.1.B: a. As homologous axial elements start to recognize each other they become more evident. One well-spread nucleus at late leptotene stage is shown in Fig. 3.1.B: b and c.

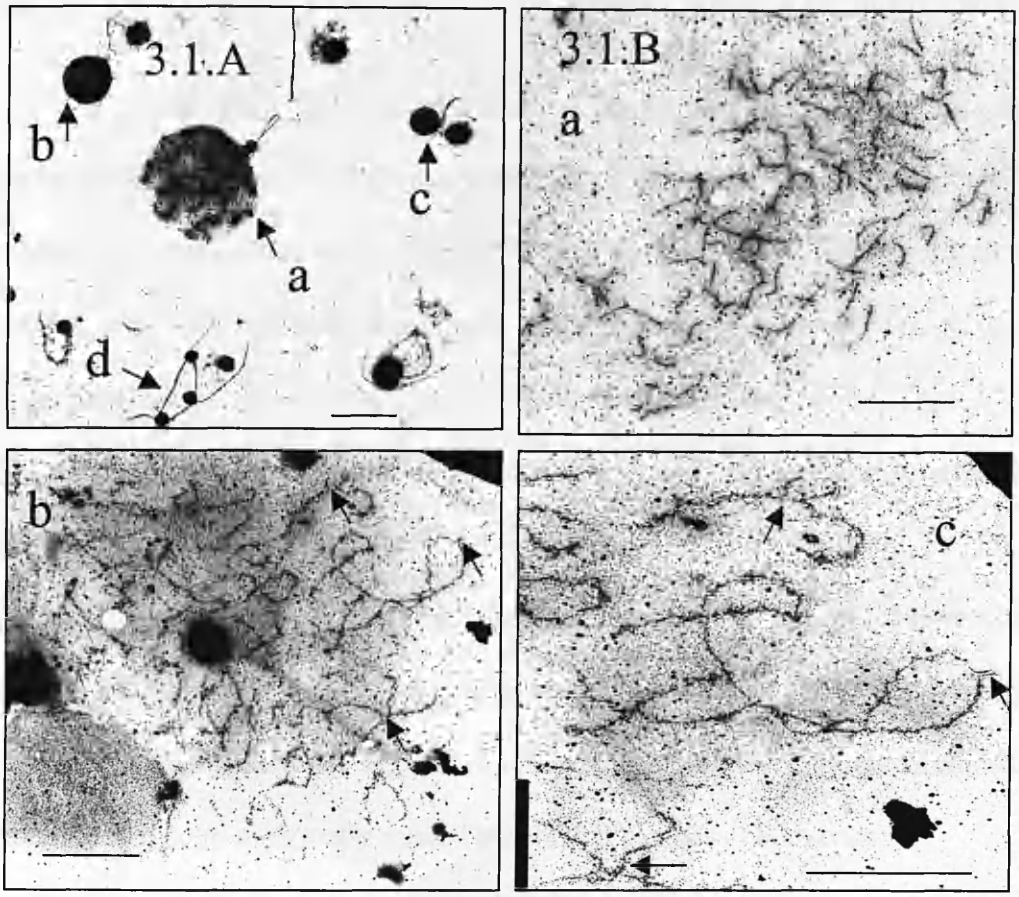


Figure 3.1.A. General observation of meiotic cells during spermatogenesis: (a) Primary spermatocytes. (b) Secondary spermatocytes. (c) Spermatids, and (d) Spermatozoa. Bar represents10 μm.

Figure 3.1.B. Leptotene. (a) Occurrence of axial elements randomly distributed during early leptotene (male *O. niloticus.*). (b) Leptotene stage showing presynaptic alignments of homologous chromosomes; black arrow (in the middle) points to the beginning of the formation of the SC in one end of bivalent 1, black arrow (above) points two axial elements approaching each other at a subterminal region; black arrow (below) points two axial elements synapsing each other at a median region. (c) Magnification of (b) (male *O. niloticus.*). Bar represents10 μm.

3.4.2. Zygotene stage

As a result of the presynaptic alignments and the chromosome recognition sites in leptotene, synapsis and the formation of the SC continues along the lateral elements (LEs) towards both extremities. Attachment plaques are observed in Figure 3.1.C: a and b in females. The LEs between attachment plaques are not apparent due to nuclear envelope interference. From early zygotene there is some degree of polarization of the LEs, in which the telomeres gather at one side. This polarization was only observed in females of the three species (Fig. 3.1. C: a,b,c).

At middle zygotene, synapses still advances and the LEs between attachment plaques become more apparent (Fig. 3.1.C: d,e,f). Axial element threads surrounding the lateral elements can be distinguished in Fig. 3.1.C: e,f. In some completely paired chromosomes, it is possible to recognise strongly stained attachment plaques at both ends.

During late zygotene, all the LEs were well defined. The SCs have nearly formed along the homologous chromosomes, but some regions that are still not synapsed can be observed (Fig. 3.1.C: g,h,i,j).

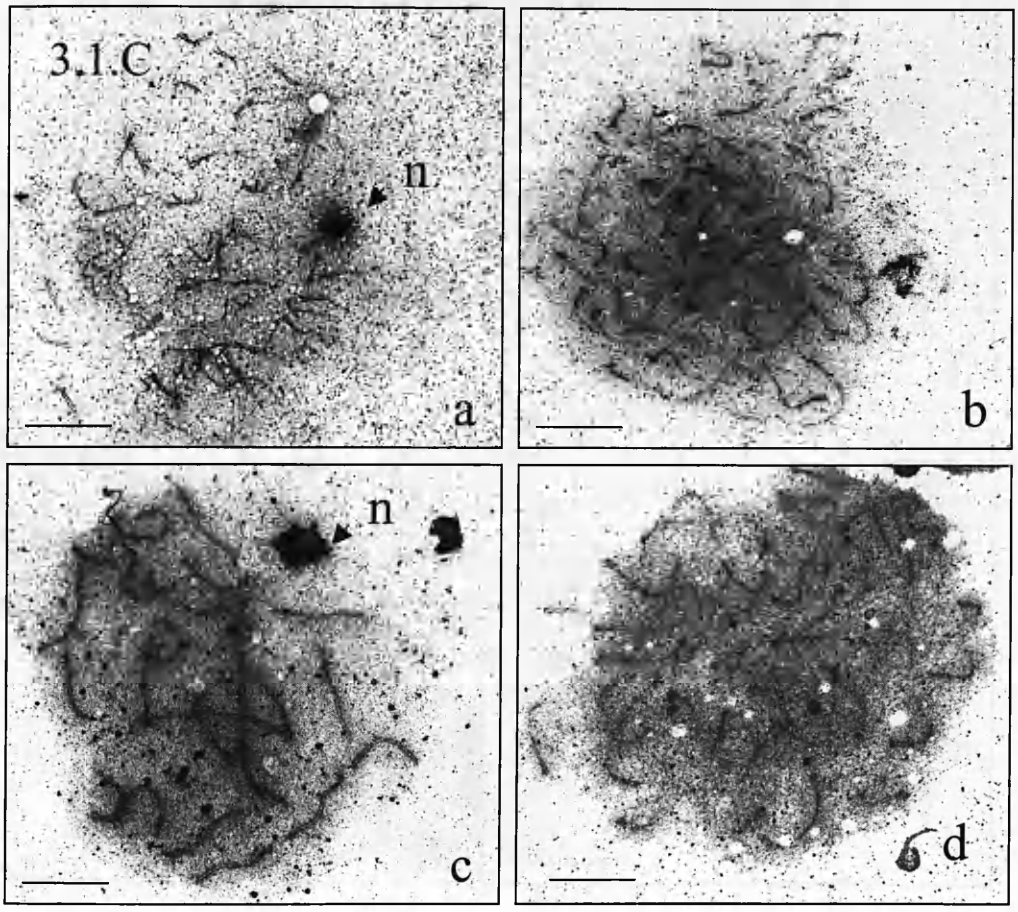


Figure 3.1.C. Zygotene. Polarization of lateral elements and bivalents in females of *Oreochromis* species: (a) observation of attachment plaques gathering at one side in *O. aureus*, (b) observation of attachment plaques gathering at one side in *O. mossambicus*, (c) observation of bivalents in pachytene gathering at one side in *O. niloticus*. (d) Female *O. aureus* in middle zygotene without polarization. n = nucleolus. Bar represents10 μm.

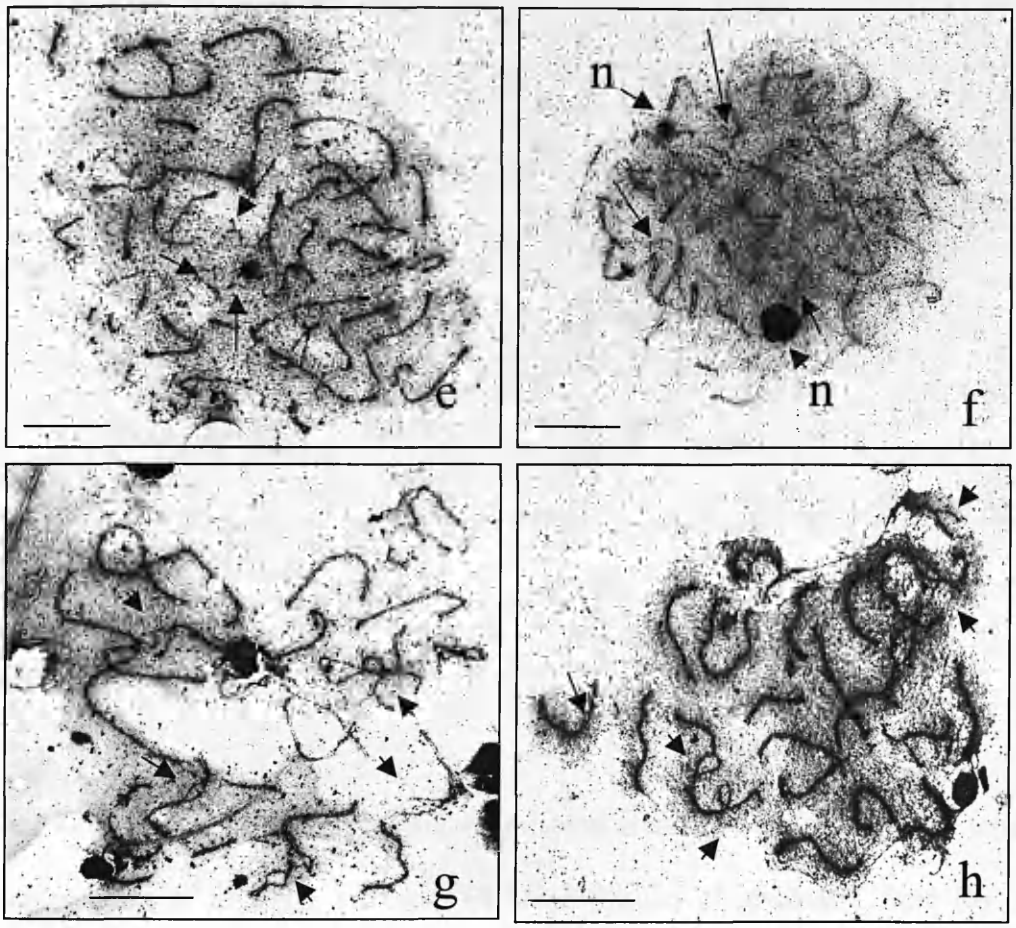


Figure 3.1.C. (continued):

(e) Male *O. mossambicus* and (f) Female *O. aureus* (two nucleoli) in middle zygotene, black arrows indicate axial element threads surrounding the lateral elements. (g) and (h) Males of *O. mossambicus* in late zygotene showing lateral elements well defined and the regions still not synapsed (arrows). n = nucleolus. Bar represents10 μm.

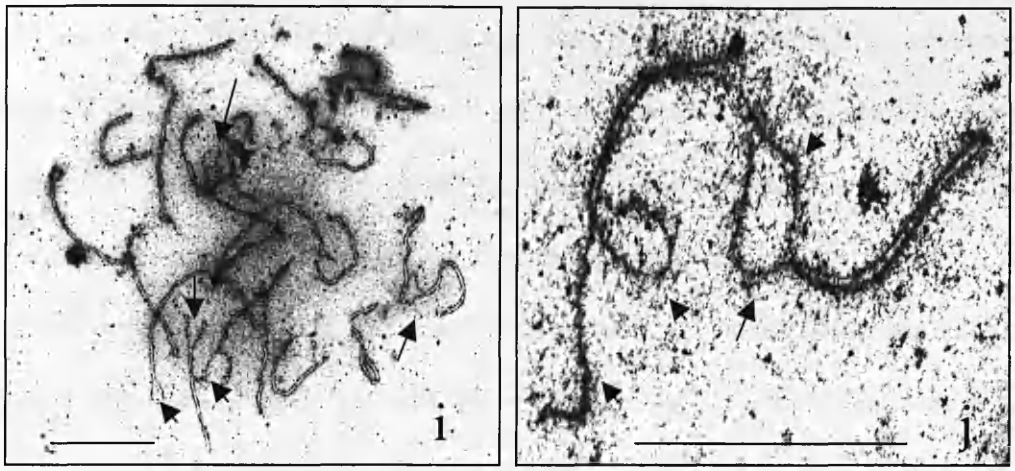


Figure 3.1.C. (continued):

(i) Male *O. aureus* in late zygotene showing lateral elements well defined and terminal unpaired regions of bivalents (arrows). (j) Magnification of bivalent 1 from (h) in *O. mossambicus*. Black arrows point unsynapsis of the lateral elements in the middle and terminal regions. Bar represents10 μm.

3.4.3. Pachytene Stage

At the pachytene stage, SC spreads in all three species contained 22 silver-stained bivalents (Fig. 3.1.D: a-k). The LEs are well differentiated and they span the bivalents from telomere to telomere without clearly revealing the central region of the SCs or the kinetochores. At the end of each SC, a distinctly stained plaque was observed, which presumably represents the point of attachment to the inner membrane of the nucleus. A bouquet arrangement of the bivalents is shown in Fig. 3.1.D: f. Interlocking was observed on a few occasions and always in only one bivalent (Fig. 3.1.D: g). In some bivalents two or three nodes were commonly observed along the SCs of males as they occur when two LEs twist, but they were fewer in SCs of females. Nucleolus Organizer Regions (NORs) were identified in some cells (Fig. 3.1.D: g,h,i,j,k), their appearance is like a tiny "mushroom" attached in a bivalent. There are two chromosomes bearing NORs in *O. mossambicus* and *O. aureus*. In *O. aureus* one NOR is located in the longest bivalent (Fig. 3.1.D: j), but there may be some polymorphism since both NORs can be located in two of the short bivalents (Fig. 3.1.D: h). One or two nucleoli are usually observed dispersed in the nucleoplasm during zygotene and pachytene of both sexes (e.g. Fig. 3.1.D: b (male), and d (female). In females one of these has a large size, characteristic of the phenotypic sex, in comparison to the two nucleoli of very similar size in males. Nucleoli were almost well rounded and they show a small size variation, however sometimes they can be confounded with granular background. Sometimes both nucleoli were not seen. Only in pictures without background nucleoli were easily distinguished. Pachytene stage continues with the progressive shortening of bivalents and then a separation of the homologues begins, except at the chiasmata. This does not happen simultaneously for all bivalents (Fig. 3.1.E: a).

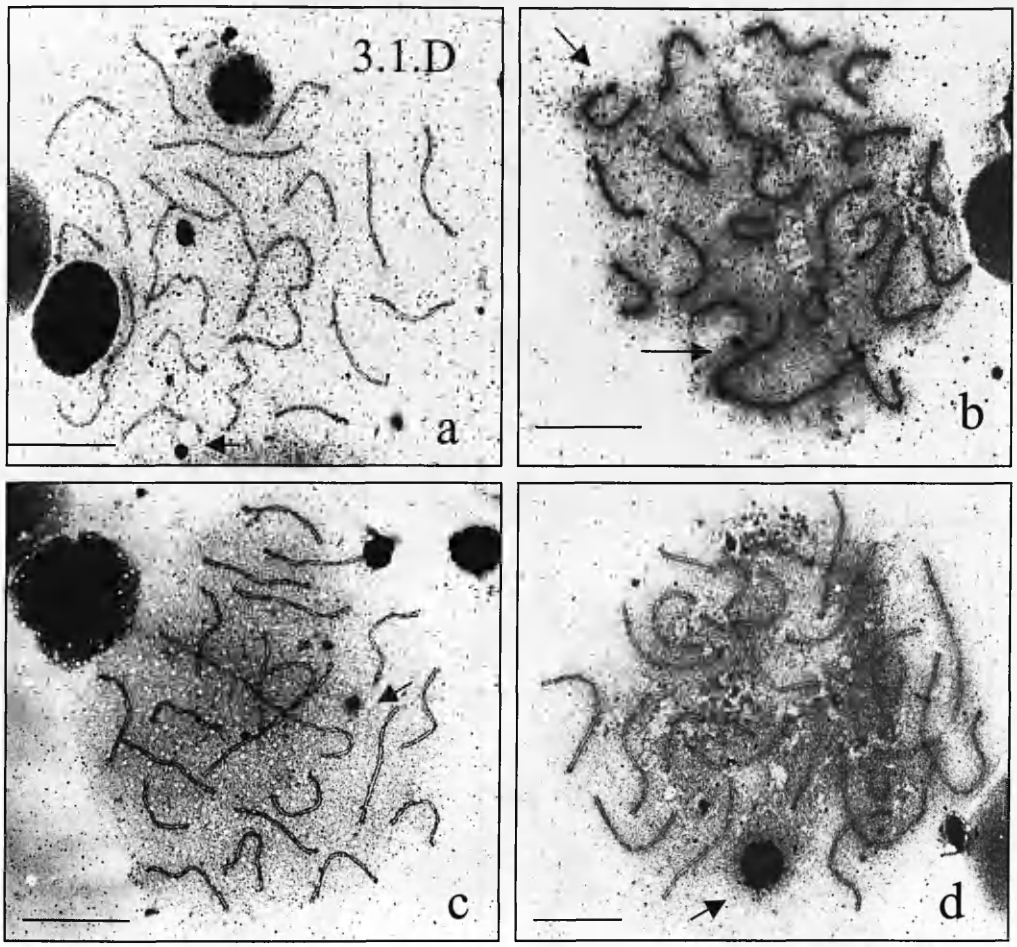


Figure 3.1.D. Pachytene. Stage showing 22 bivalents synapsed. (a) Male *O. aureus.* (b) Male *O. mossambicus.* (c) Male *O. niloticus.* (d) Female *O. mosssambicus*. Black arrows point to nucleoli. Bar represents10 μm.

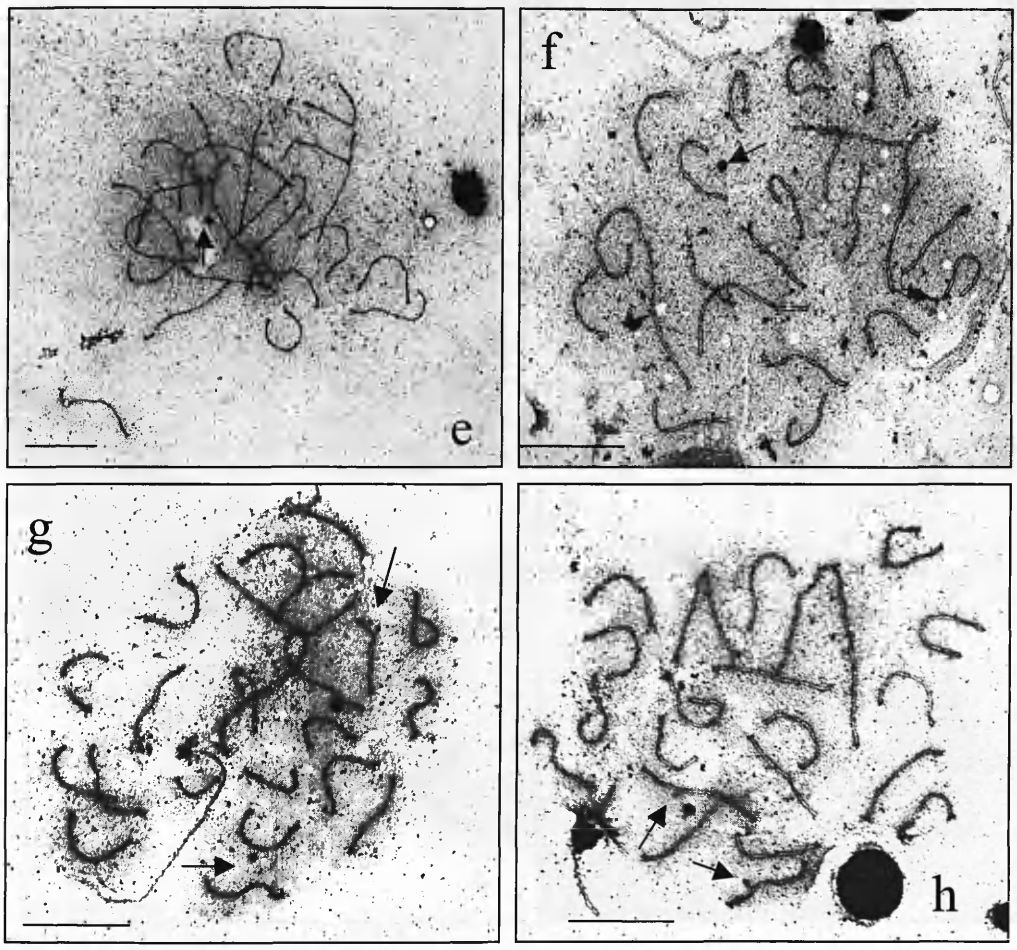


Figure 3.1.D. (continued):

(e) Female *O. niloticus*, black arrow points to the small nucleolus. (f) A bouquet arrangement of bivalents in male *O. aureus,* black arrow points to one nucleolus. (g) Interlocking in the longest bivalent of male *O. mossambicus*, arrows point to Nucleolus Organising Regions NORs. (h) NORs (black arrows) in male *O. mossambicus*. Bar represents10 μm.

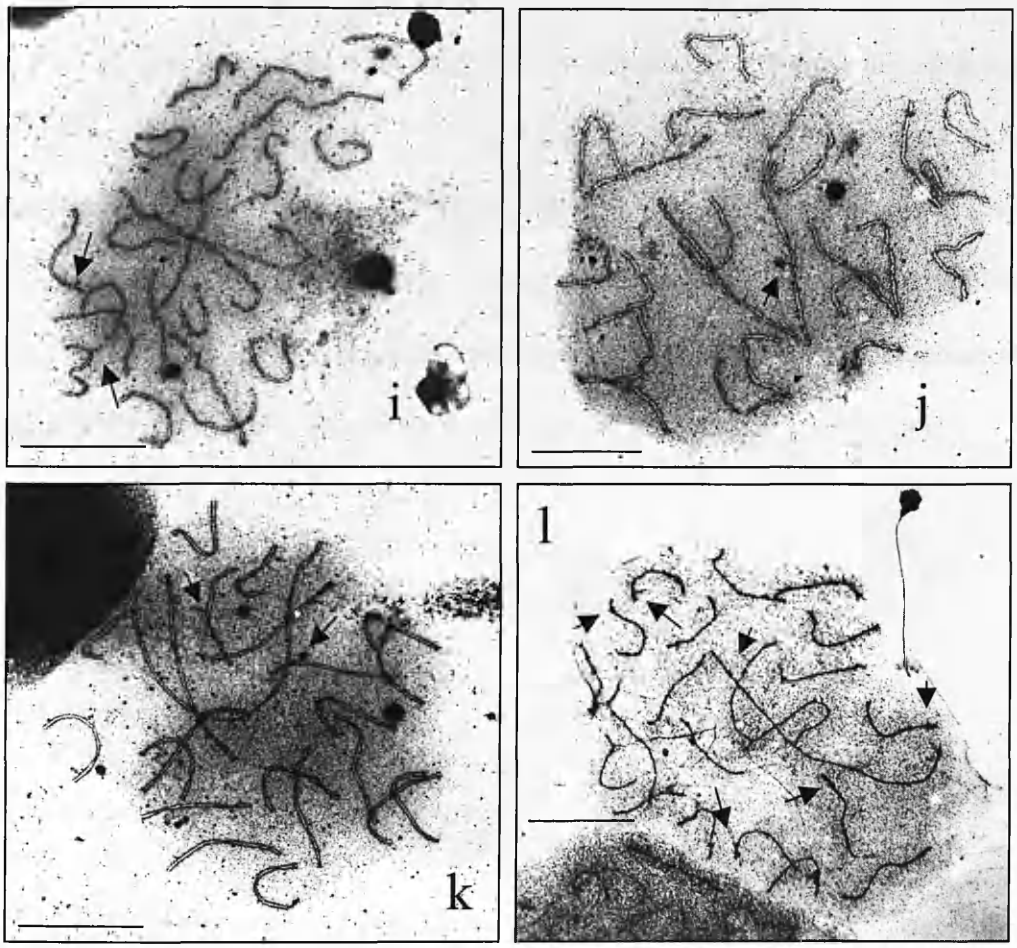


Figure 3.1.D. (continued):

(i) NORs (black arrows) in male *O. aureus* in two small bivalents. (j) NORs (black arrow) in male *O. aureus* one located in the longest bivalent. (k) Neomale WZ *O. aureus* showing two nucleoli and NORs (black arrows) in two small bivalents. (l) SC spread from male *O. mossambicus* in which the kinetochores are clearly recognised in each bivalent, arrows point to kinetochores. Bar represents10 μm.

3.4.4. Diplotene-metaphase I

Figure 3.1.E: (b), shows diplotene stage (light microscope), in which the separation of homologue is observed. Figure 3.1.E: (c and d), shows a diakinesis stage where each pair of homologous chromosomes begins to pull apart and Figure 3.1.E: (e and f) shows a more advanced diakinesis-metaphase I stage with more condensed chromosomes observed as 22 thick, highly condensed and stained bodies. Diakinesis-metaphase stages were also observed in the three species in the transmission electron microscope.

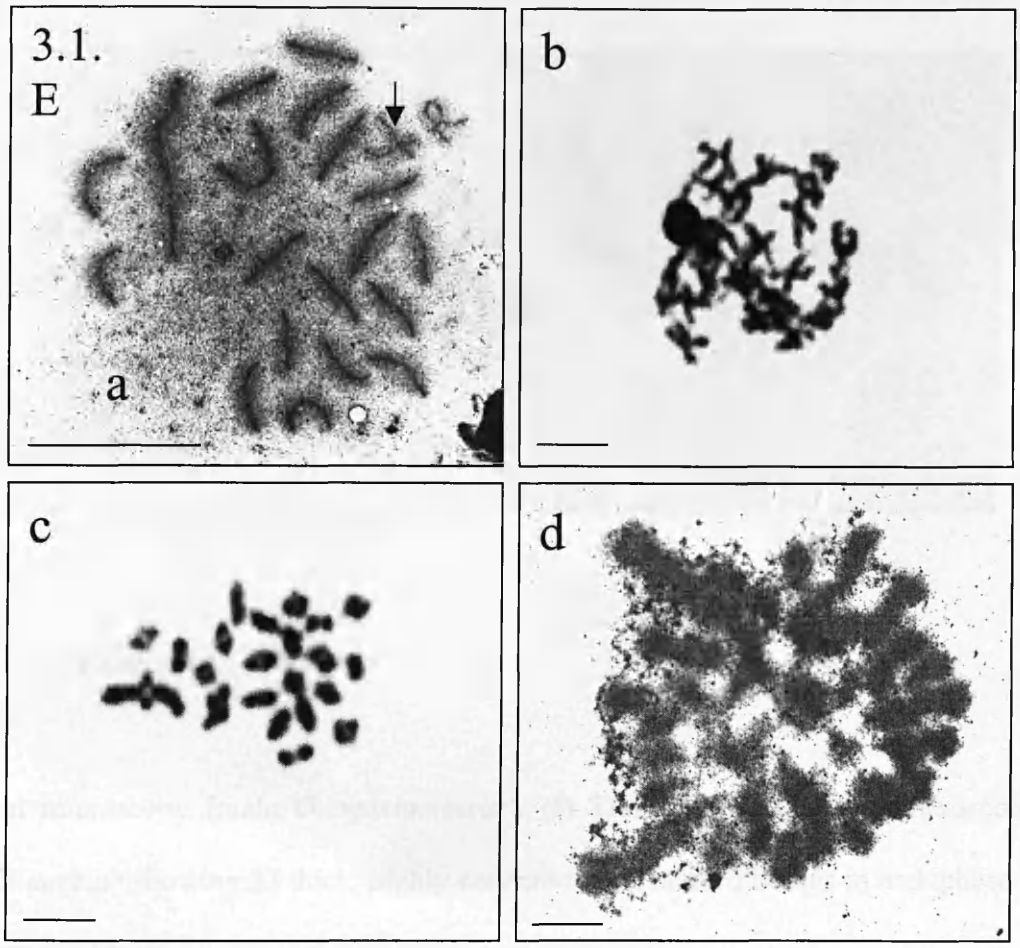


Figure 3.1.E. Diplotene-Diakinesis-metaphase I. (a) Pachytene-diplotene stage in female *O. niloticus*, in which one bivalent (black arrow) has separated in a submedian region. (b) Diplotene stage showing 22 bivalents separated by chiamata in median and submedian regions (male *O. mossambicus*). (c) Diakinesis stage where homologue chromosomes begin to be pulled apart from each other (male *O. mossambicus*). (d) Diakinesis-metaphase I, observed as 22 thick, highly condensed and stained bodies (female *O. niloticus*.). Bar represents10 μm.

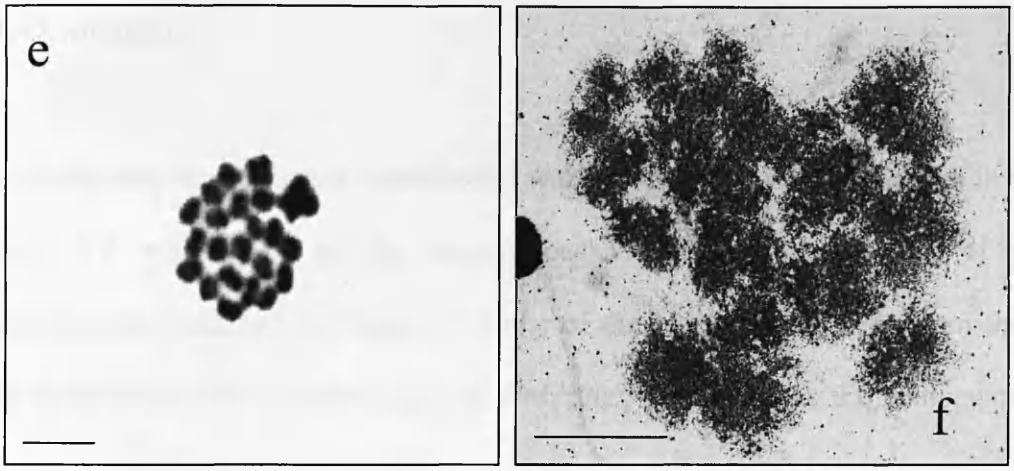


Figure 3.1.E. (continued):

(e) Light microscopy (male *O. mossambicus*). (f) Transmission electron microscopy (male *O. aureus*) showing 22 thick, highly condensed and stained bodies in metaphase I. Bar represents10 μm.

3.4.5. Sex chromosomes

3.4.5.1. *O. niloticus*

The partially unsynapsed largest chromosome was observed in 5 out of 12 nuclei in one individual XY male (42% of the observations). The observed patterns of sex chromosomes are described in Chapter 4. None of the 12 pachytene cells from the 6 females analysed showed unpaired regions, confirming the female as the homogametic sex.

3.4.5.2. *O. mossambicus*

A total of 60 meiotic pachytene nuclei from 6 males, 12 meiotic pachytene nuclei from 6 females and 60 meiotic pachytene nuclei from 6 neomales were analysed. None of them presented unpaired LEs in pachytene stage. However, in one SC spread of one male a region in bivalent 1 showed both LEs together but with a region of diffuse chromatin and differential staining (see Chapter 6). This observation also was observed in the XX female *O. niloticus* x XY male *O. mossambicus* hybrid (see Chapter 7).

3.4.5.3. *O. aureus*

Two unpaired putative sex chromosomes were found in WZ females and WZ neomales. They are described in Chapter 5. They were observed as a subterminal unpaired region or

a loop in the longest bivalent and an unpaired or partially paired small bivalent. No unpaired regions were observed in SC spreads from ZZ males, confirming the female as the heterogametic sex.

3.4.6. Synaptonemal complex complement length and packing density

The SCCL for the different species and genotypes is shown in Table 3.1. In *O. niloticus* there was a significant difference between the SCCL of males and females ($F = 11.41$, $P = 0.0$ significant). In *O. mossambicus* there was a significant difference between the SCCL of females when compared with males, neomales and males-T ($F = 28.90$, $P = 0.0$ significant). Males-T were significantly different from males but not from neomales. In *O. aureus* there was a significant difference between the SCCL of females when compared with males, neomales and males-T ($F = 32.92$, $P = 0.0$ significant). Males-T, males, and neomales were not significantly different when compared with each other.

Table 3.1. Mean synaptonemal complex complement total length ± standard deviation (SCTL ± S.D.), sample size (N), maximum (Max) and minimum (Min) values, range (Max/Min), mean length of bivalent one ± S.D. (B1 ± S.D.), and packing density (PD) expressed as pg DNA/one chromatid/micrometer SC, of three *Oreochromis* species.

Same letters in SCTL indicate no significant difference between groups within each species ($P<0.05$)

*Data from Carrasco, 1998; Carrasco *et al.*, 1999

Sexual genotype/ phenotype	**N**	**SCTL ± SD (μm)**	**Max (μm)**	**Min (μm)**	**Range**	**B1 ± SD(μm)**	**PD**
O. niloticus							
Female	12	193 ± 37(a)	233	121	1.92x	22 ± 7	4.9×10^{-3}
Male	10	144 ± 19(b)	164	106	1.54x	17 ± 5	6.5×10^{-3}
Male*		124				14	
Neomale*		132				15	
Male-MT*		173				17	
O. mossambicus							
Female	12	187 ± 22(a)	219	151	1.45x	20 ± 5	5.3×10^{-3}
Male	18	127 ± 17(c)	162	96	1.68x	14 ± 3	7.8×10^{-3}
Neomale	21	146 ± 13(b)	160	117	1.36x	15 ± 3	6.8×10^{-3}
Male-MT	13	145 ± 19(b)	191	116	1.64x	17 ± 3	6.8×10^{-3}
O. aureus							
Female	12	194 ± 30(a)	242	152	1.59x	21 ± 6	6.2×10^{-3}
Male	18	134 ± 13(b)	156	109	1.43x	16 ± 3	9.0×10^{-3}
Neomale	20	138 ± 13(b)	171	113	1.51x	16 ± 3	8.7×10^{-3}
Male-MT	20	141 ± 14(b)	168	121	1.38x	17 ± 3	8.5×10^{-3}

O. niloticus:

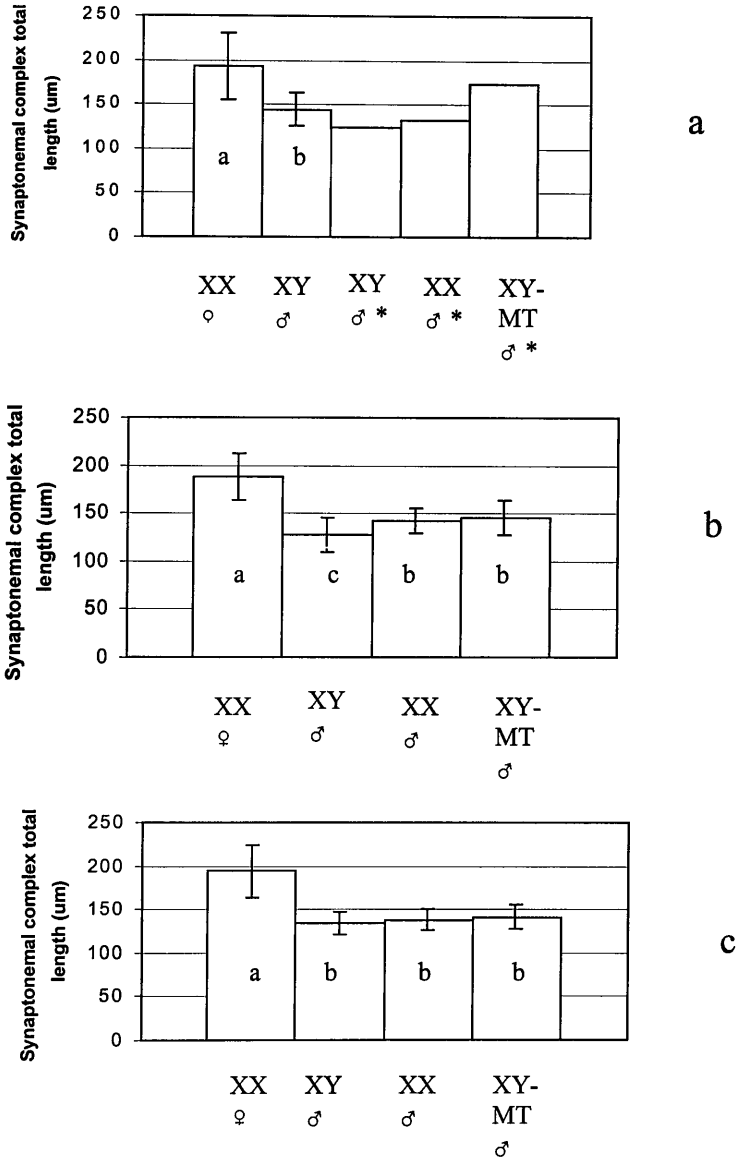
The mean total length in females was 193 ± 37 μm (n=12), with a maximum of 233 and a minimum of 121 and a range (max/min) of 1.92x. The longest bivalent measured 22 ± 7 μm. The chromosome packing density for females was 4.9 x 10^{-3}. In males, the mean was 144 ± 19 μm (n=10), with a maximum of 164 and a minimum of 106 and a range of 1.54x. The longest bivalent measured 17 ± 5 μm. The chromosome packing density for males was 6.5 x 10^{-3} (Fig. 14a).

O. mossambicus:

The mean total length in females was 187 ± 22 μm (n=12), with a maximum of 219 and a minimum of 151 and a range (max/min) of 1.45x. The longest bivalent measured 20 ± 5 μm. The chromosome packing density for females was 5.3 x 10^{-3}. In males, the mean was 127 ± 17 μm (n=18), with a maximum of 162 and a minimum of 96 and a range of 1.68x. The longest bivalent measured 15 ± 3 μm. The chromosome packing density for males was 7.8 x 10^{-3}. In neomales, the mean was 146 ± 13 μm (n=21), with a maximum of 160 and a minimum of 117 and a range of 1.36x. The longest bivalent measured 15 ± 3 μm. The chromosome packing density for neomales was 6.8 x 10^{-3}. In males exposed to MT, the mean was 145 ± 19 μm (n=13), with a maximum of 191 and a minimum of 116 and a range of 1.64x. The longest bivalent measured 16 ± 3 μm. The chromosome packing density for males exposed to MT was 6.8 x 10^{-3} (Fig 14b).

O. aureus:

The mean total length in females was 194 ± 31 μm (n=12), with a maximum of 242 and a minimum of 152, with a range (max/min) of 1.59x. The longest bivalent measured 21 ± 6 μm. The chromosome packing density for females was 6.2 x 10^{-3}. In males, the mean was 134 ± 13 μm (n=18), with a maximum of 156 and a minimum of 109 and a range of 1.43x. The longest bivalent measured 16 ± 3 μm. The chromosome packing density for males was 9 x 10^{-3}. In neomales, the mean was 138 ± 13 μm (n=20), with a maximum of 171 and a minimum of 113 and a range of 1.51x. The longest bivalent measured 16 ± 3 μm. The chromosome packing density for neomales was 8.7 x 10^{-3}. In males exposed to MT, the mean was 141 ± 14 μm (n=20), with a maximum of 168 and a minimum of 121 and a range of 1.38x. The longest bivalent measured 17 ± 3 μm. The chromosome packing density for males exposed to MT was 8.58 x 10^{-3} (Fig 14c).



Same letters indicate not significant difference between genotypes ($P < 0.05$)

Figure 3.2. Synaptonemal complex complement total lengths in pachytene stage nuclei from different species, genotypes, and phenotypes: (a) *O. niloticus*. (b) *O. mossambicus*. (c) *O. aureus*. Bars denote standard deviation. * Data from Carrasco *et al.*, 1999.

3.5. Discussion

As expected, SCs from spermatocytes were abundant and suitable for analysis. However, SCs from oocytes were difficult to obtain and few spreads were available per individual. Nevertheless, obtaining SCs from young females represents a considerable advance on previous studies. SCs from oocytes were obtained only from samples taken before the first pre-vitellogenesis stage, about 3 months after fertilisation. Once vitellogenesis advanced, it was not possible to obtain SCs from females. It was also noted that maintaining these fish in low light conditions during rearing resulted in higher quality preparations, direct light (photoperiod of 12:12 light:darkness) apparently acting as a trigger to accelerate the first vitellogenesis.

3.5.1. Leptotene stage

Presynaptic alignments are reported for the first time in fish, occurring where axial elements are aligned in parallel and overlapping in time with the development of the SC, as described by Loidl (1994). After these presynaptic alignments, synapsis commenced in each LE in one or more sites in subterminal, terminal, or middle regions without any synchronism between LEs, as occurs in rainbow trout (Oliveira *et al.*, 1995). In Fig. 3.1.B: (b and c) it can be observed that only the longest bivalent has begun SC formation in one end. This occurs when homologous chromosomes have approached each other in that region to the critical distance of 300 nm, initiating formation of the tri-partite SC which extends like a zipper along the chromosomes (Loidl, 1994). Above the longest

bivalent in the same figure, there are two axial elements that are approaching each other to start SC formation in a subterminal region. Below the longest bivalent there are two axial elements approaching each other to start SC formation at a submedian region. It can be suggested that this is a classical leptotene stage with no synapsis but completed LEs (von Wettstein *et al.*, 1984).

3.5.2. Zygotene stage

Polarization is referred as the gathering of bivalents at one side of the nucleus where each bivalent is attached to the inner membrane. It has been theorised that these "polarization attachments" are part of the organisation inside the nucleus for a more efficient way to synapse homologous chromosomes. Thus, polarization has been proposed to play a role in synaptic initiation (Loidl, 1990, 1994). By early zygotene, highly stained terminal plaques were observed in the three tilapia species, possibly representing the points where the telomeres are attached to the nuclear membrane (Loidl, 1990). However, polarization was only observed in females of the three species studied (Fig. 3.1.C: a) and it was not as conspicuous as that observed by Oliveira *et al.* (1995) in the same stage in the rainbow trout.

Zygotene was clearly identified by Van Eenennam *et al.* (1998) in the white sturgeon, Oliveira *et al.* (1995) in the rainbow trout and Dias *et al.* (1998) in *Prochilodus lineatus*. In late zygotene stage, it was possible to recognise that some of the latest synapsis can occur in different points along the chromosomes due to the different regions of synapsis since the leptotene stage. According to Liu and Yu (1991), the initiation point of an SC

varied depending on the chromosome morphology: bi-armed chromosomes started at two telomeres and continued to the middle where at some point the kinetochore was present, while in uni-armed chromosomes synapsis started at one end and then continued to the other end in one direction, towards the kinetochore. However, this seems not to be a "morphology rule". In Fig. 3.1.C: (g-j), unsynapsed interstitial lateral elements as well as unsynapsed terminal regios can be observed. This suggests that synapsis was not exclusively initiated at the telomeres, as has been observed in the white sturgeon, *Acipencer transmontanus* Richardson (Van Eenennam *et al.*, 1998).

3.5.3. Pachytene stage

The observation of 22 silver-stained bivalents (Fig. 3.1.D) is in accordance with the chromosome complement described for these species, 2n=44 from mitotic chromosomes (Majumdar and McAndrew, 1986) and n = 22 from SC studies (Liu and Yu, 1991; Foresti *et al.*, 1993; Carrasco *et al.*, 1999). As shown in Figures 3.1.D: b (female) and 3.1.D: d (male), in SCs from both sexes two nucleoli are usually observed dispersed in the nucleoplasm during zygotene and pachytene. In females one of these is larger, which is characteristic of the phenotypic sex, while the two nucleoli of males are of similar size. This large nucleolus in females is equivalent to the early stage 1 (chromatin nucleolar stage) of *Tilapia zillii* (Coward, 1997), or the stage 2a of oocyte development described by Bromage and Cumaranatunga (1988) in the rainbow trout, which disappears in subsequent stages. The presence of large and small nucleoli (females) or two equal size nucleoli (males) was not useful as a cytological structure to identify the genotypic sex.

WZ males (neomales) of *O. aureus* and XX males (neomales) of *O. mossambicus* showed one or two nucleoli of the same size as normal males. There are two chromosomes bearing NORs in *O. mossambicus* and *O. aureus*, the same number as was found in *O. niloticus* (Foresti *et al.*, 1993). There appears to be a polymorphism in the location of NORs in the bivalents of *O. aureus*. A late pachytene stage was observed with the progressive shortening of bivalents until a condensed state is reached (Fig. 3.1.E).

3.5.4. Diplotene stage

This stage is characterised by the separation of homologous chromosomes except for specific regions where actual physical crossing over (chiasmata) has taken place between non-sister chromatids (Fig. 3.1.E: a and b). This separation does not seem to occur simultaneously in all bivalents. Most of the tilapia chromosomes are subtelocentric and telocentric. In Fig. 3.1.E: (b), it is observed that most of the chromosomes present an "X" shape, representing a single chiasmata in median/submedian regions of each chromosome pair. Shortening of diplotene chromosomes continues as they begin to be pulled apart from each other during diakinesis-metaphase 1 (Fig. 3.1.E: e and f), where they are finally observed as 22 highly stained bodies. Liu and Yu (1991) mentioned the breakage and disappearance of the LEs after diplotene. We have clear evidence that the LEs continue shortening, passing through diplotene until they reach a maximum condensation stage. They do not break and disappear. According to Liu and Yu (1991), at early diplotene stage, the LEs of most SCs also begin to separate from the terminal or medial regions, and resulted in autosomal SCs presenting different separated states (Y, X, 0 or

8). Their diplotene photograph lacks a scale bar and it is not possible to clearly distinguish most of the bivalents or to compare their size against pachytene bivalents. In this study, "X" shapes were observed during diplotene only, in concordance with one chiasma presented in subtelocentric or telocentric homologue chromosomes. Furthermore, we observed that at early zygotene the LEs between attachment plaques were sometimes not apparent due to interference from the nuclear envelope (Fig. 3.1.C: a). The telomeres become thicker and highly stained making a contrast against the nuclear envelope that "hides" the LEs. If the lateral elements twist after the attachment plaque it would give the appearance of a small bivalent with an X or Y or 8 shape. This stage was identified in this study as early zygotene, possible misinterpreted by Liu and Yu (1991).

3.5.5. Kinetochores

Because kinetochores were not consistently observed in each LE, it was not possible to describe karyo-morphology. Without an explanation, only one SC spread clearly showed the kinetochrores (Fig.3.1.D: k). The recognition of the position of each kinetochore by the attachment plaque as described by Wise *et al.* (1987) in *Gambusia affinis* was not possible. This was due to *G. affinis* presenting attachment plaques at only one end of each bivalent, whilst tilapias present these plaques at both ends. Previous reports in tilapia have not shown kinetochores in meiotic prophase I (Foresti *et al.*, 1993; Carrasco *et al.*, 1999; Campos-Ramos *et al.*, 2001), apart from the study of Liu and Yu (1991). In the present study, PTA staining (Chapter 4) also did not reveal the presence of

kinetochores. In several other fish species kinetochores have not been observed: *Prochilodus lineatus* (Dias *et al.*, 1998), *Leporinus lacustris* (Mestriner *et al.*, 1995), and four species of *Poecilia* (Rodionova *et al.*, 1996). They were not consistently found in *Oncorhynchus mykiss* (Oliveira *et al.*, 1995). In *Acipenser transmontanus* Richardson, however, kinetochores were clearly observed in all the bivalents (Van Eenennaam *et al.*, 1998).

3.5.6. Synaptonemal complex complement length

The SCCL of females was significantly longer than that of males. In genetic females as neomales (through MT or ET treatment), the SCCL was reduced significantly compared to that of normal females and in males-T it had a tendency to increase compared to control males. Hypothetically, it might be expected that a male sex reversed to female (neofemale) would show an increase in SCCL, but this was not studied.

Testosterone is known to regulate protein synthesis in the germ cells (Sharpe *et al.*, 1993; McKinnell and Sharpe, 1995) and may act to decrease or increase the SCCL during meiotic prophase I. Possibly, testosterone regulates male specific proteins to pack the DNA more densely along the lateral elements. It is known that the female SCCL is always longer than males, e.g. the total SCCL in females of the amphibian *Xenopus laevis* (Loidl and Schweizer, 1992) and in humans *Homo sapiens* (von Wettstein *et al.*, 1984) exceeds that of the males by a factor of two. In *Oreochromis* species this factor is around 1.4. However in the rainbow trout the SCCL has been reported as being similar between males and females (Oliveira *et al.*, 1995), which could be due to the small

number of nuclei analysed in females (n=3). Data from Carrasco *et al.* (1999) showed that the SCCL in some nuclei of a particular individual in *O. niloticus* from the male-T group were longer than all the nuclei of neomales and normal males, but the sample size was too small to be conclusive. From the present study it is clear that there is a significant increase in the length of the bivalents in males-T of *O. mossambicus*.

The most important feature of a reduction or an increase in SC length is the density at which DNA is packed along the lateral elements, which may reduce or increase the frequency of recombination events. It has been hypothesised that with a lower DNA density the recombination rate is higher (Loidl, 1994). At a low packing density chromatin presents more and smaller loops along the axes of pairing chromosomes, bringing a bigger proportion of the DNA into direct contact with the axial elements, leading to the interaction with corresponding sequences on the homologue. Therefore, hypothetically the less packed the DNA is along the axial elements, the more frequent is the frequency of crossing over because there are more sites at which homologous DNAs can interact (Loidl, 1994). It is not known if the reduction or increment of SC length in *Oreochromis* species could affect the recombination rates and hence, alter the genetic constitution of the offspring as suggested by Carrasco *et al.* (1999). A mating study on a colour gene of *Oryzias latipes* (medaka) in which both parents were sex-reversed to the opposite sex (neomales and neofemales) showed that the frequency of crossovers from the oocytes of neofemales was 1%, while the estimated frequency of crossovers from genetic males was 0.2%, which is five times less (Yamamoto, 1961: cited by Solari, 1994). The conclusion of Yamamoto was that in fish, crossing-over between the X and Y chromosomes occurs more frequently in oocytes than in spermatocytes. Solari (1994) interpreted this result as the presence of some crossover restriction operating in the heterogametic sex. If the SC total length is increased in medaka neofemales as it has been theorised to happen in tilapia neofemales, then the DNA packing density may be a factor to consider respecting the frequency of crossovers. Of course this is only theoretical since this has not been studied in tilapia.

Alterations to the SC have been used in mammals for genotoxicity studies and as a biomarkers (Moses, 1979; Allen *et al.*, 1987). Particular attention has been given to aquatic environments where exogenous compounds such as hormones have entered natural water bodies. The main question is whether these compounds have had an effect on fish populations, especially as inductors of sex reversal, partial sterility, or intersexes. It seems that the SC total length of *Oreochromis* species could not be used as a reliable indicator of these alterations as suggested by Carrasco *et al.* (1999). Although the difference between total SC lengths of males and females is very obvious, the MT-males changed little with respect to normal males and the effect of oestrogen would also need to be investigated.

3.6. Conclusions

1) It has been found that meiotic prophase I in *Oreochromis* shares similar patterns of synapsis to other eukaryotes.

2) Through the SC technique it is possible to recognise putative sex chromosomes (the SC technique has been little utilised for this purpose in fish).

3) The SC length varies between the sexes and is altered by the process of sex reversal.

4) This work can be useful as a guide to the events of meiotic prophase I for further research on fish meiosis.

CHAPTER 4

PUTATIVE SEX CHROMOSOMES IN *O. niloticus*

4.1. Introduction

4.1.1. Sex determination in the Nile tilapia

The Nile tilapia has a primary monofactorial sex determination system (XX/XY) with male heterogamety (Jabalert *et al.*, 1974). However, this species also shows autosomal influence (Mair *et al.*, 1991a; Hussain *et al.*, 1994; Müller-Belecke and Hörstgen-Schwark, 1995). In this species Hussain *et al.* (1994) suggested an autosomal sex recombination locus named sex determination locus 2 (SDL2) and calculated a recombination rate of r = 0.85 between this locus and the centromere. This value was based on the low proportion of males observed among the meiotic gynogenetic offspring derived from one female. Müller-Belecke and Hörstgen-Schwark (1995) assumed a higher recombination of r = 1 between the centromere and SDL2 on the basis of the absence of males among 163 meiotic gynogenetics produced by two females which had males among the mitotic gynogenetic offspring in their study. However, Sarder *et al.* (1999) observed that both inbred and outbred clonal lines of *O. niloticus* exhibited 100 % female meiotic gynogenetics with the exception of one inbred line. This inbred line could be fixed genetically for some allele, or combination of alleles at different loci that cause female to male sex reversal but with limited penetrance.

A polygenic system has also been suggested in this species, based on hybrid sex ratios (Wohlfarth and Wedekind, 1991) and possibly parental influence (Shelton *et al.*, 1983; Sarder *et al.*, 1999; Tuan *et al.*, 1999 and Kwon, 2000).

Environmental sex determination is also present, mainly seen as high temperature overruling genetic sex determination in this species, as evidenced by masculinization and feminization effect in XX and YY genotypes respectively (Baroiller *et al.*, 1995a; Abucay *et al.*, 1999; Kwon, 2000). There is no low temperature effect on progeny sex ratios in the Nile tilapia (Baroiller *et al.*, 1995b). The major sex differentiation period lies between 13 and 24 days post-fertilisation in this species (Kwon, 2000; Kwon *et al.*, 2001).

Gynogenesis studies from neofemales of *O. niloticus* (Mair *et al.*, 1991a) showed recombination between the centromere and the sex-determining locus. It was calculated that sex genes were located distantly from the centromere (r = 0.68).

A study of the meiotic chromosomes of male *O. niloticus* identified a size heteromorphism in the lateral elements of the largest bivalent, which was associated with the presence of an incompletely paired segment in the terminal region of this chromosomal pair (Foresti *et al.*, 1993). Analysis of three different sexual genotypes of *O. niloticus* (XX, XY, and YY) identified this unpaired segment in the largest chromosome pair in normal males (XY), but not in XX neomales (genetic females sex-reversed to males to allow SC analysis) or in YY "supermales" (Carrasco *et al.*, 1999). This unpaired segment was observed in 25.7% of XY SC spreads, and Carrasco *et al.* (1999) suggested that the fully paired copies of bivalent 1 may represent later stages of pachytene (comparable to axial equalization in birds where the Z and W chromosome pair by late pachytene). This strongly suggests that the unpaired region is associated with heterogamety, and that the unpaired region of the largest chromosome pair (chromosome 1) is the location of the major sex determining loci in the *O. niloticus* XX/XY system.

These findings can be further interpreted as evidence that *O. niloticus* is at an early stage of sex-chromosome differentiation (Carrasco *et al.*, 1999), with the unpaired region indicating a reduction or cessation of recombination.

Wise *et al.* (1987), also observed this terminal unpairing in bivalent 1 in the mosquito fish, *Gambusia affinis*. They identified the synaptonemal complex spread as late zygotene. The mosquito fish has been reported to have slight differences in chromosome one in females and is considered to present a ZZ/ZW system (reviewed by Solari, 1994). Thus, it appears that the sex determining system in the mosquito fish should be reevaluated.

From examination of meiotic prophase I it is clear that terminal synapsis is a characteristic of late zygotene. Thus, is this an event that occurs at late zygotene, at early pachytene where all autosomal bivalents have synapsed but bivalent 1 or does it occur in an advanced pachytene stage? Why is this bivalent observed to have the terminal region in the unsynapsed and synapsed conditions in the same individuals? Is heteromorphism clearly involved?

It is difficult to stage SC spreads into early and late pachytene, but bivalent length may reduce during pachytene due to contraction, so these questions will be analysed in this Chapter.

4.2. Aims

The aims of this research were:

1) To analyse if the terminal unpairing of bivalent 1 is occurring in late zygotene, early or late pachytene stage.
2) To analyse the stages of bivalent 1 when is observed in both unsynapsed and synapsed conditions.
3) To explore Phosphotungstenic acid (PTA) staining for better resolution of the structure of bivalents, such as kinetochores and recombination nodules.

4.3. Materials and methods

4.3.1. Species studied

The Nile tilapia *Oreochromis niloticus* used in this study came from populations derived from the Lake Manzala, Egypt. This species are held in the Tilapia Reference Collection at the Institute of Aquaculture, University of Stirling.

4.3.2. Fish used for SC analysis

One *O. niloticus* progeny tested XY male and six females were analysed during meiosis in Chapter 3. Synaptonemal complex analysis at pachytene stage for unpaired regions in the heterogametic sex are described in this Chapter.

The male *O. niloticus* was used for the SC silver stain technique and the observation of the unpaired region in the subterminal or terminal position of the largest chromosome pair. A total of 12 meiotic pachytene nuclei were analysed from this male.

Another XY male *O. niloticus* was used to explore Phosphotungstenic acid (PTA) staining. A total of 6 meiotic pachytene nuclei were analysed from this male.

4.3.3. Preparation of SC spreads

Fish were killed with anaesthesia (by immersion in a 0.01% benzocaine solution) followed by destruction of the brain before dissection. Preparation of fish SC spreads for

the observation in the transmission electron microscope were made accordingly with the protocol shown in Chapter 2 (section 2.1.4).

PTA staining was made with slides coated with Falcon plastic (0.75% w/v) and rinsed in Photoflu solution. Slides were immersed in 1% PTA solution in 75 % ethanol for 2-5 minutes, rinsed twice in ethanol and air dried (Moses, 1977).

4.3.4. Statistical analysis for lengths of bivalent 1 and 2

In order to investigate in which sub-stage of pachytene the putative sex chromosomes are observed in males (terminal unpaired lateral elements), the lengths of bivalent one and the second longest chromosome (bivalent 2) were measured using Image Pro 3.0 software.

The lengths of bivalent 1 in those nuclei showing a terminal unpaired region were compared against those nuclei showing fully synapsed lateral elements. The lengths of bivalent 2 in those nuclei having unpaired region in bivalent 1were compared against those nuclei having fully synapsed lateral elements in bivalent 1. Both bivalents were analysed independently using a single ANOVA test.

4.4. Results

4.4.1. Siver staining

The partially unsynapsed largest chromosome was observed in 5 out of 12 nuclei in one individual XY male (42 % of the observations). One nucleus presented a median unsynapsis possibly near to the kinetochore that seems not to be related to the sex-unpaired mechanism (Fig. 4.1.a). One nucleus showed median and subterminal unsynapsis (Fig. 4.1.b). One nucleus showed subterminal unsynapsis (Fig. 4.1.c). One nucleus presented both subterminal and terminal unsynapsis (Fig. 4.1.d). One nucleus showed terminal unsynapsis (Fig. 4.1.e) in which one lateral element was synapsed with a broken autosomal showing an aberrant synapsis. Finally, one nucleus showed median, subterminal and terminal unsynapsis (Fig. 4.1.f). No heteromorphic size was observed. The other 7 cells had a complete synapsis as a typical pachytene stage.

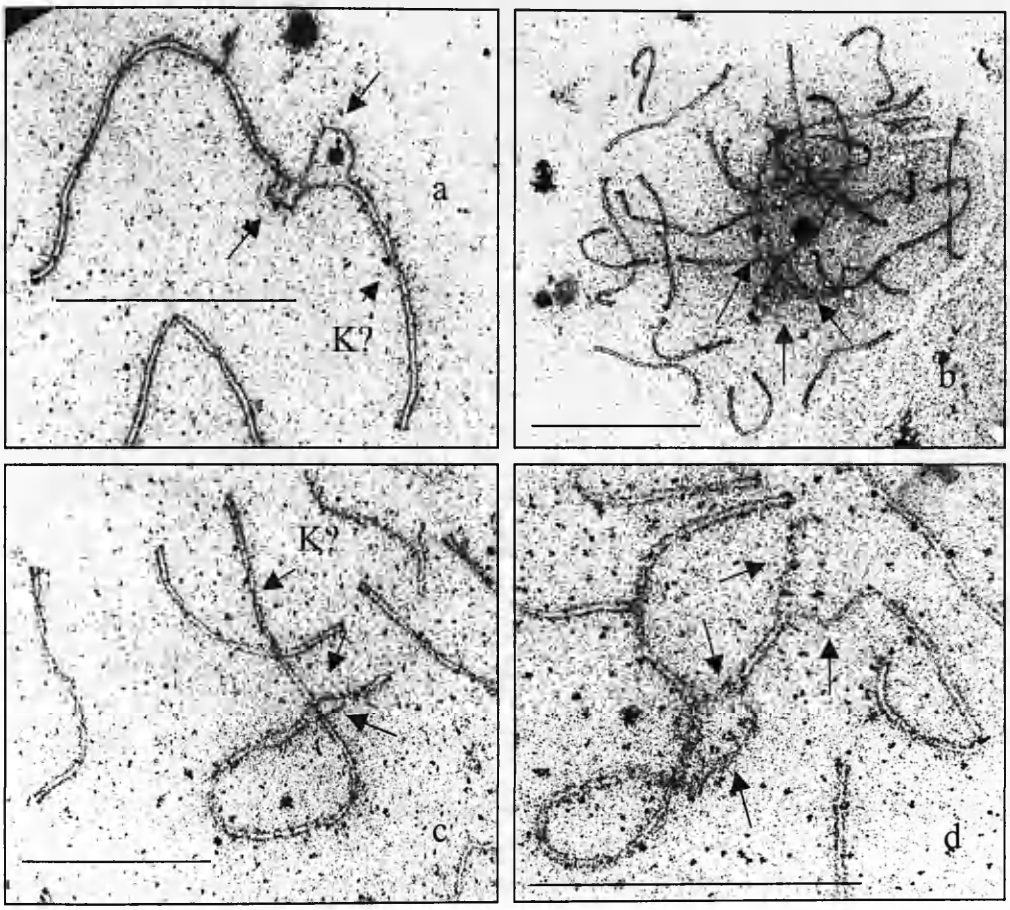


Figure 4.1. Unpaired regions in bivalent 1. (a) SC spread showing a median unsynapsis possibly near to the kinetochore that seems not to be related with sex-unpaired mechanism. (b) SC spread showing median and subterminal unsynapsis. (c) SC spread showing subterminal unsynapsis. (d) SC spread showing both subterminal and terminal unsynapsis. Black arrows point to unpaired lateral elements. K? = kinetochore?. Bar represents10 μm.

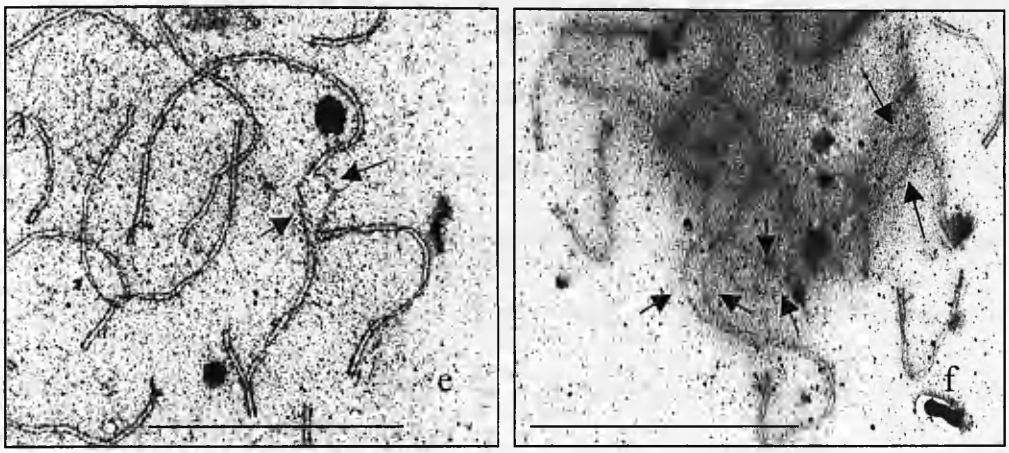


Figure 4.1. (continued):

(e) SC spread showing left lateral element of bivalent 1 synapsed with a broken autosome. (f) SC spread showing median, subterminal and terminal unsynapsis. Black arrows point to unpaired lateral elements. Bar represents10 μm.

4.4.2. PTA staining

The partially unsynapsed largest chromosome was observed in 2 out of 6 nuclei in one individual XY male (33 % of the observations). Two nuclei presented both subterminal and terminal unsynapsis (Fig. 4.2. a and b). No heteromorphic size was observed. The other 4 cells had a complete synapsis as a typical pachytene stage. The kinetochores were not observed with this technique. In Figure 4.2a it was possible to observe two recombination nodules in autosomes (kindly interpreted by Dr A.J. Solari, pers. comm.).

4.4.3. Synaptonemal complex lengths of bivalents 1 and 2

Figure 4.3a shows the lengths of bivalent 1 where the putative sex chromosomes were observed (black arrows). There was not a significant difference between the lengths of bivalent 1 (partially unpaired) and bivalent 1 (fully paired). The mean values for these two groups were 19.29 and 17.75 μm respectively ($F = 0.67$; $P = 0.43$).

Figure 4.3b shows the lengths of bivalent 2 where the putative sex chromosomes were observed in bivalent 1 (black arrows). There was not a significant difference between the lengths of bivalent 2 (in which bivalent 1 was partially unpaired) and bivalent 2 (in which bivalent 1 was fully paired). The mean values for these two groups were 11.81 and 9.68 μm respectively ($F = 3.93$; $P = 0.07$).

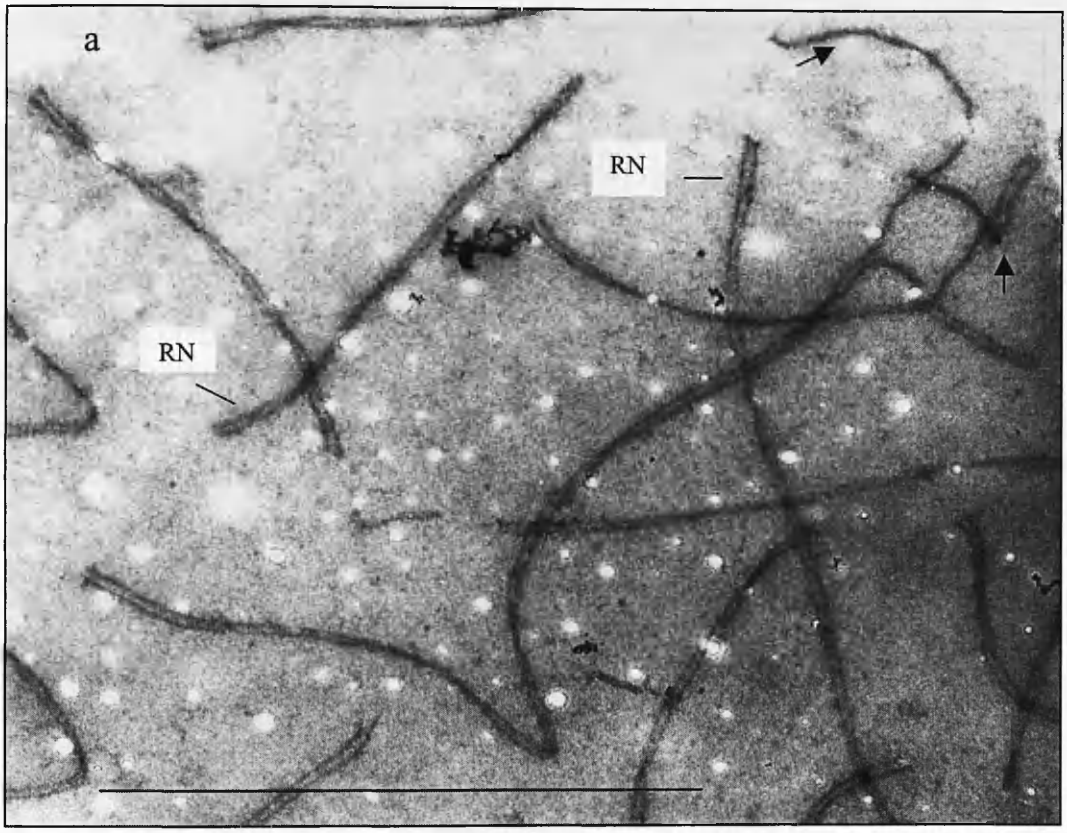


Figure 4.2 a. Putative sex chromosomes in *O. niloticus* stained with PTA. Black arrows point to unpaired lateral elements. RN: recombination nodules. Bar represents10 μm.

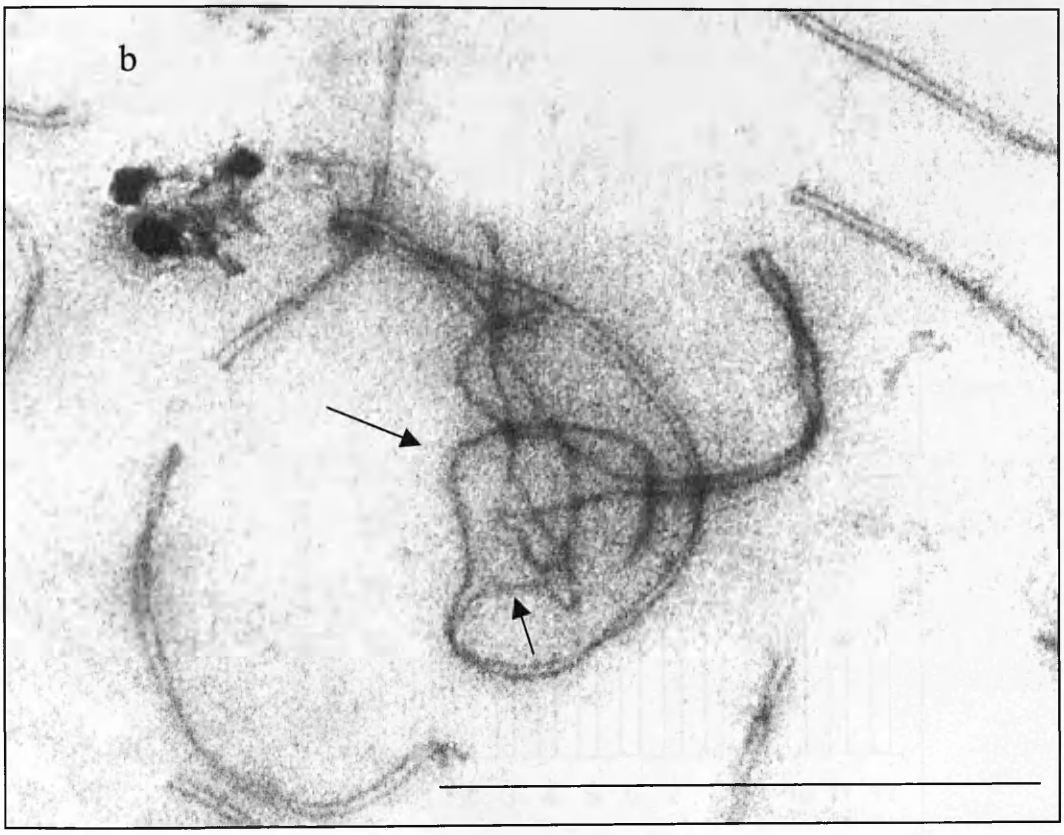
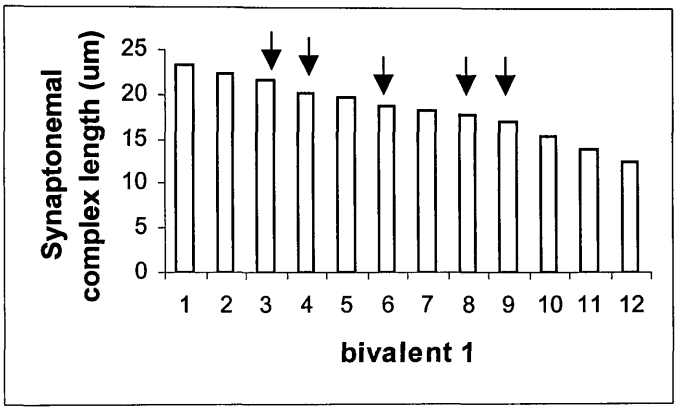


Figure 4.2 b. Putative sex chromosomes in *O. niloticus* stained with PTA. Black arrows point to unpaired lateral elements. Bar represents10 μm.

a



b

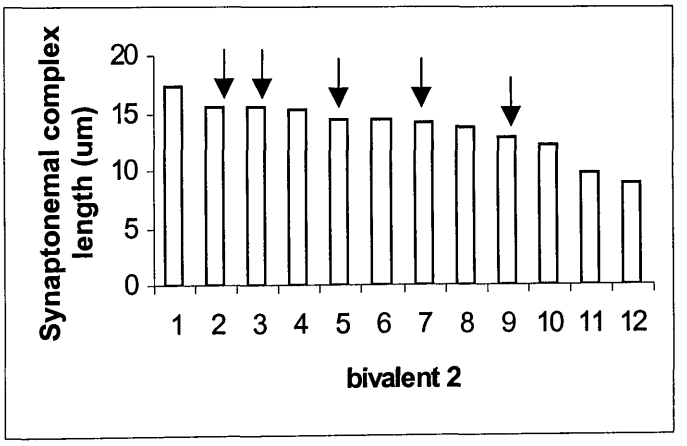


Figure. 4.3. Synaptonemal complex complement measurements of bivalent 1 (a) and 2 (b) ranking by size. Black arrows indicate those bivalents showing putative sex chromosomes.

4.5. Discussion

In general, these observations agree with those of Foresti *et al.* (1993) and Carrasco *et al.* (1999) and are compatible with the recombination rate of 0.68, which indicated that the sex determination region is located distally to the centromere (Mair *et al.*, 1991a).

In Figure 4.3 (a and b), it seems that bivalent 1 may reach full pairing first and then unsynapse in the subterminal and terminal regions. The heteromorphic sex chromosome bivalent behaviour of fully paired, unpaired and paired again is reported in pachytene stage in the rainbow trout by Oliveira *et al.* (1995). However, this interpretation in *O. niloticus* depends on the SC contracting during pachytene stage. On the other hand, bivalent 1 and 2 were longer in the partially unpaired group and bivalent 2 presented a marginal non significance ($P = 0.07$) and this could be interpreted as a stage beginning at late zygotene-early pachytene.

The presence of both subterminal and terminal unsynapsis separated by a paired region in the longest bivalent (Fig. 4.1.d) could be due to an intermediate stage to again reach full synapsis. Under this hypothesis, bivalent 1 remains subterminally and terminally unpaired from late zygotene, then a synapsis forms in the middle of the unpaired region, leaving behind an open loop, a fully paired segment and an unpaired terminal region. The fully paired segment then continues synapsis in both directions, ending in a typical pachytene stage.

It should be noticed that late zygotene is characterized by unpaired terminal regions and interstitial loops in the majority of bivalents as has been described in Chapter 3. During late zygotene stage the terminal unpaired segments are present in most of the bivalents,

being an event that precedes pachytene stage, in which the formation of the SC finished in all bivalents. Thus, the situation in which subterminal and terminal unsynapsis occur only in bivalent 1 (whilst the other bivalents are fully synapsed) can not be misinterpreted with late zygotene stage. Carrasco *et al.* (1999) observed a few unsynapsis events in neomales (XX males) restricted to the central or subterminal regions that could be explained because of a delay in SC formation of the longest bivalent at late zygotene stage. Nevertheless, if delayed synapsis in bivalent 1 were due to size alone, it would be expected to observe terminal unpairing in females or neomales, which does not occur. Therefore, it seems feasible to propose that this subterminal and terminal unsynapsed stage is very short in time.

The previous observations of heteromorphic size in the longest bivalent of XY males were not confirmed in this study. These are still not understood. Two theories have been proposed. The first is a structural polymorphism unrelated to sexual differentiation (Foresti *et al.*, 1993). In fact, through the analysis of mitotic chromosomes, the longest pair does show some size differences in both sexes of tilapias (Prasad and Mana, 1976; Kornfield *et al.*, 1979; Majumdar and McAndrew, 1986). The second one is related to the presence of a mechanism of axial equalization as occurs in birds (Carrasco *et al.*, 1999). In this case the fully-paired LEs of the longest bivalent represents non-homologous association of the terminal region of the sex chromosome, which would increase the stability of this bivalent by the end of pachytene stage. However, axial equalization is not occurring in bivalent 1 when unsynapsed terminal LEs have the same size prior to undergoing synapsis. Furthermore, if axial equalization did occur in bivalent 1, then the lateral element that is "adjusting" or "equalising" should be thicker as occur in birds and

the guppy (Traut and Winking, 2001). Sex chromosomes from the rainbow trout show heteromorphic size and later on they present a complete synapsis without axial equalization (Oliveira *et al.*, 1995). Axial equalization has been observed in birds (Solari, 1992; 1994). There is no clear evidence that axial equalization occurs in the Nile tilapia. Therefore, further observations are required to understand this phenomenon.

The absence of unpaired terminal regions in bivalent 1 in the Nile tilapia in the study of Liu and Yu (1991) could be due to chance (e.g. the 15 cells analysed from one individual fish were all in middle-late pachytene stage) or, and perhaps less likely, the fish could have been a genetic female sex-reversed to male (as can occur for example if fry are reared at high temperatures: Baroiller *et al.*, 1995a).

4.6. Conclusions

Three pachytene stages can be proposed in *O. niloticus*:

(1) Early pachytene, in which bivalent 1 remains subterminally and terminally unpaired from late zygotene, followed by the beginning of synapsis of the unpaired lateral elements in the middle leaving a loop behind, a fully paired segment, and an unsynapsed terminal region. This stage is not presented in the XX or YY genotypes.

(2) Middle pachytene in which synapsis is complete although X-Y crossing over has been avoided previously. This seems to take the longest time. Heteromorphism is not clear in this stage.

(3) Late pachytene where synapsis remains and a progressive shortening of the whole set of chromosomes occurs.

CHAPTER 5

Identification of putative sex chromosomes in *Oreochromis aureus*

5.1. Introduction

5.1.1. Sex determination in the blue tilapia

In common with most species of fish, the blue tilapia has no visible mitotic sex chromosomes (Majumdar and McAndrew, 1986; Klinkhardt *et al.*, 1995). Comparison of the mitotic karyotype of *O. niloticus*, *O. mossambicus* and *O. aureus* does not indicate that there are any significant differences in chromosome number or morphology between the species (Majumdar and McAndrew, 1986). However, analysis of the sex ratios of gynogenetic fish and the progeny sex ratios produced by sex-reversed fish has demonstrated that sex determination is primarily genetic. Female *O. aureus* have been shown to be heterogametic (WZ) and males homogametic (ZZ), with sex primarily determined by a single locus located distally to the centromere (Guerrero, 1975; Penman *et al.*, 1987; Penman, 1989; Avtalion and Don, 1990; Mair *et al.*, 1991b). However, there is evidence of other genetic factors affecting sex determination, and Mair *et al.* (1991b) proposed a multi-factorial mechanism of sex determination, with an underlying primary mechanism of female heterogamety. Mair *et al.* (1991b) proposed a genetic explanation for aberrant ratios of 3:5 and 1:3 (male: female) from single pair matings of normal broodstock, in which a Mendelian inheritance of an autosomal recessive gene, F,f, epistatic to the major sex –determining loci W Z. When this gene is homozygous for the recessive allele it causes the fish to develop as a female. However, they noticed other unexplained sex ratios. Avatalion and Don (1991) observed the existence of gynogenetic males among first, second and third generations of gynogenetic fish. From the sex ratios

of the offspring observed in the second-generation (F2), two different maternal types were proposed, the first type gave rise to both gynogenetic males and females and the second type to females only. The authors proposed a model of genetic recombination in which crossover between sex determining genes (WY) and the centromere enables three different genotypes among gynogenetic offspring. From gametes in which a single crossover occurs, only WY would be obtained. From gametes in which double crossover occurs or in which crossing-over does not occur, WW females and YY males would be produced in 1: 1 sex ratio. Thus, in contrast to both *O. niloticus* and *O. mossambicus*, in which sex is determined by a XX female / XY male system (Jalabert *et al.*, 1974; Penman *et al.* 1987; Varadaraj and Pandian, 1989; Mair *et al.*, 1991b), *O. aureus* presents a primarily WZ female/ ZZ male sex determination system.

5.1.2. Female to male sex reversal of the blue tilapia and neofemales reproduction: two constraints for aquaculture in this species?

The blue tilapia unfortunately, apart from the complexity of its sex determination system, it is not an easy species to sex reverse from female to male. McAndrew and Majumdar (1989) ranked the species *O. mossambicus* > *O niloticus* > *O. aureus* for their response to 17 α methyltestosterone (MT). They found only 65 % males after a treatment of 40 days with 40 mg/kg of MT. However, there are reports of 98 % males (Guerrero, 1975) and 83-99 % males (Mélard, 1995). It may be worth noting that this species also exhibits temperature sex determination, which increases the proportion of male progeny

at higher temperatures. For example, the maintenance of *O. aureus* fry at 34°C during sexual differentiation resulted in 97.8% males (Desprez and Melard, 1998).

The use of neofemales (ZZ females) is an alternative technique for producing monosex genetically male tilapia offspring. Desprez *et al* (1995) found that the percentage of non-spawing neofemales (F2) was 40 % against 20 % of normal females. On average females spawned three times whilst neofemales spawned once. The breeding crosses between a ZZ neofemale and a ZZ male gave on average 91 % males (range: 60-100 %).

These two factors described above are definitely constraints for fish farmers to produce monosex offspring of the blue tilapia and create less satisfactory results when compared with Nile tilapia monosex male culture.

5.1.3. Cytogenetic meiotic analysis in tilapia

As mentioned in Chapter 4, the analysis of meiotic chromosomes using the high power resolution of the electron microscope potentially represents a more powerful method of identifying differences between the karyotypes of different species or sexes (Wise *et al.*, 1987). In the introduction to Chapter 4, it was mentioned that bivalent 1 in males of *O. niloticus* presents an unpaired region associated with heterogamety, which appears to be the location of the major sex determining loci in the *O. niloticus* XX/XY system (Foresti *et al.*, 1993; Carrasco *et al.*, 1999). Therefore, the Nile tilapia has a monofactorial system where morphological undifferentiated sex chromosomes are operating in *O. niloticus* as a primary "decision" of sex differentiation. From the results of Chapter 4 it was also suggested that bivalent 1 of *O. niloticus* remains subterminal-terminally unsynapsed from

late zygotene, early pachytene and then fully synapses in middle pachytene. As can be seen, cytogenetic meiotic analysis has given important sex determination information in the Nile tilapia. Since in *O. aureus*, the presence of sex chromosomes has not previously been investigated by analysis of the meiotic chromosomes, this Chapter covers this topic.

5.2. Aims

The aims of this research were:

(1) To analyse pairing in the synaptonemal complex during pachytene stage in *O. aureus*

(2) To determine if unpaired lateral elements are related to chromosomal sex determination in females.

5.3. Materials and methods

5.3.1. Species studied

The blue tilapia used in this study came from populations derived from the Lake Manzala, Egypt. This species are held in the Tilapia Reference Collection at the Institute of Aquaculture, University of Stirling.

5.3.2. Fish used for SC analysis

Six *O. aureus* males, six females and six neofemales were analysed through meiosis in Chapter 3, section 3.3.2. Synaptonemal complex analyses of these fish at pachytene stage are described in this Chapter.

5.3.3. Sex reversal

5.3.3.1. Preparation of diets for sex reversal

Diets were prepared according to the method given in Chapter 2 (section 2.1.3).

5.3.3.2. Male to female sex reversal (ZZ neofemales)

The fry were sex-reversed with 17 ∝ Ethynylestradiol (Sigma-Aldrich) at 150 mg/kg, during 35 days from first feeding (Melard, 1995).

5.3.3.3. Female to male sex reversal (WZ neomales)

Fry were fed from first feeding with doses of 30 mg/kg for 30 days, 40 mg/kg for 40 days and 50 mg/kg for 60 days with 17∝-Methyltestosterone (MT) (Sigma-Aldrich). Fry were also fed from first feeding with 50 mg/kg for 60 days with 17 ∝ Ethynyltestosterone (ET) (Sigma-Aldrich).

5.3.4. Preparation of SC spreads

Fish were killed with anaesthesia (by immersion in a 0.01% benzocaine solution) followed by destruction of the brain before dissection. Preparation of SC spreads for observation in the transmission electron microscope were made accordingly with the method described in Chapter 2 (section 2.1.4).

5.3.5. Statistical analysis for lengths of bivalent 1 and 2

The lengths of the SC bivalents of each pachytene stage spermatocyte and oocyte were measured for subsequent analyses. As mentioned in Chapter 4, it is difficult to stage SC

spreads into early and late pachytene, but bivalent length may reduce during pachytene due to contraction. Using the length of the second largest bivalent as a measure independent of bivalents showing unpairing (see Results), statistical tests were carried out to compare the nuclei exhibiting unpairing in large or small bivalents with those not exhibiting any unpairing. To test if the extent of pairing was associated with length differences in the bivalents concerned, measurements of the lengths of bivalent 1 were grouped according to whether the bivalent was fully paired or showed any unpaired regions. The bivalent lengths of the two groups were then compared by ANOVA.

The frequencies of unpaired putative sex chromosomes from females and neomales of *O. aureus* were compared by an exact test for 2 x 2 tables. As this was not significant (see Results), female and neomale data were pooled to allow calculation of the overall frequency at which unpairing in the two types of putative sex chromosomes could be observed (Sokal and Rohlf, 1987; Zar, 1996; Bailey, 1997).

5.4. Results

5.4.1. Male to female sex reversal (ZZ neofemales)

Table 5.1 shows the results of sex reversal to female using150 mg/kg of 17 ∝ Ethynylestradiol for 35 days. The treated group showed a sex ratio significantly different from 1:1.

Table 5.1. Male sex reversal to female using150 mg/kg of 17 ∝ Ethynylestradiol for 35 days.

	Female	Male	% female	χ^2
Control	14	10	58	0.66
Ethynylestradiol	28	0	100	28.00*

* significant at $P < 0.001$.

5.4.2. Female to male sex reversal (WZ neomales)

Table 5.2 shows the results of sex reversal to male using 30 mg/kg of 17∝-Methyltestosterone (MT) for 30 days and 40 mg/kg MT for 40 days. The controls and treated groups showed a sex ratio not significantly different from 1:1.

Table 5.2. Sex reversal to male using 30 mg/kg of 17∝-Methyltestosterone (MT) for 30 days and 40 mg/kg MT for 40 days.

	Female	Male	% male	χ^2
30 mg/kg				
Control	16	13	45	0.31 ns
MT	19	12	39	1.58 ns
40 mg/kg				
Control	12	8	40	0.81 ns
MT	10	7	41	0.52 ns

ns: not significantly different from 1:1 sex ratio

Table 5.3 shows the results of sex reversal to male using 50 mg/kg of 17∝-Methyltestosterone (MT) for 60 days and 50 mg/kg of 17 ∝ Ethynyltestosterone (ET) for 60 days. In the case of MT, the control and treated groups showed a sex ratio not significantly different from 1:1. In the case of ET, the treated group showed a significant difference.

Table 5.3. Sex reversal to male using 50 mg/kg of 17∝-Methyltestosterone (MT) for 60 days and 50 mg/kg of 17∝- Ethynyltestosterone (ET) for 60 days.

	Female	Male	% male	χ^2
Control	11	6	35	1.47
MT	4	11	73	3.26
Control	9	13	59	0.72
ET	2	9	81	4.45 *

* = significant at $P < 0.05$.

5.4.3. Progeny testing of fish for SC analysis

Table 5.4 shows the progeny sex ratios from males and neomales. None of the twelve fish produced sex ratios significantly different from a 1:1 sex ratio, expected from ZW x ZZ crosses (however, these sex ratios are re-examined in the Discussion in the light of the results of the SC analysis).

Table 5.4. Progeny sex ratios from males (crossed with a normal female) and neomales (crossed with a ZZ neofemale) used for SC analysis. See Discussion for further interpretation of sex ratio results.

Group	Individual	Number of progeny sexed	Number of females	Number of males	Proportion of females (%)	χ^2 comparison with 1:1 sex ratio	χ^2 comparison with 3:1 sex ratio
Males	1	30	17	13	56.7	0.53	5.38*
	2	31	14	17	45.2	0.29	14.72***
	3	30	15	15	50.0	0.00	10.00**
	4	38	20	18	52.6	0.11	10.14**
	5	30	18	12	60.0	1.20	3.60
	6	33	20	13	60.1	1.48	3.65
Pooled		**192**	**104**	**88**	**54.2**	**1.33**	**44.44*** **
Neomales	1	28	18	10	64.3	2.28	1.71
	2	40	26	14	65.0	3.60	2.00
	3	40	24	16	60.0	1.60	4.50*
	4	40	18	22	45.0	0.40	19.20***
	5	32	18	14	56.3	0.50	6.00*
	6	36	16	20	44.4	0.44	17.92***
Pooled		**216**	**120**	**96**	**55.5**	**2.67**	**43.56*** **

*= significant at $P < 0.05$; ** = significant at $P < 0.01$; *** = significant at $P< 0.001$.

5.4.4. Identification of putative sex chromosomes

No unpaired bivalents were observed in the SC spreads of males. The most common observations of unpaired chromosomes in females and neomales were a subterminal unpaired region or a loop in the longest bivalent (a terminal unpaired region was observed once) and an unpaired or partially paired small sized bivalent. Table 5.5 summarises these observations. Although no individual SC spread showed more than one bivalent with some degree of unpairing, one of the six females and three of the six neomales (a total of four out of twelve individuals studied) showed both types of unpairing phenomena (in large and small bivalents) in different SC spreads.

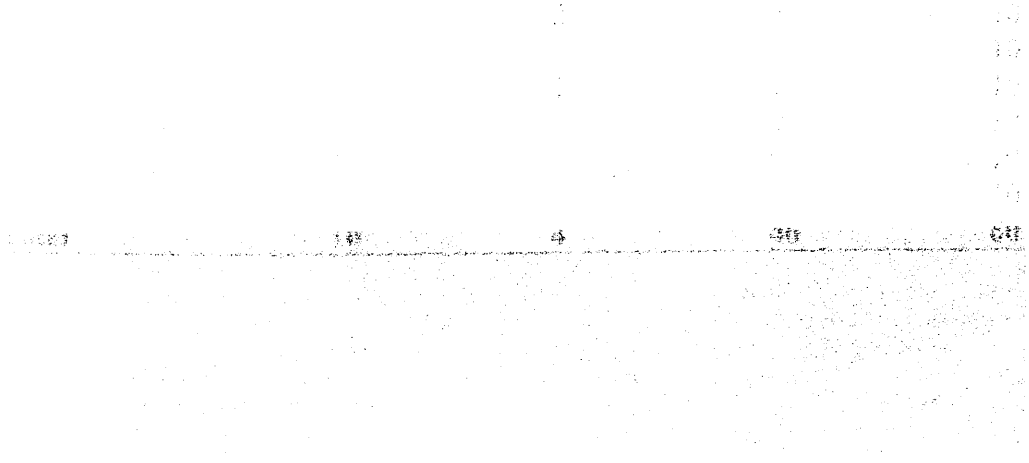


Table 5.5. Number of observations of unpairing in bivalent 1 and in a small bivalent in pachytene stage synaptonemal complexes in *O. aureus*.

Group	Individual	Number of observations of unpairing in bivalent 1	Number of observations of unpairing in a small bivalent	Number of observations of full pairing in all bivalents	Total number of SC spreads analysed
Males	1	0	0	10	10
	2	0	0	10	10
	3	0	0	10	10
	4	0	0	10	10
	5	0	0	10	10
	6	0	0	10	10
Total		**0**	**0**	**60**	**60**
Females	1	0	0	2	2
	2	0	0	2	2
	3	0	1	1	2
	4	0	0	2	2
	5	0	2	0	2
	6	1	1	0	2
Total		**1**	**4**	**7**	**12**
Neomales	1	2	2	6	10
	2	2	1	7	10
	3	2	1	7	10
	4	1	0	9	10
	5	1	0	9	10
	6	2	0	8	10
Total		**10**	**4**	**46**	**60**

5.4.5. Description of putative sex chromosomes at pachytene stage

A total of 60 meiotic pachytene nuclei from 6 progeny tested males of the blue tilapia were analysed. None of them presented unpaired lateral elements (LEs) in pachytene stage regarding the male homogametic sex. A total of 12 meiotic pachytene nuclei from 6 females were analysed, from which seven nuclei showed a complete synapsis in all the bivalents. However, a completed or partially unpaired small size subtelocentric bivalent was observed in 4 nuclei, which represented 33% of all nuclei analysed, and one nucleus (8%), showed a subterminal unpaired region in the longest bivalent. The LEs of each bivalent presented the same size. The meiotic behaviour of the small size bivalent begins with completely unsynapsed LEs (3 nuclei) (Fig. 5.1. a-f). Then it goes to a progressive synapsis of the LEs from the centromere in both directions, ending first in the short arm and further on in the other extreme of the long arm, showing by this time half of the bivalent synapsed (Fig. 5.1. g), until synapsis is completed. In the longest bivalent only subterminal unsynapsis was observed (Fig. 5.1. h).

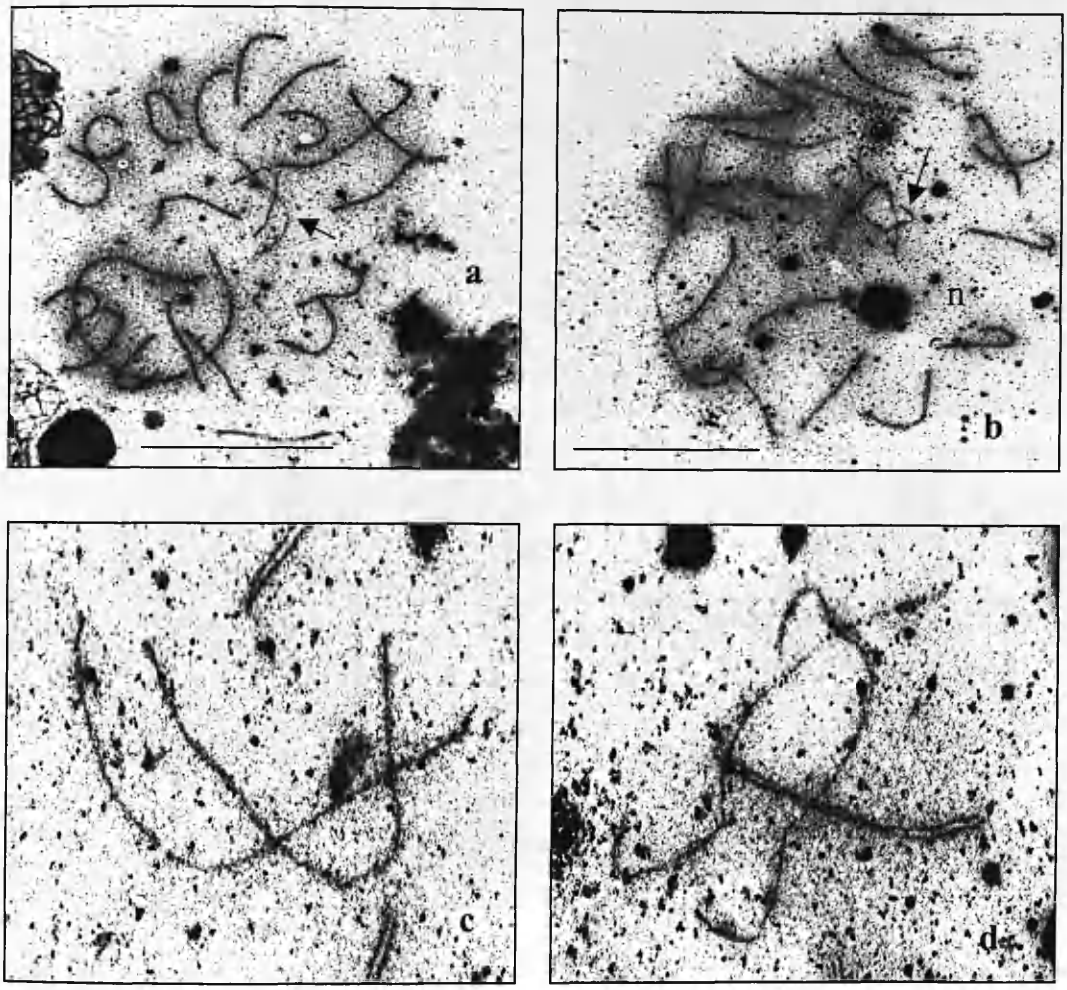


Figure 5.1. (a) SC spread from *O. aureus* female 5 (see Table 5.5) showing unpairing in a small bivalent. 5.1. (c) Detail of unpaired bivalent inverted 90 ° (from 5.2. a). 5.1. (b) SC spread from *O. aureus* female 6 (see Table 5.5) showing unpairing in a small bivalent. 5.1. (d) Detail of unpaired bivalent inverted 90 ° (from 5.1. b). Arrows point unpaired LEs. n: nucleolus. Bar represents10 μm.

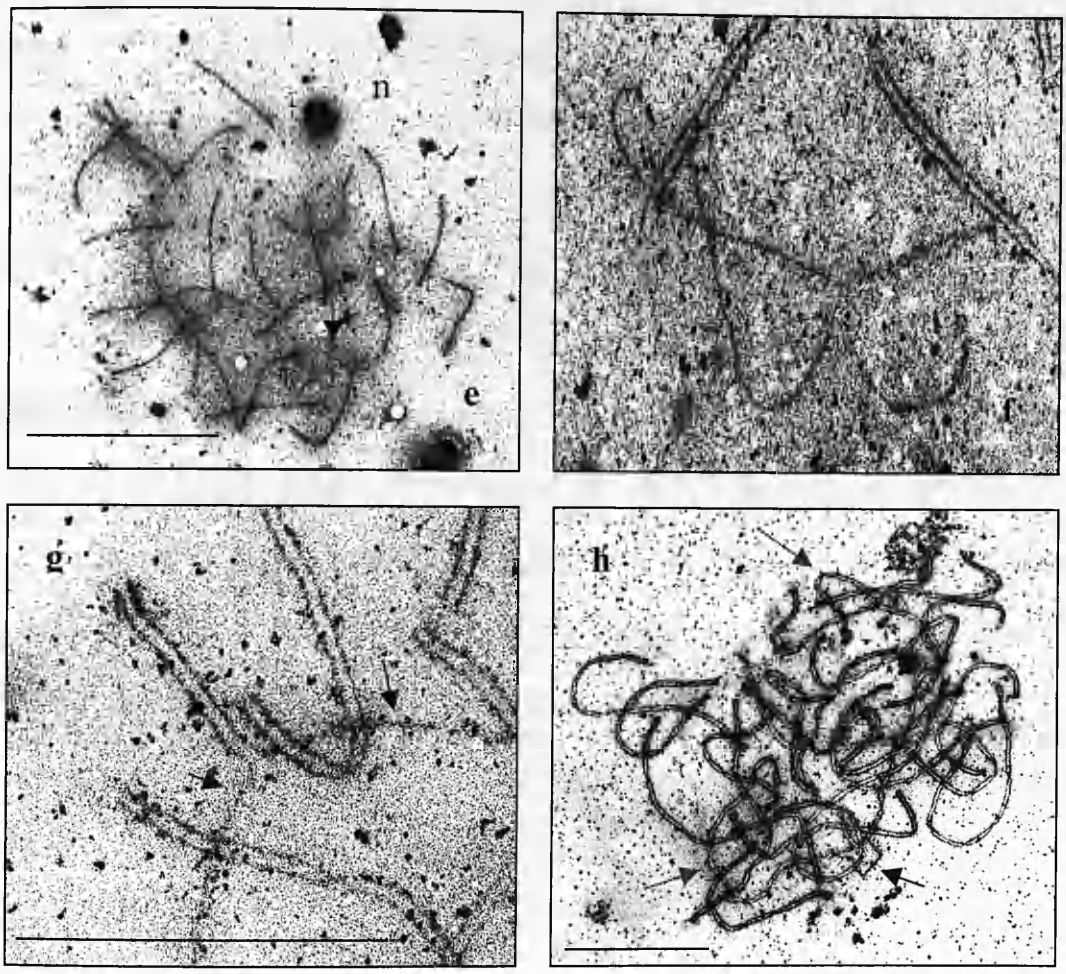


Figure 5.1. (continued):

(e) SC spread from *O. aureus* female 3 (see Table 5.5) showing unpairing in a small bivalent. 5.1. (f) Detail of unpaired bivalent inverted 90 ° (from 5.2. e). 5.2. (g) SC spread from *O. aureus* female 5 (see Table 5.5) showing unpairing in a small bivalent. 5.1. (h) SC spread from *O. aureus* female 6 (see Table 5.5) showing unpairing in bivalent 1 (black arrow), grey arrows indicate bivalent 1. Arrows point unpaired LEs. n: nucleolus. Bar represents10 μm.

A total of 60 meiotic pachytene nuclei from 6 progeny tested WZ neomales were analyzed. Neomale 1 showed two nuclei with a small size bivalent partially unsynapsed (not showed) and two nuclei with a subterminal unsynapsed region in the longest bivalent (Fig 5.2. a, b, c). Neomale 2 showed one nucleus with a small size bivalent partially unsynapsed (Fig. 5.2. d, e), one nucleus with a subterminal unsynapsed region (Fig. 5.2. f, g) and one with a terminal unsynapsed region (Fig. 5.2. h). Neomale 3 showed one nucleus with a partially unsynapsed small size bivalent (Fig. 5.2. i, j) and two nuclei with a subterminal unsynapsed region in the longest bivalent (Fig. 5.2. k, l, m, n). Neomale 4 showed one nucleus with a medium and subterminal unsynapsed region in the longest bivalent (not showed). Neomale 5 showed one nucleus with a subterminal loop in the longest bivalent (Fig. 5.2. o, p). Finally male 6 showed two nuclei, one with a subterminal unsynapsed region in the longest bivalent (not showed), and the other with a subterminal loop in the longest bivalent (Fig. 5.2. q).

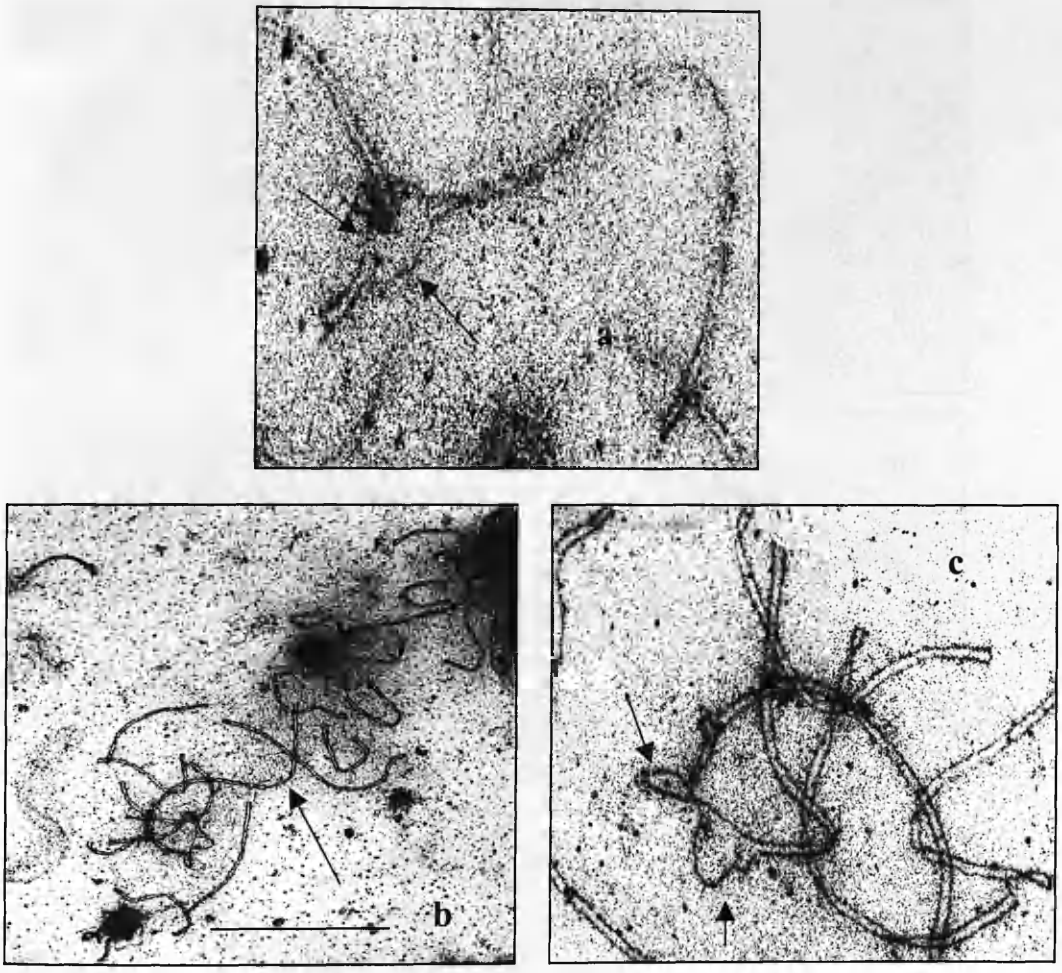


Figure 5.2. *O. aureus* neomale 1 (see Table 5.5). (a) SC spread showing a subterminal unsynapsed region in the longest bivalent. (b) SC spread with black arrow pointing to the longest bivalent. (c) Detail of unpaired subterminal region inverted 90 ° (from b) of bivalent 1. Bar represents10 μm.

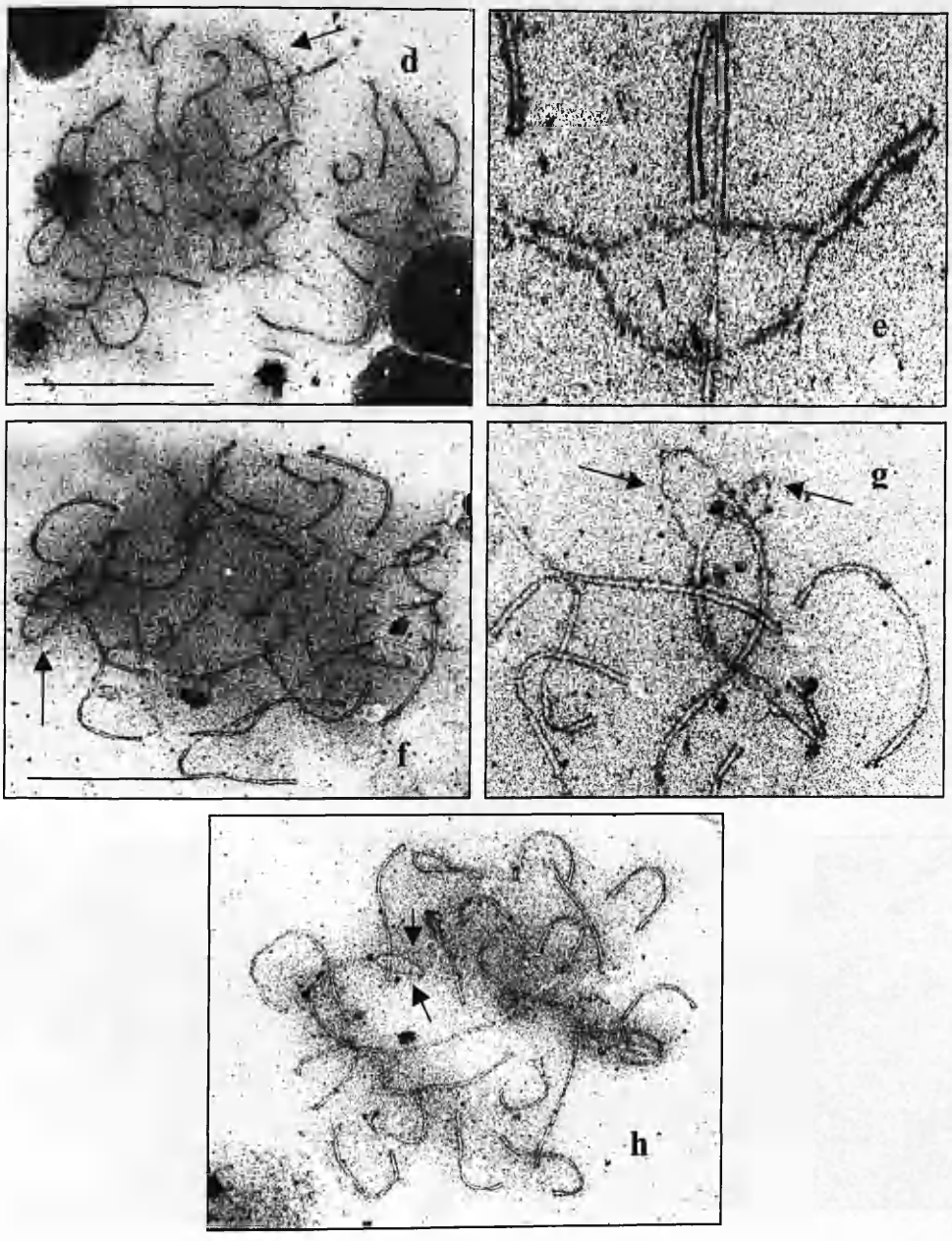


Figure 5.2. (continued):

O. aureus neomale 2 (see Table 5.5). (d) SC spread showing unpairing in a small bivalent. (e) Detail of unpaired bivalent inverted 90 ° (from d). (f) SC spread showing a subterminal unpairing in bivalent 1. (g) Detail of unpaired bivalent inverted 90 ° (from f). (h) SC spread showing a terminal synapsis in bivalent 1. Arrows point unpaired LEs. Bar represents10 μm.

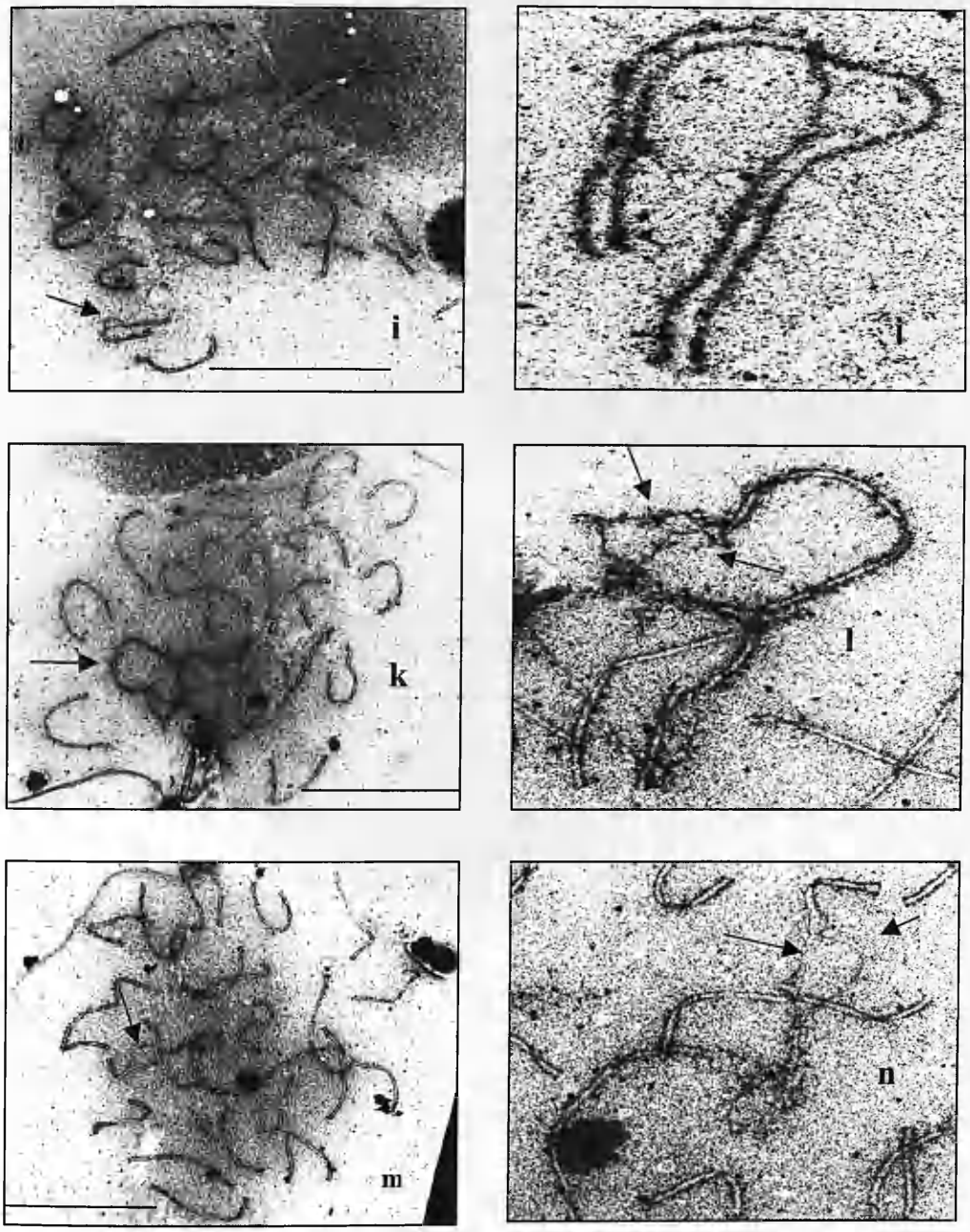


Figure 5.2. (continued):

O. aureus neomale 3 (see Table 5.5). (i) One nucleus with a partially unsynapsed small size bivalent amplified and inverted 90 ° in (j). (k and m) Two nuclei with a subterminal unsynapsed region in the longest bivalent amplified and inverted 90 ° in (l) and (n) respectively. Arrows point unpaired LEs. Bar represents10 μm.

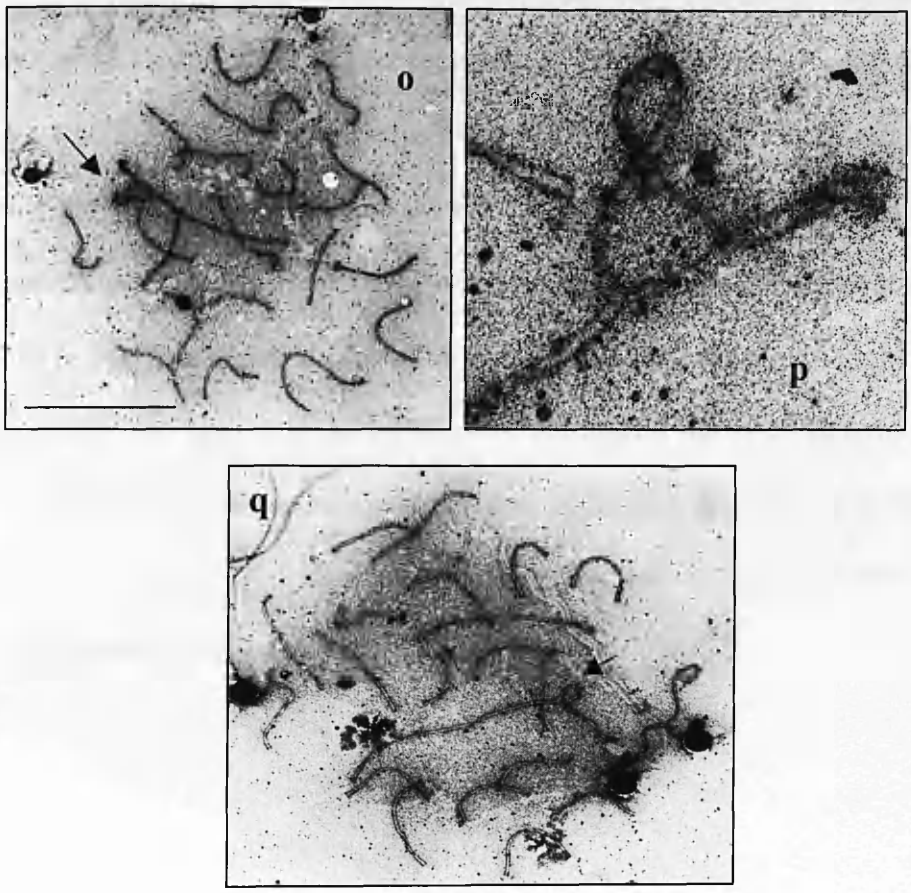


Figure 5.2. (continued):
O. aureus neomale 5 (see Table 5.5). (o) SC spread showing a subterminal loop (arrow) in the longest bivalent amplified and inverted 90 ° in (p). Bar represents10 μm.

Figure 5.2. (q) *O. aureus* neomale 6 (see Table 5.5). SC spread showing a subterminal loop (arrow) in the longest bivalent. Bar represents10 μm.

There were no significant differences between the mean lengths of bivalent 2 from SC spreads with fully paired bivalents and those with one bivalent showing some degree of unpairing ($F = 0.751$, $P = 0.39$ (n.s.) for neomales; $F = 0.032$, $P = 0.86$ n.s. for females). There was a significant difference between the lengths of fully paired copies of bivalent 1 and partially unpaired copies of this bivalent in neomales (mean values 15.64 and 19.20 μm respectively in neomales, $F = 9.449$, $P = 0.00$ significant; the data from females was not analysed as only one unpaired copy of bivalent 1 was observed).

Determination of the position in the karyotype of the small unpaired bivalent by ranking all of the individual bivalent measurements within each nucleus, showed that in females it is either the third, fifth or sixth bivalent whilst in neomales it is either the fifth or sixth position in decreasing order of size (Figure 5.3.).

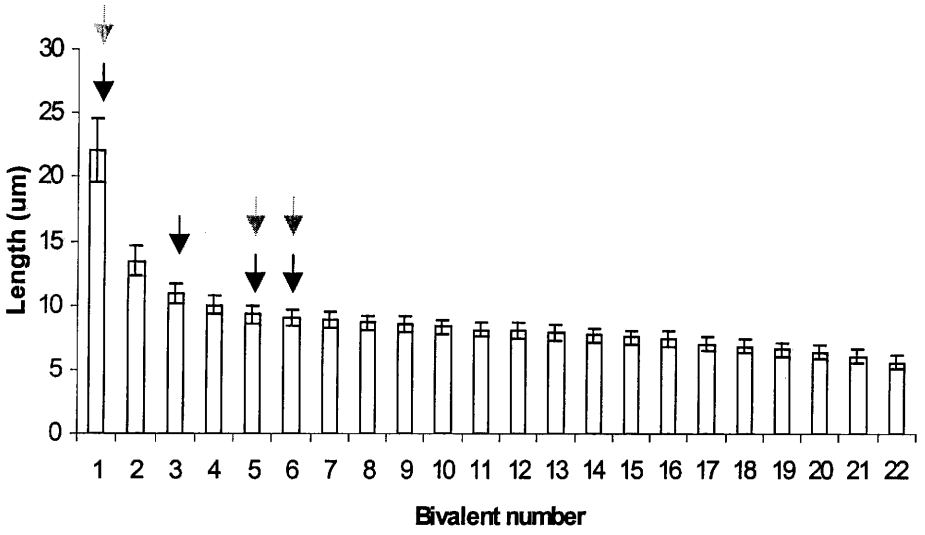


Figure 5.3. Lengths (mean ± S.D.) of each bivalent in the pachytene stage synaptonemal complex of *O. aureus* females. Within each SC spread, bivalents were measured individually, ranked by length, then labelled 1-22. Arrows denote the position (rank) in the karyotype of unpaired bivalents (putative sex chromosomes) observed in females (black arrows) and neomales (grey arrows).

To check if the observed frequencies of unpairing differed between females and neomales, a 2 x 2 table of the frequencies of pairing and unpairing was analysed for each sex chromosome (Table 5.6 a and 5.6 b). This indicated that the frequency of unpairing did not differ between the females and neomales (at $P<0.01$), allowing the frequencies to be combined, yielding overall frequencies of 11/72 for the longest bivalent and 8/72 for the small size bivalent. Although unpaired large and small bivalents were not observed in the same nucleus (see above and Table 5.5), they were observed in different nuclei from the same individuals in the sample examined in this study. Calculation of the expected probability of observing both regions of unpairing within the same nucleus, on the basis of the overall frequencies of the two events, showed that with 72 observations, this would only be expected to happen approximately once (calculated number of expected observations = 0.86) (Table 5.6 c).

Table 5.6 a. Comparison of frequencies in bivalent 1:

	Unpaired	Paired	Total
Females	1	11	12
Neomales	10	50	60
Total	11	61	72

Formula:

$\{(12!)\,(60!)\,(11!)\,(61!) / (72!)\,(1!)\,(11!)\,(10!)\,(50!)\} \times 100 \times 2 = 60$ ($P = 0.60$ ns).

Table 5.6 b. Comparison of frequencies in small bivalent:

	Unpaired	Paired	Total
Females	4	8	12
Neomales	4	56	60
Total	8	64	72

Formula:

$\{(12!)\ (60!)\ (8!)\ (64!) / (72!)\ (4!)\ (8!)\ (4!)\ (56!)\ \}\ 100 \times 2 = 4$ ($P = 0.04$; significant at $P<0.05$, not significant at $P<0.01$).

Both frequencies were added obtaining 11/72 for the longest bivalent and 8/72 for the small size bivalent. Here it is considered that each putative sex chromosomes event is independent from each other.

Because both putative sex chromosomes were not observed at the same time in any of the pachytene nuclei of females and neomales, the expected probability to see both putative sex chromosomes in one pachytene nucleus was obtained as follows:

Table 5.6c. Observed and expected frequencies of sex chromosomes.

	Observed frequencies	Expected frequencies
Longest bivalent (L)	11/72 = 0.153	L(1-S) = 0.136
Small size bivalent (S)	8/72 = 0.111	S(1-L) = 0.094
	(1-L)(1-S) = 0.752	(1-L)(1-S) = 0.782
	(L)(S) = 0.016	(L)(S) = 0.012
TOTAL	1.00	1.00

This means that the probability to observe one nucleus having both putative sex chromosomes at the same time would be:

(0.012)(72) = 0.86

i.e. 1 in 72 observations, which is a very low probability, which could explain the absence of such observations in this study.

5.5. Discussion

Analysis of the SCs in the homogametic males did not reveal the presence of any unpaired regions, while in contrast, analysis of the heterogametic females and neomales revealed the existence of two separate regions of unpairing (Figures 5.1. and 5.2.). Both types of unpairing were observed in four out of the twelve individuals analysed, although not in the same SC spreads. The first type of unpairing, in the subterminal region of the longest bivalent, is similar to that found in the closely related species *O. niloticus* (Foresti *et al.*, 1993; Carrasco *et al.*, 1999). However, in *O. niloticus* this region is associated with the heterogametic XY male genotype rather than the WZ female genotype as is the case in *O. aureus*. That these chromosomes are homologous, as suggested by karyotype analysis (Majumdar and McAndrew, 1986), has been confirmed by the hybridisation of *O. niloticus* chromosome 1 paint to chromosome 1 of *O. aureus* (Campos-Ramos *et al.*, 2001). It is therefore intriguing that this same chromosome appears to be associated with both XX / XY and WZ / ZZ sex determination systems in such closely related species. The second region of unpairing present in one of the small bivalents is also intriguing, and suggests that two separate pairs of sex chromosomes exist in *O. aureus*. No such observations have been made in other fish species.

In order to investigate if the observations of putative sex chromosomes as unpaired LEs correspond to an early pachytene stage, the lengths of the second longest chromosome (bivalent 2) were measured in SC spreads showing either fully paired bivalents or one bivalent with some degree of unpairing. As no significant difference was found between these groups in females or neomales, it seems that the size of chromosomes is not related

to the pairing behaviour of the putative sex chromosomes. However, it has not been clearly demonstrated that the size of the bivalents is an indicator of progress through pachytene, so it is still possible that putative sex chromosomes show unpairing in early pachytene and complete synapsis later.

It is not possible to clearly identify the small-unpaired bivalent to an individual chromosome pair. The ranking of unpaired small bivalents in the karyotype, according to the bivalent measurements, showed that in females it ranked as the third, fifth or sixth bivalent whilst in neomales it was measured in the fifth or sixth position in decreasing order of size (Figure 5.3.). However, this does not provide a precise identification because: (i) the standard deviations from the third to the twenty-second bivalents overlap; and (ii) the significantly larger mean length of the partially unpaired copies of bivalent 1 than the fully paired copies suggests that the length of the unpaired small bivalent may be artefactually increased relative to the other, fully paired, small bivalents in the same SC spreads. The method of construction of the data for Figure 5.3 (calculating the means of values with similar ranks in each SC spread) may also produce artefactual differences in size among bivalents 3 to 22, which cannot be independently identified.

The existence of the unpaired region in bivalent 1 of *O. niloticus* has been used to suggest that the X and Y chromosomes are in the initial of stages of divergence (Foresti *et al.*, 1993; Carrasco *et al.*, 1999). In *O. aureus*, the fact that the small bivalent, when observed, showed in most cases zero or minimal pairing suggests that this part of the primary sex determining mechanism in the *O. aureus* is well established and may indicate that high levels of sequence divergence exist between the two copies of this chromosome.

These data suggest that *O. aureus* females have two pairs of sex chromosomes, (although it has not been conclusively demonstrated that only one small size bivalent is involved), implying that the primary sex determination genes are located on different chromosomes. If only two sex determining regions are assumed, one present in the longest bivalent and the other one in a small sized bivalent, then *O. aureus* should have a WZ/ZZ, W'Z'/Z'Z' sex chromosome system. By denoting W and Z as the sex chromosomes in the longest bivalent, and W' and Z' as the sex chromosomes in the small size bivalent, then WZ W'Z' females would produce the following gametes: WW', WZ', ZW' and ZZ'. Therefore, the resulting progeny genotypes would be WZ W'Z', WZ Z'Z' and ZZ W'Z' (females) and ZZ Z'Z' (males). Because of the dominance of the W chromosome, this cross would yield a sex ratio of 3 females: 1 male. However, the WZ Z'Z' and ZZ W'Z' females would yield sex ratios of 1:1 in crosses with ZZ males.

Testing of the sex ratios observed in progeny from the neomales used in this study showed that none of these differed significantly from 1:1 (Table 5.4.). Testing of the sex ratios from the neomales showed that in two of the three fish in which both types of SC unpairing was observed (neomales 1 and 2), these were also not significantly different from 3:1 (females: males), the expected sex ratio from a WZ W'Z' x ZZ Z'Z' cross. However, the sex ratios from progeny testing of the males also showed a similar pattern (not significantly different from 1:1 but two individual crosses also not significantly different from 3:1). As these fish were all killed after progeny testing to obtain the SC spreads, it was not possible to carry out further progeny testing (e.g. crossing to different test fish, or increasing sample sizes). Thus these results do not provide clear support for the model suggested for two pairs of independently segregating sex chromosomes, but do

perhaps suggest a slight excess of females. Mair *et al.* (1991b) observed an excess of females in a population of *O. aureus* derived from earlier generations of the one used in this study.

Another problem with the model suggested above is that a WZ W'Z' x ZZ Z'Z' cross would only give one quarter of the progeny with the WZ W'Z' genotype, while no other crosses would result in this genotype. The frequency of the WZ W'Z' genotype could thus only decline. Under this model, the two females which gave rise to the females and neomales respectively for SC analysis (Table 5.4.) would both have been WZ W'Z', as both types of unpairing were observed among their offspring. This would appear to be unlikely on a probability basis from a population consisting of a mixture of all three female genotypes, with a declining proportion of the WZ W'Z' genotype. Thus another model may have to be developed to account for the apparent involvement of two pairs of chromosomes involved in sex determination. That model could be suggested under the condition that there is a natural sex reversal of some females into males (neomales), Thus, the three genotypes could be maintained in a population.

Under this model it is suggested that the male genotype is ZZ Z'Z' and then, the variations of genotypes are a consequence of two pairs of sex chromosomes in females segregating independently during meiosis. These genotypes are:

(1) WZ W'Z'

(2) WZ Z'Z' and

(3) ZZ W'Z'

When crossing with a ZZ Z'Z' male, genotype (1) gives 3:1 female:male:

Gametes; female: W'W', WZ', W'Z and ZZ' ; male: ZZ'

Genotype proportions: 1 WZ W'Z' : 1 WZ Z'Z' : 1 ZZ W'Z' : 1 ZZ Z'Z'

When crossing with a ZZ Z'Z' male, genotype (2) gives 1:1 female:male:

Gametes; female: WZ' ; male: ZZ'

Genotype proportions: 1 WZ Z'Z' : 1 ZZ Z'Z'

When crossing with a ZZ Z'Z' male, genotype (3) gives 1:1 female:male:

Gametes; female: ZW' ; male: ZZ'

Genotype proportions: 1 ZZ W'Z' : 1 ZZ Z'Z'

Assuming that all three female genotypes are equally sensitive to sex reversion we have in Table 5.7 crosses between neomales and females.

Table 5.7. Crosses between neomales and females in *O. aureus*.

Neomale (1) x female (1) gives 15:1 female:male: WZ W'Z' x WZ W'Z' Gametes: WW', WZ', ZW', and ZZ' Genotypes: $2^n = 2^4 = 16$ Genotype proportions: Current genotypes in the population: 4 WZ W'Z' 2 WZ Z'Z' 2 ZZ W'Z' 1 ZZ Z'Z' New genotypes in the female population: 2 WW W'Z' 2 WZ W'W' 1 WW W'W' 1 WW Z'Z' 1 ZZ W'W'	Neomale (1) x female (2) gives 7:1 female:male WZ W'Z' x WZ Z'Z' Gametes: neomale :WW', WZ', ZW', and ZZ' ; female: WZ' ZZ' Genotypes: $2^n = 2^4 = 16$ Genotype proportions: Current genotypes in the population: 2 WZ W'Z' 2 WZ Z'Z' 1 ZZ W'Z' 1 ZZ Z'Z' New genotypes in the female population: 1 WW Z'Z' 1 WW W'Z'	Neomale (1) x female (3) gives 7:1 female:male WZ W'Z' x ZZ W'Z' Gametes: neomale :WW', WZ', ZW', and ZZ' ; female: ZW' ZZ' Genotypes: $2^n = 2^4 = 16$ Genotype proportions: Current genotypes in the population: 2 WZ W'Z' 2 ZZ W'Z' 1 WZ Z'Z' 1 ZZ Z'Z' New genotypes in the female population: 1 WZ W'W' 1 ZZ W'W'
Neomale (2) x female (2) gives 3:1 female:male WZ Z'Z' x WZ Z'Z' Gametes: WZ' and ZZ' Genotypes: $2^n = 2^4 = 16$ Genotype proportions: Current genotypes in the population: 2 WZ Z'Z' 1 ZZ Z'Z' New genotype in the female population: 1 WW Z'Z'	Neomale (2) x female (3) gives 3:1 female:male WZ Z'Z' x ZZ W'Z' Gametes: neomale : WZ', ZZ' ; female: ZW' ZZ' Genotypes: $2^n = 2^4 = 16$ Genotype proportions: Current genotypes in the population: 1 WZ W'Z' 1 WZ Z'Z' 1 ZZ W'Z' 1 ZZ Z'Z' New genotypes in the female population: None	Neomale (3) x female (3) Gives 3:1 female:male ZZ W'Z' x ZZ W'Z' Gametes: ZW' and ZZ' Genotypes: $2^n = 2^4 = 16$ Genotype proportions: Current genotypes in the population: 2 ZZ W'Z' 1 ZZ W'W' 1 ZZ Z'Z' New genotypes in the female population: 1 ZZ W'W'

In summary, the three original female genotypes would be conserved through generations via neomales in the population. The female genotypes WW W'W', WW W'Z' and WZ W'W' are rare genotypes within the population and the genotypes WW Z'Z' and ZZ W'W' are less rare. However these rare or less rare genotypes when crossed with genetic males give:

Rare genotypes:

WW W'W' x ZZ Z'Z' = 100 % WZ W'Z' genotype (1)

WW W'Z' x ZZ Z'Z' = 50 % WZ W'Z' (genotype 1) and 50 % WZ Z'Z' (genotype 2)

WZ W'W' x ZZ Z'Z' = 50 % WZ W'Z' (genotype 1) and 50 % ZZ W'Z' (genotype 3)

less rare genotypes:

WW Z'Z' x ZZ Z'Z' = 100 % WZ Z'Z' genotype (2)

ZZ W'W' x ZZ Z'Z' = 100 % ZZ W'Z' genotype (3)

Therefore the three original genotypes are also conserved through the crosses of these rare genotypes with genetic males. There is no evidence that low temperatures could sex reverse a male into female, but if could be the case, the progeny would be 100 % or nearly (due to some autosomal influences) males.

This is the first report in which primary sex determination in fish (and maybe in the animal kingdom) appears to be achieved by two separate pairs of sex chromosomes. It appears to be different from those that present a multiple gonosomal system (rearrangements involving sex chromosomes and autosomes, see Chapter 1, section 1.2.6.3.4) and also different from those having a "three-factor" sex determining system

(reviewed by Solari, 1994): in these systems sex at the level of the individual is still determined by only one pair of sex-determining factors. *O. aureus* could be one of the rarest cases of sex determination system in vertebrates, in which sex genes are operating in two different sex chromosomes. The net result of this system appears to skew the overall progeny sex ratios towards females, unlike most sex determining systems which tend to produce balanced sex ratios (Fisher, 1930).

5.6. Conclusions

1) Putative sex chromosomes in WZ females and WZ neomales were observed as a subterminal unpaired region or a loop in the longest bivalent and an unpaired or partially paired small bivalent.
2) No unpaired regions were observed in SC spreads from ZZ males, confirming the female as the heterogametic sex.
3) It is suggested that there are two pairs of sex chromosomes in the heterogametic sex of *O. aureus*.
4) The model of two pairs of sex chromosomes in the heterogametic sex of *O. aureus* supports the skewed sex ratios to females reported in the literature.

CHAPTER 6

SEX DETERMINATION IN *Oreochromis mossambicus*

6.1. Introduction

6.1.1. Sex determination in the Mozambique tilapia

This species has been the subject of sex reversal and induced gynogenesis for sex determination studies (Penman *et al.*, 1987), cytogenetics studies (Majumdar and McAndrew, 1986; Liu and Yu, 1991) and the production of monosex-male populations for culture (Varadaraj and Pandian, 1989; Pandian and Varadaraj, 1990).

6.1.2. Sex reversal

Sex reversal by hormone treatment in tilapia is well established, with it being more than three decades since Clemens and Inslee (1968) succeeded in obtaining 95-100% males in *O. mossambicus* by treatment with 10-40 mg/kg of 17 $\propto$ Methyltestosterone (MT) during the 69 days from first feeding. In addition to this female to male sex reversal, they observed that some male individuals presented external characteristics, such as coloration and genital papilla, but had female gonads. More recently, several authors have carried out experiments dealing with the hormone concentration and the timing of feeding (see McAndrew, 1993; Pandian, 1993 for reviews). However, in the case of *O. mossambicus*, it is not know why similar doses and feeding times produce different or contradictory results and why complete masculinisation is not always achieved.

Reviewing the literature it is clear that Pandian and Varadaraj (1988) have identified the minimum period (11 days) and hormone concentration (5 mg/kg) required achieving

100% masculinisation. However, in general terms the literature as a whole suggests that a longer treatment (30-60 days) and a higher hormone concentration (>30 mg/kg) can ensure a better masculinisation rate (Clemens and Inslee, 1968; Guerrero 1979; Macintosh *et al.*, 1985; Das *et al.*, 1987; McAndrew and Majumdar, 1989; Basavaraja *et al.*, 1991).

A further problem is that of paradoxical feminisation, a phenomenon that has been reported three times. Nandeesha *et al.* (1990) observed that almost complete masculinisation of *O. mossambicus* was obtained by treatment with between 0.5 and 0.75 mg/kg of mibolerone and paradoxical feminisation combined with sterility by treatment with between 1 and 50 mg/kg. Basavaraja *et al.* (1991) found that with different MT doses (50 to 150 mg/kg) a 100% of males or a range of intersexes, a high percentage of feminisation or sterility could be obtained with results dependent on the size of the fry and the hormone concentration. In this report 50 mg/kg gave 60 % of males, 26 % of females and 14 % of intersex; 75 mg/kg gave 29 % males, 45 % females and 26 % sterile fish; 100 mg/kg gave 100 % males; and 150 mg/kg gave 0 % males, 58 % females and 42 % sterile. Finally, Nakamura (1975) reported paradoxical feminisation with a very high dose of 1 g/kg of MT.

6.1.3. Cytogenetic meiotic chromosomes analysis

As for *O. niloticus*, *O. aureus* and other tilapia species, *O. mossambicus* does not present differentiated mitotic sex chromosomes (Majumdar and McAndrew, 1986). The synaptonemal complex analysis in males of *O. mossambicus* studied with the high power

resolution of the electron microscope showed that there are not unpaired bivalents or heteromorphism during pachytene stage (Liu and Yu, 1991).

6.1.4. Induced gynogenesis

Penman *et al.* (1987) obtained only female meiotic gynogenetics in *O.niloticus* and *O. mossambicus*. However, in some XX females of *O. niloticus*, male meiotic gynogenetics and mitotic gynogenetics have been observed (Mair *et al.*, 1991a; Hussain *et al.*, 1994; Müller-Belecke and Hörstgen-Schwark , 1995; Sarder *et al.*, 1999). In the literature, there is no indication of the presence of male meiotic gynogenetics in *O. mossambicus* from XX females or the presence of males from crosses between XX males (neomales) and genetic XX females.

According to Varadaraj and Pandian (1989), a recombination rate of zero for the sex determining region was obtained from meiotic gynogenetic progeny of XY neofemales of *O. mossambicus*. This was calculated from sex ratio data; only XX females and YY males were produced through gynogenesis. This suggests that sex genes in this species are located in a region near to the centromere where the probability of recombination is low, or there is recombination suppression along the entire chromosome arm.

In the population of Mozambique tilapias used by Varadaraj and Pandian (1989), the production of YY males through gynogenesis was a valuable technique to obtain only males for culture, after crossing YY males with normal XX females. Because no recombination occurred, this technique allowed the production of YY males in the F2 generation, instead of the F3 (by a normal cross between an XY male and a XY

neofemale, and then progeny testing of males at F3). Also, it increased the production of YY males from 25 to 50 % without the need for progeny testing, and finally, if YY males are sex reversed to females using a synthetic estrogen (YY neofemales), then the production of YY males can be achieved generation to generation.

6.2. Aims

The aims of this research were:

Sex reversal:

1) To analyse the effects of treatment period and hormone concentration on the proportions of males and intersexes.

Cytogenetics:

2) To analyse pairing in the synaptonemal complex during pachytene stage in females, neofemales and males of *O. mossambicus*.

Gynogenesis:

3) To induce gynogenesis in XY neofemales of *O. mossambicus* to produce meiotic gynogenetics.

4) To analyse if there is recombination between the X and Y chromosomes.

5) To estimate the location of sex locus (loci) on their chromosome.

6.3. Materials and methods

6.3.1. Species studied

The Mozambique tilapia individuals used in this study derived from the River Zambezi, Zimbabwe. Broodstock of this species are held at the Institute of Aquaculture, University of Stirling.

6.3.2. Preparation of diets for sex reversal

Diets were prepared according to the protocol given in Chapter 2, section 2.1.3.

6.3.2. 1. Female to male sex reversal in the Mozambique tilapia (XX neomales)

Diets for hormonal masculinization of tilapia were supplemented with 20, 30, 40 or 50 mg/kg of Methyltestosterone (MT) or 50 mg/kg of Ethynyltestosterone (ET).

6.3.2. 2. Male to female sex reversal in the Mozambique tilapia (XY neofemales)

Diets for hormonal feminization of tilapia were supplemented with 150 mg/kg of Diethystilbestrol (DES), (Pandian and Varadaraj, 1990).

6.3.2. 3. Laboratory conditions for sex reversal and statistics employed

As recommended by Pandian and Varadaraj (1988), 7 days post-hatching (dph) fry were randomly assigned to 6 l plastic aquaria (50-70 fry each), and kept with a 12:12 (light:dark) photoperiod, 27 °C of temperature, and aeration. Fry were fed *ad libitum*, with food added 4 to 6 times a day and excess food removed, by siphon, one hour after each feeding. A partial water exchange was made twice a day (morning and evening). After hormone treatment, fish were transferred to 20 l plastic containers until they reached 60-150 dph.

Table 6.1. shows the hormone treatments, concentration (mg/kg), duration of each treatment (days) and the number of replicates for each treatment. Each replicate was Chi-square tested (P = 0.05) and then all the replicates in a given treatment were tested for heterogeneity Chi-square analysis, if they were not significantly different from each other then replicates were pooled.

Table 6.1. Experimental hormone treatments.

Hormone treatments	Concentration (mg/Kg)	Duration of treatment (days) (age in dph)	No of replicates
1) MT	20	16 (7- 22)	3
2) MT	30	30 (7- 36)	4
3) MT	40	40 (7- 46)	4
4) MT	50	60 (7- 67)	2
ET	50	60 (7- 67)	2
DES	150	30 (7- 36)	2

6.3.3. Fish used for SC analysis

Six *O.mossambicus* males, six females and six neofemales were analysed through meiosis in Chapter 3, section 3.3.2. Synaptonemal complex analysis at pachytene stage is described in this Chapter.

6.3.4. Preparation of SC spreads

Fish were killed with anaesthesia (by immersion in a 0.01% benzocaine solution) followed by destruction of the brain before dissection. Preparation of fish SC spreads for the observation in the transmission electron microscope were made accordingly with the protocol showed in Chapter 2; 2.1.4.

6.3.5. Identification of XX genotypes (XX neomales)

The fry were previously sex-reversed with 17 ∝ Ethynyltestosterone (ET) or 17∝-Methyltestosterone (MT) (Sigma-Aldrich) at 50 mg/kg, during 60 days from first feeding (see Chapter 2; section 2.1.3.). The treated *O. mossambicus* males were crossed with normal (XX) females to check if the progeny sex ratio was 1:0 female: male that would indicate a putative neomale or 1:1 that would indicate a normal male.

6.3.6. Identification of XY genotypes (XY neofemales)

Fish fry that were subjected to DES hormone treatment were reared at 27 °C for 7 months. Adult female individuals were crossed preferentially with two XX neomales (previously progeny tested). However, sperm from these neomales was not enough to fertilise more than one female per day and sometimes it was necessary to wait a few days before using them again. Therefore, it was decided to use additionally an XY male (previously progeny tested) to carry on with the identification of the genotypes.

6.3.7. Gynogenesis

There are two methods in the literature for the production of meiotic gynogenetics in *O. mossambicus*. One is reported from Penman (1989) the second from Varadaraj and Pandian (1989) and Varadaraj (1990), summarised as follows:

		Penman (1989)*	Varadaraj and Pandian (1989) Varadaraj (1990)
UV	UV dose rate	No indicated	254 nm; 420 $\mu W/cm^2$
	Sperm dilution	0.2 ml of sperm and 1.8 ml of Cortland salt solution	No indicated
	Duration of UV treatment	2 min	7-10 min
Heat Shock*	Time after fertilisation for heat shock	5 min	2.5 min
	Heat shock	39.0- 40.1 °C	42.0 °C
	Duration of heat shock	3 min	3 min

* The heat shock used by Penman (1989) was based on the data of Chourrout and Itskovich (1983).

These two methods were established within the conditions of each experimental laboratory, therefore, some preliminary adjustments were made at the Genetics Aquarium of the Institute of Aquaculture.

Because the quantity of sperm from the neomales was not enough for the induction of gynogenesis, it was decided to use an XY male.

Initially, thick sperm was obtained from the male urogenital papilla using a capillary tube and immediately placed into 1 ml of cold Hank's physiological solution. The sperm was checked under the microscope to ensure that it was not activated, and then a drop of water was added to check that the sperm was activated. The concentration of the sperm solution was calculated using a hematocytometer and then diluted with Hank's solution to obtain a sperm concentration between 2.5-3.0 X 10^7 / ml. 2 ml of the diluted sperm were placed in a plastic Petri dish (5 cm in diameter) and then placed on a stirrer below the UV lamp (254nm UV lamp) at a distance at which the radiometer read a dose rate of 260 $\mu W/cm^2$ (Hussain *et al.*, 1993). For the final procedure selected the sperm was UV irradiated for two minutes and 15 seconds, except for female No 38 where sperm was irradiated for 2 minutes.

An ovulated female (identified through swelling of the papilla) was anaesthetised and then stripped gently into a 20 l bucket full of water. The eggs were recovered using a plastic pipette and placed randomly into three plastic petri dishes (12 cm in diameter) with water covering them. The first petri dish was labelled as "control" (50 eggs, except in female no 4 where the control cross was based on 100 eggs), the second as "UV control" (30 eggs) and the third as "diploid gynogenesis" (200 eggs). The control batch was fertilised with normal sperm and the UV control and the "diploid gynogenesis "

batches were fertilised with the irradiated sperm (1 ml each). After 4.5 min the diploid gynogenesis batch was introduced into a re-circulation water bath at 39 °C for 3 min, then the eggs were transferred to a jar incubator with water at 27 °C and placed in the incubation system of the laboratory until they hatched and absorbed their yolk sacs. Finally fry were transferred to 20 l tanks and reared for 6 months.

6.3.7.1. Temperature

To determine the temperature to apply the heat shock (3 min), 40 eggs were placed into each of 7 Petri dishes and labelled as: 1) control; 2) UV control; 3) 38 °C; 4) 39 °C; 5) 40 °C; 6) 41 °C; and 7) 42 °C. This procedure was carried out with three different females.

6.3.7.2. UV exposure time of sperm

To determine the optimum UV exposure time of sperm, a heat shock of 3 min at 39 °C 5 min after fertilisation was employed using only one female. Again, 40 eggs were placed into each of 7 Petri dishes and labelled as: 1) control; 2) 15 sec; 3) 1 min; 4) 2 min 5) 2.5 min; 6) 3 min and 7) 4 min.

6.3.7.3. Time after fertilisation to apply heat shock

There is a big difference reported about the time after fertilisation at which the heat shock should be applied. These are: 2.5 min (Varadaraj and Pandian 1989; Varadaraj

1990) and 5 min (Penman *et al.*, 1987). A gap of 2.5 min between the two may be a "huge time" in terms of the time at which the second meiotic division of the egg takes place. Therefore the timing between fertilisation and heat shock were tested. To determine this, a 2.25-min UV irradiation time of sperm and a heat shock of 3 min at 39 °C were employed using only one female. Again, 40 eggs were placed into each of 8 Petri dishes and labelled as: 1) control; 2) UV control; 3) 1 min; 4) 2 min; 5) 3 min 6) 4 min; 7) 5 min; and 8) 6 min.

6.3.7.4. DNA extraction

After 4 months of rearing meiotic gynogenetics and control fish, a sample of blood was taken from each fish of each family. After each fish was anaesthetised, blood was collected from the caudal vein using 23g size sterile needles and 1 ml syringes. The DNA extraction protocol is given in Chapter 2, section 2.1.5.

6.3.7.5. Microsatellites DNA loci

The first fingerprinting approach was the use of microsatellite primers to amplify by PCR DNA fragments that could give some individual profiles, having polymorphic fragments in size (alleles). These microsatellites were screened for all the broodstock (14 females and one male) used for the production of meiotic gynogenetics. Originally, a large number of microsatellites were isolated from *O. niloticus* by Lee and Kocher (1996). Six of those microsatellites were chosen by Sobolewska (1999) to make cross-

species amplification in various Tilapiine fish species. From those six, only two showed polymorphism in *O. mossambicus* from 26 individuals; UNH207 with two alleles and a heterozygosity of 0.54 and UNH203 with 4 alleles and a heterozygosity of 1.0, giving an average species heterozygosity of 0.77 for these two loci. Therefore these two microsatellites and 4 four more from Lee and Kocher (1996) were tested (Table 6.2). Genotypes were obtained by automated sizing of fluorescently –tagged alleles amplified via the polymerase chain reaction.

6.3.7.5.1. PCR amplification

The PCR amplification protocol is given in Chapter 2, section 2.1.6.

Table 6.2. Microsatellites tested in *O. mossambicus* broodstock from the Tropical Aquarium, Institute of Aquaculture.

Microsatellites	Sequence	Expected PCR product size
UNH157	F: 5'-CCC AAA ACA GTA ACA TTG TAA-3' R:5'-CAT ATA ATA CAC AGG TTA CAT GC-3'	130-134
UNH203	F: 5'-CAC AAA GAT GTC TAA ACA TGT-3' R: 5'-GAA TTT GAC AGT TTG TTG TTT AC-3'	86-107
UNH211	F: 5'-GGG AGG TGC TAG TCA TA-3' R: 5'-CAA GGA AAA CAA TGG TGA TA-3'	130-160
UNH127	F: 5'-TCA ATG GTT CTT ATT ATC TCA-3' R: 5'-CTA TAC ATG ATT GTG CAA TAA ATA A-3'	126-128
UNH228	F: 5'-ACA CCT TCA CAC AAC TAC G-3' R: 5'-GAT AAT AAT GAT AAT GTC GCC T-3'	214-216
UNH207	F: 5'-ACA CAA CAA GCA GAT GGA GAC-3' R: 5'-CAG GTC TGC AAG CAG AAG C-3'	111-117

6.3.7.6. Multilocus DNA fingerprinting

The second approach for fingerprinting was by using the NICE probe 33.15 (Jeffreys *et al.*, 1985). Three families were analysed by fingerprinting: from female no.38 (XX), 4 and 25 (XY).

6.3.7.6.1. Fingerprinting (Jeffreys *et al.*, 1985)

The fingerprinting protocol is given in Chapter 2, section 2.1.7.

6.3.8. Progeny testing of male meiotic gynogenetics

The approach to find the genotypes of the male meiotic gynogenetics was by crossing them with XX females and analysing the progeny F_2 through Mendelian sex ratios. The criterion to identify a genotype was based on the methodology of Mair *et al.* (1997), in which different significance levels are taken into account.

6.4. Results

6.4.1. Sex reversal

The results of the different sex reversal trials are presented in Table 6.3. Sex ratios of all control groups (and replicates) were not significantly different from 1:1 (female:male). Treatment 1 (20 mg/kg, 16 days) resulted in 80 % (significantly different from 1:1) of males and no intersex fish, the highest proportion of masculinisation achieved. Treatments 2 and 3 (20 and 30 mg/kg, 30 and 40 days) resulted in sex ratios not significantly different from 1:1 (female:male) and some intersex fish were observed; 10 % in treatment 2 and 23 % in treatment 3. Treatment 4 showed different results with a low percentage of males (15 and 2%) and a high percentage of intersex fish (19 and 53%). The first replicate of ET resulted in a high proportion of males (78%) whilst the second one presented a low percentage (29%). In both the percentage of intersex fish was high (19 and 17%). Treatment with DES completely reversed males into females in both replicates.

Figures 6.1 shows a graph of the percentages of males and intersex fish from MT treatments. Despite increasing dose rate and duration, treatments beyond 20 mg/kg for 16 days resulted in fewer males and more intersexes.

Table 6.3. Effect of MT, ET, and DES hormones on the sex ratio of *O. mossambicus* fed different doses and times of treatment.

Treatment 1 *O. mossambicus*: 20 mg/Kg/16days (3 replicates)

	Female	Intersex	Male	χ^2 value	Female %	Intersex %	Male %
Control	35	0	43	0.82	55	0	45
	22	0	17	0.64	56	0	44
	25	0	30	0.45	45	0	55
Total	**82**	**0**	**90**	**0.37**	**47**	**0**	**53**
MT	10	0	69	44.0*	13	0	87
	18	0	55	18.75*	24	0	76
	12	0	40	15.07*	23	0	77
Total/mean	**40**	**0**	**164**	**75.37***	**20**	**0**	**80**

* Significant at P< 0.001

Treatment 2 *O. mossambicus*: 30 mg/Kg/30days (4 replicates)

	Female	Intersex	Male	χ^2 value	Female %	Intersex %	Male %
Control	18	0	18	0	50	0	50
	22	0	17	0.64	56	0	44
	32	0	38	0.51	46	0	54
	15	0	21	1.00	42	0	58
Total	**87**	**0**	**94**	**0.27**	**48**	**0**	**52**
MT	16	3	12	0.57	52	10	38
	22	7	19	0.22	45	15	40
	25	1	19	0.81	56	2	42
	15	5	21	1.00	37	12	51
Total/mean	**78**	**16**	**71**	**0.32**	**47**	**10**	**43**

Control *XX* O. niloticus	10	0	0	0	100	0	0
MT XX *O. niloticus*	0	0	10	0	0	0	100

Table 6.3. Continued.

Treatment 3, *O. mossambicus*: 40 mg/Kg/40 days (4 replicates)

	Female	Intersex	Male	χ^2 value	Female %	Intersex %	Male %
Control	11	0	9	0.20	55	0	45
	15	0	18	0.27	46	0	54
	22	0	26	0.33	46	0	54
	31	0	27	0.27	53	0	47
Total/mean	**79**	**0**	**80**	**0.00**	**49**	**0**	**51**
MT	9	6	7	0.25	41	27	32
	14	9	19	0.75	33	21	46
	13	4	8	1.19	52	20	28
	6	5	4	0.20	40	33	27
Total/mean	**42**	**24**	**38**	**0.20**	**40**	**23**	**37**

Treatment 4, *O. mossambicus*: 50 mg/Kg/60 days

	Female	Intersex	Male	χ^2 value	Female %	Intersex %	Male %
Control	16	0	14	0.13	53	0	47
	12	0	18	1.20	40	0	60
Total/mean	**28**	**0**	**32**	**0.26**	**47**	**0**	**53**
MT	21	6	5	9.84*	66	19	15
	18	21	1	15.21**	45	53	2
Total/mean	**39**	**27**	**6**	**24.2***	**54**	**37.5**	**8.5**
ET	1	5	22	19.17**	3	19	78
	19	6	10	2.79	54	17	29
Total	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**
DES***	33	0	0	0	100	0	0
	35	0	0	0	100	0	0
Total/mean	**68**	**0**	**0**	**0**	**100**	**0**	**0**

* significant at P< 0.01
** significant at P< 0.001
*** 150 mg/Kg/30 days
NA: not applicable because the two replicates were significantly different from each other.

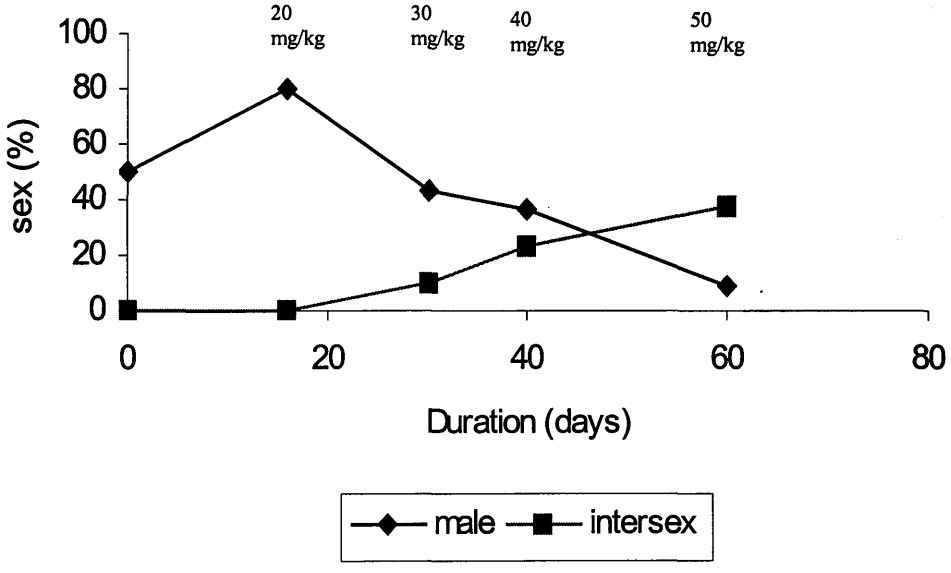


Figure 6.1. Sex reversal to male and intersex fish in the Mozambique tilapia *Oreochromis mossambicus* feeding 17∝ methyltestosterone with different hormone concentrations (mg/kg) and duration of treatment.

6.4.2. Progeny testing of fish for SC analysis

Table 6.4 shows the progeny sex ratios from males in which none of the 6 fish produced sex ratios significantly different from a 1:1 sex ratio and neomales in which all the fish produced sex ratios significantly different from a 1:1 sex ratio, expected from XX x XX crosses.

Table 6.4. Progeny sex ratios from males and neomales (crosses with a normal female) used for SC analysis.

Group	Individual	Number of progeny sexed	Number of females	Number of males	Proportion of females (%)	χ^2 comparison with 1:1 sex ratio
Males	1	35	16	19	45.7	0.25
	2	31	13	18	41.9	0.80
	3	30	17	13	56.6	0.53
	4	36	20	16	55.5	0.44
	5	39	17	22	43.6	0.64
	6	36	19	17	52.7	0.11
Pooled		**207**	**102**	**105**	**49.3**	**0.04**
Neomales	1	30	30	0	100	30*
	2	30	30	0	100	30*
	3	30	30	0	100	30*
	4	30	30	0	100	30*
	5	30	30	0	100	30*
	6	30	30	0	100	30*
Pooled		**180**	**180**	**0**	**100**	**180**

* = significant at $P < 0.001$

6.4.3 Cytogenetics, synaptonemal complex analysis at pachytene stage

A total of 60 meiotic pachytene nuclei from 6 progeny tested males, 60 meiotic pachytene nuclei from 6 progeny tested neomales and 12 meiotic pachytene nuclei from 6 females were analysed. None of them presented unpaired lateral elements in pachytene stage. However, in one male an evidence of an incompatible region in bivalent 1 was observed. In this region, the lateral elements were diffused and differential staining was observed (1 of 60 spreads). The length of this region was about 1/5 (20%) of the total length of the longest bivalent, and it was possibly skewed towards the kinetochore (Fig. 6.2).

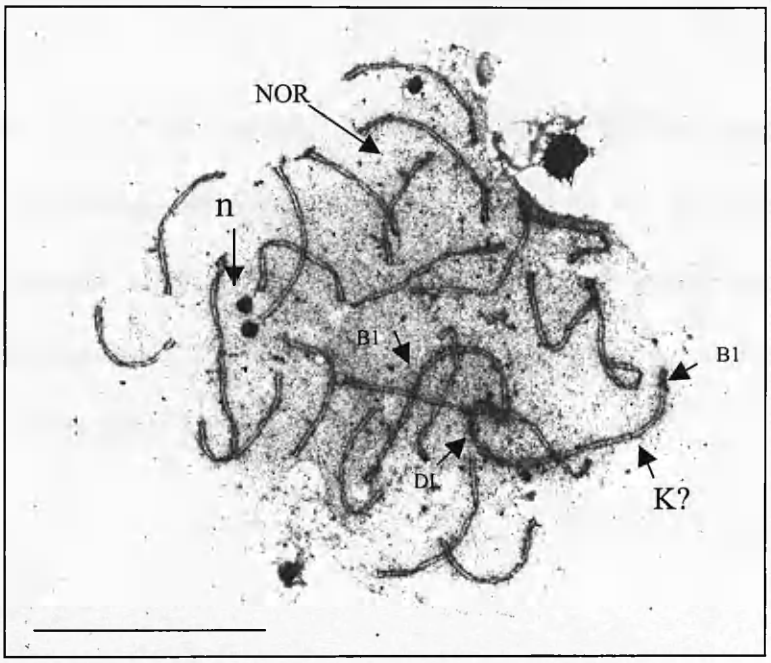


Figure 6.2. Chromosome spread of *O. mossambicus* showing 22 bivalents fully paired. The longest bivalent (B1) presents a region with diffuse lateral elements (DL) (Arrow). (n) nucleoli. (NOR) nucleolar organiser regions. (K?) kinetochore? Bar represents10 μm.

6.4.4. Induced gynogenesis

6.4.4.1. Identification of XY genotypes (XY neofemales)

Among the females (39 individuals), 14 were identified as XY neofemales and 22 were identified as XX females, being very near to the 50 % (χ^2 = 1.78, not significant from 1:1) expected from the sex reversal (Table 6.5.a,b) and 3 individuals were not identified. A frequency distribution of XX/XY genotypes is given in Fig. 6.3 a (from Table 6.5.a) and Fig. 6.3. b (from Table 6.5. b).

Table 6.5a. Results of progeny testing of DES treated females mated with a XY male and identification of XY genotypes.

Female number	Sex ratio ♀:♂	χ^2 against 1♀:3♂ sex ratio	χ^2 against 1♀:1♂ sex ratio	Female (%)	Genotype
2	19:23	9.17 **	0.38	45	XX
3	11:30	0.07	8.80 **	27	XY
4	8:36	1.09	17.82 ***	18	XY
5	80:50	92.56 ***	6.92 **	62	XX
6	18:35	2.27	5.45 *	34	?
10	19:16	16.00 ***	0.26	54	XX
12	8:29	0.22	11.92 ***	22	XY
13	18:24	7.14 **	0.86	43	XX
17	13:15	6.85 **	0.14	46	XX
23	24:20	20.48 ***	0.36	54	XX
24	30:24	26.88 ***	0.67	55	XX
25	9:38	0.85	17.89 ***	19	XY
26	41:66	10.12 **	5.84 *	38	?
27	15:45	0	15.00 ***	25	XY
29	28:36	12.00 ***	1.00	44	XX
36	24:29	11.62 ***	0.47	45	XX
37	20:23	10.61 **	0.21	47	XX

χ^2 against 1♀:3♂ sex ratio	χ^2 against 1♀:1♂ sex ratio
Not significantly different from 1♀:3♂ = XY	Not significantly different from 1♀:1♂ = XX
* significant at P< 0.05 = ?	* significant at P< 0.05 = ?
** significant at P< 0.01 = XX	** significant at P< 0.01 = XY
*** significant at P< 0.001 = XX	*** significant at P< 0.001 = XY

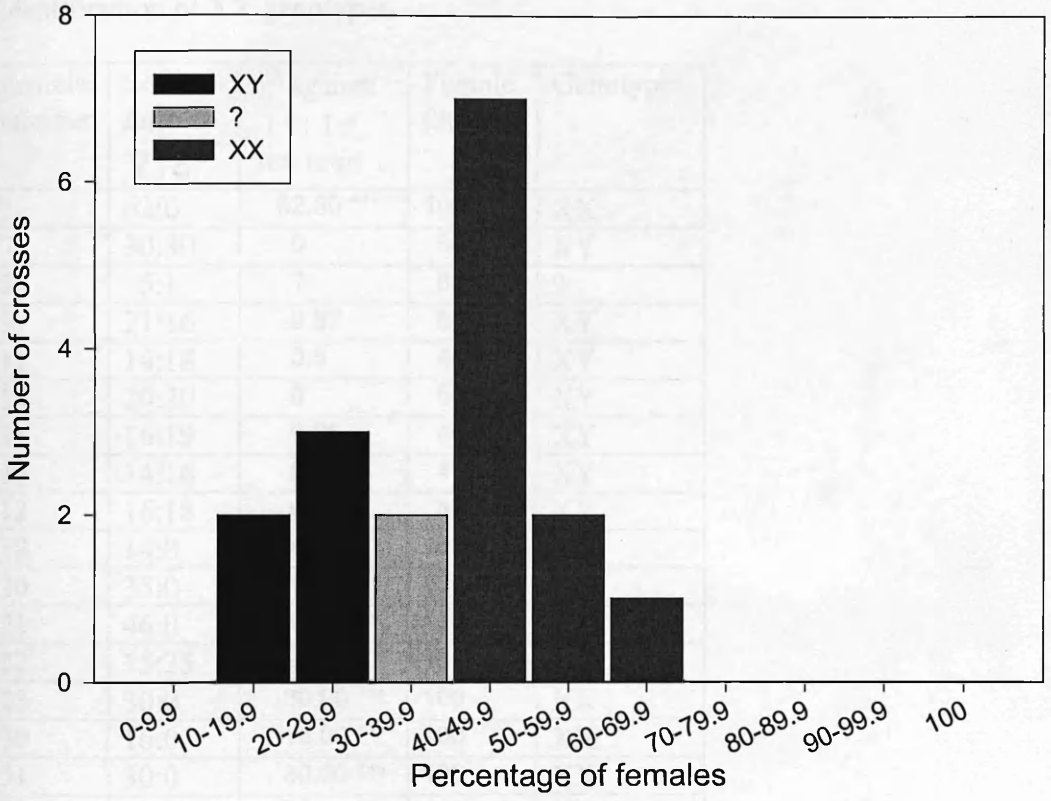


Figure 6.3a. Frequency distribution of XX/XY genotypes after progeny testing of DES treated females mated with a XY male.

Table 6.5b. Results of progeny testing of DES treated females mated with XX males and identification of XY genotypes.

Female number	Sex ratio ♀:♂	χ^2 against 1♀:1♂ sex ratio	Female (%)	Genotype
1	62:0	62.00 ***	100	XX
7	30:30	0	50	XY
8	5:1	?	83	?
9	21:16	0.67	57	XY
11	14:18	0.5	44	XY
14	20:20	0	50	XY
15	16:19	0.26	46	XY
16	14:16	0.13	47	XY
18	16:18	0.12	47	XY
19	14:0	14.00 ***	100	XX
20	35:0	35.00 ***	100	XX
21	46:0	46.00 ***	100	XX
22	15:25	2.5	38	XY
28	30:0	30.00 ***	100	XX
30	16:0	16.00 ***	100	XX
31	30:0	30.00 ***	100	XX
32	32:0	32.00 ***	100	XX
33	34:0	34.00 ***	100	XX
34	14:15	0.34	48	XY
35	42:2	36.00 ***	95	XX
38	30:0	30.00 ***	100	XX
39	29:0	29.00 ***	100	XX

not significantly different from 1♀:1♂ = XY
*** significant at P< 0.001 = XX
? not enough data

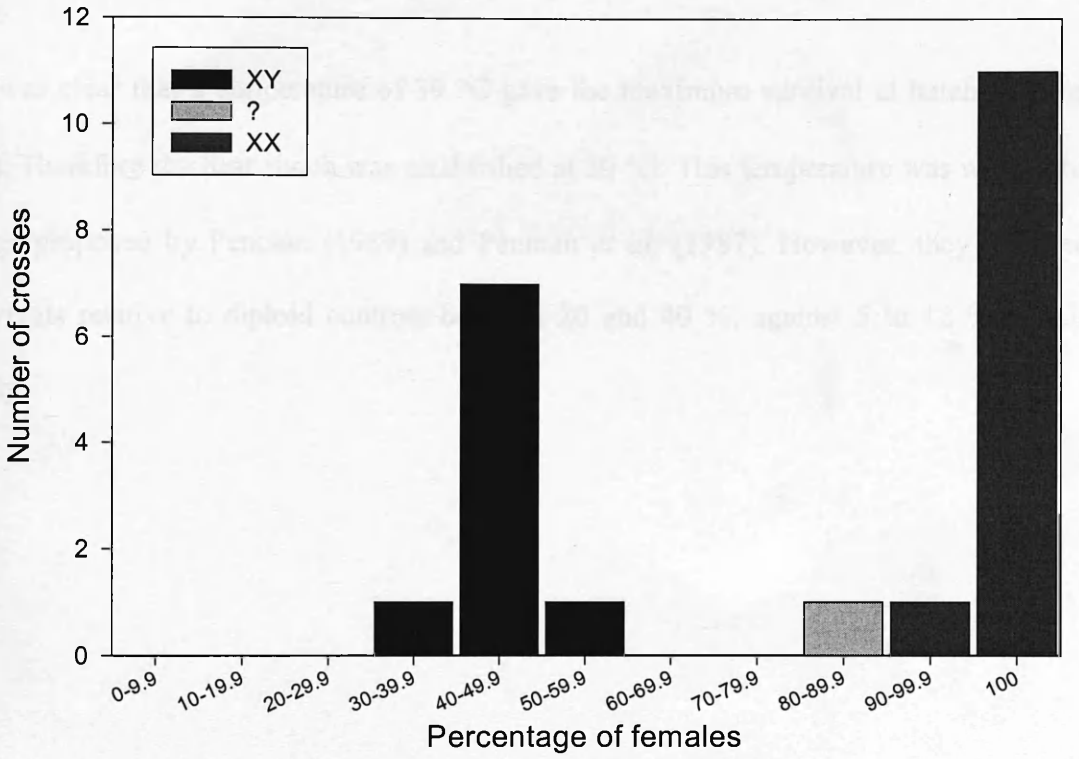


Figure 6.3b. Frequency distribution of XX/XY genotypes after progeny testing of DES treated females mated with XX males.

6.4.4.2. Temperature

It was clear that a temperature of 39 °C gave the maximum survival at hatching (Fig. 6.4). Therefore the heat shock was established at 39 °C. This temperature was within the range proposed by Penman (1989) and Penman *et al.* (1987). However, they reported survivals relative to diploid controls between 20 and 40 %, against 5 to 12 % in this study.

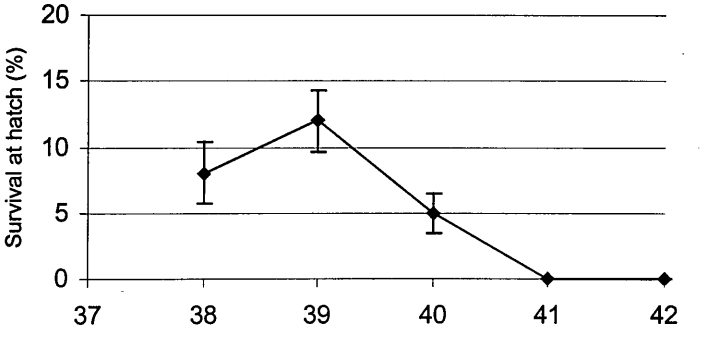


Figure 6.4. The effects of varying the temperature of the heat shock (3 min.) at hatching time. Survival rate is relative to diploid controls.

6.4.4.3. UV exposure time of sperm

The results showed a similar pattern to that found by Penman (1989). Figure 6.5 shows that at 15 seconds there was a drastic fall in survival to zero and a subsequently recuperation with the maximum peak at 2 minutes, followed by another drastic fall to zero at 2.5 minutes.

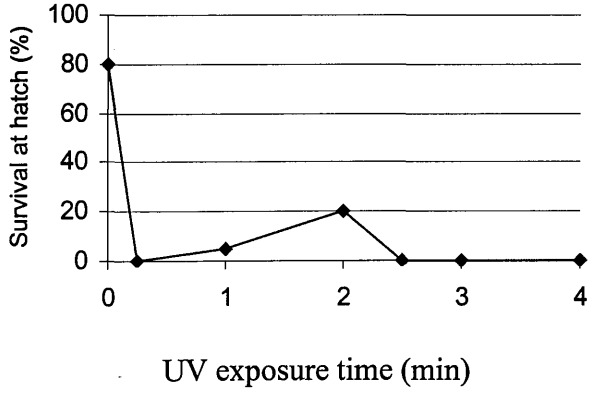


Figure 6.5. The graph shows effect of different UV sperm exposures on egg survival to hatch.

The first batch of meiotic gynogenetics (female no 38, see Table 6.6a) were induced with 2 minutes of UV irradiation of the sperm and 39 °C at the heat shock. After observing the UV control group, it was noticed that 2 fry out of 30 (7 %) looked like a normal fry, whilst the rest presented the typical curved body deformation. All fry die few days after hatching. However, it was decided empirically to increase the UV exposure time by 15 seconds.

6.4.4.4. Time after fertilisation to apply heat shock

The results indicated that between 4 to 5 min after fertilisation resulted in the optimal survival of eggs to introduce them into the heat shock (Fig. 6.6).

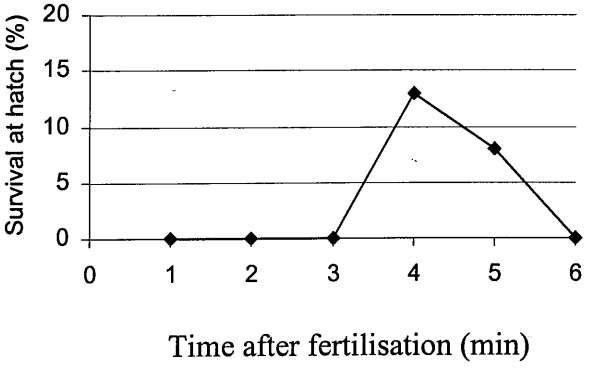


Figure 6.6. Optimisation of time after fertilisation to apply heat shock. Survival rate is relative to diploid controls.

6.4.4.5. Production of meiotic gynogenetics

Once the optimised conditions to induce gynogenesis were established, both genotypes (XX and XY) were subjected to the procedure. The male used for the production of meiotic gynogenetics and the crosses with the control group was a XY male (as mentioned in Materials and Methods).

Tables 6.6a and b show the sex ratios of the meiotic gynogenetics and the control groups. It was only possibly to produce gynogenetic progeny from 9 XX females and 3 XY neofemales. In Table 6.5a, female no 3 was identified as XY, however, it gave 9 females and 1 male meiotic gynogenetic and 1:1 sex ratio in the control group, shown in Table 6.5a. Therefore, this female was crossed again with an XX neomale and gave 15:0 female:male, being finally identified as an XX female. Female no 38 (table 6.6a) was again crossed with an XX male since she gave a considerable quantity of male meiotic gynogenetics, however this cross gave only 38 females, reconfirming the XX genotype.

Table 6.6a shows a total of 53 females (82 %) and 12 males (18 %) obtained from genetic XX females. In contrast, Table 6.6b shows a total of 25 males (100 %) obtained from XY neofemales.

6.4.5. Progeny testing of male meiotic gynogenetics

The males that were produced in the XX and XY groups were then crossed with genetic XX females.Tables 6.7a and 6.7b show the results of the sex ratio of the offspring from those males observed from XX females (Table 6.7a) and XY neofemales (Table 6.7b). A

frequency distribution of XX/XY genotypes is given in Fig. 6.7a (from Table 6.7a) and a frequency distribution of XY/YY genotypes is given in Fig. 6.7b (from Table 6.7b).

Table 6.6a. Survival at yolk sac resorption (y.s.r.) and at 4 months after fertilisation (a.f.) and sex ratio of meiotic gynogenetics and control groups from XX females. Brackets indicate number of fish (n) at each stage. Differential survival (D.F.) was calculated as: total survival at 4 months a.f./total survival at y.s.r.

	Meiotic gynogenetics			Controls		
Female number	Survival at y.s.r. % (n)	Survival at 4 months a.f. % (n)	Sex ratio ♀:♂	Survival at y.s.r. % (n)	Survival at 4 months a.f. % (n)	Sex ratio * ♀:♂
03**	7.5 (15)	5 (10)	9:1	76 (38)	60 (30)	16:14
13	2.5 (5)	2.5 (5)	5:0	44 (22)	34 (17)	07:10
20	3 (6)	1 (2)	2:0	52 (26)	38 (19)	06:13
29	0.5 (1)	0.5 (1)	1:0	82 (41)	64 (32)	16:16
31	8.5 (17)	4.5 (9)	8:1	28 (14)	26 (13)	6:7
35	4 (8)	2.5 (5)	5:0	78 (39)	72 (36)	19:17
36	5 (10)	3 (6)	6:0	62 (31)	38 (19)	07:12
38**	20 (40)	13 (26)	16:10	96 (48)	96 (48)	25:23
39	1 (2)	0.5 (1)	1:0	54 (27)	32 (16)	9:7
Total	104	65	53:12	286	230	111:119
Mean	5.8	3.6		64	51.1	
D.F.	62			81		

* χ^2 ($P < 0.05$) not significantly different from 1 ♀ : 1 ♂

** Females 03 and 38 were crossed again with a XX male and gave 15: 0 and 39:0 female: male sex ratio respectively.

Table 6.6b. Survival at yolk sac resorption (y.s.r.) and at 4 months after fertilisation (a.f.) and sex ratio of meiotic gynogenetics and control groups from XY females. Brackets indicate number of fish (n) at each stage. Differential survival (D.F.) was calculated as: total survival at 4 months a.f./total survival at y.s.r.

	Meiotic gynogenetics			Controls		
Female number	Survival at y.s.r. % (n)	Survival at 4 months a.f. % (n)	Sex ratio ♀:♂	Survival at y.s.r. % (n)	Survival at 4 months a.f. % (n)	Sex ratio * ♀:♂
04	6.5 (13)	4 (8)	0:8	92 (92)	82 (82)	21:61
16	4.5 (9)	3.5 (7)	0:7	76 (38)	70 (35)	08:27
25	6 (12)	5 (10)	0:10	48 (24)	42 (21)	04:17
Total	34	25	0:25	154	138	33:105
Mean	5.6	4.16		72 **	64.6 **	
D.F.	74			90		

* χ^2 ($P < 0.05$) not significantly different from 1 ♀ : 3 ♂

** Survival data from female no 4 was based over 100 eggs. To obtain the mean survivals, the survival of female no 4 was divided by 2 and then added to the other survivals and divided by 3.

Table 6.7a shows that one out of the 12 males was identified as an XY male (1:1 sex ratio), whilst the other 11 males were identified as XX neomales (sex ratio different from 1:1). This is graphically summarised in Fig 6.7a. In contrast, Table 6.7b shows that there were 7 XY individuals (28 %), 6 YY males that gave 100 % males (24 %) and 12 YY males that gave some females in their progeny (48 %). From the last group, 5 out of 12 gave 90 to 96 % of males, 6 out of 12 gave 80 to 86 % of males and 1 out of 12 gave 70 % of males. This is graphically summarised in Fig 6.7b.

Table 6.7a. Sex ratios of the offspring of male meiotic gynogenetics crossed with genetic XX females. These males were produced from XX females (see Table 6.5a).

XX female number	Male offspring number	Sex ratio ♀:♂	χ^2	Female (%)	Genotype
03	01	28:11	7.41 **	72	XX?
31	01	63:05	49.47 ***	93	XX
38	01	32:03	24.02 ***	91	XX
	02	25:34	1.37	42	XY
	03	35:07	18.67 ***	83	XX
	04	28:06	14.24 ***	82	XX
	05	110:03	101.30 ***	97	XX
	06	80:09	56.64 ***	90	XX
	07	147:13	112.20 ***	92	XX
	08	48:02	42.32 ***	96	XX
	09	53:05	39.72 ***	91	XX
	10	92:07	72.98 ***	93	XX
Total	12	741:105		88	

not significantly different from 1♀: 1♂ = XY
** significant at P< 0.01 = ?
*** significant at P< 0.001 = XX

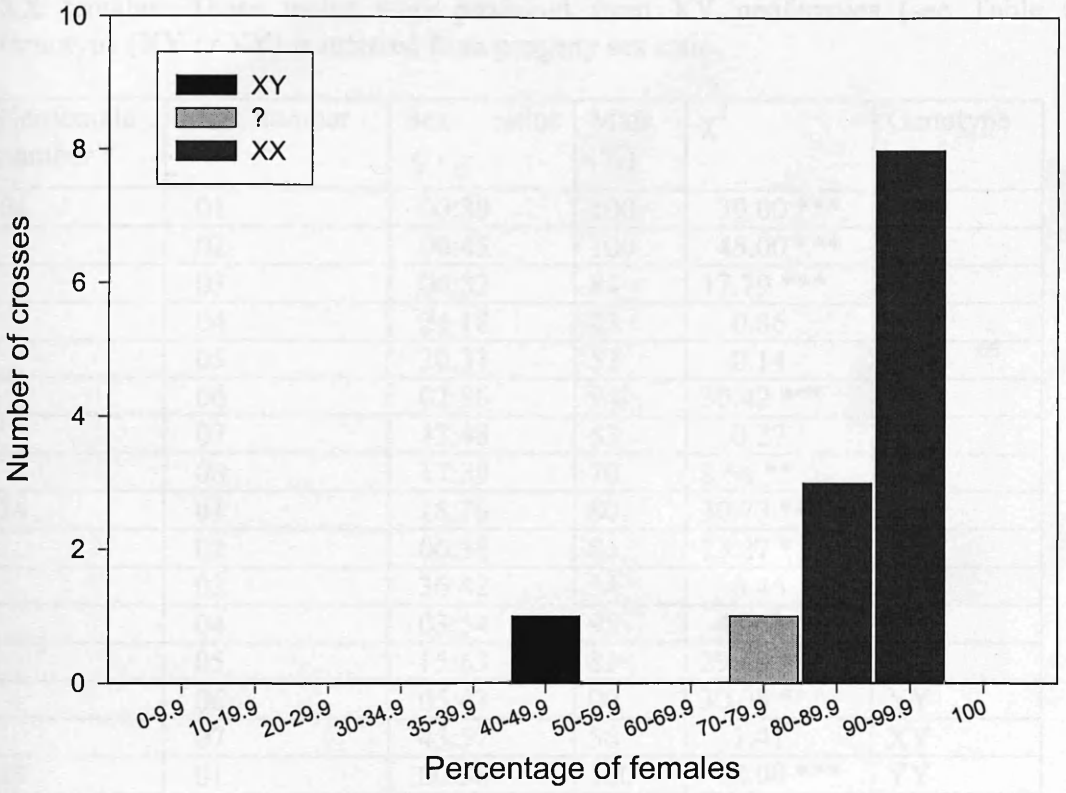


Figure 6.7a. Frequency distribution from progeny testing of gynogenetic males produced from XX females. Shading shows interpretation of these males as XY or XY genotypes.

Table 6.7b. Sex ratios of the offspring of male meiotic gynogenetics crossed with genetic XX females. These males were produced from XY neofemales (see Table 6.5b). Genotype (XY or YY) is inferred from progeny sex ratio.

Neofemale number	Male number	Sex ratios ♀:♂	Male (%)	χ^2	Genotype
04	01	00:39	100	39.00 ***	YY
	02	00:45	100	45.00 ***	YY
	03	06:32	84	17.79 ***	YY
	04	24:18	43	0.86	XY
	05	30:33	52	0.14	XY
	06	02:36	94	30.42 ***	YY
	07	43:48	53	0.27	XY
	08	17:39	70	8.64 **	YY?
16	01	18:70	80	30.73 ***	YY
	02	06:38	86	23.27 ***	YY
	03	36:42	54	0.46	XY
	04	03:54	95	45.63 ***	YY
	05	15:63	81	29.58 ***	YY
	06	05:43	90	30.08 ***	YY
	07	45:57	56	1.41	XY
25	01	00:38	100	38.00 ***	YY
	02	00:44	100	44.00 ***	YY
	03	11:45	80	20.64 ***	YY
	04	03:78	96	69.44 ***	YY
	05	00:37	100	37.00 ***	YY
	06	00:72	100	72.00 ***	YY
	07	32:43	57	1.61	XY
	08	12:14	54	0.15	XY
	09	06:28	82	14.23 ***	YY
	10	04:48	92	37.23 ***	YY
Total	25	318:1104	78		

* not significantly different from 1♀: 1♂ = XY
** significant at P< 0.01 = ?
*** significant at P< 0.001 = XX

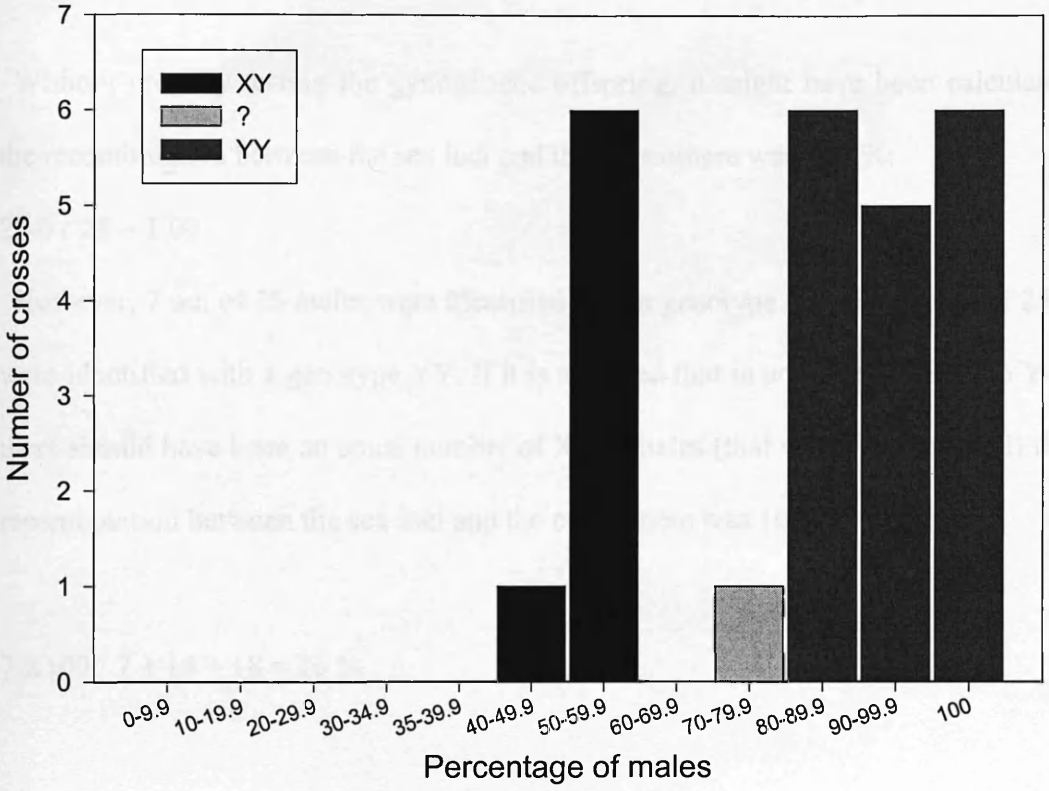


Figure 6.7b. Frequency distribution from progeny testing of gynogenetic males produced from XY neofemales. Shading shows interpretation of these males as XY or YY genotypes.

6.4.6. Recombination rates

Without progeny testing the gynogenetic offspring, it might have been calculated that the recombination between the sex loci and the centromere was 100 %:

25-0 / 25 = 1.00

However, 7 out of 25 males were identified with a genotype XY and 18 out of 25 males were identified with a genotype YY. If it is assumed that in addition to the 18 YY males, there should have been an equal number of XX females (that were not observed) then the recombination between the sex loci and the centromere was 16 %:

7 X100 / 7 + 18 + 18 = 16 %

6.4.7. Microsatellite DNA loci

In Tables 6.8a and 6.8b allele sizes from XX females and XY neofemales respectively were found to share at least one allele from the father, which prevented clear identification of meiotic gynogenetics.

Table 6.8a. Allele sizes found in 8 XX females and one XX male of *O. mossambicus* broodstock.

GENO-TYPE XX		FEMALE No.	FEMALE No.	FEMALE No.	FEMALE No.	FEMALE No.	FEMALE No.	FEMALE No.	FEMALE No.
LOCI	FATHER	38	30	35	29	39	31	13	03
UNH211	147 158	 158	147 158	147	147 158	 158	147 158	 158	147 158
UNH157	129 141	129 141	129 141	129 141	129 141	129 141	129 141	129 141	129 141
UNH203	87 89	87 91	87	 89	 89 91	87 89	 89 91	87 89	87 89
UNH207	114	114	114	114	114 117	114	114	114	114
UNH228	NA	NA	NA	NA	NA	NA	NA	NA	NA
UNH127	NA	NA	NA	NA	NA	NA	NA	NA	NA

NA: no PCR amplification.

Table 6.8b. Allele sizes found in 3 neofemales (which gave meiotic gynogenetics) and one XY male of *O. mossambicus* broodstock.

GENO-TYPE XY		FEMALE No.	FEMALE No.	FEMALE No.
LOCI	FATHER	04	25	16
UNH211	147 158	147 158	147 158	147 158
UNH157	129 141	129 141	129 141	129 141
UNH203	87 89	87 91	87	89
UNH207	114	114 117	114	114
UNH228	NA	NA	NA	NA
UNH127	NA	NA	NA	NA

NA: no PCR amplification

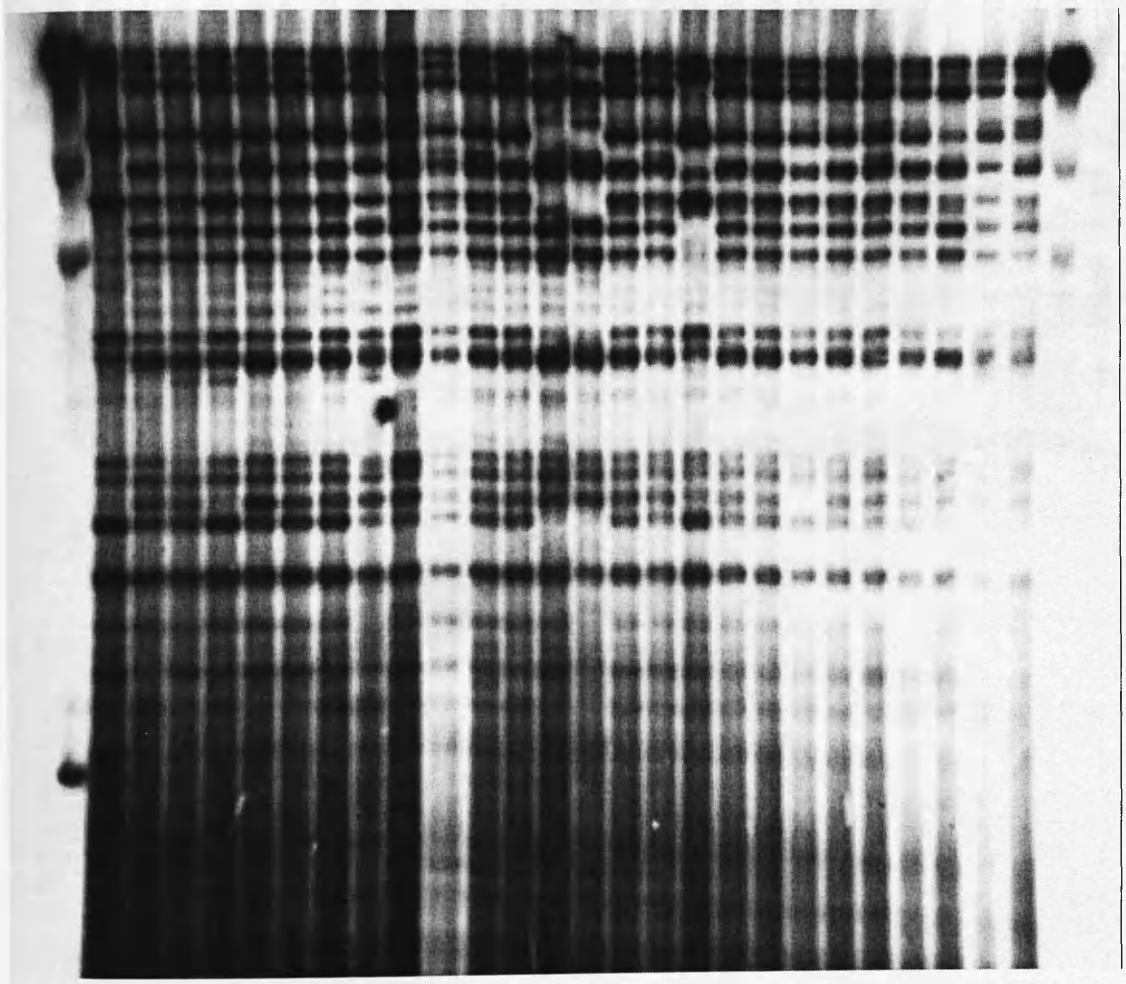
6.4.8. Multilocus fingerprinting

Figure 6.9a shows the fingerprint of mother XX female no 38, along with the father, the control group and the meiotic gynogenetic group. Figure 6.9b shows the fingerprint of mothers (XY neofemales no. 4 and no. 25) along with the father, the control group and the meiotic gynogenetic group of each mother. The fingerprinting analysis did not show any differences between the parents so could not be used.

Figure 6.9a. Fingerprinting; XX female no. 38.

CONTROL GROUP MEIOTIC GYNOGENETICS? GROUP

λ ♀ ♀ ♀ ♀ ♂ ♂ ♂ ♂ F M ♂ ♂ ♂ ♂ ♂ ♂ ♂ ♂ ♂ ♂ ♀ ♀ ♀ ♀ F M λ



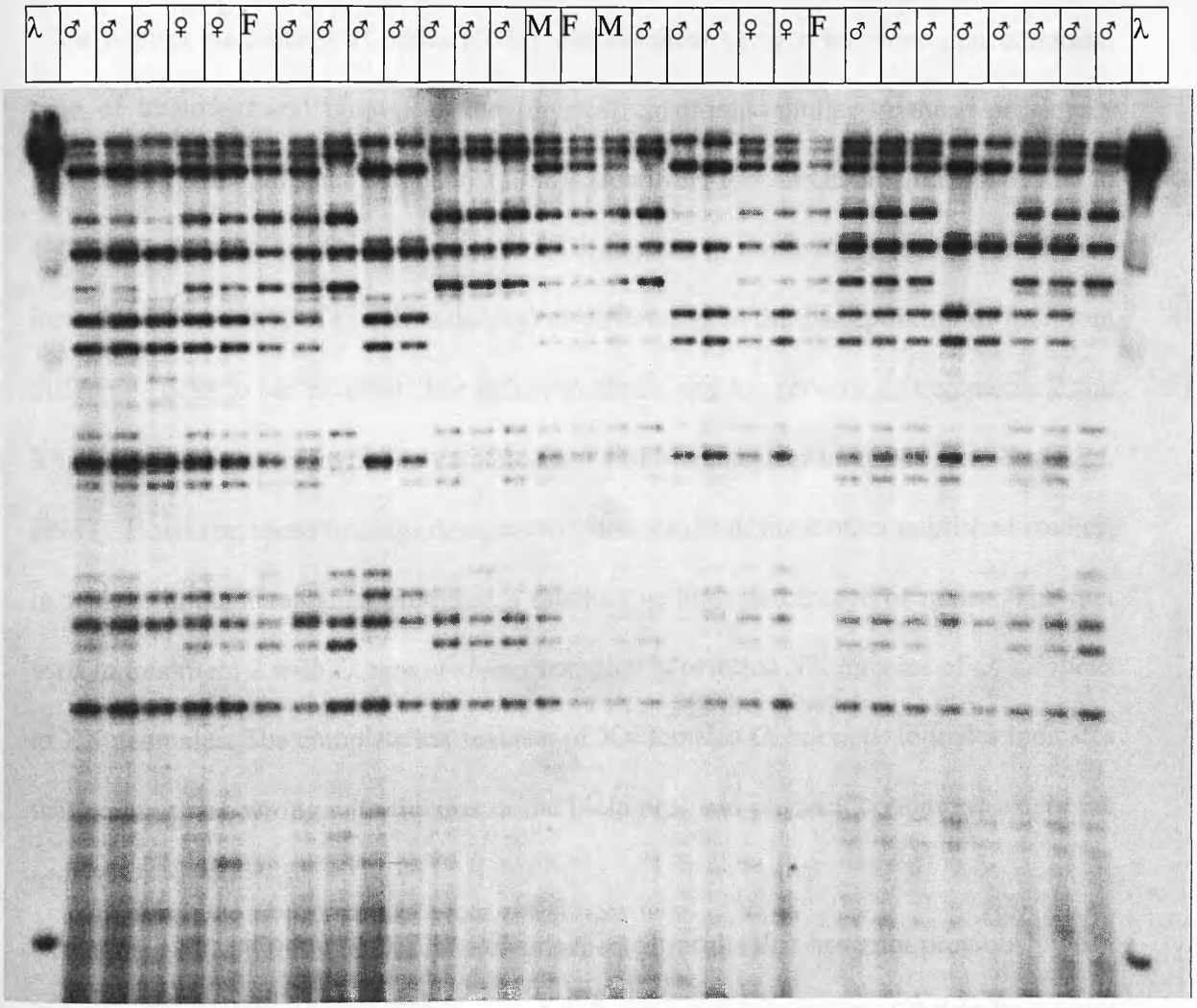
λ: standard marker (λ α Hind III)

F: Father

M: Mother

Figure 6.9b. Fingerprinting; XY females no. 4 and no. 25.

FEMALE NO. 4		FEMALE NO. 25	
CONTROL GROUP	MEIOTIC GYNOGENETICS? GROUP	CONTROL GROUP	MEIOTIC GYNOGENETICS? GROUP



λ: standard marker (λ α Hind III)

F: Father

M: Mother

6.5. Discussion

6.5.1. Sex reversal

The highest percentage of males (80%) was obtained using a hormone concentration, time of treatment and biological and physical conditions similar to those previously reported by Pandian and Varadaraj (1988). However, this treatment did not result in 100% masculinisation. This may be due to differences in the feeding regime (*ad libitum* instead of 20 to 30 % of the biomass/day) or differences in the susceptibility of fish from different stocks to sex reversal. The failure to obtain any sex reversal in treatments 2 and 3 agrees to some extent with the variable results of Macintosh *et al.* (1985) and Das *et al.* (1987). However, these findings disagree with the results of most other published studies, in which similar treatments produced a medium or high percentage of males. The diet used in treatment 2 with *O. mossambicus* completely reverted XX females of *O. niloticus* to XX neomales. The complete sex reversal of XX females *O. niloticus* to males indicates that nothing was wrong with the diet or the biological and physical conditions where the trials were performed.

Treatment 4 (MT for 60 days) showed unexpected results that have not previously been reported. Firstly, more intersex fish were found and secondly, in one of the replicates the percentage of females was significantly higher (66 %), which suggests that some of these females were the result of paradoxical feminisation. An unexpected result was also obtained in replicates 1 and 2 of treatment 4 with ET. Replicate 1 showed a high percentage of males (78 %), while replicate 2 showed a low percentage of males (29 %).

The two replicates treated with DES showed complete sex reversal from male to female. These fish were from the same batch used for MT and ET (treatment 4), again indicating that the preparation of the diet and the conditions of the trials were appropriate. Therefore, a prolonged treatment with a high hormone concentration may or may not lead to the production of intersex fish and a variable percentage of males. However there is a clear trend that prolonged treatment leads to a high percentage of intersex fish. It seems that one way to avoid confusing results is to use low concentrations of hormone, e.g. 5-20 mg/Kg, and a short duration of time, dependent of course on the biological and physical condition of each research laboratory or hatchery for production. This means that an experimental sex-reversal curve has to be determined for each lab or hatchery in order to define the best conditions for reversing females to males at that site, for instance, testing different low hormone concentrations, different feeding practises and different short times (10-25 days) of treatment. Pandian and Sheela (1995) present a review in which general information can be obtained for hormonal induction in fish.

Possibly *O. mossambicus* is more sensitive to variations in treatment parameters than *O. niloticus* so it simply requires less hormone and earlier treatment. Varadaraj *et al.* (1994) explored conditions such as photoperiod, density, feeding rates, temperature, storage conditions of the diet with the hormone, and stocking density, giving good recommendations of how to achieve complete female to male sex reversal, especially when applied to large-scale systems. However, it is clear that unsuccessful attempts to obtain a high percentage of males in this species, even using the best recommendations, have not been adequately explained. For instance: the hormone brands and "interference of other factors" (Pandian, 1993); differences between females in their resistance to MT

(Macintosh *et al.*, 1985; Das *et al.*, 1987) and long treatments (Macintosh *et al.*, 1985; Das *et al.*, 1987). Furthermore, Pandian (1993) published a list of studies described as "confusing reports on the induced sex-reversal in tilapias with reference to dose and treatment duration", in terms of the wide variety of results, most of which refer to *O. mossambicus*.

The result of this study is that, as stated by Macintosh *et al.* (1985), the effectiveness of MT varies with the duration of treatment. To this conclusion it can be added that there is a high possibility of obtaining a high percentage of intersex fish with prolonged treatment. This variation can even result in a high, medium or small percentage of males. Such results may be dependent on the metabolic pathways in which the hormone excesses are eliminated through excretion or conversion through aromatisation (androgen to oestrogen). Increasing the hormone concentration has two main disadvantages. The first one is the possibility to increase hormone levels in plasma that may lead to produce intersex fish or sterility. The second one is the waste of hormone and subsequent environmental pollution since about 99 % of the hormone impregnated into the diet is released into the water within 24 hours from feeding (Johnstone *et al.*, 1983). Hormone concentration can also be lowered and has been successfully dropped to 5 mg/kg (Pandian and Varadaraj, 1988) in this species. A further conclusion is retaken from Pandian and Varadaraj (1988) in which the labile period of sex differentiation in *O. mossambicus* occurs within two weeks or from the 9^{th} to the 20^{th} dph. They found that a delay in the treatment resulted in incomplete masculinisation. However, it seems that there may be some variation in the hormone susceptibility of broodstocks from different laboratories. Exposure to low temperature (20 °C) between zero to five or six to ten days

after hatching induce a high proportion of females whilst exposure to high temperature (32 °C) between eleven and fifteen days after hatching induces to a high proportion of males (Wang and Tsai, 2000). This again indicates that the critical period for sex determination occurs at an early stage in this species.

As had previously been thought, *O. mossambicus* is not an easy species to sex reverse to male when compared to *O. niloticus*. Several conditions have to be taken in account and will have to be studied and explored in each lab in order for the process to be successful.

6.5.2. Cytogenetics

The analysis of the synaptonemal complex, without showing any unpaired lateral elements at pachytene stage agree, with the observations of Liu and Yu (1991). Therefore it seems that the Mozambique tilapia does not present cytological evidence of sex chromosomes such as *O. niloticus* and *O. aureus* (Chapter 4 and 5). Only in one nucleus a diffuse chromatin region with differential staining possibly near the kinetochore of bivalent 1 was observed in a XY male. This could be an artefact of the technique, however in Chapter 7, the hybrid between male *O. mossambicus* x female *O. niloticus* showed similar patterns in bivalent 1 that could support the possibility that this is a phenomenon related to sex determination. These observations could support that the sex-determining genes are located within the pericentric region of bivalent 1, or very close to the centromere.

For example, the gene-centromere recombination rate from six meiogenetic channel catfish *Ictalarus punctatus* families derived from XY females were 3.5%, indicating that

the sex determining locus resides close to the centromere (Liu *et al.*, 1996). A study of Giemsa C-banding applied to the mosquito *Aedes aegypti* (Newton *et al.*, 1974) revealed an essential difference between chromosomes X and Y. The Y chromosome unlike the X and the autosomes is not C-banded in the centromere region, which indicates that the sex locus occurs some where within the pericentric region. Although the precise position of the sex locus M/m in chromosome number 1 is uncertain, M and m were always linked with a particular type of centromere. The chromosome between M/m and the centromere was achiasmate and fully sex linked. The sex locus is within a region bounded by points marking the proximal limit of crossing over, therefore the sex determining-gene(s) may be located in a short length of chromosome.

The unsynapsis of the X and Y when the autosomes are fully paired could be the mechanism restricting exchange during meiotic prophase I (Moses *et al.*, 1975; Ashley and Moses, 1980; Solari, 1980) as occurs in *O. niloticus* (Chapter 4). This may serve to prevent genetic exchange along an extensive portion of the unsynapsed region (Ashley, 1987). Alternately the composition and positioning of chromatin such as dark bands may physically suppress crossing over in this region (Ashley and Russel, 1986). Heterochromatin is usually devoid of chiasmata. The exclusion of recombination events from heterochromatin or other specific chromosome regions could be attributed to a particular chromatin conformation, which prevents the access of recombinatory enzymes. Heterochromatic regions can exert effects beyond their borders and even in other chromosomes (Loidl, 1994).

Because a previous report and this study have not shown any evidence of putative sex chromosomes in *O. mossambicus*, it can be suspected that a non-homologous association

occurs near the centromere where there is no chiasmata formation so sex genes are maintained in the Y chromosome. For example, a non-homologous synapsis occurs in the guppy *Poecilia reticulatus* in the terminal region of chromosome 1 (Rodionova *et al.*, 1996), even though the guppy shows a C-band heteromorphism in chromosome 1 in males which is sex-specific (Nanda *et al.*, 1990).

Majumdar and McAndrew (1986) showed that *Oreochromis* species possess centromeric chromatin in all chromosomes. Additionally, one pair of short submetacentric chromosomes presents an additional interstitial heterochromatin band in the long arm near the centromere. Only in *O. mossambicus* an additional telomeric band is observed in a short subtelocentric chromosome pair. The study mentioned that there is no suggestion of polymorphism for this band in each sex, therefore this band is not sex linked in this species.

6.5.3. Gynogenesis

The methods employed for the induction of meiotic gynogenetics (UV dose, heat shock and time) agree with the methodology of Penman (1989) and Penman *et al.* (1987) and disagree with the methodology of Varadaraj and Pandian (1989). Nevertheless, many factors such as the species strain, the pre-shock incubation temperature, the sperm concentration at which it is UV irradiated in a given time and UV dose rate, the age of the fish, the egg diameter and possibly other laboratory conditions have to be taken into account.

The methodology published for tilapia species with a time of 2 min UV dose, a heat shock 5 min after fertilisation and a temperature of the heat shock between 38 and 42 °C, are good initial parameters for tilapia species studied through gynogenesis. These methodologies are based on the work of Chourrout and Itskovich (1983) and are similar in *O. niloticus* (Mair *et al.*, 1991a), *O. aureus* (Mair *et al.*, 1991b) and *O. mossambicus* (Penman *et al.*, 1987). Variation of these parameters such as 7 to 10 min sperm UV dose and the time when the heat shock is applied (2.5 min after fertilisation) in *O. mossambicus* (Varadaraj and Pandian, 1989) were presumably empirically the best established experimental conditions found for a particular fish laboratory.

In most cases the progeny testing to identify the XX females and XY neofemales in *O. mosssambicus* consistently give the expected sex ratios of 1:3 (female:male) when crossed a XY male x XY neofemale or 1:0 (female:male) when crossed a XX neomale x XX female or 1:1 (female:male) when crossed a XY male x XX female. Only in one case (female no. 35), a cross XX neomale x XX female, gave 2 males out of 44 individuals (Table 6.5b) and one case (female no. 5), a cross XY male x XX female gave 80:50 (female: male) skewed to females and significantly different at $P<0.01$ (Table 6.5a).

Nevertheless, caution has to be taken when crossing a XY male x XY neofemale if the progeny sample size is small. It will be difficult to statistically differentiate between 1:3 and 1:1 sex ratios.

After the identification of neofemales was complete, gynogenesis was induced to produce meiotic gynogenetics offspring, which in control crosses gave the expected 1: 3 (female:male) sex ratio, confirming for a second time the genotype XY.

The induction of meiotic gynogenetics from XY neofemales was only possibly in 3 out of 17 individuals (18 %) against 9 out of 21 (43 %) in the XX female group. Although both genotypes showed the same dilated pink coloured genital papilla previous to spawning, neofemales were more difficult to strip since the belly was usually tighter than genetic females where eggs were always loose when stripping.

The presence of males from genetic XX females is reported for the first time in this species. As shown in Table 6.6a, a total of 53 females (82 %) and 12 males (18 %) were obtained. Female no. 38 contributed most of the males present (10 out of 12). If female no. 38 is not taken in account a total 37 females and 2 males was obtained, giving an overall sex ratio closer to that expected. The survival of the progeny of female no 38 from yolk sac reabsorption to 4 months was 62 %, so possibly some female mortality occurred. This female was the first one to be induced to produce meiotic gynogenetics with 2 min UV sperm radiation. At the beginning it was thought that this UV dose was too low due to the observation of some normal fry (7 %) in the haploid gynogenesis group. However, in Table 6.7a it is shown that all the males but one (no.2) gave 82 to 97 % of females when crossed with a XX female. In the case of the male from female no. 03, it gave a chi-square value significantly different from 1:1 sex ratio (72 % of females), however it gave the lowest percentage of female progeny observed. The high percentage of females in the progeny of most of the males (none gave 100 % females) gave enough evidence to consider them as a XX neomales (Table 6.7a). They showed more male progeny than the "XX" x "XX" crosses (Table 6.5.b), which suggests a genetic cause. Therefore, an autosomal sex influence that reversed females into males is suggested.

The autosomal influence on sex determination observed in *O. mossambicus* could be the same locus reported as an epistatic gene or sex determining locus 2 described in *O. niloticus*. This locus (or loci) is not consistent, the sex ratio varies through different crosses perhaps due to a natural sex reversal or an epistatic recessive influence and limited penetrance (Mair *et al.*, 1991a; Hussain, 1994; Sarder *et al.*, 1999).

An isolated observation was "caught" in a meiotic gynogenetic male from XX female no 31 (Table 6.7a). When this male was crossed with a genetic XX female to identify its genotype, this male fertilised the eggs and at the same time picked up about half of the eggs to mouthbrood them. This is not a common observation in *Oreochromis* species and maybe its maternal genetic content contributed towards this behaviour, since this male was identified as an XX neomale.

In contrast, only males were present in gynogenetic progeny from XY neofemales. There is no obvious biological explanation for the absence of females. It would be expected that the number of YY males would have been matched by an approximately equal number of XX males (the two non-recombinant groups). The absence of females could have been due to very high a differential mortality (i.e. females were produced but they all die). However the survival from yolk sac resorption to 4 months of age was 74 % (Table 6.6b), which would appear to rule this out as we would have expected equal numbers of YY males (n = 18) and XX females. It has to be considered that the number of meiotic gynogenetics per neofemale was small (n = 10, 8 and 7) and possibly this (sampling error) could be the strongest argument to explain the absence of females. However the probability of not producing even a single meiotic gynogenetic female out of 25 is very low and this could require further interpretation. The high percentage of

males in the progeny of most of the males gave enough evidence to consider them as a YY males. Therefore, an autosomal sex influence that reversed a few males into females in the progeny of these YY males is suggested, similar to those observed in the Nile tilapia (Mair *et al.*, 1997).

Since female meiotic gynogenetics were not produced, it was not possible to calculate the gene-centromere recombination rate, therefore, it is not possible to give the location of the sex gene(s) on the chromosome. However, it can be suggested that this sex gene(s) are located within the proximal part of the long arm of the chromosome. These results agree to some extent with the observations of Varadaraj and Pandian (1989). Both results suggest that sex determining genes are located in a region near to the centromere where the probability of recombination is low. Nevertheless, these results also disagree with Varadaraj and Pandian (1989) who found only XX and YY fish through gynogenesis while in the present study there was a proportion (28 %) of XY recombinants.

Therefore, it is difficult to agree with them that it is easier and faster in time to produce YY individuals in *O. mossambicus* by gynogenesis. As all male meiotic gynogenetics have to be progeny tested first to identify the YY males and then, to cross them with XX females to produce genetically all-male tilapia. The availability of a molecular sex-specific marker would obviate simply this process. The implication of a small degree of autosomal sex determination influence would still have to be taken into account even with this scenario.

Sex determination in tilapia is mainly monofactorial with some environmental (temperature) and autosomal influences. The present results indicate that the Mozambique tilapia is not an exception to this.

Microsatellites and fingerprinting analysis could not confirm the gynogenetic nature of any of the individuals analysed. This does not mean that the techniques employed were not useful. The microsatellites used originated from *O. niloticus* and cross-amplified in *O. mossambicus*. The absence of at least one allele of different size in the male employed for gynogenesis was not found, so in all cases at least one allele was shared with the females. The ideal scenario would be if UNH203 were homozygous for one allele in the father (e.g. 87) different from the allele sizes (e.g. 89 and 91) of the mothers independently if the mother alleles were heterozygous or homozygous. It would have been necessary before inducing gynogenesis to find one or more males of different genotypes to the female and neofemale broodstock. However this could have taken a long time and was outside the main study. Nevertheless, a previous microsatellite screening of the same broodstock (Sobolewska, 1999) revealed that from 6 microsatellite loci only one (UHN203) had 100 % heterozygosity (26 individuals scored) and 4 different allele sizes. In this study this locus gave 3 alleles with 66 % heterozygosity (12 individuals), (Table 6.8a,b). Jeffreys's Fingerprint probe suggested that the broodstock of *O. mossambicus* is inbred. Therefore no meiotic gynogenetics could be identified.

6.6. Conclusions

1) A short treatment period combined with a low hormone concentration seems to be adequate to sex reverse females into males and may reduce or nullify the presence of intersex fish. In contrast, a high hormone concentration combined with a long treatment period does not guarantee a high male percentage and it is more likely to produce intersex fish.

2) There is no cytogenetic evidence of putative sex chromosomes as unpaired lateral elements in the Mozambique tilapia. Nevertheless, a diffuse chromatin region with differential staining of bivalent 1 possibly near to the kinetochore might indicate that bivalent 1 is related to sex determination.

3) Sex loci could be located within the proximal part of the long arm of chromosome 1.

4) There is a small autosomal influence in sex determination in genetic XX females that reverses some XX females into XX males in *O. mossambicus*.

5) There is a small autosomal influence in sex determination in genetic XY males that reverses some XY males into XY females in *O. mossambicus*.

6) These autosomal influences seem to be very similar to those observed in the Nile tilapia.

CHAPTER 7

SYNAPTONEMAL COMPLEX OF INTERSPECIFIC HYBRIDS OF TILAPIA OF THE GENUS *Oreochromis*

7.1. Introduction

One way to analyse the degree of homology between the chromosomes of closely related species is to observe the zygotene and pachytene stages of meiosis prophase I. This has been done in different animals and plants e.g. buffalo (Dai *et al.*, 1994; Guimaraes *et al.*, 1995), muntjac (Liming and Pathak, 1981), cattle (Dollin *et al.*, 1991; Scavone *et al.*, 2000), equines (Andrea *et al.*, 2001), mice (Hale *et al.*, 1993; Forejt, 1996; Safronova *et al.*, 1999), wheat and rye (Cuñado and Santos, 1999). This analysis permits us to observe if the chromosomes of the parental species of the hybrid present sufficient differentiation to avoid chromosome synapsis, through the observation of gross abnormalities like partial synapsis, multivalents, or unsynapsed axial elements.

The karyotypes of a number of *Oreochromis* species have been stated to vary in chromosome morphology, but to have the same highly conserved chromosome number of 44 (Majumdar and McAndrew, 1986; Klinkhardt *et al.*, 1995). Some apparent differences stem from the highly condensed state of mitotic chromosomes that makes accurate karyotyping difficult. For example, the mitotic karyotype (2n) of *O. mossambicus* Peters has been described as 22 m (Natarajan and Subrahmanyami, 1968); 3 st +2 t + 17 T (Prasad and Manna, 1976; Krishnaja and Rege, 1980) and 3 sm + 4 sm-st + 15 st (Majumdar and McAndrew, 1986), while the meiotic karyotype (n) has been described as 4sm + 17 st + 1t (Liu and Yu, 1991). Comparing *O.mossambicus* with other *Orechromis* species, the Nile tilapia *O. niloticus* (L.) 2n = 1 m + 9sm + 5 sm-st + 7 st and the blue tilapia *O. aureus* Steindachner 2n = 7 sm + 8 sm-st + 7 st (Majumdar and McAndrew, 1986) can be cited. However there seems to be agreement that most of the chromosomes

are submetacentric, subtelocentric or acrocentric in morphology. Furthermore, the overall karyotype structure, with a large subtelocentric chromosome 1, a medium size subtelocentric chromosome 2, and the rest of the karyotype composed of small chromosomes, seems to be well conserved. Exceptions to this karyotype exist and it has recently been observed that the tilapia *O. Nyasalapia karongae* Trewavas has a karyotype of 2n = 38 chromosomes with the tentative karyotype (2n) = 2 m + 11sm-st + 8t. This karyotype has a unique morphological characteristic. The longest pair (chromosome 1) and a medium size pair (chromosome 2) are present as in the other species. However, there are an additional 2 pairs of subtelocentric chromosomes and one metacentric pair, all about the same size as chromosome 2, with the rest of the karyotype composed of small chromosomes (Harvey *et al.*, 2001; 2002). *O.N. karongae* has been investigated as a new aquaculture candidate (Msiska and Costa –Pierce, 1997a,b), being part of a group of closely related species known as the "chambo" in Malawi (Turner and Robinson, 1991). In comparison to the karyotypes of the other species, the medium size metacentric pair that is observed in *O. karongae* is distinctive. The presence of such a metacentric pair could be the result of a Robertsonian fusion of two small acrocentric chromosomes. In contrast, the 2 medium sized subtelocentric chromosome pairs could be the results of two arm breakages, then an inter-chromosome translocation. Thus, one Robertsonian fusion and two translocation events could have reduced the *O. N. karongae* diploid chromosome number from 44 to 38.

Therefore, if chromosome number and possibly chromosome morphology are well maintained within the genus *Oreochromis*, those species presenting a different chromosome number are likely to be the result of chromosome rearrangements. Further,

the chromosomes of hybrids resulting from species with the same chromosome number should match perfectly during pachytene stage, otherwise both homologues should present at least some heteromorphism or an aberrant synapsis resulting from the different morphology. The former might imply that all *Oreochromis* species having 44 chromosomes could have the same morphology and that apparent differences between the karyotypes could just be artefacts of observation.

No evidence of sex chromosomes has been founded in analysis of mitotic karyotypes from several typical (2n =44) *Oreochromis* species (Majumdar and McAndrew, 1986) and the analysis of the mitotic karyotype in males and females of *O. N. karongae* does not reveal sex chromosomes either (Harvey *et al.*, 2001; 2002). No analysis of meiosis has been reported in this species.

In the genus *Poecilia*, Rodionova *et al.* (1996) made a synaptonemal complex analysis of interspecific hybrids of the species *P. reticulatus* (guppy), *P. sphenops* (black molly), and *P. velifera* (marble molly). Mitotic karyotypes not vary in these three species, all of them contain 23 pairs of acrocentric chromosomes. *P. reticulatus* (Yosida and Hayashi, 1970, cited in Rodionova *et al.*, 1996), *P. sphenops* (Prehn and Rasch, 1969, cited in Rodionova *et al.*, 1996) and *P. velifera* (Post, 1965, cited in Rodionova *et al.*, 1996). Meiotic chromosomes of the pure species and black molly x marble molly hybrid showed complete pairing at pachytene, without any indication of chromosomal heteromorphism. Most of the pachytene cells of the hybrid between the marble molly and the guppy showed various signs of pairing failure such as univalents, interlocks, multiple non-homologous pairing and end-to-end associations. However a few cells were found with completely paired homomorphic bivalents, which made the authors conclude that the

pairing failures were due to genetic incompatibility of the species-specific mechanisms controlling meiotic prophase in the parental species, rather than loss of homology between their chromosomes. Indeed, the finding of a very small number of mature sperm demonstrated that meiotic arrest in the hybrids was not absolute, and some cells were able to surmount it and complete meiosis. The largest bivalent (chromosome 1) in males of the guppy showed lateral elements of equal length, uniformly stained and completely paired with each other in the majority of the pachytene cells. No bivalent had axes of unequal size. Some bivalents displayed a temporary asynapsis of the terminal segments at early and late pachytene, but usually there were several of these. Therefore, sex chromosomes were not observed during meiosis, although it has been reported in guppies that there is a difference in the C band patterns between the telomeres of the largest chromosome and that the species has a XX/XY system (Nanda *et al.*, 1990, 1992). Recently, Traut and Winking (2001) observed putative sex chromosomes in males of the guppy through the synaptonemal complex technique and fluorescence *in situ* hybridisation analysis. This putative sex chromosome was observed in one of the small bivalents instead of bivalent 1. The authors did not mention or discussed the previous work cited in the literature, nor did they analyse any females. Therefore, the existence of putative differentiated sex chromosomes in the guppy is not clear.

There is a vast literature about tilapia hybrids (see McAndrew, 1993). Hybrid growth performances have been analysed by McAndrew and Majumdar (1989). The early work on tilapia hybrids gave clear indications of the sex determination systems. Nevertheless, none of the investigations of hybrid sex ratios could be used to adequately explain the primary sex-determination mechanism in the parental species, largely due to the great

variability observed in the sex ratios (e.g. Mair *et al.*, 1991a, 1991b). One interesting feature of hybridisation in *Oreochromis* is that the progeny are normally not sterile. Therefore, observations of SCs would be expected to show either no chromosome pairing failures, or only a few minor anomalies. A previous investigation analysed the hybrid mitotic karyotype of *O. niloticus* x *O. mossambicus* (Crosseti *et al.*, 1988) without observing any major differences in karyotypes. At a meiotic level, the SC approach opens the possibility of identifying non-homologous regions that could be related to sex chromosomes in interspecific hybrids. The hybrid between female *O. niloticus* and male *O. mossambicus* (and the reciprocal) is an XY x XX cross. Because *O. niloticus* males present an unsynapsed region in bivalent 1, these two hybrids should allow the existence of any pattern in bivalent 1 of the hybrid males with either paternal chromosome of *O. mossambicus* or *O. niloticus* to be discerned. The hybrid between female *O. niloticus* and male *O. aureus* is a ZZ x XX cross. This hybrid should thus assess the degree of homology between the species only.

The analysis of the male *O.N. karongae* would confirm the diploid chromosome number found by Harvey *et al.* (2001; 2002). The hybrid between female *O. niloticus* and male *O.N. karongae* would allow analysing the chromosome rearrangements during meiosis.

7.2. Aims

The aims of this research were:

(1) To analyse meiotic pachytene stage of bivalent 1 of the hybrid between female *O. niloticus* and male *O. mossambicus* and the reciprocal cross.

(2) To analyse meiotic pachytene stage of the hybrid between female *O. niloticus* and male *O. aureus* to assess the degree of homology between the species only.

The male of *O.N. karongae* and the hybrid between female *O. niloticus* and male *O.N. karongae* would allow analysing the following aims:

(3) To analyse the meiotic chromosomes of male *O.N. karongae*, following the recent finding that the diploid chromosome number is 2n = 38.

(4) To assess the degree of homology between the chromosomes of *O.N. karongae* and *O. niloticus* and the chromosome rearrangements between them.

(5) To make some interpretations about chromosome evolution in *O.N. karongae*.

7.3. Materials and methods

7.3.1. Species studied

The fish used in this study came from populations derived from the River Zambezi, Zimbabwe (*O. mossambicus*), Lake Manzala, Egypt (*O. niloticus* and *O. aureus*) and Lake Malawi, Malawi (*O.N. karongae*). These species are held in the Tilapia Reference Collection at the Institute of Aquaculture, University of Stirling.

7.3.2. Fish used for SC analysis

The SC analysis was made at pachytene stage of the following male individuals:

(1) Six male hybrids of *O. niloticus* (female) x *O. mossambicus* (male).

(2) Six male hybrids of *O. mossambicus* (female) x *O. niloticus* (male).

(3) Six male hybrids of *O. niloticus* (female) x *O. aureus* (male).

(4) One *O.N. karongae* male (only one male was analysed because the other broodstock held in the Tropical Aquarium subsequently died after a technical failure of the water recirculation system during the night).

(5) Six male hybrids of *O. niloticus* (female) x *O. N. karongae* (male) and two female hybrids.

The number of pachytene cells analysed for groups 1-3 above were 6 per individual with a total of 36 per hybrid cross.

The gonads of the hybrids of *O.N. karongae* were analysed from a pooled sample (4 males and 2 females) because they were small and less developed, so 42 SCs were analysed from 4 males and 2 SCs from 2 females.

All hybrids were analysed at between four and six months old.

7.3.3. Preparation of SC spreads

Fish were killed with anaesthesia (by immersion in a 0.01% benzocaine solution) followed by destruction of the brain before dissection. Preparations of fish SC spreads for the observation in the transmission electron microscope were made accordingly with the protocol showed in Chapter 2 (section 2.1.4).

7.3.5. Statistical analysis

The bivalent lengths of the male *O. N. karongae* and the *O. niloticus* (female) x *O. N. karongae* (male) hybrids were measured. Progeny sex ratios were assessed by chi square test ($P = 0.05$).

7.4. Results

7.4.1. The hybrid between *O. mossambicus* female and *O. niloticus* male

This cross resulted in 12 male and 16 female progeny, a sex ratio not significantly different from 1:1 (χ^2 = 0.57). From the 36 SC spreads observed in male hybrids, only one presented a subterminal unpaired region in bivalent 1 (Fig. 7.1. b,c). The rest of the spreads showed complete synapsis (Fig.7.1. a) with all nuclei presenting 22 bivalents.

7.4.2. The hybrid between *O. niloticus* female and *O. mossambicus* male

This cross resulted in 20 male and 18 female progeny, a sex ratio not significantly different from 1:1 (χ^2 = 0.42). From the 36 SC spreads observed, 9 presented diffuse lateral elements and differential staining in one region of bivalent 1. The rest of the observations showed a complete synapsis. Usually three nucleoli were observed instead of the two seen in the parental species (Fig. 7.2. a-g). All nuclei presented 22 bivalents.

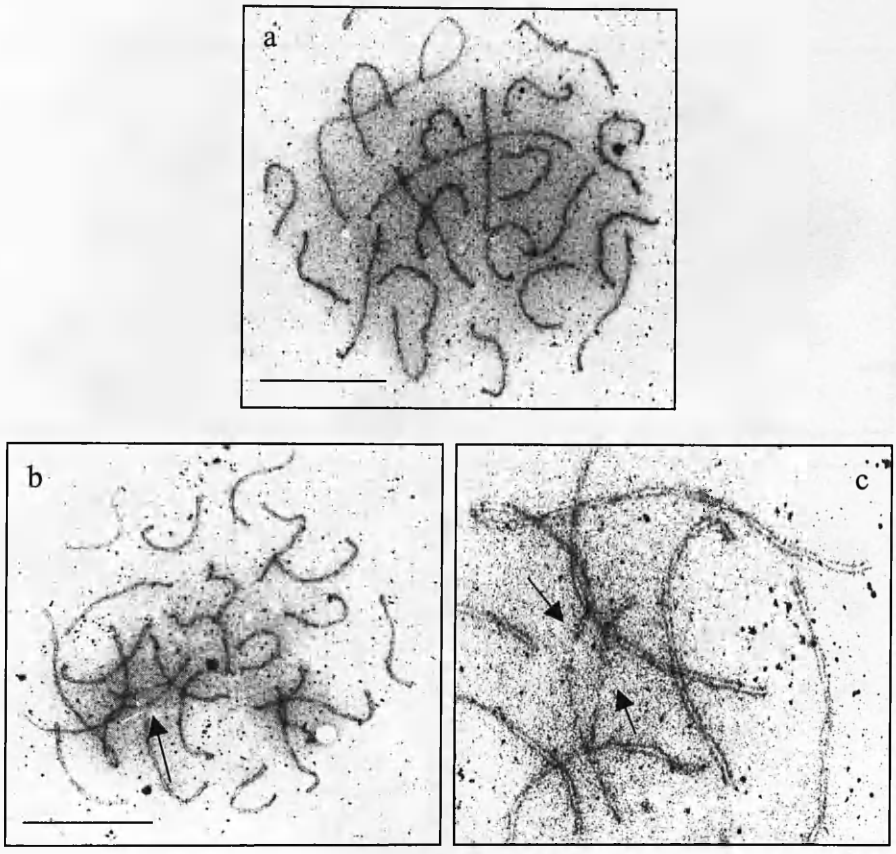


Figure 7.1. Pachytene stage of the hybrid between *O. mossambicus* female and *O. niloticus* male. (a) Complete synapsis of 22 bivalents. (b) Complete synapsis except for the terminal region of bivalent 1 (arrow). (c) Amplification (rotated 90 °) of the unpaired region from (b). Bar represents10 μm.

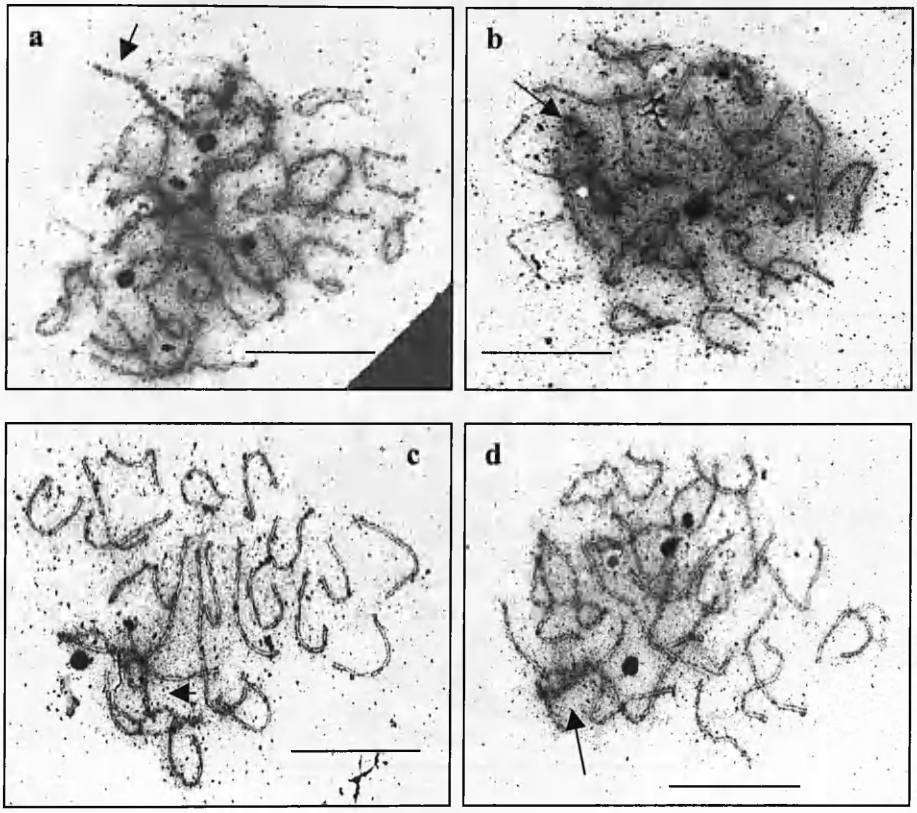


Figure 7.2. Pachytene stage of the hybrid between *O. niloticus* female and *O. mossambicus* male. Figures a to d: Diffuse lateral elements and differential staining is observed in one region of bivalent1 (arrows) from different SC spreads. The presence of 3 nucleoli can be observed in a and d. Bar represents10 μm.

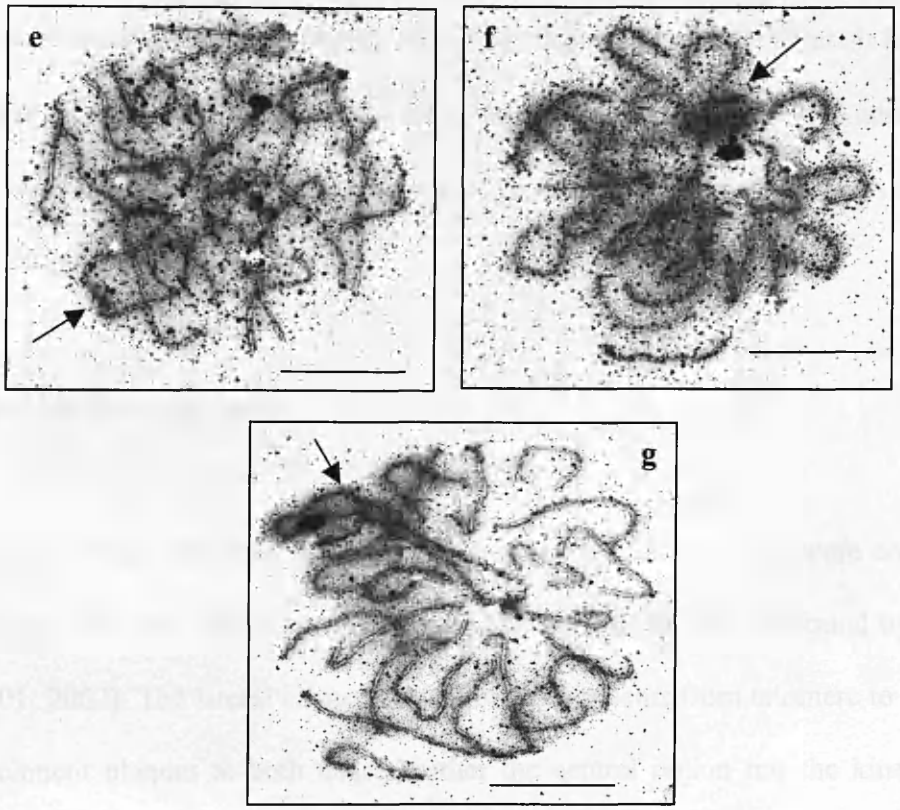


Figure 7.2. (continued):

Figures e to g: Diffuse lateral elements and differential staining is observed in one region of bivalent1 (arrows) from different SC spreads. Bar represents10 μm.

7.4.3. The hybrid between *O. niloticus* female and *O. aureus* male

This cross resulted in 14 male progeny only, a sex ratio significantly different from a 1:1 sex ratio ($\chi^2 = 14$), but agreeing with the expected 0:1 female:male (see McAndrew, 1993 for review). All 36 observations in pachytene stage showed a complete synapsis of the 22 bivalents (Fig.7.3. a,b).

7.4.4. The *O.N. karongae* male

At pachytene stage, the male analysed presented 19 bivalents, which were completely synapsed (Fig 7.4. a,b). This confirmed the diploid number of 2n = 38 found by Harvey *et al.* (2001; 2002). The lateral elements spanned the bivalents from telomere to telomere with attachment plaques at both ends. Neither the central region nor the kinetochores were revealed.

The total length of the synaptonemal complex complement was 115 μm (2 nuclei measured). The longest bivalent was 11.5 μm. There were four medium size chromosomes in a range from 7.60 to 9.70 μm. Finally 14 small bivalents were in a range from 3.90 to 6.20 μm (Fig.7.5.).

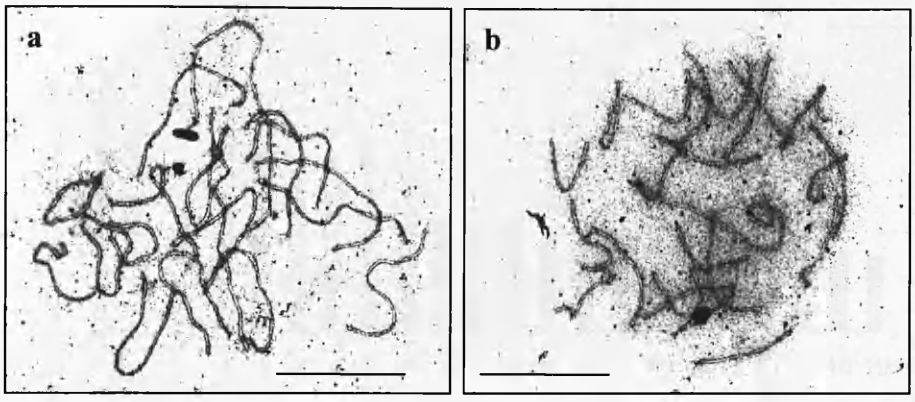


Figure 7.3. Pachytene stage. Hybrid between *O. niloticus* female and *O. aureus* male. (a) and (b) complete synapsis of all 22 bivalents. Bar represents10 μm.

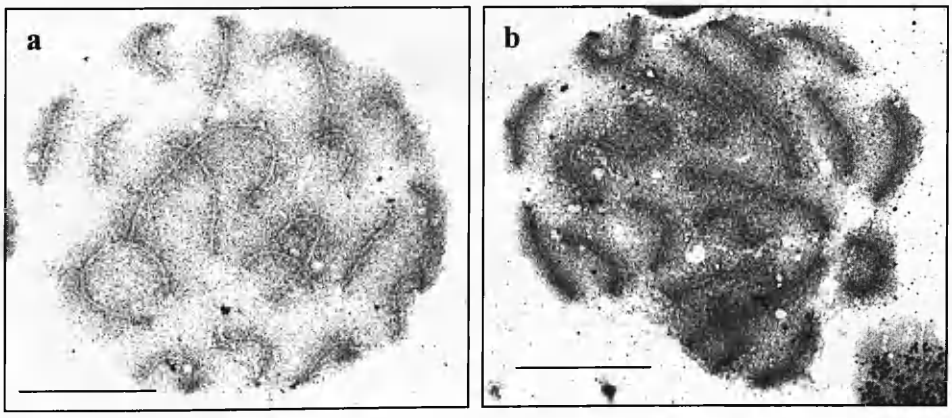


Figure 7.4. Synaptonemal complex at pachytene stage. (a and b) *O.N. karongae*, showing 19 bivalents. Bar represents10 μm.

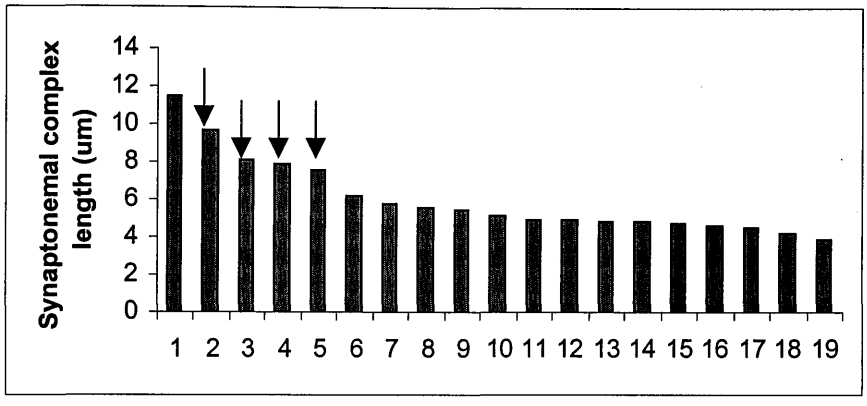


Figure 7.5. Mean synaptonemal complex complement length of each bivalent of *O.N. karongae*. Arrows point to the four middle size chromosomes. Based on 2 spreads from one male.

7.4.5. The hybrid between *O. niloticus* female and *O.N. karongae* male

It should be mentioned that the control cross (*O. niloticus* female x *O. niloticus* male) gave 4 females and 26 males a sex ratio not significantly different from 1:3 ($\chi^2 = 2.17$) and significantly different from 1:1 sex ratio ($\chi^2 = 16.13$). The hybrid cross gave 5 females and 30 males, a sex ratio also not significantly different from 1:3 ($\chi^2 = 1.05$) and significantly different from 1:1 ($\chi^2 = 17.85$). Because the control cross resulted in a sex ratio of 1:3, it was assumed that the Nile tilapia female was an XY neofemale. A second cross of this hybrid was produced later on in the Tropical Aquarium in which the control cross (XX x XY) gave 13 females and 14 males (not significantly different from 1:1; $\chi^2 = 0.03$) and the hybrid cross gave 5 females and 37 males (significantly different from 1:1; $\chi^2 = 24.38$, not significantly different from 1:3; $\chi^2 = 2.95$). The second hybrid was not analysed through meiosis.

By 8 + months old the male hybrids did not show well-developed testes and hence the quantity of sperm was very low in all the males, suggesting that there was a degree of meiotic segregation failure during spermatogenesis. At late zygotene in both sexes it can be seen that some lateral elements are still unsynapsed whilst others have synapsed from one of the telomeres going through the beginning or middle of the bivalent (Fig.7.6.).

From the analysis of 42 pachytene SC spreads, 3 nuclei (7.14 %) were observed to contain 16 synapsed bivalents and three trivalents (Fig. 7.7. a,b,c, d, e); 5 nuclei (11.9 %) were observed with 18 bivalents and one trivalent; 2 nuclei (4.76 %) were observed with 17 bivalents and two trivalents; 5 nuclei (11.9 %) were observed with nineteen bivalents

fully synapsed and no identifiable trivalents. This makes a total of 15 nuclei that were fully synapsed (36 %).

Multiple bivalent associations (the typical observation was a star-shape) and unpaired or partially unpaired lateral elements only in the bivalents involved in the association were observed in 11 nuclei (26 %) (Fig.7.8. a,b,c). In the remaining 16 SCs (38.0 %) the arrangement of all the bivalents could not be clearly identified.

The total length of the synaptonemal complex complement was 130 μm (3 nuclei measured). The longest bivalent was 13.7 μm and the second 11.49 μm. The three medium-sized chromosomes were in a range from 8.50 to 10.30 μm. Finally the 14 small bivalents were in a range from 4.30 to 6.60 μm (Fig.7.9.).

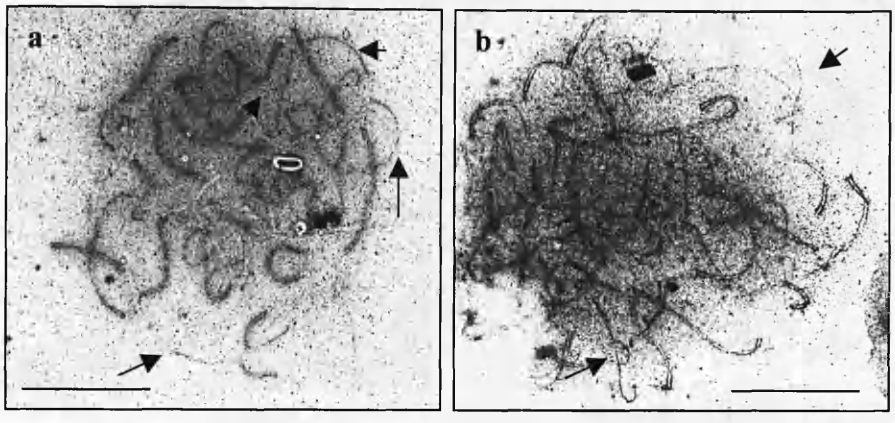


Figure 7.6. Hybrid between *O. niloticus* female and *O.N. karongae* male. Late zygotene in a female (a) and in a male (b). Both sexes show that some lateral elements are still unsynapsed whilst others have synapsed from one of the telomeres going through the beginning or middle of the bivalent (arrows). Bar represents10 μm.

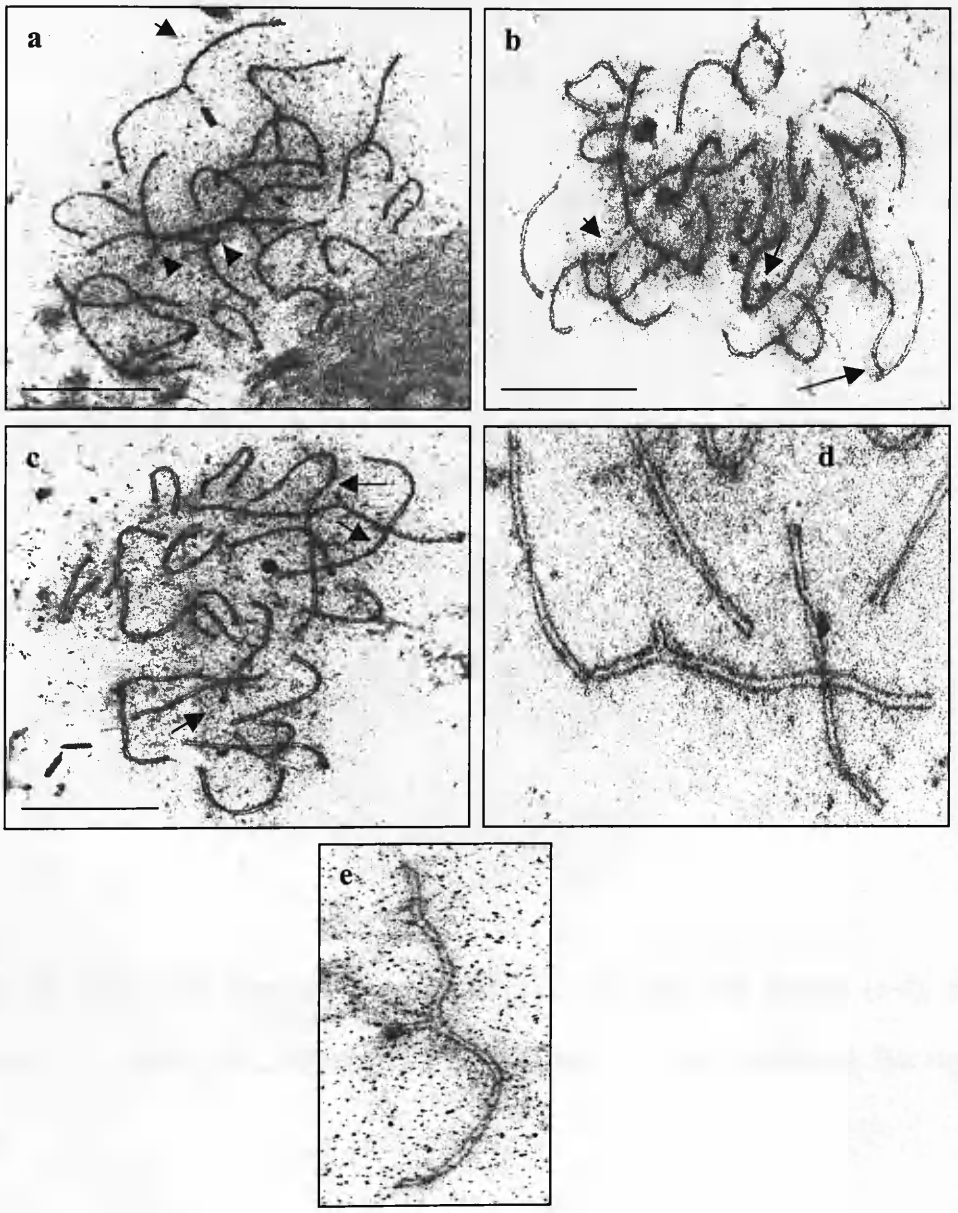


Figure 7.7. Pachytene stage (a,b,c) from *O. niloticus x O. N. karongae* hybrid showing the three trivalents (arrows); d and e: amplification of trivalent. Bar represents10 μm.

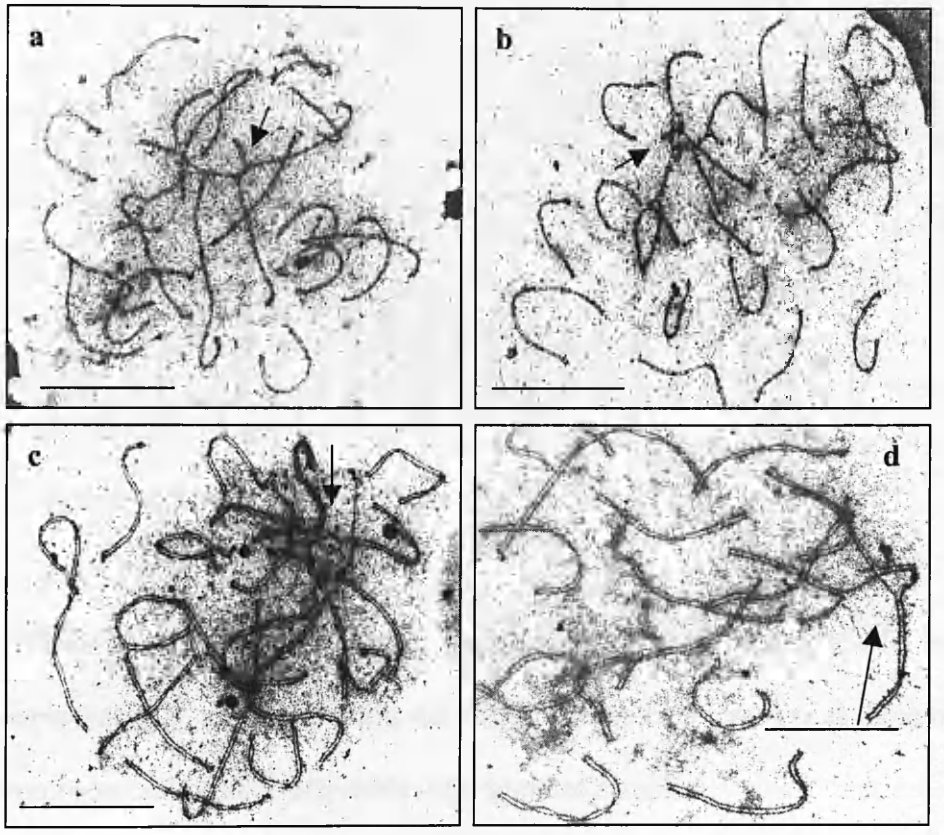


Figure 7.8. Pachytene stage of *O. niloticus x O. N. karongae* hybrid (a-d), showing multiple associations (like "star shapes") of the lateral elements (arrowed). Bar represents 10 μm.

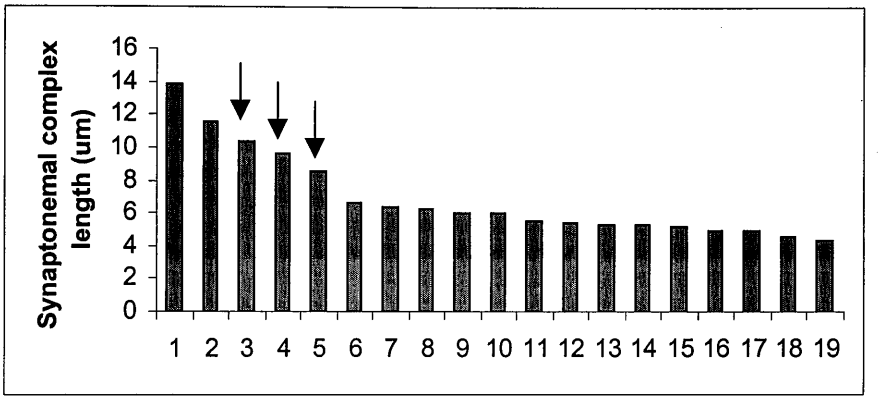


Figure 7.9. Mean synaptonemal complex complement length of each of the 16 bivalents of *O.N. karongae* x *O. niloticus* hybrid and the three trivalents (arrows) as three medium sized chromosomes. Based on 3 spreads from a pooled sample.

7.5. Discussion

It can be suggested that the morphology in the parental *Oreochromis* species having 44 chromosomes (22 bivalents) is very similar. Unfortunately in the SC spreads the kinetochores could not be visualised. Nevertheless the total synapsis and recognition of homologous chromosomes and the similar SC complement length when compared to the parental species (see Chapter 3) in the *O. mossambicus* x *O. niloticus* and *O. aureus* x *O niloticus* crosses strongly suggest that no major differences in morphology exist. If the morphology were different, a noticeable pairing "adjustment" would have been expected along the bivalents.

A remarkable situation is present between the Y chromosome of *O. mossambicus* and the X chromosome of *O. niloticus*. It seems that a delay of recognition in some region along bivalent 1 occurs and this is observed as diffuse lateral elements and differential staining. It seems that this is similar to that observed in one spread of a male *O. mossambicus* (Chapter 6) and possibly is related to sex determination. It is suggested then, that some structural change or a mutation has taken place in the Y chromosome of *O. mossambicus*.

The reciprocal hybrid (female *O. mossambicus* x male *O. niloticus*) presented an unpaired subterminal terminal region of bivalent 1 in one case. Therefore, the synapsis of the Y chromosome of *O. niloticus* with the X chromosome of *O. mossambicus* possibly show a similar sex chromosome pattern as reported by Foresti *et al.* (1993) and Carrasco *et al.* (1999) and Chapter 4.

The hybrid between *O. aureus* male and *O. niloticus* female gave only males, due to the ZW genotype. The homology of all bivalents matched perfectly without any abnormality. Hence, nothing else can be inferred from analysis of the SCs from this hybrid.

The sex ratios obtained from the female *O. niloticus* x male *O.N. karongae* are difficult to interpret (1:3 sex ratio in both hybrid groups) therefore nothing can be inferred about the sex determining system in *O. N. karongae*. This will have to be established by gynogenesis, sex reversal, sex-linked markers or the observation of putative sex chromosomes through synaptonemal complex analysis.

During meiosis in the female *O. niloticus* (2n = 44) x male *O. karongae* (2n = 38) hybrid (2n = 41), three trivalents were observed, each composed one large chromosome from the father and two small chromosomes from the mother. In other spreads failures in chromosome synapsis were observed as aberrant pairing and multivalents.

The low quantity of sperm of most of the hybrid males is likely to be the result of aberrant pairing such as the multiple associations observed. According to the observed percentage of normal SCs, only about 36 % of viable meiosis is occurring in these males, which would agree with the observation of very small testes. Several rearrangements lead to germ cell death and chromosome non-disjunction failing in the production of gametes, reducing the testes weights and lowered the sperm counts, e.g. in mice (Cattanach and Moseley, 1973). However, it is reported that sexual maturation in *O. N. karongae* is reached at a larger size than in other *Oreochromis* species (Msiska and Costa –Pierce, 1997a). Therefore, it is not clear if the low quantity of sperm is a consequence of problems on meiosis or slower gonad development. In fact after 14 months, all hybrid males produced viable sperm.

The SCs in this hybrid are from the synapsis of 22 chromosomes from *O. niloticus* with 19 chromosomes from *O.N. karongae*. The addition of both makes 41 chromosomes (Harvey *et al.*, 2001; 2002). The average of 20.5 makes it appear likely that after meiosis I an unequal segregation will occur, e.g. 20 to one nucleus and 21 to the other nucleus or 19 and 22. It could be interesting to study backcrosses to observe individuals with different chromosome numbers in the offspring.

It was clear that these chromosome rearrangements were not associated with the longest bivalent in which chromosomal sex determination is achieved (at least in *O. niloticus*). Also, it was quite clear that the second longest bivalent was not associated either. By the observation of the karyogram in Fig. 7.9, chromosome rearrangements have occurred in *O. karongae* in pairs 3, 4 and 5. Therefore is possible to detect the first and second bivalents, the three "new" medium sized bivalents and the 14 small bivalents in *O.N. karongae* species (Fig. 7.5).

The viability of this hybrid suggests that possibly karyotype evolution in tilapias is the result of chromosome fusion and chromosome rearrangements. We observe 2n = 48 chromosomes in *Oreochromis alcalicus* (Post, 1965; Denton, 1973; Park, 1974), then 2n = 44 in most tilapias species e.g. *Tilapia rendalli*, *T. zillii*, *O. niloticus*, *O. mossambicus* and *O. aureus* (see Majumdar and McAndrew, 1986 for review). Then 2n = 42 in *T. sparrmanii* (Thompson, 1981), 2n = 40 in *Tilapia mariae* (Thompson, 1981) and 2n = 38 in *O.N. karongae* (Harvey *et al.*, 2001; 2002) and possibly in one strain of *Tilapia zillii* (Badr and El-Dib, 1977).

There is only one previous report of *Poecilia* fish hybrids analysed through the synaptonemal complex (Rodionova *et al.*, 1996). In the genus *Poecilia* gross

chromosomal rearrangements have not occurred, but there is a low degree of homology between the chromosomes of the marble molly and the guppy. In tilapia hybrids it is clear that chromosomal rearrangements have occurred maintaining their homology, at least between the four species studied.

This kind of analysis could be used to study the degree of homology of closely related species, to understand karyotype evolution, chromosomal rearrangements and possibly the behaviour of sex chromosomes.

In aquaculture the possibility of mostly male hybrids between an XX female *O. niloticus* and a ?? male *O. karongae* that produces a low quantity of sperm during at least 8 months of culture (either due a meiotic failure or a late maturation stage) should be investigated further in terms of growth rate, maturation and reproduction stages compared with the parental species.

7.6. Conclusions

1) The hybrid between *O. mossambicus* female and *O. niloticus* male showed a complete homology, as judged by complete synapsis of bivalents in pachytene stage.
2) It is suggested that the Y chromosome of the Mozambique tilapia presents some structural change or some mutations that makes it different from the Y chromosome of *O. niloticus*, from the diffuse chromatin and differential staining observed in bivalent 1.
3) The hybrid between *O. niloticus* female and *O. aureus* male showed a complete homology, as judged by complete synapsis of bivalents in pachytene stage.
4) The haploid chromosome number of *O.N. karongae* was n = 19. This observation confirms the diploid chromosome number of 2n = 38.
5) There is chromosome homology in *O. niloticus* female x *O. karongae* male hybrids. Nevertheless, during meiosis three trivalents were observed, each composed of one large chromosome from the father and two small chromosomes from the mother. In other spreads failures in chromosome synapsis were observed as aberrant pairing and multivalents.
6) The *O. karongae* karyotype appears to have arisen from the typical *Oreochromis* one by three fusions.

CHAPTER 8

GENERAL DISCUSSION

8.1. Meiotic studies in tilapias

The events during prophase I in tilapia, the formation of the axial elements and the synapsis behaviour of the homologous chromosomes, was similar to the patterns observed in other eukaryotes. The observation of sex chromosomes in the heterogametic sex, using the electron microscope, showed essential cytogenetic differences in the three *Oreochromis* species studied. These differences are considered of great importance and increase our understanding of sex-determination studies of tilapias and fish in general. It expands our knowledge on the evolution of undifferentiated sex chromosomes in lower vertebrates and clearly suggests the necessity to analyse fish chromosomes under mitotic and meiotic stages.

In Chapter 3 it was possible to analyse both male and neomale genotypes in *O. niloticus* as previously realised by Carrasco (1998), but it was also possible to analyse both sexes directly (females) and indirectly (neomales) and males in the three tilapia species. These observations have identified that the synaptonemal complex complement length of females is about 1.4 (on average) greater than males and that the process of sex reversal (to induce neomales) has an effect on this length.

8.2. Sex chromosomes behaviour during Meiosis

In Chapter 3, prophase I was described basically for some confusion arose when comparing the well documented progressive synapsis and desynapsis of the homologous chromosomes in eukaryotes with a previous published report in fish (Liu and Yu, 1991).

This description was also meant to present the progressive synapsys and desynapsys events of the homologous chromosomes involving the observation of sex chromosomes during pachytene stage in the heterogametic sex of each *Oreochromis* species. The undifferentiated sex chromosomes in tilapia mean that it is important to distinguish between real unpairing events in pachytene stage rather than being confused with the presence of unpaired regions during late zygotene.

8.3 Sex chromosomes in *O. niloticus*

In Chapter 4, it was suggested that the unpaired bivalent 1 in XY *O. niloticus* can be considered as an early pachytene stage where bivalent 1 remains terminally unpaired and later on synapses again at mid pachytene. However, the non-significant ANOVA test between the total length in each of the two longest bivalents (unpaired against unpaired) did not support this statement. However, the sample size in the ANOVA analysis was n = 12 (5 unpaired and 7 fully paired), which was considered to be small and not statistically representative. In the other hand, both bivalents were longer when bivalent 1 showed partial synapsis, which indicated a delay in synapsis of bivalent 1 through the progressively shortening of chromosomes during prophase I. The marginal non-significance ($P = 0.07$) of bivalent 2, suggests that if the sample size is increased, there would be a significance between the mean lengths. Anyway, it can be recommended to measure more SCs spreads (bivalents 1 and 2) to reach a statistically based conclusion.

The observation made by Wise *et al.* (1987) in the mosquito fish male, (*Gambusia affinis*), is controversial as they observed an unpaired terminal region in bivalent 1 that they identified as late zygotene.

8.4. Sex chromosomes in *O. aureus*

Testing of the sex ratios observed in progeny from *O. aureus* neomales showed that none of these differed significantly from 1:1 (Table 5.4.). Testing of the sex ratios from the neomales showed that in two of the three fish in which both types of SC unpairing was observed (neomales 1 and 2), these were also not significantly different from 3:1 (females: males), the expected sex ratio from a WZ W'Z' x ZZ Z'Z' cross. However, the sex ratios from progeny testing of the males also showed a similar pattern (not significantly different from 1:1 but two individual crosses also not significantly different from 3:1). These results do not provide clear support for the model suggested for two pairs of independently segregating sex chromosomes.

Another problem with the model suggested in Chapter 5 is that a WZ W'Z' x ZZ Z'Z' cross would only give one quarter of the progeny with the WZ W'Z' genotype, while no other crosses would result in this genotype. The frequency of the WZ W'Z' genotype could thus only decline. Under this model, the two females which gave rise to the females and neomales respectively for SC analysis (Table 5.4.) would both have been WZ W'Z', as both types of unpairing were observed among their offspring. This would appear to be unlikely on a probability basis from a population consisting of a mixture of all three female genotypes, with a declining proportion of the WZ W'Z' genotype.

Of course it was not expected to observe two pairs of sex chromosomes and as fish were all killed after progeny testing to obtain the SC spreads, it was not possible to carry out further progeny testing.

Chapter 5 lacks the Mendelian evidence of offspring derived from three different genotypes in females. It is well documented that the sex ratios in the blue tilapia are some times skewed to females (Mair *et al.*, 1991b).

In Chapter 5 it was also mentioned that this species exhibits temperature sex determination, which increases the proportion of male progeny at higher temperatures: maintenance of *O. aureus* fry at 34 °C during sexual differentiation resulted in 97.8% males (Desprez and Melard, 1998). Furthermore, constant high temperature and fluctuating temperatures (simulating the natural environment) have a masculinizing effect, with the fluctuating temperatures having a lower masculinizing potential (Baras *et al.*, 2000). In their research, Baras *et al* (2000) simulated the natural variations of temperature conditions occurring in the blue tilapia habitat with fluctuations of 35 °C during the day going progressively down to 27 °C at night. They discussed the evolutionary advantage of thermosensitivity in *Oreochromis* species in which males have a faster growth rate with the advantage to escape from predation and capability to colonise more niches. The condition of mostly males migrating and colonising new areas was a strategy of survival and one way to reproduce successfully through generations.

This suggests that in nature a certain number of females are sex reversed to males (neomales). The presence of males and 3 genotypes of females and neomales, all carrying the genetic primary sex determination, could sustain maleness might be an important evolutionary strategy.

The first question that arises from the above is whether each genotype has the same sensitivity to be sex reversed by high temperature or even by hormone treatment. This could explain why some batches are more difficult to sex reverse as was mentioned in the introduction to Chapter 5. It is not necessarily a high temperature that is needed to produce these neomales, possibly there are some minor autosomal sex determining genes that are expressed equally or distinctly in the three female genotypes, so maintaining the different genotypes. If some minor sex determining genes are acting in the progeny of WZ W'Z' genotype it could be possible to disguise the progeny sex ratios from males and neomales presented in Chapter 5. The presence of three different genotypes in the blue tilapia skewing the sex ratios toward females could be an adaptive reproductive strategy that allowed a faster recovery of females in the population after a hot and dry year where most of the population became males and neomales. Baras *et al* (2000) refers to maleness as an important reproductive strategy in *Oreochromis* species. In the case of XX/XY systems, the presence of XX neomales carrying the female genotype makes a simple model to understand when compared with the blue tilapia. However, XX/XY systems could not increment the presence of females in the population after a hot and dry year as fast as the blue tilapia.

The presence of three genotypes in WZ W'Z' females will have to be proved by Mendelian genetics or hopefully by molecular markers in the future.

8.5. Sex chromosomes in *O. mossambicus*

In Chapter 6, it was found that in one of the males analysed showed a region where the lateral elements appeared diffuse. This observation was considered as possibly an artefact of the technique and its frequency in the total of the cells analysed was very low (1 in

60). However, in the hybrid between female *O. niloticus* x male *O. mossambicus* (Chapter 7) 9 out of 36 nuclei presented a similar pattern and always in bivalent 1. From these observations, the cytogenetic evidence in a male of *O. mossambicus* was re-analysed. The region with diffuse lateral elements was located in the first third of the chromosome, it comprise about 1/5 (20 %) of the total length of bivalent 1 and it was skewed towards the kinetochore (structure not 100 % identifiable in the bivalent). The analysis of recombination rates between the sex genes and the centromere (Chapter 6) suggested that sex genes were near the centromere (its exact location was not possible to calculate). The cytogenetic evidence appears to be similar to the gynogenetic evidence. The diffuse lateral elements patterns in bivalent 1 of the hybrid (Chapter 7) were variable, however, it was clear that three photographs (Figures 7.2.c,d and e) presented the same pattern as that found in the pure species male. This suggests that bivalent 1 in *O. mossambicus* could be related with sex determination as *O. niloticus* and *O. aureus*.

8.6. Crossover restriction in *Oreochromis*

Meiosis involves the production of gametes through two divisions. The first division reduces to half the chromosome number, segregating independently the homologue chromosomes and the second division separates both chromatids that were initially formed through a semi-conservative replication of DNA. Meiosis is a process that is involved in the diversity of species through evolution. The variability that each species and population present nowadays has been the result of DNA crossover events and mutations during or after the DNA replication that are passed on through the gametes to

the next generation. The unpaired regions observed in the heretogametic sex of *O. niloticus* and *O. aureus* implies that crossing over restriction is taking place in sex chromosomes. As has been covered in Chapter 1, crossing over restriction between the sex factor region is considered the first step in the evolution of sex chromosomes (Bull, 1983; Solari, 1994; Charlesworth and Charlesworth 2000). The Muller's ratchet and Genetic hitchhiking (see Chapter 1, section 1.2.5.1.1 and 1.2.5.1.2) and other theories reviewed by Bull (1983) and Charlesworth and Charlesworth (2000), consider that the most plausible hypothesis in crossover suppression derives from the fact that recombination is disadvantageous for sex-linked alleles with opposite effects in the two sexes. For example, consider a mutation with a slightly beneficial dominant effect in males, but a highly deleterious dominant effect in females. Such a mutation could not increase if it was inherited autosomally because the large disadvantage of females would outweigh the small advantage in males. However, if the mutation was closely linked to sex determining region of the Y-chromosome, it could increase in frequency by virtue of the superior benefit conferred nearly exclusively on males. Solid evidence suggests that pigment genes in Poecillid fish are beneficial in males but not in females (reviewed by Bull, 1983). Bright colours are disadvantageous to females because of increased predation. Brightly coloured males also experience increased predation, but this disadvantage is offset by female preference for bright males.

It can be suggested that in *O. niloticus* and *O. aureus* a primitive stage of sex chromosome differentiation is observed through a genetic mechanisms of crossover suppression. However, in a closely related species, *O. mossambicus*, the unpaired lateral elements were not observed. During pachytene of the Mozambique tilapia, it was

expected to observe that the unpaired region of the lateral elements could be even larger than those observed in *O. niloticus*, since this species was not reported to present recombination between the sex locus and the centromere (Varadaraj and Pandian 1989). In Chapter 6, it was suggested that sex loci might be near the centromere region (instead of being distantly from it, as occurs in *O. niloticus*). It also was suggested that crossover suppression could occur because of the presence of heterochromatin blocks (C-bands) that are devoid of chiasmata and prevent recombination beyond their borders. So how do some tilapia species present distal sex loci whilst the Mozambique tilapia present sex loci closer to their centromere? It can be suggested that a paracentric inversion occurred in the Y chromosome of the Mozambique tilapia that included sex genes and then located them near the centromere region. Chromosomal rearrangements may be evolutionary events in tilapias as observed in *O. karongae* (Chapter 7).

As argued and reviewed by Bull (1983), X-Y crossover may be suppressed through chromosomal rearrangements. Chromosome rearrangements such as inversions and translocations may have two effects. First, a rearrangement may interfere with the synapsis of homologous chromosome regions and thereby inhibit crossing-over in these segments, consequently, sex chromosome rearrangements may be selected for suppression of X-Y recombination. Second, a possible consequence of rearrangements is that they are deleterious, because they often complicate meiosis and lead to aneuploid gametes (e.g. Chapter 7). However, in *Drosophila* a special mechanism in oogenesis reduces or eliminates crossover products resulting from an inversion that do not overlap the centromere (paracentric inversion); crossing-over is rare or absent in the male, so inversions are without deleterious effects in either sex. Consequently, paracentric

inversions act as crossover suppressers without deleterious effects even though they do not necessarily inhibit the formation of crossovers. An inversion that is free from crossing- over has no deleterious effect in the production of gametes.

8.7. Evolution of sex determination systems in tilapias

Cichlid fish have been much studied at the anatomical, behavioural and molecular levels in attempts to identify their phylogenetic relationships. The tilapiines have been grouped into a tribe within the family Cichlidae and despite more than one century of research into this group the evolutionary and taxonomic relationships are still unclear (reviewed by McAndrew, 2000). Trewavas (1983) proposed the present classification of the tilapiines with the biparental and parental mouthbrooding *Sarotherodon* and maternal *Oreochromis* as separate genera that arose from a single or separate split from substrate and guarding spawning Tilapia. However, Peters and Berns (1982) believed that there have been a number of splits from the ancestral substrate brooders, the more ancient of these having had time to evolve into maternal mouthbrooders (*Oreochromis*) compared with the more recently separated lineages, which have only progressed to the less efficient paternal and biparental mouthbrooding state (*Sarotherodon*). Therefore, Peters and Berns (1982) proposed that the various species should all be *Tilapia* and at best given subgeneric status. However, using allozyme variation McAndrew and Majumdar (1984) studied 9 species from these three genera using 25 allozyme loci and a follow up study was conducted (Sodsuk and McAndrew, 1991), which included 15 species analysed for 44 allozyme loci, in an attempt to discriminate between the two main hypotheses,

Trewavas vs. Peters and Berns, on the evolution of mouthbrooding in tilapiines. Both a phenetic and cladistic analysis of the allozyme data clearly showed a dichotomy between the substrate spawning and guarding *Tilapia* species and the mouthbrooding *Sarotherodon* and *Oreochromis*. Furthermore, Franck *et al* (1994) analysed the SATA satellite DNA family to derive phylogenetic relationships from consensus sequence data from five species within three genera (*Oreochromis*, *Sarotherodon* and *Tilapia*). They obtained a tree with the most parsimonous explanation in which the mouthbrooding *Sarotherodon* and *Oreochromis* species formed a single clade separated from the *Tilapia* species analysed. Sodsuk (1993) using morphological traits and allozyme data to produce a cladogram with a consensus phylogeny of 14 different *Oreochromis* species, placed *O. mossambicus* close to *O. spilurus* and *O. niloticus* close to *O. aureus* but in separate branches.

Cytogenetic meiotic analysis, shows that meiotic prophase I had a similar pattern of synapsis of the lateral elements between the three *Oreochromis* species. The only cytogenetic differences were observed in the heterogametic sex of *O. aureus* (WZ, ZZ; W'Z', Z'Z') and of *O. niloticus* (XX, XY), which were different to that observed in *O. mossambicus* (XX, XY). These differences were related with the behaviour of sex chromosomes during meiosis. It is confirmed that bivalent 1 in *O. aureus* is homologous to bivalent 1in *O. niloticus*. Both chromosomes share a similar meiotic constraint in bivalent 1, observed as a subterminal unsynapsis of the lateral elements in pachytene stage present only in the heterogametic sex (Campos-Ramos *et al.*, 2001). Recently it was also confirmed that bivalent 1 in *O. niloticus* is homologous to bivalent 1 in *O. mossambicus* (Harvey, pers comm), but the latter does not show unpaired subterminal

lateral elements in bivalent 1. These cytogenetic differences between the species may exist in the location of sex genes in the Y-chromosome.

The presence of two pairs of sex chromosomes in females of *O. aureus* may indicate that the longest sex chromosome (bivalent1) is a common ancestor for sex determination in *Oreochromis* species. The small chromosome pair in *O. aureus* may be related with the evolution of a WZ ZZ; W'Z' Z'Z' female sex determination system in this species.

Hybrids of the three species show homology in all the bivalents with no irregularities during synapsis except for a diffuse chromatin surrounding the synapsis of bivalent 1 between the Y-chromosome of *O. mossambicus* and the X-chromosomes of *O. niloticus* (Chapter 7). In the *O. niloticus* x *O. karongae* hybrid chromosomes rearrangements involved small chromosomes, in all cases bivalent 1 showed homology without any irregularity. From an evolutionary point of view, the Y-chromosome in this genus has been little differentiated from its X homologue. There are possibly a few allelic differences restricted in an obligatory way to the heterogametic sex. There is no evidence of sex-linked inheritance such as colour as happens in the male guppy *Poecilia reticulatus* (Chourrout and Chevassus, 1998) that means that a few mutations have occurred in the vicinity of the alleles. Consequently, there has been little accumulation of heterochromatin since both chromosomes present only centromeric bands (C-bands) (Majumdar and McAndrew, 1986) and so gene inactivation or loss of functional genes have not occurred.

Reproductive behaviour has been used widely for taxonomy and evolution of tilapias; this behaviour had to emerge since the first gonochorist tilapia fish appeared and that seems to be related with chromosome rearrangements and the evolution of sex determination systems of each species.

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Appendix

List of Communications

Poster Presentation

Penman D.J., Griffin, D. K., Masabanda, J., Harvey S.C., Campos-Ramos R., Carrasco L.A.P. and Bromage N.R. 2000. Microdissection of *Oreochromis niloticus* putative sex chromosomes and DOP-PCR produces chromosome-specific FISH probes. In: *Proceedings in Genetics in Aquaculture VII*, Townsville, Australia.

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Campos-Ramos R., Carrasco L.A.P., McAndrew B.J., and Penman D.J. 2001. Description of meiotic Prophase I and potential sex chromosomes in the tilapias *Oreochromis aureus, O. mossambicus* and *O. niloticus,* through a synaptonemal complex analysis. *Chromosome Research* 9: 127.

Oral Presentation and Abstract Published

Harvey S.C., Campos-Ramos R., Kennedy D.D., Masabanda J., Griffin D.K., Bromage N.R. and Penman D.J. 2001. Karyotype analysis of *Oreochromis karongae*, a species with an unusual chromosome complement, and cytogenetic analysis of *O. karongae* x *O. niloticus* hybrids. *Chromosome Research* 9: 37.

Publication

Campos-Ramos R., Harvey S.C., Masabanda J.S., Carrasco L.A.P., Griffin D.K., McAndrew B.J., Bromage N.R. and Penman D.J. 2001. Identification of putative sex chromosomes in the blue tilapia, *Oreochromis aureus*, through synaptonemal complex and FISH analysis. *Genetica* 111 (1-3): 143-153.

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