VARIATION IN SEX DETERMINATION AND THE APPLICATION OF THE YY MALE TECHNOLOGY FOR THE PRODUCTION OF ALL-MALE POPULATIONS OF THE TILAPIA *OREOCHROMIS MOSSAMBICUS*

Assignment presented in partial fulfilment of the requirements for the degree Master of Philosophy (Livestock Industry Management: Aquaculture) at the University of Stellenbosch

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DECLARATION

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

Signature: _____ Date: 25-11-2004

ABSTRACT

Tilapia is presumed to have a well-defined genetic mechanism of sex determination, but not all sex ratios are compatible with a monofactorial sex determination model. A theory of autosomal gene influence, as well as temperature sex determination (TSD) has been proposed in order to explain large variations in sex ratios. This study assessed the variation in progeny sex ratio in *O. mossambicus* as a basis for the application of YY male technology in the production of all-male progeny groups.

Three populations of *O. mossambicus* were sampled that are representative of the geographical distribution of the species in Southern Africa. Progeny groups were produced from randomly selected parents and maintained at constant temperature during the labile period of sex differentiation. Variation in sex ratios between different families of the same strain as well as between different strains was calculated. No significant differences were observed in sex ratio between strains, though a significant intra-group variation was identified. This study identified both male and female-biased sex ratios. The data in general conform to a monofactorial sex determination model. Male-biased sex ratio observed in one strain can possibly be ascribed to temperature sex determination (TSD). This strain of *O. mossambicus* originated from an area with different annual temperature patterns and the possibility of TSD having an adaptive advantage is discussed.

This thesis also presents the results of a program to produce monosex male tilapia through the application of the YY male technology in *O. mossambicus*. Viable XY female and YY male genotypes were produced. XY females sired progenies ranging from 68-94% male ofspring, while YY males sired a mean progeny of 94% male phenotypes. From these results it is concluded that YY male technology provide a viable method for the production of all-male progeny in *O. mossambicus*. Once available on a commercial scale, the technology can be made more reliable through the application of the appropriate selection methods.

OPSOMMING

Tilapia spesies is oorwegend onderworpe aan ‘n goed gedefinieerde enkelfaktor model van genetiese geslagsbepaling, hoewel afwykings van sodanige geslagsbepalingsmodel soms waargeneem word. Die invloed van outosomale gene, sowel as omgewings temperatuur word voorgehou as verklaring van die waargenome variasies in geslagsverhoudings. Hierdie studie behels die evaluasie van variasie in geslagsdifferensiasie van *O. mossambicus* as basis vir die implementering van die YY manlike tegnologie vir die produksie van slegs-manlike nageslag groepe.

Monsters is bekom van drie populasies van *O. mossambicus* verteenwoordigend van die geografiese verspreiding van die spesie in Suider Afrika. Nageslag groepe is geproduseer vanaf ewekansig gekose ouerpare en by ‘n konstante temperatuur gehuisves tydens die sensitiewe tydperk van geslagsdifferentiasie. Variasie in geslagsverhouding tussen verskillende families binne dieselfde subpopulasie sowel as binne verskillende populasies is bepaal. Geen betekenisvolle verskille is waargeneem in die geslagsverhouding tussen die verskillende populasies nie terwyl betekenisvolle verskille tussen families binne ‘n populasie waargeneem is. Die data voldoen aan ‘n enkelfaktor genetiese geslagsbepalingsmodel, met die uitsondering van een populasies waar ‘n moontlike omgewingsverwante geslagsbepalingseffek waargeneem is.

Die tesis sluit ook in die resultate in van ’n program om YY-tegnologie in *O. mossambicus* te vestig ten einde manlike nageslag groepe te produseer. Funksionele vroulike XY- en manlike YY genotipes is ontwikkel. Die vroulike XY-genotipes het manlike nageslag groepe opgelewer wat wissel van 68%-94% manlik, terwyl manlike YY-genotipes nageslag groepe gelewer het wat gemiddeld 94% manlik was. Die resultate bevestig die lewensvatbaarheid van YY-tegnologie vir die kommersiële produksie van manlike nageslaggroepe in *O. mossambicus*.

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INTRODUCTION

1.1 GLOBAL AQUACULTURE

Worldwide fisheries statistics indicate that the global yield in marine products have reached a plateau, while the demand for aquatic protein sources have been rising steadily. This increase in demand has resulted in a fast expanding aquaculture industry, with an increasing amount of fisheries products resulting from aquaculture output (Gilbert, 2002). Aquaculture's contribution to total global fisheries has increased from 5.3% in 1970 to 32.2% of total fisheries landings by weight in 2000 (FAO, 2003) as presented in Figure 1.

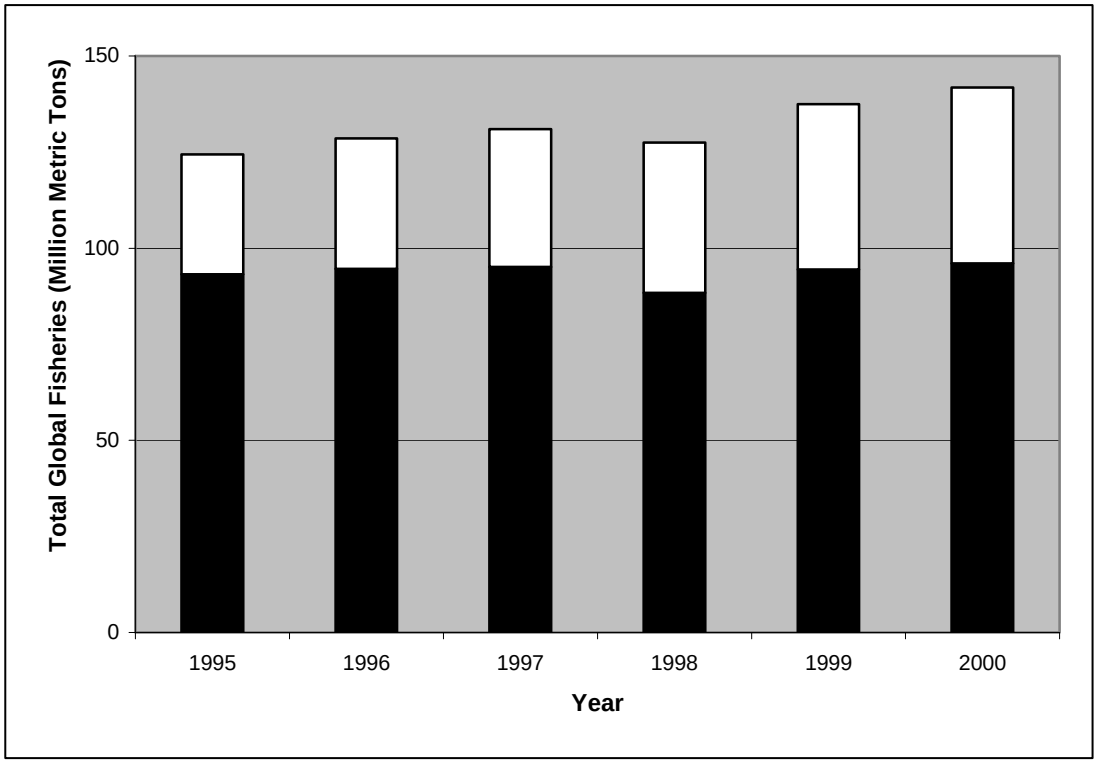

Figure 1.1: Global statistics for fisheries and aquaculture produce from 1995 to 2000. ■ = fisheries output, ڤ = aquaculture production (Gilbert, 2002).

Total aquaculture production in 2000 was reported as 45.71 million metric tons (mmt) by weight and valued at US$56.47 thousand million, with production increasing by 6.3 % by weight and 4.8% by value since 1999 (FAO, 2003). Over half of the total global aquaculture production in 2000 was in the form of finfish (50.4% of total production), followed by molluscs (23.5%), aquatic plants (22.2%), crustaceans, (3.6%), amphibians and reptiles (0.22%) and miscellaneous aquatic invertebrates (0.08%).

Freshwater species dominate global finfish production with 85.8% of total production, followed by diadromous species and marine species at 9.8% and 4.4%, respectively. The major finfish groups and species cultivated in 2000 are summarized by weight and value in Table 1.1

Table 1.1: Production figures and values of major finfish groups and species cultivated in 2000 (FAO, 2003).

Group	Production (mt)	Value (million US $)
Freshwater species		
Cyprinids	15 707 109	15 251
Tilapia	1 265 780	1 706
Catfish	421 709	655
Diadromous species		
Salmonids	1 533 842	4 875
Milkfish	461 857	715
Eels	232 815	975
Marine species		
Marine finfish	1 009 663	4 072

1.2 TILAPIA AQUACULTURE

Judging by FAO production statistics, it is evident that tilapia plays an important role in freshwater fish production, with different species of tilapia being successfully produced in many parts of the world. World harvest of commercially farmed tilapia has surpassed 1 000 000 MT per annum and tilapia is second only to carps as the most widely farmed freshwater

fish in the world (Popma and Lovshin, 1994; FAO, 2003). The suitability of tilapia as an aquaculture candidate for a variety of production systems, ranging from extensive to intensive systems, is well known. Tilapias are tolerant to a wide range of environmental factors, as well as the stress factors typical of most production systems, e.g. high densities, handling, etc. Tilapias are also prolific breeders and are generally disease resistant (Popma and Masser, 1999; Roberts and Sommerville, 1982).

Tilapias are endemic to Africa, but their potential as aquaculture species has led to a nearly worldwide distribution in the past 50 years. *O. mossambicus* was the first tilapia species to be exported from Africa to the Indonesian island of Java in 1939 (Popma and Lovshin, 1994). During the 1950's and 1960's many people and organizations, encouraged by its ready production in ponds, resistance to disease and tolerance of adverse environmental conditions, introduced *O. mossambicus* to numerous countries in Central America, South America, Southeast Asia, India, Pakistan and north Australia. However, enthusiasm for its culture as food fish was curbed as soon as the problems associated with mixed-sex populations, such as overpopulation and stunted growth, became apparent.

Attempts to overcome these and other problems was made in the form of introducing other tilapia species which grew faster and larger under many culture conditions and reached sexual maturity at a later age and larger size than *O. mossambicus*. Two other species introduced outside Africa, *O. niloticus* and *O. aureus* are currently responsible for virtually all large-scale commercial culture of tilapia (Popma and Lovshin, 1994). The switch from *O. mossambicus* to *O. niloticus* has been one of the major reasons for the impressive production levels now seen in countries such as Taiwan and the Philippines (pers. comm. Eric Roderick [1]).

[1] University of Wales, Swansea

1.3 THE RATIONALE FOR THE DEVELOPMENT OF THE YY-MALE TECHNOLOGY

Many popular aquaculture species exhibits a sexual growth dimorphism, making the culture of a monosex population an attractive option for most aquaculturists. In the culture of tilapias, male is the preferred sex for a production system due to their faster growth rate and larger size (Baroiller and Toguyeni, 1996; Mair et al., 1997; Gale et al., 1999).

An equally important problem in the culture of tilapia is the paradoxical consequences of the prolific breeding that is characteristic of a mixed sex population of this species (Popma and Lovshin, 1994; Baroiller, 1996; Mair and Abella 1997; Ezaz et al., 2004). As soon as fish become sexually mature, reproduction begins and the recruits begin to compete with the stocked fish for resources, with the result being a lower yield from the production system. Linked to the problem of early sexual maturity and uncontrolled breeding, is the energy that is wasted in the synthesis of reproductive products. The production of ova requires more metabolic resources than sperm, so producing a monosex male population results in more resources that can be devoted to somatic growth (Patino, 1997).

Initially monosex populations were produced through manual sexing of juvenile fish and the subsequent removal of female individuals. This technique is both labour-intensive and not 100% accurate (Baroiller and Toguyeni, 1996). Experimental work with hybrid crosses that yield 100% male progeny was initially thought to be the answer to monosex male production, but the inconsistency of this approach is the main reason why it has not been implemented on a commercial scale (Wolfarth, 1994).

Hormone-induced sex reversal in tilapia has shown great promise and with the optimization of both masculinization and feminization protocols, it is possible to create a monosex population in the excess of 95% (Shelton et al., 1981; Rosenstein and Hulata, 1993;

Vera Cruz and Mair, 1994; Panadian and Sheela, 1995; Gale et al., 1999). Factors affecting hormone-induced sex reversal are discussed in Chapter Three (Table 3.1).

However, the large-scale use of synthetic hormones has raised several concerns over human as well as environmental health (Baroiller and Toguyeni, 1996; Gale et al., 1999, Baras et al., 2001). There are both ethical and legal implications when large amounts of any synthetic hormone are used on a continuous basis, such as in hormonal sex reversal. Several important questions still remain unanswered as to what the effect of continuous exposure to these chemicals is on both the environment and the personnel working with it. Furthermore, in the case of a chemical such as diethylstilboestrol (DES), where toxicity to humans has been proven and the necessary regulations concerning its use are in place, lesser or underdeveloped countries are still at risk (Gale et al., 1999). Under such conditions it is often the case that these chemicals may be used without regulation, handled incorrectly due to a lack of knowledge and applied without the necessary care, as operators have not been properly trained.

1.4 OBJECTIVES OF THIS STUDY

A. The main objective of this project is to transfer the YY male technology from *O. niloticus* to *O. mossambicus*, a tilapia species indigenous to South Africa. The successful development of a YY-strain could improve the viability of tilapia culture in Southern Africa, as it provides a better alternative to traditional hormonal sex reversal. Hormonal sex reversal is subject to growing consumer and market resistance.

B. To conclude a literature study on the mechanisms of sex determination in fish and the occurrence of environmental sex determination (ESD) in tilapia. There are several reports of temperature sex determination (TSD) in different fish species.

Understanding the mechanism and effect of this phenomenon is likely to have commercial value (Mair et al., 1990; Baroiller et al., 2001).

C. To assess the variance in sex ratio between different strains of *O. mossambicus* at a set temperature. This will form the basis of future work on the occurrence and magnitude of ESD in *O. mossambicus*.

CHAPTER 2
GENERAL AND REPRODUCTIVE BIOLOGY OF TILAPIAS

2.1 GENERAL BIOLOGY

The group Tilapia, which consists out of approximately 110 species, belongs to the family Cichlidae. Originally considered as one genus, the tilapias have now been divided into three distinct genera – *Oreochromis*, *Sarotherodon* and *Tilapia* (Baroiller and Jalabert, 1989). All tilapias can readily be identified by an uninterrupted lateral line characteristic of the Cichlid family, while different species can be identified by different banding patterns on the caudal fin (Popma and Masser, 1999). Colouration varies between species, with *Oreochromis niloticus* characterized by grey or pink colouration on the throat area. *Oreochromis mossambicus* males have a concave head and distinct black colouration with red margins on the caudal and dorsal fin during breeding time (Popma and Lovshin, 1994). Tilapias are typically deep-bodied and laterally compressed with long dorsal fins. The forward portion of the dorsal fin is heavily spined, with spines also found on the pelvic and anal fins (Popma and Masser, 1999).

Tilapias are generalist feeders which can utilize a large variety of food sources. Zooplankton such as copepods, as well as a variety of plant material forms an important part of the natural diet of tilapia. In addition to microorganisms, detritus is digested and is a major nutritional resource for tilapias (Pullen and Lowe-McConnel, 1982). Tilapias require the same essential amino acids as other warm water fish, and protein requirements for maximum growth are a function of protein quality and fish size (Popma and Masser, 1999).

2.2 REPRODUCTIVE BIOLOGY

Several characteristics distinguish the three different genera, but possibly the most important relates to reproductive behaviour. All *Tilapia* species are substrate spawners, with the male

engaging in nest building behaviour, followed by spawning and the guarding of the fertilized eggs in the nest by a brood parent. Substrate spawning species ventilate their eggs by constantly stirring the water with their caudal fins (Baroiller and Jalabert, 1989). The *Oreochromis* and *Sarotherodon* species are mouth brooders, with the female picking up the fertilized eggs and incubating them in her buccal cavity until several days after hatching (Pullen and Lowe-McConnel, 1982; Popma and Masser, 1999). Mouth brooding behaviour in females is often combined with a migration of the breeding fish to a planted and thus protected area (Baroiller and Jalabert, 1989). In *Oreochromis* species only females practice mouth brooding, while in *Sarotherodon* species both the male and female are mouth brooders (Popma and Masser, 1999).

The majority of commercially important tilapia species is classed under the genus *Oreochromis* and are maternal mouth brooders. Most tilapia species reach sexual maturity at six months or earlier and can breed at sizes as small as 40 grams (Mair and Abella, 1997). Sexual maturity in tilapia species is a function of age, size and environmental conditions. *O. mossambicus*, in general, reaches sexual maturity at a younger age and smaller size than *O. niloticus* and *O. aureus* (Popma and Masser, 1999).

Tilapia populations in nature mature at a later age and larger size than the same species reared in a production environment. For example, in several East African lakes, *O. niloticus* was found to mature at 10-12 months and 350-500 grams. The same population, under conditions of near maximum growth in an intensive production system, will reach sexual maturity at an age of 5-6 months and 150-200 grams (Popma and Lovshin, 1994).

Upon reaching sexual maturity, males become very territorial and both sexes demonstrate hierarchy (Mackintosh, 1985, as cited in Mair and Abella, 1997). During breeding time, the males are highly aggressive, but under natural conditions most interactions do not result in serious injury. Under favourable breeding conditions and suitable stocking

densities, breeding in a population is continuous but fluctuating (Baroiller and Jalabert, 1989). No synchronization exists between spawning females within the population, with a female being able to produce fry every four to six weeks. An exception to this would be populations from areas exposed to significant seasonal variation (Moreau, 1979, as cited in Baroiller and Jalabert, 1989).

The reproductive cycle can be divided into 5 phases as indicated in Figure 1.

(1) Nest building and courtship,

(2) Ovulation and spawning,

(3) Incubation,

(4) Nursing, and

(5) Feeding and recovery.

At the onset of breeding, the fish naturally seeks out shallow areas with a firm substrate where the males tend to group together. The male then excavates a nest in the pond bottom and exhibits nest-protecting behaviour. Nests are constructed in areas of sparse to moderately dense vegetation and at different depths. Average nest size was found to increase in size up to a water depth of 55cm, but remained constant in size in deeper water (Trewavas, 1983). Females ready to spawn enter the nesting area and after a short mating ritual the female spawns in the nest and the male proceeds to fertilize the eggs. A male will mate consecutively with several different females.

The total number of eggs produced is a function of body size with 1-2 eggs produced per gram of bodyweight (Trewavas, 1983; Popma and Lovshin, 1994). After spawning the female picks up the fertilized eggs and incubates them in her buccal cavity for approximately two weeks until hatching. Newly hatched fry remain in the female's mouth until yolk sack absorption. This period of "maternal dependency" lasts between 10 and 30 days and varies in duration depending on the environment (Macintosh 1985, as cited in Mair et al., 1997). As

fry get older, they are released for longer and longer periods of time until they become completely independent.

The development of tilapia from fertilized egg to free-swimming juvenile fish is characterized by several different developmental stages. Fertilized eggs hatch after three to five days and fry are released by their mother 10 to 14 days after spawning (Trewavas,1983). Knowing the exact age of the fish at the onset of every stage is difficult as development is influenced by ambient temperature. Furthermore, as the young are incubated in the buccal cavity of the female, it is extremely difficult if not impossible to witness the development from one stage to the next.

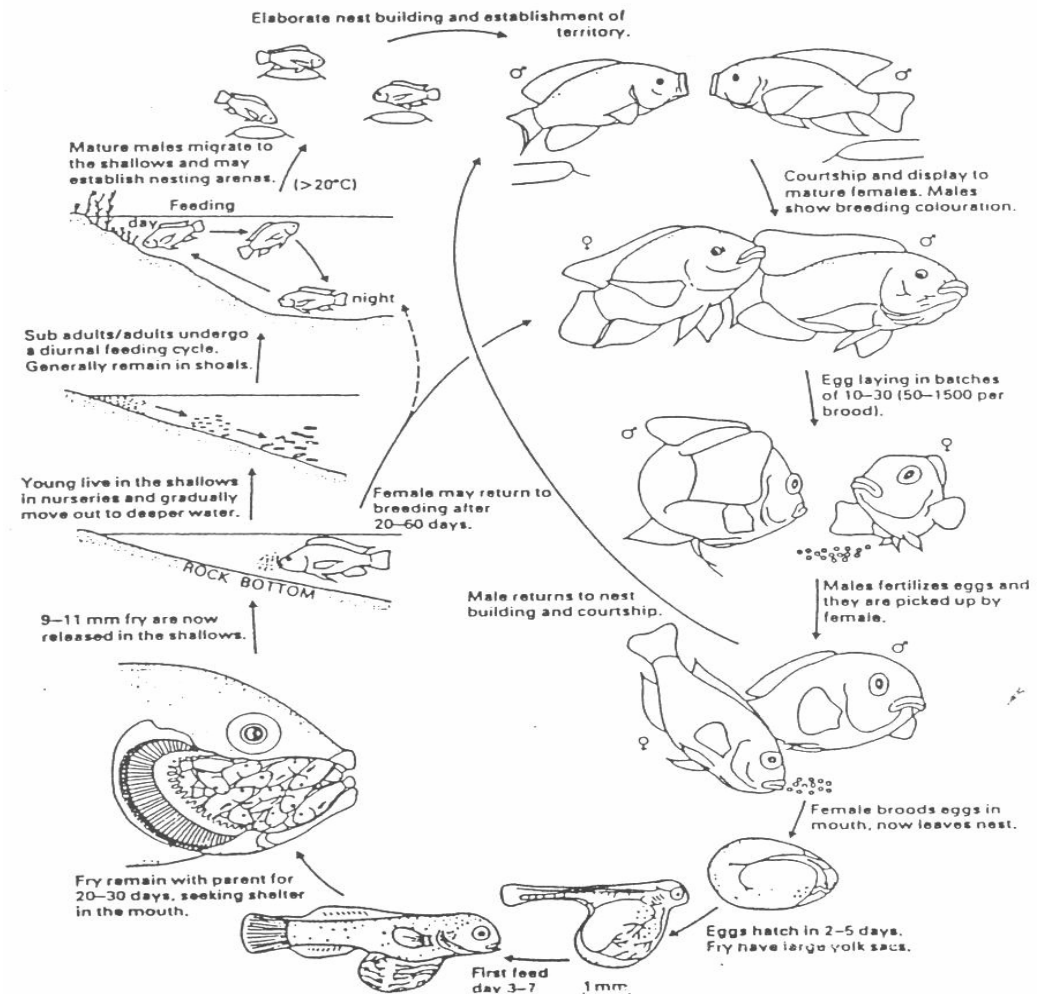

Figure 1: Diagram showing the breeding cycle of a typical mouth brooder such as *O. mossambicus*. (Mason, 1984)

2.2.1 DEVELOPMENTAL STAGES

The early developmental stages of tilapia can be classified as:

1. Eyed ova: This is the stage at which the fertilized egg is far enough developed for the eyespots of the embryo to become visible. Healthy tilapia eggs are a dark yellow colour with the eyespots visible as two dark brown to black spots. Unviable eggs are a light opaque yellow in colour and are easily distinguished from healthy ones.
2. Yolk-sack fry (early stage): This stage follows the eyed ova stage and commences when the juvenile fish start to hatch from the egg. The developing embryo can be seen as a head and tail attached to the yolk sac, which at this stage is very big in relation to the developing fish. Mobility of the newly hatched fish is minimal.
3. Yolk-sack fry (late stage): When development has progressed to this stage the fish are actively moving and attempting to swim up to the surface. The yolk sack is still present, but much smaller in relation to the fish's body size.
4. Swim-up fry/first feeding fry: By now the yolk sack is completely absorbed and the fish are highly mobile. They tend to aggregate at the water's surface, hence the term swim-up fry. It is also at this stage that they are ready to start feeding on whatever food source is available to them. In hatchery system a commercially available powder feed is normally offered as soon as the fish will accept it. At this stage the fish are ready to be stocked for sex reversal treatment.

An understanding of reproduction of tilapias, especially that of the commercially important species such as *O. mossambicus*, is critical to achieving the objectives set out in Chapter One. Most importantly, implementing the YY breeding program depends upon successful hatchery management and fry rearing. Optimizing reproductive output and hatchery management stands central to economic viability of any aquaculture operation and presents one of the most challenging scientific and technological challenges facing the tilapia industry today.

CHAPTER 3

SEX DETERMINATION AND NATURAL VARIANCE OF SEX RATIO IN TILAPIA, *OREOCHROMIS MOSSAMBICUS*

3.1 INTRODUCTION

The study of sex determination in fish is of importance both from an academic and commercial point of view. The biology and ecology of fish is sufficiently diverse to provide a vast array of sex determination and differentiation mechanisms, yet these mechanisms possess many of the same processes and pathways that are typical of other vertebrate systems (Devlin and Nagahama, 2002). Due to their suitability to artificial culture and experimental investigation, fish also provide unique opportunities to investigate and test different theories of sex determination, ranging from biochemical processes to evolutionary mechanisms (Devlin and Nagahama, 2002).

A second very important reason for studying and understanding sex determination in fish relates to the need to understand the reproductive biology of commercially important aquaculture species. As stated previously, the demand for aquatic protein sources is steadily growing, in spite of a plateau that has been reached concerning the harvesting of marine fish. In most aquatic production systems, understanding and controlling reproductive output is central to optimizing economic gain. Thirdly, fish are becoming increasingly important indicators of environmental health and play an indispensable role in environmental management and testing procedures.

Sex determination and differentiation in fish is an extremely flexible process with respect to evolutionary patterns observed among different genera and families (Devlin and Nagahama, 2002). Furthermore, a vast array of environmental factors has been proven or is suspected of influencing gender in a number of species (Baroiller et al., 1995a; Baroiller et al., 1995b; Abucay et al., 1999; Baroiller et al., 1999; Baras et al., 2001; Beardmore et al.,

2001). It is important here to differentiate between sex determination and differentiation, as these two terms are sometimes used as synonyms, in spite of having somewhat different definitions. Sex determination is the process whereby genetic sex is established, whereas sex differentiation can be observed as the process of gonad development after sex has been determined (Baroiller et al, 1999; Devlin and Nagahama, 2002). Mair et al. (1990) distinguishes between primary and secondary sex determination, where the former (primary) refers to the production of male- or female-causing gametes by parental fish, and the latter (secondary) to the differentiation of the phenotypic sex in immature fish.

3.2 MECHANISMS OF SEX DETERMINATION IN TILAPIA

Gender in all higher organisms is determined by the sex chromosomes (Bull, 1983). Fish, however, contain a vast array of sex-determining mechanisms, including sex determination by environmental factors (Trombka and Avtalion, 1993; Baroiller, 1996). In some fish species the chromosomes can be morphologically distinct, such as in rainbow trout, but tilapia does not have dimorphic sex chromosomes (Baroiller et al., 1995a). In fact, the evidence for sex chromosomes in tilapia, based mainly on sex ratio and sex inversion experiments, remain equivocal (Trombka and Avtalion, 1993). Cytogenetic examinations are rarely helpful in identifying sex chromosomes in fish due to the low occurrence of heteromorphy (Baroiller et al, 1995a). However, in the more recent work of Carrasco et al. (1999), evidence based on cytogenetic analysis was presented for the presence of sex chromosomes in *O. niloticus*.

Two distinct systems of chromosomal sex determination exist in the animal kingdom. These are the XX / XY system and the ZZ / ZW system, both of which are present in fish. The XX / XY system is characterized by female homogamety and male heterogamety. The genes that determine maleness is situated on the Y chromosome and individuals carrying this

chromosome will thus develop as males. Examples in tilapia are *O. niloticus* and *O. mossambicus* (Mair et al, 1991a).

The ZZ / ZW system is the opposite of the previous system and is characterized by female heterogamety and male homogamety. In this system, femaleness is determined by the W chromosome just as maleness is determined by the Y chromosome. An example in tilapia is *O. aureus* (Mair et al, 1991b). It is interesting to note that here both the sex determining mechanisms are present in the same genus (Desprez et al., 2003).

Tilapia is presumed to have a well-defined genetic mechanism of sex determination, but in spite of this hermaphroditism is known to occur in both natural and farmed populations of tilapia. Furthermore, in both hybrids and species-specific crosses, several authors have observed sex ratios that are not compatible with a monofactorial sex determination model (Mair et al., 1991a, 1991b; Wolfarth, 1994; Ezaz et al., 2003). Mair et al. (1991a, 1991b), based on results obtained from gynogenetic studies in *O. aureus* and *O. niloticus*, suggests that the proposed sex chromosomes may undergo a high degree of meiotic crossover whereby the sex-determining genes may be transposed from one sex chromosome to the other.

Hammerman and Avthalion (1979) proposed a theory of autosomal genes influencing sex determination in order to explain the large variations in sex ratios observed in the literature. However, with hindsight, this still fails to fully explain the large variability observed in sex ratios of different crosses of tilapias and the occurrence of environmental sex determination (ESD) must be considered. The most likely form of ESD to be encountered in tilapia is temperature dependent sex determination (TSD).

3.3 *MENIDIA MENIDIA* AS EXAMPLE OF TEMPERATURE DEPENDENT SEX DETERMINATION IN A GONOCHORISTIC FISH

Following a comprehensive study of environmental sex determination (ESD) in fish, it was demonstrated that temperature-dependent sex determination (TSD) is present in the Atlantic silverside *Menidia menidia*. Sex in this species is determined by the interaction of major sex determining genes and temperature during a distinct period of larval development (Conover and Kynard, 1981).

It was found that at lower temperatures larvae developed primarily as females and higher temperatures shifted the sex ratio to predominantly male. Sex ratios were sensitive to temperature only during a specific stage late in larval development, just before completion of metamorphosis. This illustrates the existence of a thermosensitive window in the development of this species. During this window, natural mortality was very low, indicating that temperature directly affected sex differentiation long after conception, rather than causing differential mortality of the sexes. Furthermore it was shown that sex ratios of progeny from different maternal sources vary greatly in their response to temperature (Conover and Kynard, 1981).

A likely explanation for the occurrence of TSD in *M. menidia* is the possibility of adaptive advantage. In the natural environment of this species, lower temperatures are typical of the beginning of the breeding season, while warmer temperatures occur only later. This means that earlier in the breeding season, it is mostly females that are produced, while later on when water temperatures increase, most individuals develop as males. Females (from earlier in the breeding season), therefore have a longer growing season and tend to be larger than males. Larger females have a higher fecundity and the difference in body size brought on by a longer growth period therefore brings a reproductive advantage, since male reproductive success is not affected by body size.

Populations of *M. menidia* are found in salt marshes and estuaries along the eastern North American coastline from Florida to the Magdalen Islands of Quebec, Canada (Conover and Kynard, 1981). Slightly higher temperatures are typical of the breeding season of the southern populations, and the duration of the breeding and growing seasons progressively decrease with an increase in latitude (Conover and Kynard, 1984; Middaugh, 1981).

Conover and Heins (1987) proposed two hypotheses from this geographical data combined with the adaptive sex ratio theory. First, populations that experience warmer temperatures during the breeding season compensate by shifting the functional response of sex ratio to temperature. Therefore the temperature that yields a 1:1 sex ratio is likely to be higher in southern (warmer) than in northern (cooler) breeding populations. Secondly, the magnitude of ESD (namely the maximum change in sex ratio with temperature in a given population) is expected to decrease with an increase in latitude and thus a shortening of the duration of the breeding season. This prediction is made on the grounds that the relative benefit of being born at the beginning rather than the end of the breeding season (in terms of body size) decreases with the duration of the breeding season. Furthermore, inter-annual variation in mean temperature during the breeding season is likely to be greater among the northern populations with shorter breeding seasons, causing sex ratio fluctuations that would select for an increased level of genetic sex determination (GSD).

The predictions mentioned have been confirmed by the authors in a study of several sub populations of *M. menidia*. The temperature-sex ratio response varied greatly among populations at different latitudes and ESD was completely absent in populations where the growing season was too short to offer any adaptive advantage of differential growth rates. According to Bull (1983), this temperature dependence of sex differentiation in *M. Menidia* can be classed as ESD, but according to Mair (1990) it should be considered as an environmental modification of genotypic sex determination (GSD).

3.4 OVERVIEW OF TEMPERATURE DEPENDENT SEX DETERMINATION IN *OREOCHROMIS* SPECIES

In most of the thermosensitive fish species, the male to female ratio has been found to increase with an increase in temperature, and / or ovarian differentiation is induced by low temperature. This response is representative of several freshwater and marine families, including the Atherinids, Poecilids and Ciclids (Baroiller and D'Cotta, 2001). The latter group includes all commercially important species of tilapia. The critical period for sex reversal in tilapia, both by temperature and hormone treatment, is believed to be 14 – 24 days after fertilization (Baroiller and D'Cotta, 2001), extending to 30 days (Srisakultiev, as cited in Mair et al, 1997; Alvendia-Casauay and Carino, 1998).

Mair et al. (1990) conducted a comparative study using three different species of tilapia to assess the effect of temperature on sex ratio. In al three species, *O. aureus, O. niloticus* and *O. mossambicus*, temperature was found to significantly affect sex ratio. High temperatures produced more males in *O. aureus* and *O. niloticus*, while low temperatures had the same effect in some experiments with *O. mossambicus*. Low temperatures were not found to have an effect on sex determination on *O. niloticus* (Abucay et al., 1999)

Some of these results were confirmed by Desprez and Melard (1998) who studied the effect of temperature in the blue tilapia *O. aureus*. In this species high temperatures caused a predominance of males while low temperatures were not found to have an effect on sex determination. The progenies reared at lower temperatures were treated for longer than those at higher temperatures and were also gonad squashed at a later stage. The reason for this is the fact that lower temperatures delay gonadal development. It was also found that successive progenies from the same pair yielded similar sex ratios under a given temperature regime. This illustrates that temperature sensitivity is strongly influenced by parental traits.

In the work of Baroiller et al. (1995a) and Desprez and Mèlard (1998) it was suggested that different window periods of thermosensitivity may exist. In two species of tilapia, *O. aureus* and *O. niloticus*, as well as in the Florida Red strain (which is a hybrid), low temperatures has no significant effect on sex ratios, even when applied during the period of sensitivity to hormone treatment. Similarly, in *O. mossambicus*, high proportions of males were found at elevated temperatures, but low temperatures had no effect when treatments were applied from 10 days post-hatching (Baroiller and D'Cotta, 2001). However, when exposing the same species to low temperatures at an earlier stage (from hatching or 5 days post-hatching), high proportions of females could be induced (Wang and Tsai, 2000).

The above evidence is indicative of a temperature-genotype interaction in the process of sex differentiation in tilapias, including *O. mossambicus*. Preceeding any study in the effects of temperature on sex differentiation, it would be valuable to have an indication of the degree of natural variance in sex ratio at the preferrential temperature range of the species. In determining this, it is essential that the temperature range be maintained in order to minimize any modifying effects on sex ratio at the given temperature.

3.5 MATERIALS AND METHODS

A number of progeny groups were produced to assess the extent of natural variation that occurs with regard to sex ratio of *O. mossambicus.* Three different strains were identified that represent the geographical distribution of *O. mossambicus* in Southern Africa. The three selected strains are Le Pommier, Amatikulu and Sucomba, and were collected from the Western Cape, Kwazulu-Natal and Malawi respectively.

Table 3.1 present detailed information of sampling sites of the respective strains and approximate geographic locations of populations are presented in Figure 3.1. As there is a difference in the temperature profile of the different areas, it is posible that the temperature

yielding a 1:1 ratio may differ between the different strains and may indicate the existence of temperature sex determination in *O. mossambicus*.

Table 3.1: Detail of collection sites of different strains of *O. mossambicus* in Southern Africa. (Hall, 2002).

POPULATION	APPROXIMATE MAP GRID LOCATION	POPULATION DESCRIPTION
Amatikulu*	29º South 31º 30' East	A red coloured, ornamental breeding population, collected at the Amatikulu fish farm in Natal
Bushmans	33º 30' South 26º 30' East	A wild population from the Bushmans river in the Eastern Cape, South Africa
Elands	25º 15' South 29º 15' East	A wild population from the Limpopo river system in Mpumalanga, South Africa
Kasinthula	17º South 35º 30' East	A wild population from a research station based on the edge of the Shire river system, Malawi
Le Pommier*	34º South 18º 45' East	A population introduced in 1940's into the Western Cape, South Africa
Makathini	27º South 31º 30' East	A population from the Makathini research station flanking the Pongola river in Northern Kwazulu-Natal, South Africa
Ndumu	26º 45' South 32º East	A wild population from the Pongola/Usutu river systems in Northern Kwazulu Natal, South Africa
Nick James	33º South 27º East	A red coloured, ornamental breeding population collected from an ornamental fish farm near Grahamstown, South Africa
Olifants	24º South 31º 45' East	A wild population from the Limpopo river system in Mpumalanga, South Africa
Sucomba*	17º South 35º 30' East	A wild population from the lower Shire river system, Malawi
Valley Fish Farm	25º South 31º East	A red coloured, ornamental breeding population collected from the Valley ornamental fish farm in Mpumalanga, South Africa
Verloerenvlei	34º 10' South 18º 30' East	An introduced population from a brackish water lake in the Western Cape, South Africa

* Strains used in current study.

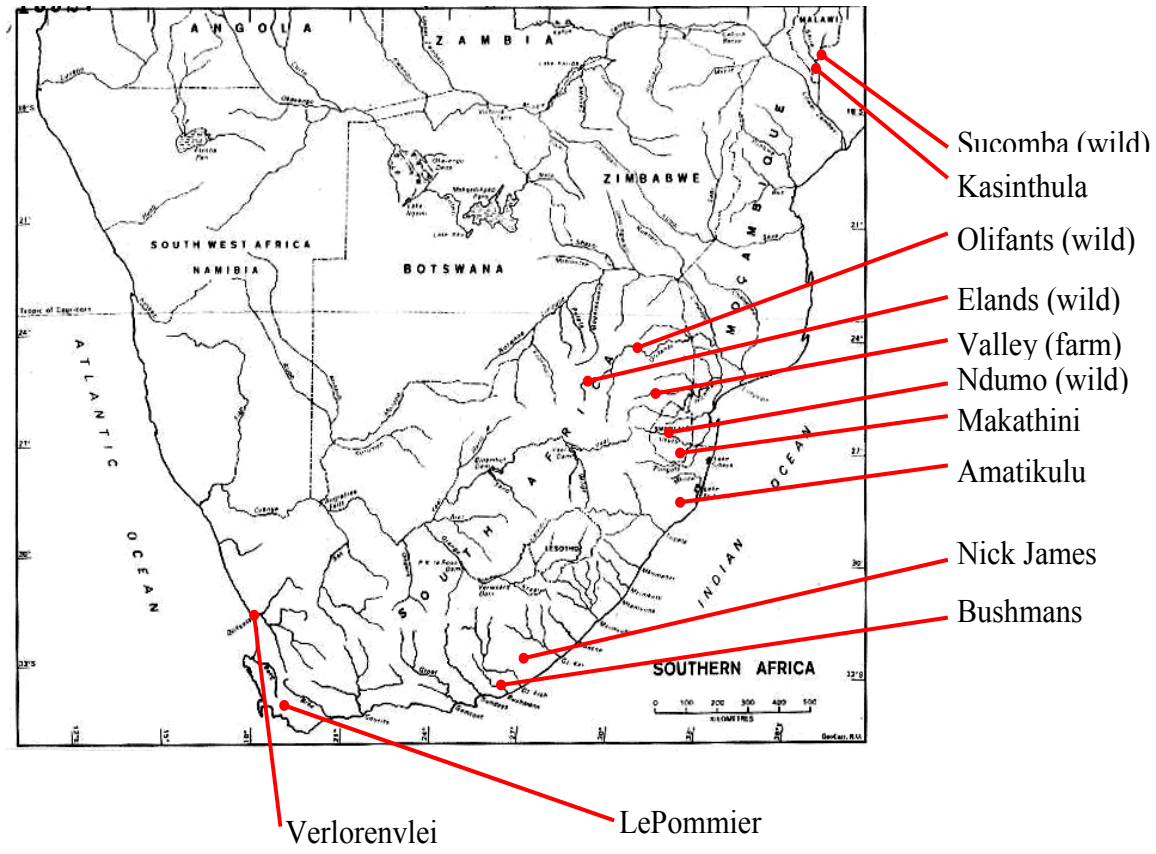

Figure 3.1: Map indicating collection sites of different populations of *O. mossambicus* (Hall, 2002).

For each of the three strains, a total of seven breeding pairs were constituted through the random selection of one male and two females per pair. These individuals were then conditioned to spawn. Conditioning consisted of maintaining water temperature in the optimal range for breeding (± 28 ^{o}C) and feeding broodfish two to three times daily on a 34% protein tilapia pelleted diet, obtained from Nutrex Pty. Ltd.

The experiment was conducted in a recirculation system within a temperature-controlled facility. The system consists of 36 x 120 liter tanks equipped with a biofiltration unit. The latter is made up of settling medium for bacteria and limestone pebbles (Aquastab) for buffering pH. The facility was maintained at 28 ± 0.93 ^{o}C throughout the breeding and progeny rearing phase, and set to 16:8 hour day /night cycle to mimic natural conditions.

Broodfish were conditioned as described until spawning had occurred. As soon as a female was seen to be carrying eggs, the other fish was removed from the tank and the female left by herself to incubate the eggs.

As soon as fry was observed to have hatched, the female was removed from the tank and the progeny left to grow for 28 days under the same temperature regime as was experienced during hatching. Water changes was kept to a minimum with less than 5% water replacement weekly. This was done to ensure that all offspring were subjected to an identical temperature regime and water quality conditions during the labile period of sex determination.

Upon completion of the trial fry were transferred to a heated glass house system and grown to sexing size. Fifty individuals were randomly selected from each progeny group at the age of 90 to 120 days post hatch for sex determination according to the gonad squash technique of Guerrero and Shelton (1974). The data obtained from this formed the basis of the analysis to determine natural variation in sex ratio of *O. mossambicus*.

3.5.1 Determination of sex in fish using the gonad squash technique.

Sex determination in fish is usually possible through examination of external characteristics, but this method is not always accurate, even when performed by experienced personnel. It usually also requires that fish attain a minimal size before sexing is possible, making the technique unsuitable for use in immature fish. In these instances, histological examination of the gonads provides an accurate way of distinguishing sex. This method, however, is time and resource intensive and may not be suitable when results are required immediately. Furthermore, this method may not always be suitable for fieldwork, as access to histology lab facilities is required.

In 1974, Gurerrero and Shelton proposed a simple and effective solution to the problem of quick and accurate sex determination in fish. The method, known as the aceto-carmine

gonad squash technique, can be applied to mature as well as juvenile fish and the technique works equally well for both fresh tissue and those fixed in formalin.

The stain is prepared by adding 0.5 gram of carmine to 100 ml of 45% acetic acid and then boiling for 2-4 minutes. The solution is then left to cool after which it is filtered through paper to remove any impurities.

For this work juvenile tilapias were gonad squashed when attaining a mean weight of ±10 grams, but it is possible to work with much smaller fish. For locating the gonads in smaller fish, it is often necessary to use a dissection microscope. For removal of gonadal tissue, a small vertical cut is made near the genital pore, followed by two horizontal cuts from the base of each pectoral fin to the edge of the first cut, resulting in an L-shaped incision. The visceral mass can now be removed using tweezers, exposing the gonadal tissue that is situated on the ventral side of the swim bladder. A small section of the gonadal tissue is then removed and placed on a glass slide. A few drops of aceto-carmine dye are added before squashing the tissue with another slide or cover slip. The slides are now ready to be examined under a light microscope using magnifications of 25 to 100 X.

The aceto-carmine is differentially absorbed by the various structures and allows a distinct contrast between the different tissues. Ovarian tissue can be readily identified by the presence of pre-vitellogenic and vitellogenic oocytes (Figure 3.2a). The lightly stained nucleus is surrounded by much darker cytoplasm. Testicular tissue is more difficult to positively identify, but usually stains a light brown colour and is characterized by a lobular configuration. Developing spermatocytes can usually be observed (Figure 3.2b).

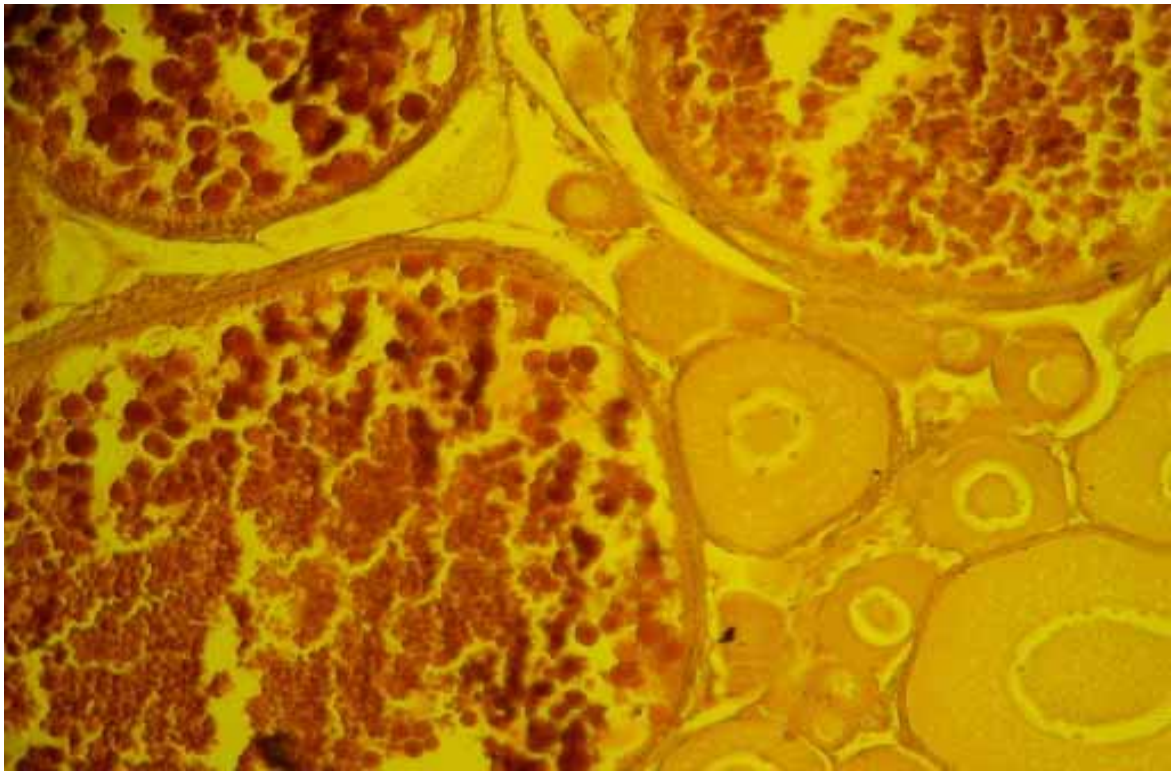

Figure 3.2a: Cross section of the gonad of a *O. mossambicus* female indicating the different stages of oocyte development in the reproductive cycle.

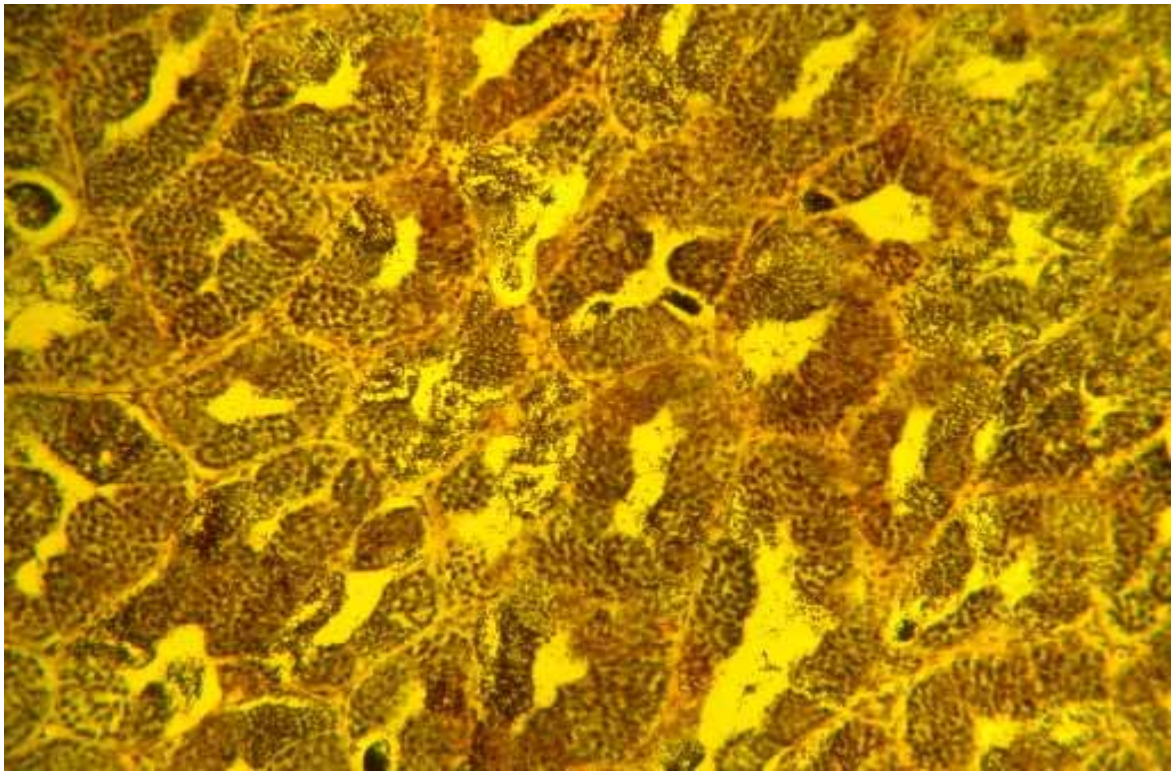

Figure 3.2b: Cross section of the gonad of an *O. mossambicus* male indicating spermatogenic cysts as well as spermatozoa.

3.5.2 Statistical Analysis

Unless otherwise indicated, normally distributed data was analyzed by calculating means and standard deviations. Chi-squared analysis was used to test for significant deviation from the expected 1:1 ratio at a 5% probability level for each progeny group as well as for the pooled data for each strain. A one-way analysis of variance (ANOVA) was performed to test for significant variation in sex ratio between different strains.

3.6 RESULTS

The sex ratio data obtained from the three strains, along with means and standard deviations for each strain are presented in Table 3.2. The sex ratio for the Le Pommier strain was approximately 1:1, with a slight excess (53.1%) of males present. The group exhibited a standard deviation of 5.76 (Figure 3.3). None of the seven groups nor the strain as whole showed a significant difference from a 1:1 ratio ($p>0.05$).

In the Amatikulu strain, one of the progeny groups (group A4), showed a female-biased sex ratio ($p<0.001$). Groups A5 and A6 were also significantly different, and showed an excess of males. The large standard deviation observed in this group (15.74) is reduced to 8.16 when omitting group A4 (Figure 3.3). The group as whole has a mean of 49.4% males (54.6% excluding groups A4) and is not different from a 1:1 ratio, whether or not group A4 is included ($p>0.05$).

The Sucomba strain shows two groups, S2 and S5 where a significant deviation from the expected 1:1 ratio can be observed. The mean percentage of males is 59.1% with a standard deviation of 5.39 (Figure 3.3). This group as a whole is different from a 1:1 ratio at a probability level of 5 %, but not at 1%.

The data was subjected to a one way analysis of variance (ANOVA), but the values for the outlier (group A4) was excluded from the analysed data. The results of the ANOVA is presented in Table 3.3 indicating that no significant differences occurred between the groups.

Table 3.2: Sex ratios obtained from three different strains of *O. mossambicus* reared at 28^{o} C. All sex ratios were tested against a predicted 1:1 ratio.

Strain:		**Le Pommier**		
Progeny group	Percentage males	Percentage females	Sample size	P-value
L1	62	38	50	0.090
L2	50	50	50	1.000
L3	52	48	50	0.777
L4	48	52	50	0.777
L5	58	42	50	0.256
L6	46	54	50	0.572
L7	56	44	50	0.396
Mean	53.14	46.86		
± S.D.	5.76			
Strain:		**Amatikulu**		
Progeny group	Percentage males	Percentage females	Sample size	P-value
A1	48	52	50	0.777
A2	54	46	50	0.572
A3	54	46	50	0.572
A4	18	82	50	<0.001
A5	64	36	50	0.048
A6	64	36	50	0.048
A7	44	56	50	0.396
Mean	49.43	50.57		
± S.D.	15.74			
Strain:		**Sucomba**		
Progeny group	Percentage males	Percentage females	Sample size	P-value
S1	50	50	50	1.000
S2	64	36	50	0.048
S3	58	42	50	0.258
S4	62	38	50	0.090
S5	64	36	50	0.048
S6	62	38	50	0.090
S7	54	46	50	0.572
Mean	59.14	40.86		
± S.D.	5.39			

Table 3.3 Table presenting the results of the one-way analysis of variance (ANOVA). No significant differences were found between the groups.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	135.152	67.576	1.620	0.226
Error	17	707.048	41.591		
Corrected Total	19	842.200			

The mean percentage of males observed in each strain are presented in Figure 3.3. Standard deviations are indicated with error bars. The largest standard deviation can be observed in the Amatikulu strain (8.16), with the smallest standard deviation in the Sucomba strain (5.39).

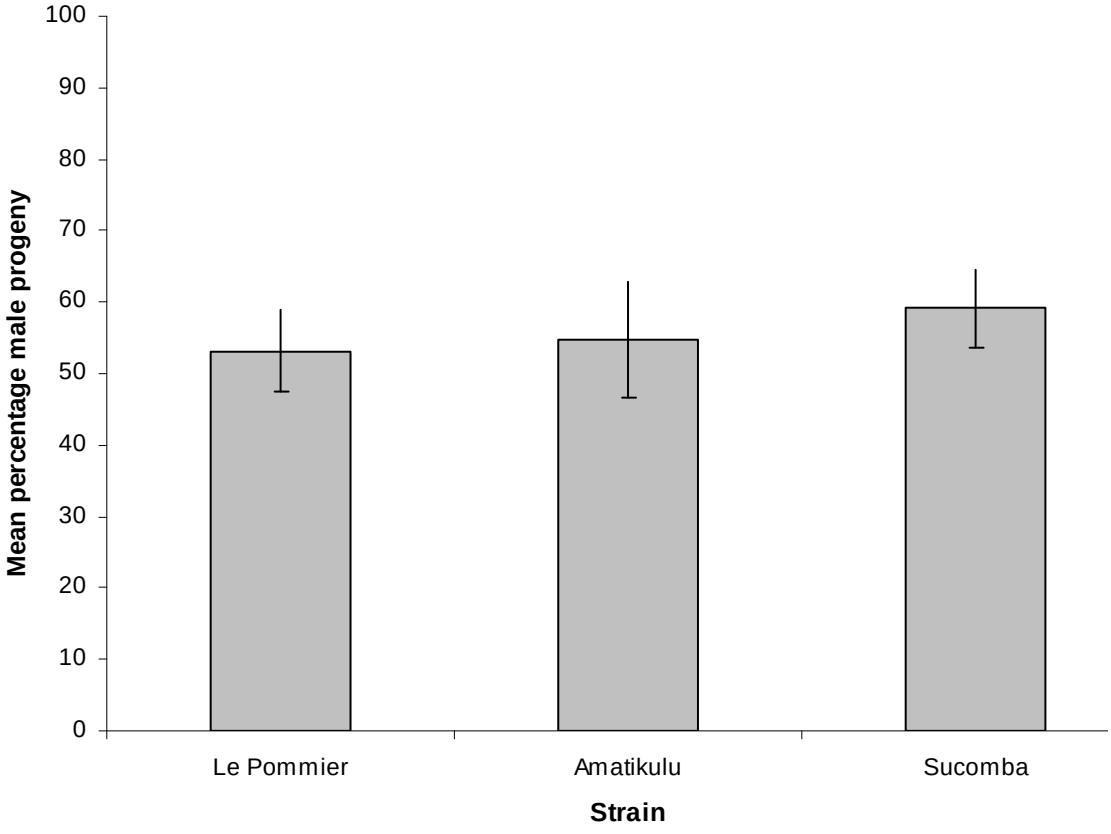

Figure 3.3: Mean percentage males observed in each strain. Standard deviations are indicated with error bars.

3.7 DISCUSSION

Mechanisms of sex determination in fishes are diverse and involve sex chromosomes, autosomal genes, polygenic sex determination, environmental influences, as well as combinations of the above (Bulmer and Bull, 1982; Baroiller et al, 1999). For the *Oreochromis* species, a considerable amount of research has been conducted on the mechanism that determines sex, resulting in a number of proposed models of sex determination in this genus. Mair et al. (1991a) proposed a predominantly monofactorial genotypic system, whereas Shelton et al. (1983) hypothesized a polygenic system of sex determination. A genotype-temperature effect on sex ratio of different *Oreochromis* species was reported by Mair et al. (1990), Baroiller et al. (1995a, 1995b) and Abucay et al. (1999).

This provides evidence for the existence of an environmental effect in the process of sex differentiation in this genus. In the work of Mair et al. (1990), some evidence was presented for temperature-dependence of sex ratio in *O. mossambicus*. Low temperatures was found to significantly increase the number of males compared to the controls, but there was no opposite response producing an excess of females at a higher rearing temperature. It was also reported that the observed temperature effect was monotonic, with the percentage of males increasing with a decrease in temperature below 28 ^{o}C.

The data obtained in this study conform to the model proposed for a predominantly monofactorial sex determination system, with the possiblility of temperature sex determination. In the Le Pommier strain, all seven progeny groups, as well as the group as a whole exhibited sex ratios that conform to the expected 1:1 ratio and there is sufficient evidence to suggest that the observed deviation can therefore be attributed to natural variation within the population. The Amatikulu strain had three progeny groups that were significantly different from the expected ratio, but there was no clear trend in the deviation (two groups

were male-biased and a single group exhibited an excess of females). In spite of this the group as a whole was not significantly different from the expected ratio.

The Sucomba group as a whole, while only having two individual groups differ from the expected, is significantly different from a 1:1 ratio and shows the higest mean percentage of males over the seven progeny groups (59.5 % versus 54.67% and 53.14% of Amatikulu and Le Pommier, respectively (Figure 3.3)).

The male-biased sex ratio observed for the Sucomba strain can possibly be explained by a temperature effect on the process of sex determination. The Sucomba strain of *O. mossambicus* originates from the lower Shire river system in Malawi and is thus exposed to a different annual temperature regime than the Le Pommier and Amatikulu strains. Thus, assuming that a 1:1 sex ratio is produced at the average temperature experienced by the population during breeding season, the temperature that yield a 1:1 ratio will be different for populations from geographically different areas.

This temperature effect on sex differentiation could pose an adaptive advantage to the species in question. In the case of the Atlantic Silverside *M. menidia*, cooler temperatures shift the sex ratio to predominantly female, thus yielding more females in the beginning of the breeding season. These females have a longer growing season and, as egg production is a function of body size, larger females (from earlier in the breeding season) have an adaptive advantage over smaller ones, produced later in the breeding season. In the *Oreochromis* species, egg production is also a function of female body size and the same adaptive advantage could be applicable as in *M. menidia.* This could explain the male biased sex ratios induced by higher temperatures in *O. niloticus* and *O. aureus*. However, the adaptive advantage theory would then require that female-biased sex ratios are produced at lower temperatures. Other authors have however, not provided conclusive evidence of lower

temperatures shifting sex ratio towards either sex. and the theory of adaptive advantage requires further study.

On the other hand, an adaptive advantage possibly exists for larger male body size, namely the territorial advantage that larger males have over smaller males during the breeding season. This was proposed by Mair et al. (1990) to explain the occurrence of a male biased sex ratio produced by lower temperatures in *O. mossambicus*.

CHAPTER 4

APPLICATION OF THE YY-MALE TECHNOLOGY IN *OREOCHROMIS MOSSAMBICUS*.

4.1 INTRODUCTION

Tilapias posses several traits such as a tolerance to lower water quality and their ability to utilize a wide variety of natural food sources that renders it a suitable species for use in aquaculture. The main biological constraints to the development of commercial tilapia farming is their inability to withstand water temperatures below 10^{o}C (Chervinski, 1982) and early sexual maturation that result in fish spawning before reaching market size (Phillipaert and Ruvet, 1982). This leads to unwanted recruitment, with the result being a large number of small unmarketable fish in the production system. The latter problem can be remedied through the production of monosex populations, which eliminates unwanted recruitment.

The development of a tilapia industry in Southern Africa will be reliant on the development and application of different methods of producing all-male populations. The YY male technology provides an alternative to hormone-based sex reversal that is both environmentally friendly and enjoys widespread consumer acceptance.

4.2 Problems associated with early sexual maturation in tilapia culture

The problem of precocious breeding and excessive population growth due to the early onset of sexual maturation is a well-known phenomenon in tilapia aquaculture (Hepher and Pruginin, 1982; Mair et al., 1997; Mair and Abella, 1997). Sexual maturity is a function of both the environmental conditions and age of a fish. If fish are kept in suboptimal conditions, as is often the case in a commercial production system environment (e.g. high stocking densities and limited access to food) their growth become stunted. Characteristics of a

stunted tilapia population include sexual maturity at an earlier age and smaller size and an increased spawning frequency (Lowe McConnell, 1982; Pullin, 1982). These fish thus become sexually mature at a smaller size than fish kept under optimal conditions. The fish kept under optimal conditions may reach sexual maturity only at sizes of 150-200g, while ones kept under poorer conditions may breed at sizes as small as 10 grams (Jalabert and Zohar, 1982). Under normal conditions of moderate growth the fish will become sexually mature at sizes of 40-60 grams (Popma and Masser, 1999). Males and females mature at approximately the same size.

Upon reaching sexual maturity, spawning will be induced. The diet of fry and small juveniles differ from that of adult fish so there will be little or no competition for resources. As the juveniles grow larger, however, they start competing for resources with the larger stocked fish. As more and more offspring are produced, overpopulation and competition becomes a problem. The result of this is that the stocked fish will not grow to optimal size and that producing a tilapia of a preferred market size in a mixed-sex population becomes extremely difficult.

4.3 Methods to control recruitment in mixed-sex populations of tilapia

4.3.1 Manipulation of environmental factors

Keeping fish under suboptimal conditions can prevent spawning to a certain extent, but continuous environmental stress can be detrimental to the health of the fish, leaving them prone to disease and negatively impacting on growth rate. There is evidence that sexual maturity in some species of tilapia, e.g. *O. aureus*, can be delayed by rearing them in salt water. In these species, nest building behaviour ceases and the gonadosomatic index (GSI) drops when the salinity equals or exceeds the salinity of seawater (35ppt) (Chervinski, 1982).

In saline intolerant species this approach would be impractical, as it would be accompanied by an increased susceptibility to disease and a negative impact on growth rate. Furthermore, controlled induction of environmental factors such as high salinities or low temperatures is difficult in all but the most intensive production system. There is also a considerable economic impact associated with manipulating environmental conditions, which puts this option out of reach of most small-scale farmers. Even when suitable conditions for inhibiting reproduction can be maintained, growth and production rates may suffer to a considerable extent.

4.3.2 Grading and manual removal of recruits

Manual separation of offspring and parents and restocking the larger fish is time-consuming and labour intensive. It is also impractical due to the asynchronous breeding cycle of tilapia. It is possible to remove fry by draining the water out of the pond, though only in intensive production systems (e.g. tank or portapool systems).

When farming tilapia in cage systems it is possible to reduce or eliminate recruitment through disruption of the natural reproductive behaviour of the fish (Pagan-Font, 1975; McGinty & Rackocy, 1996). When using a large mesh size (>3 cm), the spawned eggs fall through the bottom of the cage before the male can fertilize them and the female has time to pick them up. Even if some eggs were fertilized, the lack of parental care would preclude their normal development (Rifai, 1980). Although reproduction is inhibited, the disadvantage here is that there is still a considerable amount of energy invested in ova and sperm production that could have been directed to somatic growth (Patino, 1997; Gale et al, 1999).

4.3.3 Sterilization through chromosome-set manipulation

The sterilization of salmonid species through the induction of triploidy has been used with considerable success (Yamaziki, 1983; Thorgaard, 1992). The induction of triploidy in

salmonid species prevents a decrease in meat quality normally brought on by sexual maturation. Triploidy has been successfully induced in some tilapia species through exposing fertilized eggs to heat, cold or pressure shock (Varadaraj and Panadian, 1990; Mair, 1993). However, large-scale induction of triploidy in this manner is impractical due to the small individual clutch size of female tilapia.

Another method of producing sterile triploids is through the mating of tetraploid x diploid individuals (Myers, 1986; Maclean and Penman, 1990). Available evidence suggests that the viability of tetraploids in fish is rather low (Thorgaard, 1992), but a few pairs of mature tetraploids would be sufficient to create tetraploid populations, although these populations will only have limited genetic variability. This method could hold promise for the commercial sector, but to date there has been no direct benefit to the tilapia culture industry (Mair, 1993).

4.3.4 Establishment of high stocking densities

It has been reported that reproduction can be considerably reduced when tilapia are stocked at very high densities (>10 kg/m^3), as this brings about the disruption of normal nest building behaviour (Mair and Abella, 1997). Such high densities require intensive cultivation systems and large amounts of supplementary feed. Too high stocking densities are likely to lead to continuous stress in the fish which in turn lead to disease outbreaks and stunted growth. High-density cultures are also likely to experience stress-induced mortalities (Roberts and Sommerville, 1982)

4.3.5 The use of natural predators

The stocking of predatory fish in grow-out facilities to control tilapia fry and fingerlings has been used with varying amounts of success. Reduction in recruitment has been observed using catfish (*Clarias* species) and largemouth bass *(Micropterus salmoides)*, but success

depends on optimal predator-prey stocking rates and availability, survival and control of the predators themselves (McGinty, 1985).

4.3.6 Selection for late sexual maturation

Sexual maturity in some species of tilapia can be delayed by growing them in salt water, but suboptimal rearing conditions often leads to an increased susceptibility to disease and a negative impact on growth rate. A sustainable and more beneficial approach would be to develop late maturing strains through selection. Kronert et al. (1989) proposed that gonadal development is primarily genetically controlled and that a selective breeding program shows promising chances of developing late maturing strains. However, literature provides evidence that environmental components affect the onset of sexual maturity to a certain extent and that a possible genotype-environment interaction cannot be ignored (Jalabert and Zohar, 1982; Pullen, 1982).

Thus, when implementing an actual breeding program, it must be shown that selection results obtained in the laboratory will yield the desired results in the actual production environment (Kronert et al., 1989). Unfortunately there is evidence to suggest that selection for late maturity will result in indirect negative selection for growth (Mair and Abella, 1997). This has resulted in some doubt as to whether a selection program for late maturation would indeed be viable.

4.4 Methods for the production of monosex male tilapia populations

4.4.1 Manual sexing

The sexes can be separated manually before they reach sexual maturity and the males restocked. Sexing is done on the basis of differences in the appearance of genital papillae of male and female fish. Through using malachite green or Azorubin (a green food dye) in the water, male and female genital openings are more readily distinguished, making it easier to

accurately sex the fish (Chervinski and Rothbard, 1981). However, this process is extremely labour intensive and it is impossible to be 100 percent accurate. With some females restocked, reproduction is not likely to be completely eliminated. If females are to be discarded, only half the population is grown to market size and the resources invested in growing individuals to sexing size are wasted. The sexing process is also stressful for the fish and mortalities or disease outbreaks could result.

4.4.2 Hybridization

All- or nearly all-male hybrid populations are known to occur in the centrachids (sunfishes), but the best known examples of this phenomenon are in the tilapias (Beardmore et al., 2001). The production of all-male progeny from *O. mossambicus* (female) x *O. hornorum* (male) was first described by Hickling (1960). It has since been reported by several authors that 95-100% male progeny can be produced in a number of interspecific hybrid crosses, most consistently in (female x male) *O. niloticus* x *O. hornorum* and *O. niloticus* x *O. aureus* (Lovshin, 1982). This system is used with moderate success in the Israeli tilapia culture industry (Mair and Abella, 1997).

However, sex ratios resulting from these crosses can be highly variable between different parental strains, particularly for *O. niloticus* x *O. aureus*. The latter yielded between 52% and 100% male progeny, depending on the origin of the parental strain used (Wolfarth, 1994). Insufficient care in broodstock management, such as failing to keep the parental strains pure, can contribute to this. Stricter broodstock management can remedy this problem to a certain extent.

Another principal concern of hybridization is low and inconsistent fry production encountered in some interspecific hybrid crosses (Lovshin & Da Silva, 1975). In the case of *O. niloticus* x *O. aureus*, this low fecundity can be attributed to the specific strain of *O. niloticus* that is used (Hulata et al., 1985). Furthermore, the appearance of some hybrids

makes them less acceptable to consumers. An example of this would be the darker colour of an *O. mossambicus* x *O. hornorum* hybrid (Wolfarth, 1994).

In some instances, hybridization can offer additional benefits in the form of positive heterosis for growth in hybrids and the combination of desirable characteristics from different species. An example of this would be inherited cold tolerance in *O. niloticus* x *O. aureus* hybrids. The main disadvantage of this approach is the inconsistency of results and the effect that large-scale interspecific hybridization will have on the genetic integrity of tilapia resources. In the case of the South African tilapia populations, genetic diversity should be protected due to unique qualities such as cold tolerance and high salinity tolerance characteristic of certain populations (de Moor and Bruton, 1988)

4.4.3 Hormone-induced sex reversal

Producing 100% male tilapia populations in a production system is possible through oral administration of the male hormone 17α-methyltestosterone (MT). Sex reversal is achieved through applying androgen treatments to sexually undifferentiated fry. In order to be sure that the fry are sexually undifferentiated it is essential that hormone treatment commence as soon as possible after yolk sac absorption. Fry are then fed on a diet containing hormone at a concentration of 40-100 mg of hormone per kg of food. The optimal hormone concentration is species-specific, but 40 mg is usually considered to be adequate (Vera Cruz and Mair, 1994). The androgen is introduced into the feed by dissolving it in alcohol first and then mixing into the feed. The fry are treated for approximately 25 days, feeding 4-6 times per day.

The correct application of treatment can produce male progeny groups in the range of 95%-100%. Several factors can affect the success of hormonal sex reversal using hormone treated feeds. These are presented in Table 4.1.

Table 4.1. Factors affecting the success of sex reversal in tilapia using hormone treated feeds.

Factor	Comments
Age and size of fry	It is essential that treatment coincide with the window period where sexual differentiation is labile. This period is normally considered to be 14-24 days after yolk sack absorption (Baroiller and D'Cotta, 2001).
Feeding frequency	Initially, treated feed must be administered 4-6 times daily for the first 10 days, after which the feeding frequency can be reduced (Mair & Abella, 1997).
Treatment duration	Treatment is only effective when administered in the labile period of sexual differentiation. The optimum treatment duration for tilapia is considered to be three to four weeks
Effective dosage	Optimum concentration varies between the different tilapias and whether the hormone in question is an androgen or estrogen, but is normally in the range of 40-100mg/kg of food.
Fry density	Stocking density affects the availability of natural food (if any) and the establishment of a feeding hierarchy in the population. This quickly leads to size differences and thus differential consumption of treated food. Generally the effectiveness of sex reversal treatment increases with an increase in stocking density (Vera Cruz & Mair, 1994).
Availability of natural food	The availability of natural food may influence the consumption of treated food and thus the amount of hormone ingested (Vera Cruz & Mair, 1994).
Water quality	Feeding behaviour, and thus hormone uptake is influenced by water quality parameters such as temperature, pH and available O_2 (Mair & Abella, 1997). Temperature has an effect on sex differentiation and may influence the effect of hormone treatment (Dezprez & Melard, 1998; Mair et al., 1990).
Water flow	Sex reversal appears to be more successful in static than in flow-through systems. A possible reason for this is the increased exposure and routes of uptake (i.e. through the gills and skin) of the hormone.
Type of steroid	Several natural and synthetic steroids are used for sex reversal in fish. Usually, synthetic hormones have a more pronounced sex reversal effect than natural steroids (Piferrer, 2001)
Nature of steroid	The efficiency of a hormone is determined by its chemical nature, such as the differences in potency of aromatizable vs. nonaromatizable androgens (Piferrer, 1993; Piferrer, 2001, Baroiller & Toguyeni, 1996)

4.4.4 Genetic manipulation of the sex chromosomes

Work has been done with the objective of producing tilapia males that carry a YY genotype and as a result can only produce male or nearly all-male progeny when bred to genetically normal females. This technology, involving a series of sex reversal and subsequent progeny testing procedures, has been attempted on many tilapia species. As yet it is only in *O. niloticus* that the technology has become commercially viable.

4.5 Control of recruitment in a production system through the production of monosex male populations

The need for producing all-male tilapia populations without the use of hormones has led to research on the possibility of sex chromosome manipulation. From this the so-called YY male technology was developed. The objective of this technology is to produce, through a series of sex reversal and progeny testing procedures, a tilapia male with a YY genotype instead of normal XY genotype. These males are capable of siring only male offspring and when bred to normal females, yield an all-male progeny. The offspring of a YY male and XX female is known as Genetically Male Tilapia (GMT).

4.6 The production of Genetically Male Tilapia (GMT)

The method for producing YY males can be divided into 4 steps. A schematic representation of the different steps is given in Figure 4.1.

Stage 1: Development of sex-reversed females through estrogen treatment

The first stage involves the hormonal feminization of progenies obtained from normal males and females to produce a phenotypically female population, irrespective of the genetic sex (XX or XY). The method of feminization is similar to androgen based sex reversal to male (section 4.4.3), though here an estrogen-based hormone such as DES is used. Several authors have published on the topic of hormone-based feminization in tilapia, with optimal hormone concentrations varying from 40 mg –1000 mg hormone/kg of food (Mair et al., 1997; Piferrer, 2001).

The females who are genotypically male (XY) are designated Δ-XY to distinguish them from normal XX females. No phenotypic distinction can be made between the Δ-XY and XX females. A test cross with a masculinized (Δ-XX) female is conducted to distinguish between the two female genotypes (Δ-XY and XX) on the basis of progeny sex ratio. If the

female tested is a normal XX female, the progeny will be all- or nearly all female. If the female is a sex-reversed male (Δ-XY), the progeny will have a sex ratio of close to 1:1. To simplify this process, normal XY males can also be used in testcrosses to distinguish between Δ-XY and XX females. A cross between a Δ-XY female and a normal male will yield a 3:1 male to female sex ratio, while a normal female will yield a 1:1 ratio.

Stage 2: The development and identification of YY-males

The second stage involves crossing the Δ-XY females identified in stage 1 with normal males with a XY genotype. The expected sex ratio from this progeny is 3 males: 1 female. One third of these males are expected to have a YY genotype while the remaining two thirds are normal XY males.

Identification of the YY-males must again be done through progeny testing, as they are not morphologically distinguishable from normal XY males. A test cross with a normal XX female is conducted and the sex ratio of the progeny examined. If the male in question was a normal XY male then the offspring will have a sex ratio close to 1:1. If the male was a YY male, then the offspring will be all- or nearly all male.

The YY males are the genotypes required for the production of all-male progeny, but as it is time consuming to identify each one by progeny testing, it is necessary to mass-produce them in order to make the technology practical on a commercial scale.

Stage 3: Development of YY-females

The third stage involves the crossing of YY males, identified in Stage 2, to Δ-XY females from Stage 1. The progeny from these crosses should be all male, with one half of them expected to have the YY genotype. The progeny of these YY x Δ-XY crosses are then subjected to hormone treatment, with the aim to feminize males with the YY genotype in order to produce Δ-YY females. The procedure for progeny testing to identify Δ-YY females

is the same as for identifying Δ-XY females. The progeny of sex-reversed Δ-YY females crossed to XY males will yield a 100% male progeny, while Δ-XY females crossed to XY males will yield a 3:1 sex ratio. Progeny testing can also be done using Δ-XX males, produced through methyltestosterone treatment. In this case a sex ratio of 1:1 (Δ-XY female) versus 100% male (Δ-YY female) will be observed.

Stage 4: Production of YY-progeny groups

This stage is the final step in being able to mass-produce all male tilapia populations. Once YY males and YY females have been identified, they can be crossed to produce large quantities of YY progeny. The YY males can then be used in crosses with normal females to mass-produce all-male offspring with a natural XY genotype, known as genetically male tilapia (GMT), for commercial production.

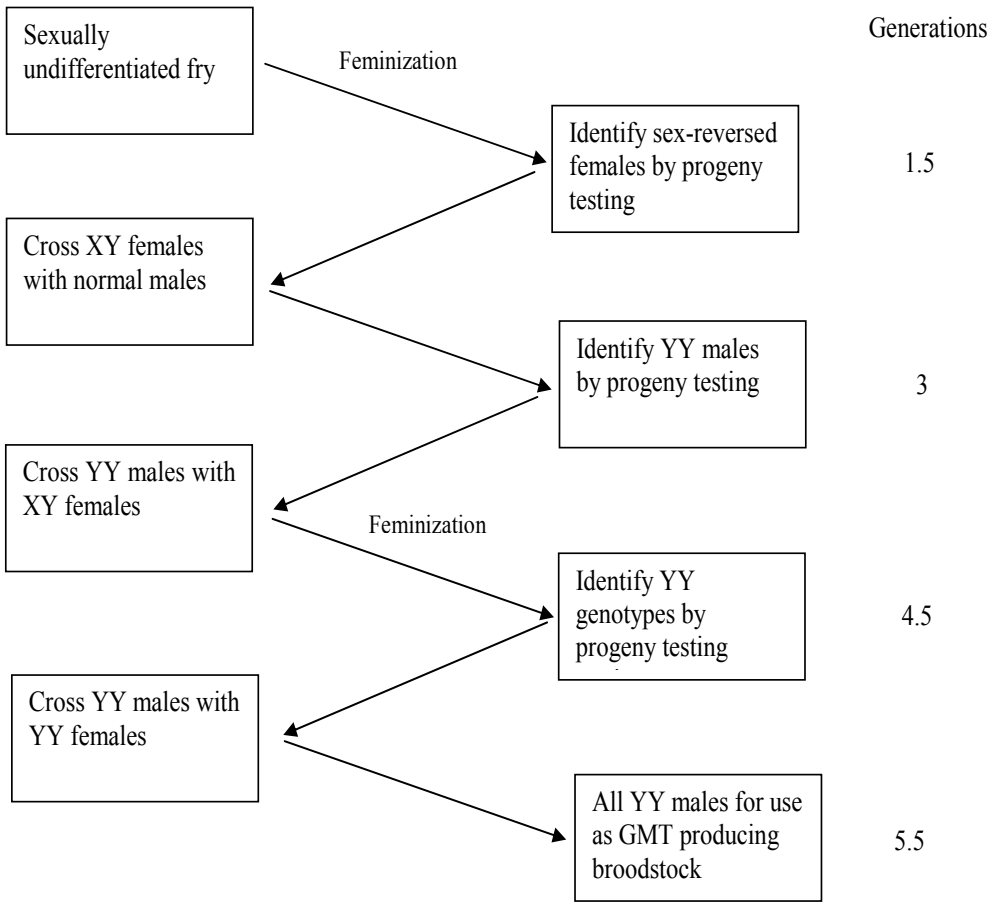

Figure 4.1: Flow diagram of the YY breeding program (Mair et al. 1997)

4.7 Problems encountered in the production of Genetically Male Tilapia

The process of producing GMT in *O. niloticus* has proved to be more difficult than it appears in theory and the occurrence of abnormal sex ratios has presented problems. In many studies conducted, sex ratios are produced that are incompatible with a simple monofactorial sex determination system (XX/XY or ZZ/ZW, depending on the species in question). Genetic and environmental effects could be presented as possible reasons for this phenomenon. See Chapter Two for a broad overview of sex determination in fish.

4.7.1 Genetic effects

To explain these abnormal sex ratios, two other models of sex determination are proposed: one suggests the involvement of an autosomal locus with two different alleles (Avtalion and Hammerman, 1978), while the other suggests a polygenic model (Majumdar and McAndrew, 1983; Mair et al., 1987). Bull (1983) defines polygenic sex determination as a mechanism controlled by a number of genes, with no single gene having a major influence. The main contradiction of this hypothesis is the results from sex reversal experiments conducted by various authors. In *O. niloticus*, 100% female offspring was observed in mass spawnings between sex-reversed males and normal sibling females (Calhoun and Shelton 1983). This is strongly indicative of a monofactorial sex determining mechanism. This hypothesis is further strengthened by the occurrence of 100% monosex hybrid progenies, mostly between *O. niloticus* and *O. aureus*.

Hybrid crosses, however, also provides the most relevant contradiction of this hypothesis. Large variation in hybrid sex ratio (52-100%) has been observed in different single-pair crosses between these two species and in most cases 100% male offspring could not be produced consistently (Wolfarth, 1994). From this it is clear to see that these hypotheses have failed to fully explain all sex ratios found in the literature.

4.7.2 Environmental effects

Thermodependent sex determination in a gonochoristic fish was first observed in the Atlantic silverside *Menidia menidia* (Conover & Kynard 1981). There is also experimental evidence of a temperature effect in different species of tilapia (Mair, 1990). The latter proposed that environmental factors could be involved in species where heterogamety cannot account for the variety of sex ratios observed.

4.8 MATERIALS AND METHODS

The development of GMT technology in *O. mossambicus* is part of a larger project aimed at the genetic improvement of indigenous fish species for utilization in the country's growing aquaculture sector. The experimental material was obtained from twelve different populations ("strains") of the tilapia *O. mossambicus* that were collected from different areas in Southern Africa and include wild and farmed populations, as well as three strains of red tilapia. The geographic origin of these populations is presented in Chapter 3, Figure 3.1 and Table 3.1. Comparative growth trials were conducted among eight of the strains in order to assess the difference in growth characteristics. Upon completion of a 147-day growth trial, the three best performing strains, Ndumu, Boesmans and Kasinthula, were selected for incorporation in the YY-male breeding program (Brink et al., 2002).

4.8.1 Implementation of the YY-male breeding program

Phase A: Production of Δ-XY females through feminization treatments.

The first phase in the production of YY males is the successful feminization of a progeny group through hormone treatment. The objective here is to feminize genetic males (i.e. those with a XY genotype that would normally develop as males) to produce functional XY genotypic females.

Progenies from five randomly selected breeding pairs from the Boesmans strain were subjected to hormone treatment as from first feeding for 28 consecutive days. The treatment was administered to fry through dissolving diethylstilboestrol (DES) in ethanol (AR grade, Merck Chemicals) prior to mixing it into food. The hormone treated food was prepared by using 60mg of DES dissolved in 500ml of alcohol per one kilogram of food. A standard tilapia grower diet, containing 40% protein was used. All feeds used were obtained from the standard commercial range supplied by Nutrex (Pty) Ltd.

Fish were fed on an *ad-lib* basis four to six times per day over the 28-day treatment period. Approximately one third of the original progeny group was sampled prior to the start of treatment and kept as a control group. The controls were subjected to the same experimental conditions, though they received food without the hormone.

All treatments were conducted in a partial recirculation system within a greenhouse. The system consists of 36 heated 200-liter circular tanks supplied with aeration. The control groups were housed in a separate greenhouse equipped with a similar system. Upon completion of the hormone treatment after 28 days, both the treatment and control groups were kept within the systems and allowed to grow to fingerling size (approximately 10cm) before being sexed on the basis of external characteristics.

Phase B: Identification of Δ-XY females through progeny testing

After approximately six months, when the hormone treated fish had grown to sexual maturity (weighing between 150 and 200 grams), individual females from each group were test crossed with normal Boesmans males. Individuals were randomly selected from each of the four groups that had feminized successfully (B2-B5) and these fish were progeny tested to identify possible Δ-XY females. Progeny testing was conducted in a heated recirculation aquarium facility as described in Chapter Three. An active charcoal filter was fitted to each set of tanks to maintain water quality and minor water changes (±10%) were done weekly. An air blower provided constant aeration to all tanks. The entire facility was maintained at 28 ±1.5 ^{o}C with the aid of a central heating system and set to a 16:8 hour day/night cycle.

For progeny testing, fish were kept in a ratio of two females to one male per tank. Females were transferred to the facility and allowed to acclimatize for five days before the male was introduced. All males were randomly selected from the initial Boesmans population. Females were fed twice a day until spawning. As soon as one female was

observed to have spawned, the other two fish were moved to a new tank to spawn, leaving the female by herself to incubate the eggs.

Once the fry had become independent from their mother, approximately three weeks post spawning, they were transferred to the glasshouse system for grow-out. All females that had produced progeny were transferred to numbered tanks where they were kept until such a time as the sex ratio of their progeny could be determined.

After a growth period of 90 to 120 days, sex ratios of juveniles were determined by means of the gonad squashed method of Guerrero and Shelton (1974), as described in Chapter Two. Ideally the entire progeny should be examined, but as this technique is extremely labour intensive and time consuming, a random sample of 50 individuals were taken from each progeny group.

Analysis of the sex ratio of the progeny was used to assign a genotype to every female tested. According to Mendelian genetics, it is expected that approximately one half of the females tested will have the XY genotype. If a progeny group exhibited the expected 3:1 ratio when tested with a normal male, it was assigned "potential YY group" status and the maternal parent could be identified as being a Δ-XY female.

Phase C: Identification of YY males through progeny testing

Progeny testing of females was conducted until at least 10 Δ-XY females and potential YY progeny groups were identified on the basis of a predicted 3:1 sex ratio. The potential YY progeny groups (i.e. the progeny of positively identified Δ-XY females) were kept for an additional 4-6 months in order to reach sexual maturity. The fish were then sexed on the basis of external characteristics and all females discarded. The males were retained and progeny tested with normal XX females in order to determine the genotype of each individual and identify possible YY males.

4.8.2 Statistical analysis

All sex ratios obtained from progeny testing of parent fish, with the exception of YY individuals, were expected to conform either to a 1:1 or 3:1 sex ratio. The statistical criterion adopted for the designation of parental genotypes when progeny testing for Δ-XY or YY genotypes was chi-squared analysis. All females were first tested according to a 1:1 sex ratio to identify normal XX females. All females of which the progeny differed from a 1:1 sex ratio at a probability level of 5% were then tested according to a 3:1 ratio of male to female, in order to identify Δ-XY females. All females with sex ratios that conformed to the expected 3:1 ratio ($p>0.05$), or those with sex ratios exceeding 75% male, were designated as Δ-XY. Identification of YY genotypes was based on a sex ratio that differ significantly from a 1:1 sex ratio, at a probability level of 0.1% ($p<0.001$). Some males exhibited a sex ratio different from 1:1 at a 5% or 1% level, but not at a 0.1% level. No genotype was assigned to these males.

4.9 RESULTS

Phase A: Sex reversal of progeny groups through DES feminization

Feminization levels higher than 95 % were obtained in four of the five progeny groups that were subjected to DES-hormone treatment (Figure 4.2). Levels of feminization in all four of these groups differed significantly from the control groups ($p<0.001$). One group (B5) showed complete sex reversal with no males present, while another (B1) did not feminize successfully and exhibited a sex ratio not significantly different from the control group ($p> 0.05$). In this group, there were slightly less females in the treatment group than in the control group, and neither the control nor the treatment group was significantly different from the expected 1:1 sex ratio ($p>0.05$). The mean feminization, calculated across all five groups was 88.2 %, with a standard deviation of 20.33. When recalculating these figures excluding

group B1, the average feminization achieved is in the excess of 97% with a standard deviation of 2.21.

All the control groups exhibited a sex ratio close to the expected 1:1 ($p>0.05$). The only exception to this was group B3 that was 39% female and significantly different from the expected 1:1 ratio ($p<0.01$). Determination of sex ratio was based on results from sexing of the fish on external characteristics and there was no histological examination of gonadal development. Even though experienced technical staff conducted the sexing process, it is possible that some mistakes could have been made and as a result a slight overestimation of the percentage feminization could have resulted.

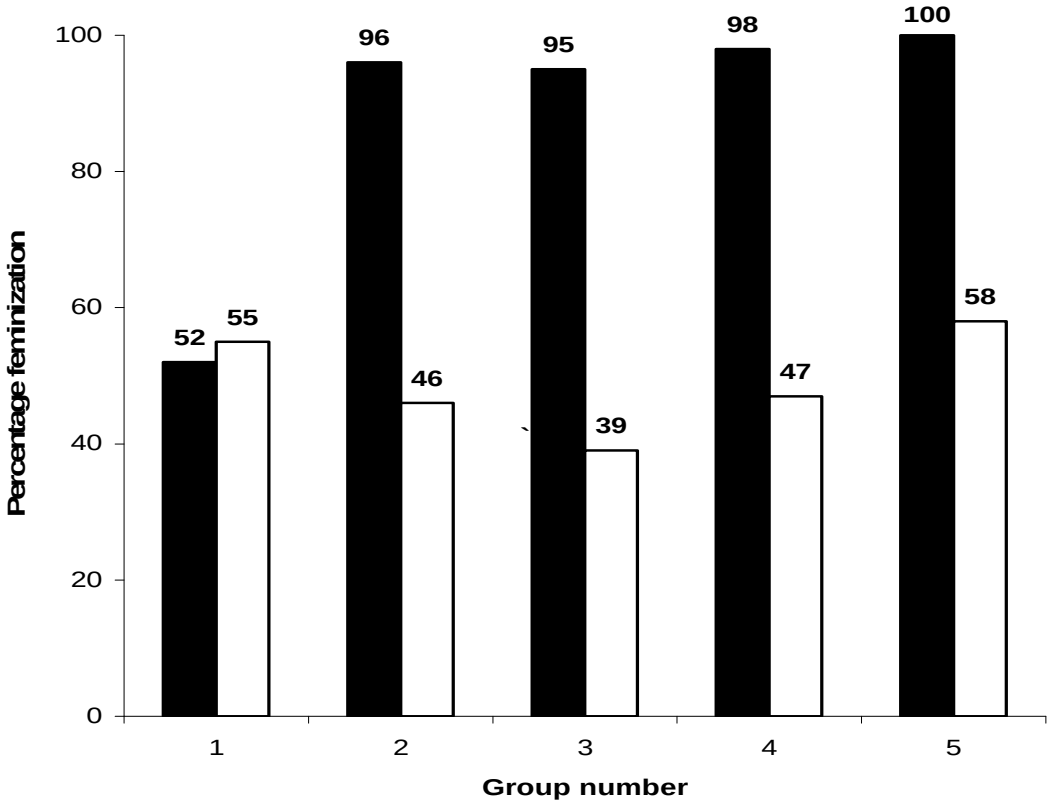

Figure 4.2: Percentage feminization in DES treated tilapia (*O. mossambicus*) versus untreated control groups. The black bars indicate DES treated groups while the white bars represent the control groups.

Phase B: Identification of sex-reversed females through test crossing with XY males

The genotype of the female parent was determined on the basis of data obtained from gonad squashing technique as summarized in Tables 4.2, 4.3 and 4.4. When a female was identified as being a Δ-XY individual, the progeny was assigned a "potential YY-group" status. This means that of the males in the remainder of the progeny, one third is expected to have the desired YY genotype.

Table 4.2 Results from progeny testing of the B3-group of DES treated tilapia for identification of potential sex reversed (Δ-XY) females. The P_1-value, as obtained when tested for a 1:1 sex ratio, is indicative of a normal XX genotype. When the P_1-value was not significant a P_2-value was obtained testing to a 3:1 sex ratio, indicative of a sex-reversed (Δ-XY) genotype. A genotype was assigned based on the significance of these P-values.

No	% Male	Sample size (n)	P_1 value (1:1 ratio)	P_2 value (3:1 ratio)	Assigned genotype
1	51	206*	0.7804	---	XX
2	54	50	0.5716	---	XX
3	56	50	0.3961	---	XX
4	56	50	0.3961	---	XX
5	48	50	0.9682	---	XX
6	48	50	0.9682	---	XX
7	54	50	0.5716	---	XX
8	42	50	0.2578	---	XX
9	67	30	0.0678	---	XX
10	52	50	0.7772	---	XX
11	58	50	0.2578	---	XX
12	76	50	0.0002	0.8702	Δ-XY

* This progeny group exhibited a large number of red coloured individuals, to that were retained for further breeding purposes. The population was therefore sexed on external characteristics.

The B1 group, although 96% female, produced only a single individual that could be classed as Δ-XY based on its sex ratio. As a result of these findings, progeny testing on this group was discontinued and instead more individuals from groups B4 and B5 were assessed.

Table 4.3 Results for Boesmans B4 group. The P_1-value, as obtained when tested for a 1:1 sex ratio, is indicative of a normal XX genotype. When the P_1-value was not significant a P_2-value was obtained testing to a 3:1 sex ratio, indicative of a sex-reversed (Δ-XY) genotype. A genotype was assigned based on the significance of these P-values.

Ind. no	% Male	Sample size	P_1 value (1:1 ratio)	P_2 value (3:1 ratio)	Assigned genotype
1	58%	50	0.2579	---	XX
2	76%	50	0.0002	0.8703	Δ-XY
3	80%	50	0.0002	0.4142	Δ-XY
4	56%	50	0.3961	---	XX
5	76%	50	0.0002	0.8703	Δ-XY
6	57%	30	0.4652	---	XX
7	80%	50	0.0002	0.4142	Δ-XY
8	54%	50	0.5716	---	XX
9	62%	50	0.0897	---	XX
10	60%	50	0.1573	---	XX
11	70%	50	0.0047	0.4142	Δ-XY
12	80%	50	0.0002	0.4142	Δ-XY
13	48%	50	0.7773	---	XX
14	90%	20	0.0003	0.1213	Δ-XY
15	35%	26	0.1167	---	XX
16	52%	50	0.7773	---	XX

Table 4.4 Results for Boesmans B5 group. The P_1-value was obtained when tested for a 1:1 sex ratio. This would be indicative of a normal XX genotype. When the P_1-value was not significant a P_2-value was obtained testing to a 3:1 sex ratio, this being indicative of a sex-reversed (Δ-XY) genotype. A genotype was assigned based on the significance of these P-values.

No of indiv.	% Male	Sample size (n)	P_1-value (1:1 ratio)	P_2-value (1:3 ratio)	Assigned genotype
1	54%	50	0.5716	---	XX
2	60%	50	0.1572	---	XX
4	60%	50	0.1572	---	XX
5	60%	50	0.1572	---	XX
6	74%	50	0.0004	0.8702	Δ-XY
7	46%	50	0.5716	---	XX
8	78%	50	0.0007	0.6242	Δ-XY
9	94%	50	<0.0001	0.0019	Δ-XY
10	56%	50	0.3961	---	XX
11	68%	50	0.0109	0.2529	Δ-XY
12	52%	50	0.7772	---	XX
13	56%	201*	0.0778	---	XX

Table 4.4 *continued*

14	80%	50	0.0002	0.4142	Δ-XY
15	48%	50	0.7772	---	XX
16	76%	25	0.0093	0.9080	Δ-XY
17	52%	50	0.7772	---	XX
18	50%	50	1	---	XX
19	80%	40	0.0001	0.4652	Δ-XY
20	84%	50	<0.0001	0.1416	Δ-XY
21	40%	50	0.1572	---	XX
22	68%	50	0.0109	0.2529	Δ-XY
23	54%	50	0.5716	---	XX
24	50%	50	1	---	XX
25	56%	50	0.3961	---	XX
26	80%	50	0.0002	0.4142	Δ-XY

* This progeny group exhibited a large number of red coloured individuals, to that were retained for further breeding purposes. The population was therefore sexed on external characteristics.

Figure 4.3 presents a summary of the numerical data of the sex ratio distribution of females tested in the B3 group presented in Table 4.2.

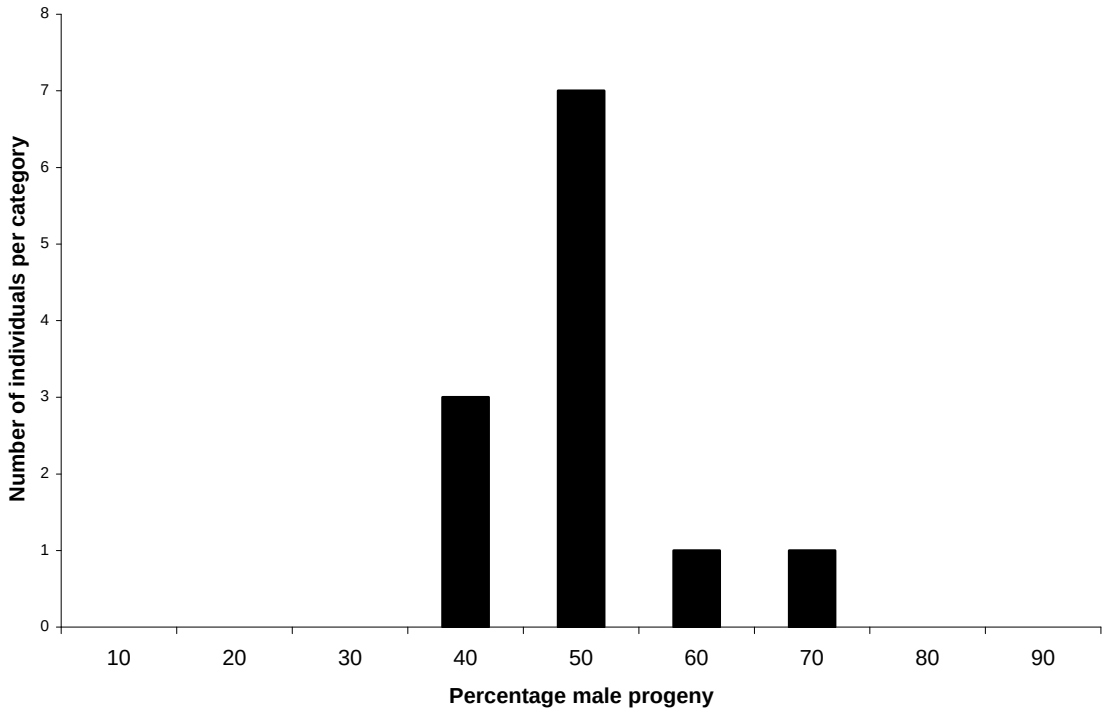

Figure 4.3 A histogram of the sex ratio distribution of females tested in the B3 group. This summarizes the data presented in Table 4.2.

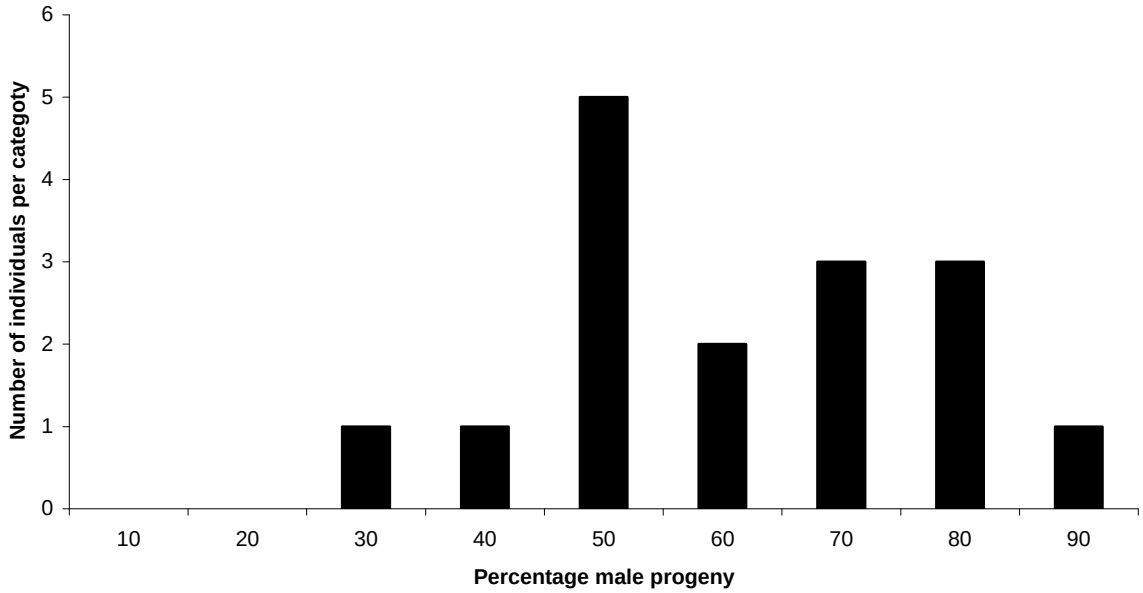

Figure 4.4 A histogram of the sex ratio distribution of females tested in the B4 group. This summarizes the data presented in Table 4.3.

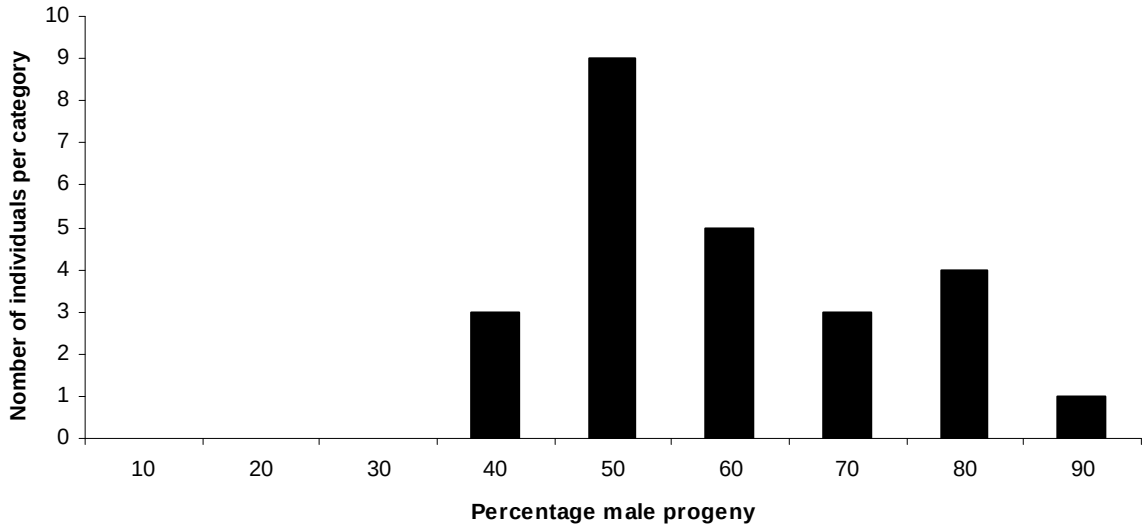

Figure 4.5 A histogram of the sex ratio distribution of females tested in the B5 group. This summarizes the data presented in Table 4.4.

Table 4.5 presents a summary of the data obtained from progeny testing of the potential XY groups obtained through DES feminization. Only data from the B4 and B5 groups were included in this table, as the B3 group did not conform to the expected results.

Table 4.5 Summary of progeny testing data of potential XY groups. Groups B3 was excluded.

Parameter	Quantity
No of groups treated with DES	5
No of family groups tested for potential XY females	3
Number of progeny sexed (Average number per female)	2 893 (53.57)
Range of sex ratios observed	35%-94%
Number of females classed as XX	36
Mean % males in XX progeny groups (±S.D.)	53.20 ±6.38
Number of females not classed	0
Number of females classed as Δ-XY	18
Mean % males in Δ-XY progeny groups (±S.D.)	78.33 ±6.66
P-value for observed vs. expected number of Δ-XY females	0.217
Total number of potential YY groups produced	18

Phase C: Progeny testing of potential YY groups to identify YY males.

When individuals from potential YY groups had reached sexual maturity, they were subjected to the same testing procedure, described in Phase B, which was used to identify Δ-XY females. In this instance a normal XX female was used as the female parent in the testcross. All males were tested to a 1:1 ratio and only males that were significantly different at a 0.1% ($p<0.001$) were designated the YY genotype.

Table 4.6 Results of progeny testing of the first YY group. The female parent was Individual no. 6 from the B5 group. The P-value was obtained when testing the observed sex ratio against the expected 1:1 ratio. A p-value greater than or equal to 0.5 is indicative of a normal XY genotype. A p-value less than 0.001 indicate the YY genotype. No genotype was assigned for P values smaller than 0.5 but greater than 0.01.

No	% male	Sample size	P value (1:1 ratio)	Assigned genotype
1	52%	50	0.7773	XY
2	88%	50	<0.001	YY
3	56%	50	0.3961	XY
4	44%	50	0.3961	XY
5	48%	50	0.7773	XY
6	72%	50	0.0019	Not assigned
7	42%	50	0.2579	XY
8	58%	50	0.2579	XY
9	68%	50	0.0109	Not assigned
10	58%	50	0.2579	XY
11	54%	50	0.5716	XY
12	54%	50	0.5716	XY
13	40%	50	0.1573	XY
14	52%	50	0.7773	XY
15	70%	50	0.0047	Not assigned
16	32%	50	0.0109	Not assigned
17	98%	50	<0.001	YY
18	42%	50	0.2579	XY
19	62%	50	0.0896	XY
20	90%	50	<0.001	YY
21	64%	50	0.0477	Not assigned
22	48%	50	0.7777	XY
23	98%	50	<0.001	YY
24	58%	50	0.2579	XY
25	60%	50	0.1573	XY
26	54%	50	0.5716	XY
27	46%	50	0.5716	XY
28	96%	50	<0.001	YY
29	40%	50	0.1573	XY
30	98%	50	<0.001	YY
31	64%	50	0.0477	Not assigned
32	96%	50	<0.001	YY
33	72%	50	0.0019	Not assigned
34	60%	50	0.1573	XY

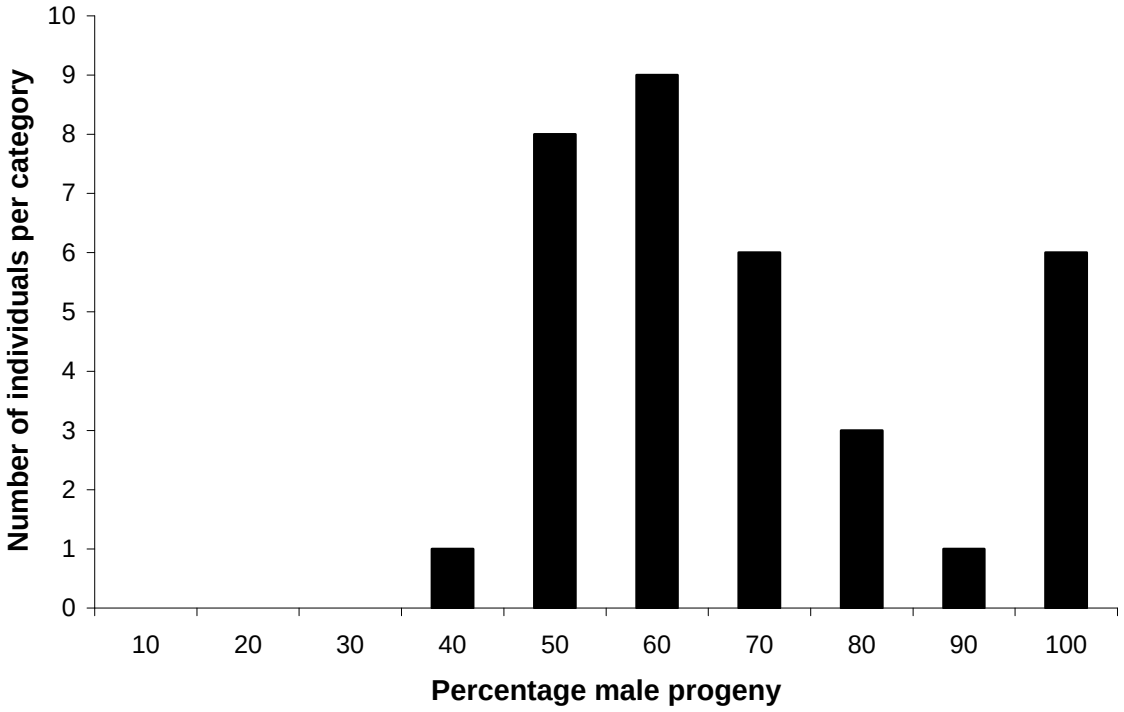

Figure 4.6 A histogram of the sex ratio distribution of males tested in the first YY group. This summarizes the data presented in Table 4.6.

Table 4.7 Summary of results obtained from the progeny testing of the first potential YY group.

Parameter	Quantity
Number of individuals tested	34
Total number of progeny sampled (mean number per group)	1700 (50)
Range of sex ratios observed	32% - 98%
Number of males classed as XY genotype	20
Mean % males in XY progeny groups (±S.D.)	51.4% (±7.17)
P-value for observed vs. expected number of XY males	0.365
Number of males not classed (no genotype assigned)	7
Mean % males in "unassigned" progeny groups (±S.D.)	63.14 % (±14.13)
Number of males classed as YY	7
Mean % males in YY progeny groups (±S.D.)	94.8% (±4.14)
P-value for observed vs. expected number of YY males	0.117

4.10 DISCUSSION

The incentive for developing a reliable technique for the production of monosex (all-male) tilapia populations was to alleviate problems associated with the production of mixed-sex populations, such as precocious breeding, inferior growth rates and inadequate market size. The approach taken was the implementation of a breeding program directed towards the production of YY male genotypes that has provided successful results under commercial conditions in a closely related species, *O. niloticus* (Mair and Abella 1997; Scott et al., 1989).

Prior to the production of YY males and the subsequent development of the GMT breeding program in *O. niloticus*, the viability of the YY genotype was demonstrated in several other species, including *O. mossambicus* (Yang et al., 1980, as cited in Mair et al., 1997; Varadaraj and Panadian, 1989). Onozato (1993) developed the YY genotype in the Amago salmon through androgenesis; while Kavumpurath and Pandian (1993) produced a female YY genotype in the guppy *Poecilia reticulata*.

The development of a YY genotype in *O. mossambicus* was expected to be successful only if the main sex determining mechanism is based on a simple monofactorial XX/XY system, as postulated by Mair et al. (1991a). The range of sex ratios observed among the progeny of DES sex-reversed fish provides evidence for such a sex determination system (Tables 4.2-4.4). DES feminization treatment was considered to be successful, as four out of the five treated groups exhibited almost complete sex reversal, and the mean percentage of females in these four groups was 97% (Figure 4.2). These results are concurrent with those of Yamazaki (1983); Rosenstein and Hulata (1993) and Tuan et al. (1997).

All DES treated females that were test crossed could be assigned a genotype on the grounds of a predicted sex ratio based on a XX/XY system. Furthermore, in the case of a monofactorial sex determination system, the number of Δ-XY individuals per group should

theoretically constitute one half of the group, as was the case in two of the three tested groups (Table 4.5).

Results obtained from control groups of the DES treated families were also in confirmation of a monofactorial sex determination system, as all but one group exhibited the expected 1:1 ratio (Figure 4.2). The single control group that did not conform to the expected ratio was significantly different at a 1% probability level. Incidentally, this group also showed the lowest level of sex reversal among all the DES treated groups (excluding Group B1).

A possible explanation for this could be offered by the temperature regime that was experienced during the labile period of sex reversal when the hormone was administered. Hormone treatment was administered in a recirculation system within a glasshouse facility during early spring. Fluctuating temperatures during this period could possibly have had an effect on hormone treatment. In *O. niloticus*, there is evidence that DES treatment is more effective at higher temperatures (Abucay et al, 1999; Mair, 2000), with hormone dosage being reduced without a subsequent decrease in the effectiveness of the feminization treatment. However, no evidence exists on the effect of lower temperatures on the effectiveness of hormone treatment. It is known that tilapias feed at a lower rate at temperatures below their optimal range, so the uptake of hormone treated food and therefore the dosage may be affected at lower temperatures.

Convincing evidence is presented in Chapter 3, which illustrate the role of temperature in sexual differentiation in the *Oreochromis* species. Baroiller et al., (1995b) and Abucay et al. (1999) have provided evidence that temperature sensitivity varies between breeding pairs and that pairs differ greatly in their response to temperature. In *O. niloticus* and the Florida Red tilapia, 0 to 98% males were produced following a constant 36 ^{o}C treatment. (The

Florida Red tilapia was originally obtained by crossing a mutant red *O. mossambicus* with three other species, *O. hornorum, O. niloticus* and *O. aureus*)

While the temperature effect on sex differentiation in *O. mossambicus* is still not fully understood, it is possible that ambient temperature during sex reversal could have influenced both the treatment and control groups. Mair et al. (1990) found strong evidence to indicate that low temperatures (± 20^{o}C) could significantly increase the proportion of males in a progeny group, compared to the control. Although there is no evidence to directly support such a hypothesis, it is possible that the lower temperature during treatment caused more males to be present in the control group and the hormone treatment to be less effective than it would have been at ambient or higher temperature. In the event of such a temperature effect on sexual development in *O. mossambicus*, the data would be indicative also of a family effect, as the other groups were exposed to a similar temperature regime and still yielded the expected results (Figure 4.2).

Of the 18 potential YY male groups that were produced, progeny testing had commenced on four of the groups. The results of only a single group were included, as the rest of the progeny groups have not yet reached the minimum size required for the gonad squashing technique of sex determination. The sex ratios produced by males that were tested were not quite in accordance with those predicted by the hypothesis of a purely monofactorial sex determination system (Table 4.6 and Table 4.7). Assuming a strictly XX/XY sex determination system, all individuals should be able to fit into a genotypic group (i.e. either XY or YY) on the basis of their sex ratio. A large percentage of males (20.5%), however, did not fit into either genotypic group and no genotype could be assigned to them.

According to the XX/XY sex determination system, all YY males are expected to sire 100% male progeny. This was not the case and all males that were designated as YY sired progenies that were in the excess of 90% male, but none sired a 100% male progeny. The

range of sex ratios observed from YY males was 88-98%, with a mean of 94.3%. This observation is in accordance with the results of Mair et al. (1997), who observed sex ratios ranging from 79.5-100%, with an average of 98.9% male for YY males in their work on *O. niloticus*. Scott et al. (1989), on the other hand, observed no females in the sexing of progenies from a single YY male crossed to 10 different females. Similarly, Varadaraj and Panadian (1989) found no females in the progeny of eight YY males in *O. mossambicus*.

There can be several possible explanations for the occurrence of the unexpected number of females in YY progeny groups. Abucay et al. (1999) proposed that these females might be an indication of the presence of a factor or factors other than the major sex determining genes that may be exerting an influence during sex differentiation. These factors are possibly autosomal sex modifying genes that, along with the sex chromosomes, determine sexual differentiation.

Rothbard et al. (1990), in their work on *O. aureus*, demonstrated that newly hatched fry carry high levels of endogenous hormones and that these levels vary among individual fish. It is possible that those fry with a higher load of male autosomal sex modifying genes may have a higher level of endogenous androgens. During hormone treatment for sex reversal, the administered exogenous hormone, combined with the high level of endogenous hormone may enable fish to be masculinized more easily. In the experiments of Baroiller (1996) on androgen sex reversed males (Δ-XX), the majority of males tested had significant proportions of unexpected males in their progeny. A possible explanation for this is that the sex reversed fish, which are carrying more male modifying genes, will cause the load of these genes to increase in the next generation, which will cause aberrant sex ratios to be observed.

This same theory could explain the presence of females in the progeny of YY males, only here it would be female modifying genes that are accumulating and causing the occurrence of some female individuals. Capilli (1995), as cited in Mair et al. (1997)

demonstrated that in *O. niloticus*, the females that arise in progenies of YY x XX crosses conform to the expected XY genotype when test crossed to a known genotype from the same stain. It was also noted that significant paternal and maternal effects exists in the occurrence of rare females in the progeny of YY males.

It is therefore concluded that the unexpected females that were present in the progeny of YY males is likely to be the result of some autosomal sex modifying genes and that these could be selected against to increase the proportion of males in YY progeny groups. Mair et al. (1997) demonstrated an increase in the mean number of males produced from selected YY males that produced 100% male sex ratios during initial progeny testing. This response to selection is further indicative of a genetic basis to the occurrence of aberrant females.

There is also a possibility that the occurrence of a small number of females in YY progenies, is due to a temperature effect. A clear temperature effect on sex differentiation has been demonstrated in the progenies of normal and sex reversed individuals from different *Oreochromis* species. Extreme temperatures have been proven to influence sex in the direction of both male and female and there appears to be a genetic basis for the sensitivity to temperature effects on sex ratio (Baroiller et al., 1995a, 1995b.; Abucay et al., 1999).

The thermosensitive window in most *Oreochromis* species is relatively early in the life cycle, 9-15 days post fertilization (Baroiller et al., 1999), and it is possible that fry from YY parents in this trial were subjected to a fluctuating temperature regime during this period. Fry were transferred to a greenhouse rearing unit shortly after hatching and, as this unit is heated by solar energy, a constant temperature was not always maintained. Fry rearing was conducted during late summer to beginning of winter. The mean temperature during fry rearing was 26.5 ^{o}C. This correlates well with the preferential temperature range of *O. mossambicus* and a major modifying effect of temperature on sex ratio is therefore unlikely. All progenies from DES treated groups were subjected to the same temperature

regime and among them only one aberrant sex ratio was observed, namely the control group of B3 that was biased towards the female sex. As discussed, all progeny tested females exhibited sex ratios that complied with a monofactorial system of sex determination and the effects of temperature on sex ratio can, in this instance, be ignored.

CONCLUSION

The application of monosex culture methods is predominant in aquaculture production where one sexual phenotype displays a significant advantage over the other, such as an all-male culture of tilapia. Monosex culture also offers other advantages such as a reduction in aggression among individuals, more uniform harvest size and the elimination of unwanted recruitment (Scott et al., 1989; Beardmore, 2001).

Monosex production of aquaculture species has conventionally been achieved through the use of hormone induced sex reversal, though problems are experienced with this method in terms of environmental effects and consumer preference. The YY male technology, as applied in tilapia, presents an alternative and more acceptable procedure to obtain monosex progeny groups for commercial application. With the YY technology commercial stocks consist of natural male genotypes which are not exposed to hormone treatment. Furthermore, all males that are produced are normal genetic males and strain and species purity is maintained. The latter is not the case when all male populations are produced through hybridization.

From the data obtained in the project it would appear that the development of the GMT technology in *O. mossambicus* is indeed viable and could find commercial application in Southern Africa, where *O. mossambicus* is endemic, as well as certain parts of the United States where *O. mossambicus* is the preferred species in commercial production systems (Beardmore et al., 2001; Wanatabe et al, 2002.). The production of both Δ-XY females and YY males have proven to be successful and the next important phase in the program will be the production of YY females in order to produce commercial quantities of YY males. Successful feminization of the YY genotype has been achieved in *O. niloticus* and is a vital step in the development of the YY technology on a commercial scale, as it makes possible the production of YY males without the need for time-consuming progeny testing. A possible

drawback here is the differential feminization of XY and YY genotypes and the increased difficulty of feminizing the YY genotype (Vera Cruz et al., 1996). If however, this feminization treatment proves to be successful, the YY male technology can be applied on a commercial scale. The performance of GMT populations has been evaluated on a commercial scale in *O. niloticus*. Results from these trials indicate GMT have considerable benefits under culture conditions, such as increased yields of up to 58% compared with mixed sex populations of the same strain (Mair et al., 1995). In addition to the negligible recruitment in GMT populations, there is the further advantage of more uniform harvest size and better food conversion ratios (Mair et al., 1995). Overall, the advantages offered by the use of GMT appear to outweigh the costs of the development of this technology.

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